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TABLE OF CONTENTS

ACKNOWLEDGEMENTS.....iii

TABLE OF CONTENTS.....v

LIST OF FIGURES.....xii

LIST OF TABLES.....xvii

LIST OF ABBREVIATIONS.....xviii

LIST OF SYMBOLS AND UNITS.....xxii

RÉSUMÉ xxiv

ABSTRACT.....xxvi

PART I –

CHAPTER I

STARCH DERIVATIVES AS SELF-ASSEMBLED BIOMATERIALS FOR PHARMACEUTICAL AND BIOMEDICAL APPLICATIONS.....2

1.1.Starch and cross-linked starch derivatives for use as pharmaceutical excipients .3

1.1.1. Gelatinized starch and cross-linked starch as pharmaceutical excipients.....3

1.1.1.1. Starch as excipient in pharmaceutical formulation 6

1.1.1.2. Cross-linked starch as excipient for drug delivery 9

1.1.1.3. Cross-linked starch as binder and disintegrant 11

1.1.1.4. Cross-linked starch as implant 12

1.1.1.5. Cross-linked starch for separation of macrophages 12

1.2.Carboxymethyl starch as multifunctional excipient 13

1.2.1. Carboxymethyl starch as disintegrant : Explotab®, Primojel®.....15

1.2.2. Carboxymethyl starch as excipient for high loaded pharmaceutical dosage form 16

1.2.3. Carboxymethyl starch as pH responsive excipient 16

1.2.4. Carboxymethyl starch as pH-independent excipient 17

1.2.5. Carboxymethyl starch microspheres for gastro-retentive formulation 17

CHAPTER II

COMPLEXES OF STARCH AND CHITOSAN.....19

2.1. Introduction 19

2.2. Unmodified starch – chitosan complexes 20

2.2.1. Unmodified starch – chitosan films.....20

2.2.1. Unmodified starch – chitosan tablets..... 24

2.2.3. Unmodified starch – chitosan scaffolds.....24

2.3. Modified starch – chitosan complex 25

2.3.1. Cross-linked starch – chitosan microspheres.....25

2.3.2. Carboxymethyl starch – chitosan physical mixture.....27

2.3.3. Carboxymethyl starch – chitosan polyelectrolyte complex (PEC) 29

2.3.4. Carboxylated starch-chitosan nanoparticles 33

2.3.5. Carboxylated starch-chitosan scaffolds.....33

2.3.6. Maleic starch half-ester acid – chitosan polyelectrolyte complex 34

CHAPTER III

MACROPHAGES IN BIOMEDICAL SCIENCE 36

3.1. Origin and Role of Macrophages.....36

3.2. Macrophages for biomedical applications 38

3.3. Techniques of macrophages separation.....40

CHAPTER IV

PROJECT PRESENTATION AND RESEARCH ACHIEVEMENTS 42

PART II - EXPERIMENTAL CONTRIBUTIONS 47

CHAPTER V

STARCH MATERIALS AS BIOCOMPATIBLE SUPPORTS AND ROCEDURE FOR FAST SEPARATION OF MACROPHAGES 48

Contribution of the main author and of the co-authors.....49
Abstract 50
5.1. Introduction 51
5.2. Materials and methods 52
5.2.1. Materials 52
5.2.2. Preparation of starch filmogenic materials 53
5.2.3. Evaluation of substitution degree of derivatives 54
5.2.4. Fourier transform infrared (FT-IR) analysis 55
5.2.5. ^{1}H NMR measurements 55
5.2.6. Scanning electron microscopy (SEM) 55
5.2.7. Film casting and macrophage culture 55
5.2.7.1. Preparation of film-forming solutions of starch materials 55
5.2.7.2. Macrophage incubation 56
5.2.7.3. Microscopy 56
5.2.7.4. Susceptibility to enzymatic hydrolysis of starch films.....57
5.2.7.5.Determination of enzymatic activity on the starch filmogenic supports 57
5.2.7.6. Macrophage cell recovery and counting 58
5.2.7.7. Macrophage activation 58
5.2.7.8. Quantitation of tumor necrosis factor (TNF-α).....58
5.2.8. Statistical Analysis 58
5.3. Results and discussions 59
5.3.1. Polymer and film characterization 59
5.3.2. Macrophage cells attachment and recovery by film amylolysis.....66
5.4. Conclusion 71
5.5. Acknowledgements 71

CHAPTER VI

AMPHOLYTIC STARCH EXCIPIENTS FOR HIGH LOADED DRUG FORMULATIONS: MECHANISTIC INSIGHTS 72

Contribution of the main author and of the co-authors 73

Abstract 74

6.1. Introduction 76

6.2. Materials and methods 78

6.2.1. Materials 78

6.2.2. Preparation of starch derivatives 78

6.2.3. Evaluation of the degree of substitution 80

6.2.4. Zeta potential (ζ) and pH determination 81

6.2.5. Viscosity determination 81

6.2.6. Fourier transform infrared (FT-IR) analysis 82

6.2.7. ^{1}H NMR measurements 82

6.2.8. Thermogravimetric analysis 82

6.2.9. Scanning electron microscopy (SEM) 82

6.2.10. X-ray diffraction 83

6.2.11. Micromeritics properties of starch derivatives powders.....83

6.2.12. Preparation of tablets 84

6.2.13. Determination of the fluid uptake and erosion 84

6.2.14. *In vitro* dissolution tests.....85

6.2.15. Release kinetic patterns.....86

6.2.16. Statistical analysis.....86

6.3. Results and discussions.....86

6.3.1. Structural insights 86

6.3.2. Fourier transform infrared (FT-IR) analysis 89

6.3.4. ^{1}H NMR measurements.....90

6.3.5. Thermogravimetric analysis.....91

6.3.6. X-ray powder diffraction.....92
6.3.7. Scanning electron microscopy (SEM) 93
6.3.8. Micromeritics properties of starch derivatives powders 95
6.3.9. Determination of the fluid uptake and erosion.....96
6.3.10. *In vitro* dissolution tests.....100
6.4. Conclusion 110
6.5. Acknowledgements 110

CHAPTER VII

SELF-STABILIZING AMPHOLYTIC STARCH EXCIPIENTS FOR SUSTAINED RELEASE OF HIGHLY SOLUBLE DRUGS: THE CASE STUDY OF METFORMIN.....111

Contribution of the main author and of the co-authors.....112
Abstract 113
7.1. Introduction 114
7.2. Materials and Methods 117
7.2.1. Materials 117
7.2.2. Preparation of ampholytic starch derivatives 117
7.2.3. Evaluation of the degree of substitution.....118
7.2.4. Preparation of tablets 119
7.2.5. Determination of the fluid uptake and erosion 119
7.2.6. Fourier transform infrared (FT-IR) analysis.....120
7.2.7. ^{1}H NMR measurements 120
7.2.8. Thermogravimetric analysis 121
7.2.9. Scanning electron microscopy (SEM).....121
7.2.10. X-ray diffraction.....121
7.2.11. *In vitro* dissolution tests.....121
7.2.12. Release kinetic patterns 122
7.2.13. Statistical analysis.....122

7.3. Results

7.3.1. Ampholytic starch characterization and structural insights 122

7.3.2. Scanning electron microscopy (SEM)..... 124

7.3.3. Fourier transform infrared (FT-IR) analysis 124

7.3.4. ^{1}H NMR measurements.....127

7.3.5. Thermogravimetric analysis..... 128

7.3.6. X-ray powder diffraction.....130

7.3.7. *In vitro* dissolution tests..... 132

7.3.8. Determination of the fluid uptake and erosion.....138

7.4. Discussions.....138

7.5. Conclusion.....142

7.6. Acknowledgements 142

CHAPTER VIII

DISCUSSION AND CONCLUSIONS..... 143

CHAPTER IX

ANNEXES: Published Articles, Abstracts and Posters:.....157

A.1. Published Articles:

A.1.1 Starch materials as biocompatible supports and procedure for fast separation of macrophages. Published in Carbohydrate Polymers, 2017, vol. 163, 108-117. 159

A.1.2 Self-stabilizing ampholytic starch excipients for sustained release of highly soluble drugs: the case study of metformin. Published in AAPS PharmSciTech, 2017, DOI: 10.1208/s12249-017-0723-3.....169

A.1.1 Ampholytic starch - excipients for high loaded drug formulations: Mechanistic insights. Published in International Journal of Pharmaceutics, 2017, vol 535, 201-216.....184

A.1. Oral presentation

A self-stabilizing ampholytic starch excipients for sustained release of highly soluble drugs: the case of metformin. 19th Annual CBGRC, November 18th, 2016, Concordia University. Montreal, Quebec, Canada. 200

A.2. Extended abstract:

New class of self-stabilizing ampholytic starch as excipients for drug sustained release. Control Release Society annual meeting, July 16-19, 2017. Boston, Massachusetts, USA. 202

A.3. Posters

A.4.1. Ampholytic starch excipients for high loaded drug formulations. 7e Colloque Pharmaquam (Université du Québec à Montréal). 31 August, 2017. Montréal, Québec, Canada. 204

A.4.2. Novel ampholytic starch derivatives as excipients for high loaded drug formulations. 7e Colloque RQRM (Réseau québécois de recherche sur les médicaments). 30 Sept – 1 Oct, 2017. Québec, Canada. 205

REFERENCES 206

LIST OF FIGURES

Figure		Page
1.1	Amylose and amylopectin in dry phase and after interaction with water	5
1.2	Schematic representation of a model for the structural arrangement of high amylose cross-linked starch with a low cross-linking degree in the dry (a) and hydrated (b) state	6
1.3	Drug release time from monolithic tablets with cross-linked starch and hypothetical assembling at various cross-linking degrees	11
1.4	Starch structure with hydroxyl groups numbered. Chemical	13
1.5	Structure of carboxymethyl starch (CM-St).	14
2.1	Chemical structure of chitosan.	19
2.2	Chemical structure of starch presenting (a) amylose and (b) amylopectin.	20
2.3	SEM micrographs of the cross-sections of the films containing or not thyme extract (TE).	23
2. 4	Scanning electron micrographs of beads prepared with 2 h or 4 h of crosslinking time.	26
2. 5	pH stability of enzyme formulations in tablets based on CM-St:Chitosan.	29
2. 6	Scanning electron microscopy micrographs of (a) CM-St, (b) chitosan-400, (c) chitosan-700, and (d) PEC at magnifications of 100× and 500× and voltage of 15 kV	31
2. 7	Photographs of CM-St (CMS), chitosan-700, 50% CMS:50% chitosan-700 and PEC tablets	32
2.8	The SEM images of composite scaffolds (a) chitosan pure, (b) 70 % chitosan and 30% starch, (c) 50 % chitosan and 50% starch, and (d) 30 % chitosan and 70% starch	34

2.9	Starch (St) maleate half-ester (MSA) structure	35
3.1	Monocyte and macrophage differentiation	36
3.2	Preparation of macrophage-based microrobots.	40
4.1	Schematic presentation of project	46
5.1	Design of device and procedure with adhesion (1) and amylolysis (2) steps for fast recovery of macrophages.	56
5.2	FT-IR spectra of Gelatinized starch (G-St), Acetate starch (Ac-St), Carboxymethyl starch (CM-St) and Amino-Ethyl starch (AE-St).	60
5.3	^{1}H NMR spectra of Gelatinized starch (Red), Acetate starch (Green), Carboxymethyl starch (Blue) and Amino-Ethyl starch (Black).	61
5.4	Water contact angle measurement for insert coating films of Gelatinized starch (G-St), Acetate starch (Ac-St), Carboxymethyl starch (CM-St) and Amino-Ethyl starch (AE-St) (n = 3).	62
5.5	Scanning electron microscopy micrographs of Native starch (Hylon VII), (a) Gelatinized starch (G-St), (b) Acetate starch (Ac-St), (c) Carboxymethyl starch (CM-St) and (d) Amino-Ethyl starch (AE-St) powders at magnifications of 100x and 1000x.	63
5.6	Scanning electron microscopy micrographs of films.	65
5.7	Confocal fluorescence microscopy images showing live cells (green) and dead macrophage cells (red).	66
5.8	(A) Release reducing sugar (µmol) after Gelatinized starch (G-St), Acetate starch (Ac-St), Carboxymethyl starch (CM-St), and Amino-Ethyl starch (AE-St) film hydrolysis by alpha-amylase; (B) Percentage of dead cells (%) incubated on cell insert coated with G-St, Ac-St, CM-St and AE-St; (C) Macrophage count with an initial number ($6.5x10^{5}$) and incubated 48 h on inserts coated with film of G-St, Ac-St, AE-St or Control (uncoated); (D) Tumor necrosis factor TNF-α (pg/mL) from recovered macrophage activated by lipopolysaccharide (LPS) 50 ng/mL.	69

6.1	Graphical abstract for ampholytic starch excipients	75
6.2	Schematical presentation of starch derivatization with sodium monochloroacetate and with chloroethylamine hydrochloride to generate ampholytic starch carrying carboxymethyl (CM) and aminoethyl (AE) functions obtained by one step (OS) or two steps (TS) preparation methods.	87
6.3	FT-IR spectra of Native Starch (red), CMAESt (OS) (blue), CMAESt (TS) (black) and AECMSt (TS) (green).	89
6.5	^1H NMR spectra in D_2O (I) and in DMSO-d6 (II) for Native Starch (1), CMAESt (OS) (2), CMAESt (TS) (3) and AECMSt (TS) (4).	90
6.6	Thermogravimetric (TG) and its derivative (DTG) diagrams of Native Starch (black), CMAESt (OS) (blue), CMAESt (TS) (green) and AECMSt (TS) (red).	92
6.7	X-ray diffraction patterns of Native Starch (A), CMAESt (OS) (B), CMAESt (TS) (C) and AECMSt (TS) (D) powders.	93
6.8	Scanning electron microscopy micrographs of Native Starch (A), CMAESt (OS) (B), CMAESt (TS) (C) and AECMSt (TS) (D) powders at magnifications of 100x and 1000x.	94
6.9	Fluid uptake (I), Erosion (II) and axial tablet swelling (diameter changes) (III) of a drug-free tablet formulated with CM-AE-St (OS), CM-AE-St (TS) and AE-CM-St (TS) as a matrix forming agents in SGF and SIF.	97
6.10	Fluid uptake (I), Erosion (II) and axial tablet swelling (III) of a drug-free tablet formulated with CM-AE-St (OS), CM-AE-St (TS) and AE-CM-St (TS) as a matrix forming agents incubated 2 h in SGF and then in SIF for 6 h.	98
6.11	Release profiles of Aspirin (I), Metformin (II), Mesalamine (III) and Acetaminophen (IV) in SGF and in SIF from tablets (60% loading) formulated with CM-AE-St (OS), CM-AE-St (TS) and AE-CM-St (TS) as a matrix forming polymers.	101

6.12	Release profiles of Aspirin (I), Metformin (II), Mesalamine (III) and Acetaminophen (IV) from tablet (60% loading) formulated with CM-AE-St (OS), CM-AE-St (TS) and AE-CM-St (TS) as a matrix forming polymers followed for 2 h in SGF then transferred to SIF.	104
7.1	Hypothetical presentation of (A) starch derivatization with sodium monochloroacetate and with chloroethylamine hydrochloride.	123
7.2	Scanning electron microscopy micrographs of (A) Native Starch, (B) CM- St (DS 0.02), (B') CM- St (DS 0.06), (C) CM-AE-St (DS 0.02-0.024), (C') CM-AE-St (DS 0.06-0.019), (D) CM-AE-St (DS 0.02-0.049), (D') CM-AE-St (DS 0.06-0.045), (E) CM-AE-St (DS 0.02-0.064) and (E') CM-AE-St (DS 0.06-0.059) at magnification of 1000.	125
7.3	FT-IR spectra of (Red) Native Starch and of (I): (Blue) CM- St (DS 0.02), (Black) CM-AE-St (DS 0.02-0.024), (Green) CM-AE-St (DS 0.02-0.049), and (Brown) CM-AE-St (DS 0.02-0.064), and of (II) (Blue) CM- St (DS 0.06), (Black) CM-AE-St (DS 0.06-0.019, (Green) CM-AE-St (DS 0.06-0.045), and (Brown) CM-AE-St (DS 0.06-0.059).	126
7.4	^{1}H NMR spectra I): (A) CM- St (DS 0.02), (B) CM-AE-St (DS 0.02-0.024), (C) CM-AE-St (DS 0.02-0.049), and (D) CM-AE-St (DS 0.02-0.064). and II) of (A') CM- St (DS 0.06), (B') CM-AE-St (DS 0.06-0.019, (C') CM-AE-St (DS 0.06-0.045), and (D') CM-AE-St (DS 0.06-0.059).	127-128
7.5	Thermogravimetric (TG) and its derivative (DTG) diagram of Native Starch (Red) and of I) (Blue) CM- St (DS 0.02), (Black) CM-AE-St (DS 0.02-0.024), (Green) CM-AE-St (DS 0.02-0.049), and (Brown) CM-AE-St (DS 0.02-0.064). and of II) (Blue) CM- St (DS 0.06), (Black) CM-AE-St (DS 0.06-0.019, (Green) CM-AE-St (DS 0.06-0.045), and (Brown) CM-AE-St (DS 0.06-0.059).	129
7.6	X-ray diffraction patterns of (A) Native Starch and of (I): (B) CM-St (DS 0.02), (C) CM-AE-St (DS 0.02-0.024), (D) CM-AE-St (DS 0.02-0.049), and (E) CM-AE-St (DS 0.02-0.064). and (II): (B') CM-St (DS 0.06), (C') CM-AE-St (DS 0.06-0.019, (D') CM-AE-St (DS	131

0.06-0.045), and (E') CM-AE-St (DS 0.06-0.059).

7.7 Release profiles of metformin from 1000 mg tablet (60% loading) of I): (A) CM- St (DS 0.02), (B) CM-AE-St (DS 0.02-0.024), (C) CM-AE-St (DS 0.02-0.049), and (D) CM-AE-St (DS 0.02-0.064). II) and of (A') CM- St (DS 0.06), (B') CM-AE-St (DS 0.06-0.019, (C') CM-AE-St (DS 0.06-0.045), and (D') CM-AE-St (DS 0.06-0.059) at pH 1.2 (left) and pH 6.8 (right) side. 133

7.8 Release profiles (I), Fluid uptake and Erosion (II), Axial and Radial tablet dimension changes (III) of Glumetza® (G) and tablet based on CM-AE-St(DS 0.06-0.059) oblong (O) of 1 g (50% loading). 137

LIST OF TABLES

Table		Page
6.1	Viscosity (cP) of starch derivatives at 1 % w/v in distilled water (W) and in SIF measured after 4 h and respectively after 48 h, at 25°C	88
6.2	Micromeritic properties of starch derivatives powders	95
6.3	Structures of bioactive molecules in neutral, SGF, and SIF media.	102
6.4	Time (h) of 90% drug release from monolithic tablet contained ampholytic starch derivatives as matrix forming agents in SGF, SIF and SGF followed by SIF.	105
6.5	Kinetic parameters of Aspirin (i), Metformin (ii), Mesalamine (iii), and acetaminophen (iv) from tablets (60% loading) formulated with CMAESt (OS) (I), CMAESt (TS) (II) and AECMSt (TS) (III) as a matrix forming excipients in SGF then transferred to SIF.	107
6.6	Kinetic parameters of Aspirin (i), Metformin (ii), Mesalamine (iii), and Acetaminophen (iv) released in SGF and in SIF from tablets (60% loading) formulated with CM-AE-St (OS) (I), CM-AE-St (TS) (II) and AE-CM-St (TS) (III) as matrix forming excipients	108
7.1	Kinetic equations and corresponding values of metformin release from tablets (60% loading) based on (A) CM-AE-St (DS 0.02-0.064), (B) CM-AE-St (DS 0.06-0.059) at pH 1.2 and pH 6.8, and from tablets (50% loading) based on (C) CM-AE-St (DS 0.06-0.059) compared to Glumetza®.	135
8.1	Biopharmaceutical classification system (BCS).	153

LIST OF ABBREVIATIONS

Ac-St	Acetate starch
ASA	Acetylsalicylic acid
AC-ST-CL	Acetate starch cross-linked
API	Active pharmaceutical ingredient
ALP	Alkaline phosphatase assay
AE-CM-St (TS)	Aminoethyl carboxymethyl starch two steps
AE-St	Aminoethyl starch
AE-ST-CL	Aminoethyl starch cross-linked
θ	Angle of repose
BCS	Biopharmaceutical classification system
ρ_{bulk}	Bulk density
CaCM-ST	Calcium carboxymethyl-starch
^{13}C NMR	Carbon-13 nuclear magnetic resonance
CM-AE-St	Carboxymethyl aminoethyl starch
CM-AE-St (OS)	Carboxymethyl aminoethyl starch one steps
CM-AE-St (TS)	Carboxymethyl aminoethyl starch two steps
CM-St	Carboxymethyl starch
CM-ST-CL	Carboxymethyl starch cross-linked
CMS-MS	Carboxymethyl starch mucoadhesive microspheres
CI	Carr's index
Cs, CH	Chitosan

CEAHC	Chloroethylamine hydrochloride
CFX	Ciprofloxacin
CSFS	Colony-stimulating factors
cld	Cross-linking degree
CLHAS	Cross-linked high amylose starch
DS	Degree of substitution
DAO	Diamine oxidase
DSC	Differential scanning calorimetry
DMSO	Dimethyl sulfoxide
DTX	Docetaxel
EMA	Electromagnetic actuation
EACM	Ethylaminocarboxymethyl
EADCM	Ethylaminodicarboxymethyl
FT-IR	Fourier transform infrared (spectroscopy)
GIT	Gastrointestinal tract
G-St	Gelatinized starch
HR	Hausner's ratio
HPMC	Hydroxypropyl methylcellulose
Hex-hGRF	Human growth hormone-releasing factor analog
Infγ	Interferon-γ
LPS	Lipopolysaccharide
MNP	Magnetic nanoparticles

MSA	Maleic starch half-ester acid
MDT	Maximal decomposition temperature
MW	Molecular weight
MMT	Montmorillonite
NPS	Nanoparticles
OS	One step method
PSDAO	Plant diamine oxidase
PEC	Polyelectrolyte complex
PLGA	Poly-lactic-co-glycolic-acid
^{1}H NMR	Proton nuclear magnetic resonance
QA	Quaternary ammonium
SEM	Scanning electron microscopy
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
NaCM-ST	Sodium carboxymethyl-starch
SMCA	Sodium monochloroacetate
St, S, ST	Starch
TA	Tannic acid
ρ_{tap}	Tapped density
TGA	Thermogravimetric analysis
3D	Three-dimensional
TNF-α	Tumor necrosis factor

TS	Two steps method
TE	Thyme extract
UV/VIS	Ultraviolet–visible spectroscopy
USP	United States Pharmacopeia
XRD	X-ray diffraction
ζ	Zeta potential

LIST OF SYMBOLS AND UNITS

h	Hour
MW	Molecular weight
w/w	Weight per weight
µm	Micrometer
mm	Millimeter
kV	Kilovolt
°C	Degree centigrade (Celsius)
rpm	Rotation per minute
M	Molar
v/v	Volume per volume
µS/cm	MicroSiemens per centimetre
W	Weight
mol	Mole
w/v	Weight per volume
mM	Millimolar
U	Unit
nm	Nanometer
ng	Nanogram
µL	Microliter
pg	Picogram
ppm	Part per million

MHz	MegaHertz
mV	Millivolt
°	Degree
Å	Angstrom
λ	Lambda
mmol	Millimole
2θ	Scattering angles

RÉSUMÉ

L'amidon est considéré comme l'un des polymères les plus intéressants et les plus utilisés dans l'industrie alimentaire, biomédicale et pharmaceutique, en raison de sa biocompatibilité, sa biodégradabilité, sa non-toxicité et ses sources abondantes.

Dans la continuité des travaux de notre laboratoire sur l'amidon et ses dérivés, le présent projet se penche sur l'ouverture de nouveaux aspects sur l'utilisation de l'amidon dans des applications biomédicales et pharmaceutiques, en se concentrant sur des dérivés de l'amidon en tant que biomatériaux pour la séparation des cellules macrophages qu'excipient potentiel pour la libération contrôlée des médicaments.

L'amidon gélatinisé (G-St), l'acétate d'amidon (Ac-St), le carboxyméthyl amidon (CM-St) et l'aminoéthyl amidon (AE-St) ont été synthétisés et évalués comme supports pour la fixation et le détachement des macrophages (ligne RAW 264.7). Les films obtenus à partir des dérivés d'amidon, présentent une susceptibilité à une hydrolyse douce par l'alpha-amylase (amylolyse), libérant ainsi les macrophages adhérés. Ce nouveau procédé permet une séparation rapide des macrophages par leurs adhésion aux films d'amidon, suivie par leur libération suite à l'hydrolyse du film avec de l'alpha-amylase, avec des rendements excellents et une grande viabilité des cellules récupérées. Meilleurs résultats ont été obtenus avec des cellules macrophages en utilisent des inserts revêtus par G-St et par Ac-St. L'interaction possible entre les macrophages et les films d'amidon a été étudiée par comptage cellulaire, pourcentage de mortalité cellulaire ainsi que par le niveau de facteur de nécrose tumorale (TNF-α). La viabilité des cellules avec la méthode présentée était sensiblement plus élevée en comparaison avec les méthodes actuelles de détachement des cellules: grattage ou vortex.

Des nouveaux excipients ont été obtenus à base **d'amidon ampholytique**: des matériaux présentant des fonctions acides (ex: carboxymethyl [CM]) et basiques (ex: aminoethyl [AE]) greffées sur la macromolécule d'amidon. Les dérivés ampholytiques d'amidon ont été préparés par deux approches différentes. La méthode à une étape (one step [OS]) consiste en l'ajout de deux réactifs (sodium monochloroacétate [SMCA] et le chlorhydrate de chloroéthylamine [CEAHC]) simultanément. Différemment, dans la méthode à deux étapes (two steps [TS]) le carboxyméthyle amidon (CM-St), et l'aminoéthyle amidon (AE-St) ont été préparés séparément dans une première étape, chacun traité avec l'autre agent de fonctionnalisation. Les polymères obtenus ont été caractérisés par infrarouge à transformée de Fourier (IRTF), résonance magnétique nucléaire du proton (^{1}H RMN), microscopie électronique à balayage (SEM), l'analyse thermogravimétrique (ATG), diffraction des rayons X et potentiel Zeta (ζ). Des comprimés monolithiques avec une charge de 60% d'acide acétylsalicylique (ASA) ou de metformine ou de mésalamine ou d'acétaminophène ont été préparés comme traceurs modèles par compression

directe de l'agent actif avec les différents dérivés ampholytiques de l'amidon. Les tests de dissolution ont été effectués dans du fluide gastrique simulé (SGF) et dans du fluide intestinal simulé (SIF). Les résultats ont montré que l'ordre d'addition des réactifs peut affecter les propriétés du polymère-excipient. Par exemple, CM-AE-St (OS) peut contrôler la libération de médicaments principalement dans le SIF, alors que l'AE-CM-St (TS) a prolongé la libération des médicaments dans la SGF. D'autre part, le CM-AE-St (TS) donne les meilleurs résultats de libération des médicaments dans le SGF et dans le SIF. Lorsque les tests de dissolution *in vitro* ont été effectués durant 2 h en SGF puis suivis par SIF, tous les dérivés ampholytiques d'amidon ont pu contrôler la libération des médicaments acides, amphotères ou neutres pendant environ 24 h.

Dans le contexte d'un intérêt croissant pour les dispositifs médicaux à libération contrôlée et pour les comprimés a contenance élevée en agents actifs fortement hydrosolubles, il y a un besoin de nouveaux excipients capables de garder la taille des comprimes suffisamment petite pour permettre d'être avalés facilement et obtenus avec une méthode de production simple. Les dérivés ampholytiques d'amidon synthétisés en deux étapes CM-AE-St (TS) ont été obtenus en différents degrés de substitution en faisant varier le nombre de groupes CM et AE sur la macromolécule d'amidon. La metformine, un agent antidiabétique largement utilisé (système de classification biopharmaceutique: BCS, classe I) a été sélectionnée comme agent actif modèle et des comprimés monolithiques avec des charges 50% et 60% ont été préparés par compression directe de l'agent actif avec diverses CM-AE-St. Les tests de dissolution des agents actifs *in vitro* ont montré que des degrés de substitution plus élevés avec des groupes CM et AE favorisent la capacité du CM-AE-St ampholytique à contrôler la libération de l'agent actif dans le fluide gastrique simulé et dans le fluide intestinal simulé. Les comprimés basés sur les dérivés ampholytiques CM-AE-St charges avec de la metformine (50% charge) ont été comparés à la forme commerciale Glumetza® (50% de charge). La libération du médicament a été contrôlée pendant 12h présentant un profil de dissolution selon le modèle Higuchi similaire pour les deux formes posologiques. Ces nouveaux dérivés ampholytiques d'amidon offrent, comme excipients, une alternative simple et pratique pour formuler et fabriquer des comprimés contenant des agents bioactifs hautement solubles, en une seule étape de compression directe.

Mots clés: Séparation des macrophages, amidon ampholytique, libération contrôlée, comprimé monolithique, formulation à haute charge.

ABSTRACT

Starch is still considered as one of the most used polymer not only in the food industry, biomedical and pharmaceutical industries, due to its biocompatibility, biodegradability, non-toxicity and abundant sources.

In continuation of our laboratory achievements on starch and starch derivatives as biomaterials, the present doctoral project is proposed to open new windows on the utilization of starch in biomedical and pharmaceutical applications, focuing on novel ampholytic starch derivatives as potential excipients for controlled drug delivery dosage forms.

Gelatinized starch (G-St), acetate starch (Ac-St), carboxymethyl starch (CM-St) and aminoethyl starch (AE-St) were synthesized and evaluated as supports for attachment and recovery of macrophages (RAW 264.7 line). These polymers were first assayed for novel macrophage adhesion materials. The films obtained from starch derivatives presented a certain susceptibility to mild hydrolysis with alpha-amylase, liberating the adhered macrophages. The possible interaction between macrophages and starch films was investigated through cell counting, cell viability and tumor necrosis factor (TNF-α) levels. This novel method allows a fast macrophage separation with excellent yields and high viability of recovered cells, especially on insert devices coated with G-St and on Ac-St. The viability of macrophage cells obtained with the presented method was higher than recorded with current methods of cells detachment: scrapping or vortex.

The Ampholytic starch represents a novel class of excipients based on the macromolecular starch backbone carrying anionic (i.e. carboxymethyl) and cationic (i.e. aminoethyl) functional groups. The ampholytic carboxymethyl aminoethyl starch (CM-AE-St) excipients were prepared by two different approaches. For One step (OS) method, where the two reactants (sodium monochloroacetate [SMCA] and chloroethylamine hydrochloride [CEAHC]) were added simultaneously. In the two

steps (TS) method either carboxymethyl starch (CM-St) or aminoethyl starch (AE-St) was prepared separately in the first step, then followed by an interaction with the second reactant: CEAHC or SMCA, respectively. The obtained polymers have been characterized by Fourier transform infrared (FT-IR) spectroscopy, Proton nuclear magnetic resonance (^{1}H NMR), Scanning electron microscopy (SEM), Thermogravimetric analysis (TGA), X-ray diffraction and Zeta potential (ζ). Monolithic tablets with 60% loading of Acetylsalicylic acid (ASA) or Metformin or Mesalamine or Acetaminophen as model drugs were prepared by direct compression of the active molecule with various ampholytic starch derivatives. The dissolution studies were done in simulated gastric fluid (SGF) and in simulated intestinal fluid (SIF). The result showed that the order of addition of reagents affects the properties of the polymeric excipients. For instance, CM-AE-St (OS) was able to control the release of medication mainly in SIF, whereas AE-CM-St (TS) prolonged the release of medication in SGF. On the other hand, CM-AE-St (TS) gives the best drug release behaviour in both SGF and in SIF. When the *in vitro* dissolution tests were carried out first for 2 h in SGF and followed by SIF, all ampholytic starch derivatives were able to modulate the release of acidic (i.e. aspirin), amphoteric (i.e. mesalamine), and neutral (i.e. acetaminophen) drugs for about 24 h.

In the context of a growing interest for sustained drug release devices and for high loaded dosage forms with medications having high water solubility (i.e. metformin), there is a need for new excipients able to keep the tablet size small enough to allow it to be easily swallowed alongside with a simple method of tablet production. The ampholytic starch derivatives CM-AE-St were obtained at different degrees of substitution by varying the number of CM and AE groups on starch macromolecule. Metformin, a largely used anti-diabetes drug (biopharmaceutical classification system: BCS, class I) was selected as a model drug and monolithic tablets with 50% and 60% loading prepared by direct compression of the active molecule with various CM-AE-St derivatives. The *in vitro* drug dissolution tests showed that higher degrees

of substitution for both CM- and AE- groups favor the ability of ampholytic CM-AE-St to control drug release in simulated gastric fluid and in simulated intestinal fluid. Tablets based on CM-AE-St ampholytic derivative were compared to the commercial Glumetza® (50% loading). The drug release was controlled for 12 h and the two dosage forms exhibited similar Higuchi's model dissolution profiles. These new ampholytic starch derivatives offer a simple and convenient alternative to formulate and manufacture highly soluble drugs in a single step process.

Keywords: Macrophage separation, ampholytic starch, controlled release, monolithic tablets, high loading formulations.

PART I

CHAPTER I

STARCH DERIVATIVES AS SELF-ASSEMBLED BIOMATERIALS FOR PHARMACEUTICAL AND BIOMEDICAL APPLICATIONS

Self-assembling can be defined as the capacity of certain molecules, macromolecules, or composite materials to associate themselves and to form complexes or networks or even other structures with novel properties. Self-assembling has been studied for the last few decades due to the ability of these polymers to offer a rich variety of morphologies as well as their potential applications in many fields, such as biomedical applications, micro-electronics, photoelectric and optical processes (Lehn, 1990; Lehn, 1993; Ikkala and ten Brinke, 2002; Janata and Josowicz, 2003; Li *et al.*, 2014). Self-assembly is scientifically interesting and technologically important for at several reasons:

a) Self-assembling is centrally important in life. Cells contain an astonishing range of complex structures such as lipid membranes, folded proteins, protein aggregates, structured nucleic acids, molecular machines, and several others self-assembled materials (Vella, 1994).
b) Self-assembly provides routes to a range of materials with regular structures for example: molecular crystals (Schwiebert *et al.*, 1996), liquid crystals (Schmidt-Mende *et al.*, 2001), and semi-crystalline and phase-separated polymers (De Rosa *et al.*, 2000).
c) Self-assembly also occurs widely in systems of super-molecular components, and there is great potential for their use in biomedical and material science (Whitesides, 1995).
d) Self-assembly seems to offer one of the most general strategies available for generating nanostructures.

The self-assembling process may occur:

1. By association (with themselves and/or with different structures) via various types of interactions (hydrogen associations, Van der Waals forces, hydrophobic stabilization, ionic interactions, click noncovalent recognition) (Mateescu *et al.*, 2015a)
2. By inclusion/complexation (structure A which includes structure B), such as inclusion complexes of starch (clathrates), like iodine blue inclusion complexes of starch (known since the early 1930s) or of cyclodextrins (Loftsson and Duchêne, 2007) and the recently studied Rotaxanes with various oligomers (a Rotaxane is a mechanically interlocked molecular architecture consisting of a dumbbell shaped molecule which is threaded through a macrocycle) (Ariga *et al.*, 2008; Harada *et al.*, 2009; Xue *et al.*, 2015).

1.1. Starch and starch derivatives for use as pharmaceutical excipients

1.1.1. Gelatinized starch and cross-linked starch as pharmaceutical excipients

Excipients are important components of conventional and novel drug products, providing specific functions in the formulation of pharmaceutical dosage forms. Starch in its native or modified forms has been widely used as a pharmaceutical excipient (Sasaki *et al.*, 2000; Massicotte *et al.*, 2008). The modifications of starch could be either physical (i.e., heating, pressure) or chemical (cross-linked or functionalized with nonpolar, polar or charged groups). The obtained polymer after modification is aimed to have a specific application. Depending on respiratory, gastric, intestinal, or colonic delivery site, starch will need specific functionalities for the targeted delivery (Mateescu *et al.*, 2015b).

Gelatinization is the simplest physical modification occurring in native starch. Also modified starch that was directly derivatized from native starch can be gelatinized. Thus, modified starches can be destabilized/stabilized via self-assembling phenomena (Mateescu *et al.*, 2015b). Interaction of amylose and amylopectin with water will generate different modifications in native, gelatinized, and retrograded phases (Fig

1.1). In the dry state, the helical arrangements involve chain–chain stabilization by self-assembling via hydroxyl groups and the degree of organization and compaction can be very high. By interaction with water and by heating, a destructuring / disassembling of starch grain occurs and amylose and amylopectin chains gain more freedom. The phenomenon, known as gelatinization, has been studied since the early 1950s by various groups, and applications are numerous, in food, paper, and textiles industries. During the past three decades, more relevant applications have been described in drug delivery systems (Biliaderis, 1991). Progressive swelling and starch grain breakage become possible via multiple interactions between -OH groups existing on both sides (glucopyranosic residues and water), resulting in the disentanglement of the initial B-type pattern of amylose by alteration of hydrogen bonding. During gelatinization water acts as a plasticizer and is first absorbed in the amorphous space of starch. In fact, three main processes occur in the starch granule: granule swelling, crystal or double-helical melting, and amylose leaching. Penetration of water increases randomness in the starch granule structure and decreases the number and size of crystalline regions. Stress caused by this swelling phenomenon interrupts structure organization and allows for leaching of amylose macromolecules to surrounding water. By heating, such regions become more diffuse and the chains begin to disassemble and separate into amorphous forms. The gelatinization temperature of starch depends on the source, amount of water, pH, types and concentration of salt, and the degree of the amylopectin branching. Once the heating is stopped, the random coiled amylose will be stabilized and spontaneous associations of amylose chains will occur, generating a self-assembled gel (gelification). This phenomenon (gelification) consists of structural modifications occurring during heating and cooling of starch. It can be followed by differential scanning calorimetry (DSC) and FT-IR (Bernazzani *et al.*, 2001), and by carbon-13 nuclear magnetic resonance (^{13}C NMR) spectroscopy (Le Bail *et al.*, 1999).

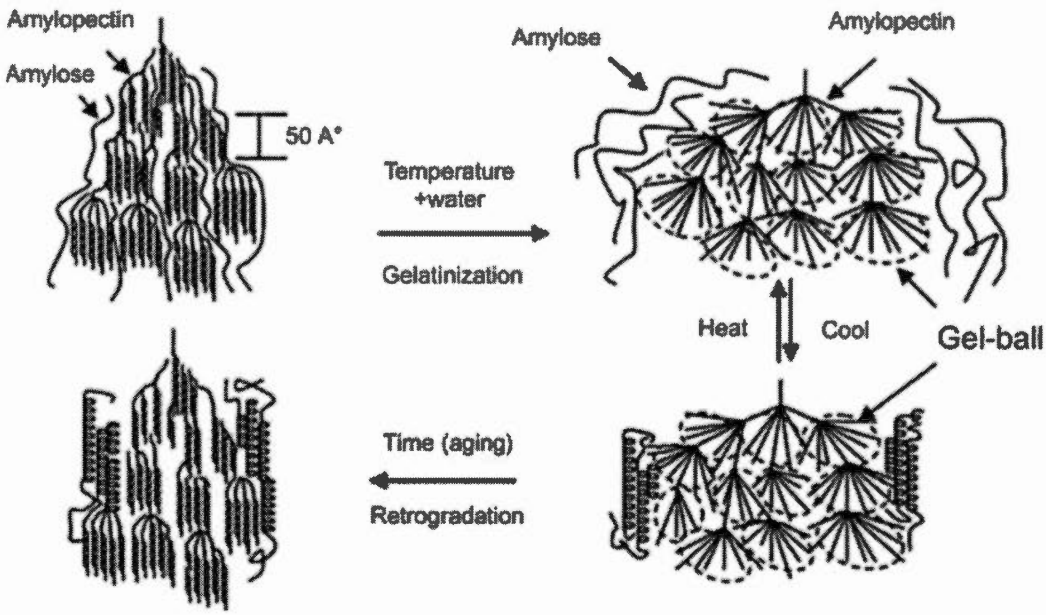


Fig 1.1 Amylose and amylopectin in dry phase and after interaction with water [from(Liu *et al.*, 2009)].

Cross-linking is a common approach to improve the performance of starch for various applications. It was first introduced in the middle of last century. It can be done with ionic or covalent cross-linkers and was applied in the past to obtain food with specific textures and for stabilization of starch gels (Fig 1.2). There are chemical and physical cross-linking methods (Hennink and Van Nostrum, 2002; Xiao, 2013).

Chemical cross-linking consists of reactions between polymer chains with suitable bi-functional or multifunctional reagents. The cross-linking agents can have either low or high molecular weight (MW). Chemically cross-linked starch hydrogels can be currently obtained by treatment with low MW of bi-functional or multifunctional compounds such as glutaraldehyde, epichlorohydrin, tripolyphosphate, and bicarboxylic or tricarboxylic acid (Lenaerts *et al.*, 1991; Dumoulin *et al.*, 1998; Hirsch and Kokini, 2002; Šimkovic *et al.*, 2004; Seker and Hanna, 2006).

Physical cross-linking of polymers involves non-covalent interactions as hydrogen bonding or, for starch ionic derivatives, electrostatic attraction between chains or groups with opposite charges, with advantages in economical, biocompatibility, and biodegradability aspects (Coviello *et al.*, 2007; Ye *et al.*, 2016).

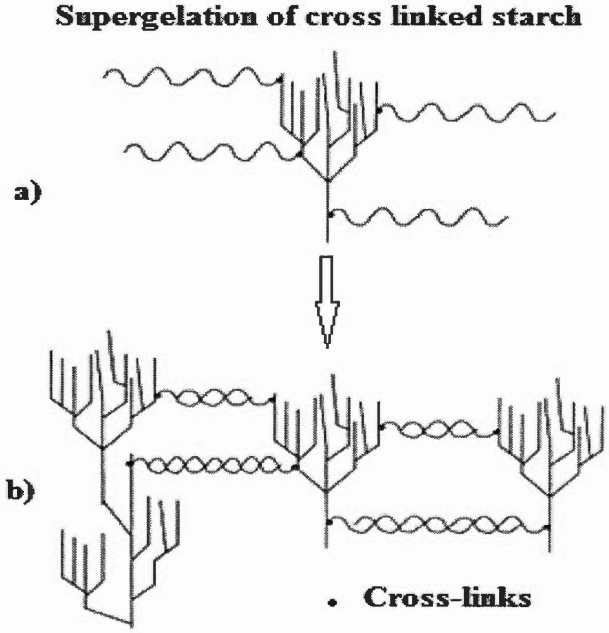


Fig 1.2 Schematic representation of a model for the structural arrangement of high amylose cross-linked starch with a low cross-linking degree in the dry (a) and hydrated (b) state [from (Lenaerts *et al.*, 1998)].

1.1.1.1. Starch as excipients in pharmaceutical formulation

Starch-based excipients have been shown to offer numerous advantages in drug production in terms of lower cost, safety, and product quality. Native starch has traditionally been used for the production of bulk granules, capsules, and tablets. It has multiple uses in different physical forms as a binder (an important excipient to be added in tablet formulation).

Binders or adhesives are the substances that promote cohesiveness. Starch is utilized either in dry form in a direct compression method. Starch powders can be converted into granules through a process known as granulation. This is an operation by which small powdery particles are agglomerated into larger entities called granules (Bos *et al.*, 1987).

Disintegrants: A disintegrant is an excipient added to a drug mix intended to be formulated into oral tablets or capsules having the role to facilitate the breakup of the tablets or hard shell capsules into smaller particles and to promote the rapid release and subsequent absorption of the active drug (Bos *et al.*, 1987; Desai *et al.*, 2014b; Akin-Ajani *et al.*, 2016).

Diluents: A diluent (also called fillers) is excipient added primarily to low dose conventional formulations such as tablets, capsules, and granules to increase weight, facilitate mixing, and improve content uniformity) (Jivraj *et al.*, 2000).

Glidants: Glidants are excipients that reduce inter-particulate friction, resulting in improved flow of granules and powders (Builders and Arhewoh, 2016). Maize starch BP (BP, 2007) has been used as a glidant in tablets and capsules production, other starch types such as cassava (Auletta *et al.*, 1999), yam (Musa *et al.*, 2003), fonio (Muazu *et al.*, 2010) have been investigated as potential glidants in tablets production

Lubricants are excipients added usually in small quantities to powders and granules during tablets and capsules production. There are three functional roles identified with lubricants:

i. decreasing the friction at the interface between a tablet's surface and the die wall during ejection by interposing at intermediate layer between the particle constituents of the tablets and the die wall during ejection,
ii. reduction the wear on punches and dies

iii. prevention of the sticking of the tablets to the face of the punch and of granules, and powder mix sticking to the dosators and tamping pins of capsule filling machines) (Bos *et al.*, 1987).

As a pharmaceutical excipient, starch is employed in different physical forms (Sasaki *et al.*, 2000). When used as a gel in the form of heat swollen aqueous dispersion, it serves as a binder in granules for tablet production. As an un-gelatinized dry, soft powder, starch can be employed as disintegrant, diluent, and glidant in conventional tablets or capsules formulations. It is also used as a carrier and lubricant for body and face powders and as a surface carrier for colors and flavors (Bos *et al.*, 1987; Sanghvi *et al.*, 1993).

Freshly made starch mucilage prepared as a gel by heat treatment of the starch dispersion in water has been extensively used as a binder in tablets and capsules production using the wet granulation technique. The starch mucilage is incorporated as a gel and functions as a glue to provide the necessary binding force that holds the powder particles together in order to form the required agglomerates. When the agglomerates are compressed under optimal load, stable robust tablets are formed (Garr and Bangudu, 1991). Another important attribute of starch when used as binder is its compatibility with other adjuvants and active pharmaceutical ingredients (APIs) used in many conventional and novel formulations. The powder mix often comprises the APIs and other excipients which are granulated to produce uniform distribution of the API. When starch gel is used as a binder, the granules are produced by wet granulation process, usually by massing the powder mix and the starch gel to form a wet mass which is then screened through sieves of appropriate mesh size (1000 - 1700 μm), depending on the range of the granule size desired (Bos *et al.*, 1987). The agglomerates are then dried in hot air at different temperatures depending on the nature of the API or other excipients or desired formulation type (Pilpel *et al.*, 1978; Joneja *et al.*, 1999; Akin-Ajani *et al.*, 2005). The major attributes of wet granulation using native starch as a binder include enhanced powder density and flow,

homogeneity of the ingredients, enhanced compaction, improved drug release, reduced dust during production and desirable tablet, and granule appearance (Joneja *et al.*, 1999). Native starch gel is incorporated at concentrations of 3–20% w/w of the granule or tablet weight. Typical concentration is usually 5–10% w/w, depending on the starch type (Sasaki *et al.*, 2000). The optimal quantity of the gel required to produce robust granules or tablets is predetermined by optimization studies using any or combinations of such parameters as granule flow, tablet friability and hardness, disintegration time, and dissolution rate (Sasaki *et al.*, 2000). When used appropriately at optimal concentrations for granule formulation. Starch will add sufficient cohesion to the powder mix to produce granules of desirable and optimal physico-mechanical properties. The granules can be packed into sachets, capsule shells or can be compressed into tablets. Among the accessible starches, corn starch is the most frequently used in conventional granulation and tablet production technology. However various studies indicate that other starches from non-conventional sources such as breadfruit and cocoyam starches have also shown potential as binders and can serve as a substitute for corn starch (Adebayo and Itiola, 1998).

1.1.1.2. Cross-linked starch as excipients for drug delivery

Beginning in the 1990s, several materials based on epichlorohydrin cross-linked starch have been introduced as excipients for drug controlled delivery (Lenaerts *et al.*, 1991; Dumoulin *et al.*, 1998; Cury *et al.*, 2008). One of these modified starches known as Contramid™ is presently used in several formulations (i.e., once-per-day tramadol tablets commercially available in the United States, Canada, and Europe) or is under development for new modified release products. Contramid™ is a brand name for a cross-linked starch used as excipient for sustained drug release (Cartilier *et al.*, 1996; Dumoulin *et al.*, 1998; Ispas-Szabo *et al.*, 1999). Tablets were prepared by direct compression of the dry mixture of cross-linked starch and drug powders. A nonmonotonous dependency of the release time upon the cross-linking degree was

found. Thus, a slightly increase of the cross-linking degree of amylose generates an extension of drug-release time, with maximal values for degrees of cross-linking (cld) (ranging from 3 up to 6). Cross-linking degree was defined as the amount (g) of cross-linker i.e. epichlorohydrin used to cross-link 100 g of polymer under specific conditions (Ispas-Szabo *et al.*, 1999). A hypothesis has been suggested that moderate cross-linking of amylose signifies a reduced frequency of cross-linking (glycerin bridges introduced by epichlorohydrin reaction) and allows the matrix to be stabilized in proper conformation. At higher degrees of cross-linking, with a higher density of glycerin bridges, the mobility and ability of the amylose chains to form an adequate water gel barrier are limited (Cartilier *et al.*, 1996). The cross-linked starch matrix allows close to linear release kinetics for periods of 24 h or even longer for some types of therapeutic agents. There are various types of drugs with lower aqueous solubility, giving total release times longer than 40 h. However, for oral administration, release times higher than 20–24 h are not worthwhile. A system that can accelerate the release rate was proposed for these drugs. It has previously been demonstrated that amylose gels, chemically modified with epichlorohydrin, can still be recognized as a substrate by α-amylase and can still be biodegradable, depending on the degree of cross-linking (Schell *et al.*, 1981). The structural hypothesis was that the double helices that arise from amylose branches can be better stabilized at a lower cross-linking degree (cld) that allows enough chain flexibility and thus enhances self-assembly by hydrogen association (Fig 1.3). At higher cld (i.e. 20), the hydrogen association is not allowed, due to a higher cross-linking between chains of amylose and amylopectin. This will limit chain flexibility and decrease hydrogen bonding (Ispas-Szabo *et al.*, 1999). Furthermore, this will expose hydroxyl groups acting as a binder in the solid state and as a fast disintegrant in dissolution fluids (Dumoulin *et al.*, 1994). The fast hydration of hydroxyl groups may keep granules insoluble (because of cross-linking), but not the shape of the tablet, which is rapidly disintegrated (Cartilier *et al.*, 1996). These drastically different properties obtained

with the same type of treatment (but a different ratio of reagents) fit well with the concept of multitask excipients (Mateescu *et al.*, 1995; Cartilier *et al.*, 1996).

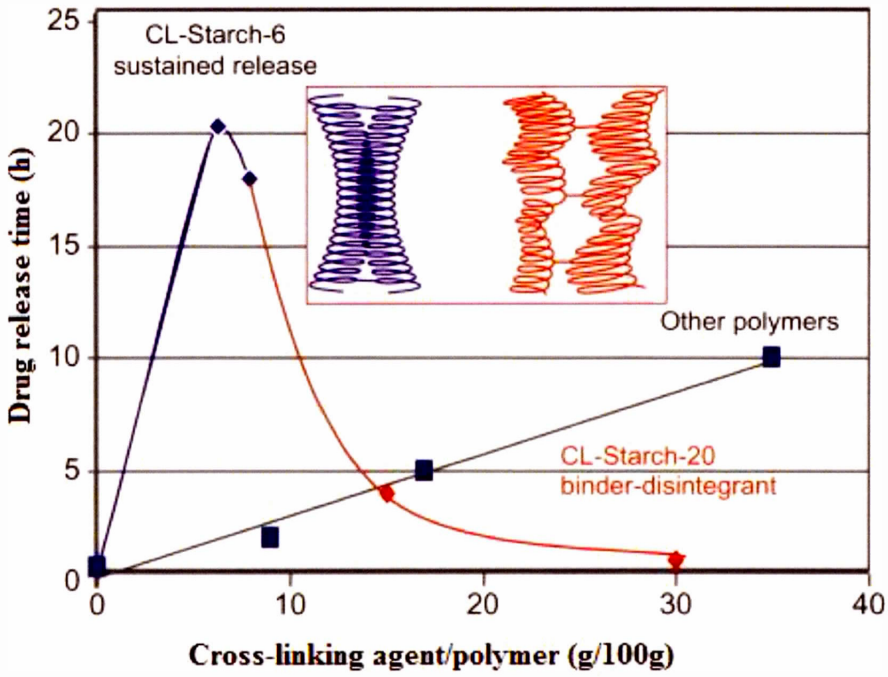


Fig 1.3 Drug release time from monolithic tablets with cross-linked starch and hypothetical assembling at various cross-linking degrees [from (Mateescu *et al.*, 2014a)].

1.1.1.3. Cross-linked starch as binder and disintegrant

Disintegrants are substances requested for some tablet formulations that promote their dispersion and resulting in a faster dissolution of the drug. Cross-linked starch was used as binder and disintegrant excipient with the brand name Liamid® for tablet preparation. It was obtained from high amylose starch by cross-linking at a higher degree (Cartilier *et al.*, 1996). Liamid® (cross-linked starch) was the first simultaneous binder and disintegrant. To the best of our knowledge, this remains a

unique excipient presenting both binder and disintegrant properties. The binding properties of cross-linked amylose are comparable or even superior in some instances, to microcrystalline cellulose (Avicel PH-102™) which is widely used as a binder in solid dosage form preparations. Certain cross-linked amyloses also present excellent disintegration properties, thus preventing the need of an additional excipient having these properties in the tablet (Rolland and Frank, 1971; Cartilier *et al.*, 1996)

1.1.1.4. Cross-linked starch as implant

Cross-linked high amylose starch (CLHAS) has been proposed as a platform for implants. Désévaux *et, al* 2002 prepared an implant for *in vitro* and *in vivo* delivery of ciprofloxacin (CFX). The results showed that CLHAS implants with 1% hydrogenated vegetable oil and 7.5% CFX provided the longest period of drug delivery without any initial burst effect (Désévaux *et al.*, 2002a). The *in vivo* results showed excellent biocompatibility and bioresorption of CFX alongside with the biofunctionality of CLHAS implants. The delivery profile of CFX with 20.0% implant loading appeared to be the best of all formulations tested and was appropriate for the intended use in local antibacterial therapy particularly for treatment of osteomyelitis (Désévaux *et al.*, 2002c). CLHAS matrix implants containing ciprofloxacin have a considerable potential as a parenteral controlled delivery system for local prevention and treatment of bone and soft tissue infections (Désévaux *et al.*, 2002c). CLHAS was also investigated as a solid implant for evaluation of host response in mice and as a possible delivery system for a human growth hormone-releasing factor analog (Hex-hGRF) in pigs (Désévaux *et al.*, 2002b). The results were also encouraged the development of CLHAS implants as a sustained delivery system for peptidic drugs (Désévaux *et al.*, 2002b).

1.1.1.5. Cross-linked starch for separation of macrophages.

Desmangles *et al,* (1992) proposed a new biomedical application of starch derivatives. Cross-linked starch (using epichlorohydrin as cross-linker) was prepared

and used as chromatographic gel support to selectively isolate, retain and detach macrophage cells. The attached macrophages were successfully detached enzymatically from the gel by liquefaction of the cross-linked starch beads by α-amylase solution. The results showed that cross-linked starch was compatible to macrophage cells and that 90% of cell population was attached to cross-linked starch with viability of the recovered cells higher than 90% (Desmangles *et al.*, 1992).

1.2. Carboxymethyl starch as a multifunctional excipient

Starch modifications mean that structural alterations at the molecular level will affect the assembling capacities (i.e., hydrogen bonding) in a controllable manner and thus will allow its applications to expand. Starch modification could be achieved through physical treatment (using heat or moisture), decomposition (acid or enzymatic hydrolysis), and derivatization such as cross-linking, etherification, esterification, or grafting of starch backbone. Chemical modifications of functional groups in the starch molecule result in marked alteration of physicochemical properties, such as gelatinization and swelling behavior. The most used derivatization procedures involve one, two or all three hydroxyl groups existing on each anhydroglucose unit (Volkert *et al.*, 2004; Heinze and Koschella, 2005). The reactivity decreases in the following order C2>C6>C3 (Massicotte *et al*, 2008) (Fig 1.4).

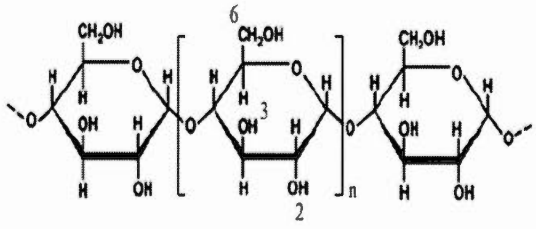


Fig 1.4 Starch structure with hydroxyl groups numbered.

The degree of substitution (DS) was defined as the average number of substituents per anhydroglucose unit; therefore, its values can be between 0 and 3, irrespective of

the nature of the newly introduced groups. Starch derivatives could be classified into two classes: nonionic derivatives and ionic compounds. In the nonionic category, starch solubility was increased by substitution with hydroxyethyl or hydroxypropyl substituents. It becomes possible that the presence of the groups introduced on the starch chain decreases the intermolecular hydrogen bonding between adjacent starch chains and new conformations, new interactions, and new stable self-assembly may occur. Hydroxyethyl starch (HES) exhibits higher aqueous solubility than starch and is less degradable by alpha amylase enzyme (Moad, 2011). In the ionic category, carboxymethyl starch was synthesized (Mulhbacher *et al.*, 2001; Heinze *et al.*, 2004; Mulhbacher and Mateescu, 2005; Mateescu *et al.*, 2006; Spychaj *et al.*, 2013). Carboxymethylation is the substitution of part of the hydroxyl groups of glucose units with anionic carboxymethyl (CM) groups, which improve the hydrophilicity of starch (Fig 1.5).

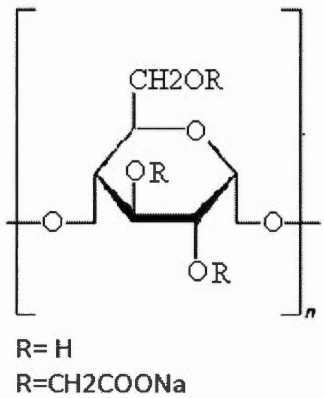


Figure 1.5 Chemical structure of carboxymethyl starch (CM-St).

Furthermore, the carboxyl groups of such excipients respond differently to pH values of gastric acidity, neutral or slightly alkaline pH values of intestinal and colonic media (Mateescu *et al.*, 2015a). They are protonated and more compact in acidic environment and are deprotonated and ionized at neutral pH in the intestinal tract, leading to hydration, erosion, and accelerated release. The preparation of

carboxymethyl starch (CM-St) was described by Chowdhury in 1924 (Sloan *et al.*, 1962). CM-St was prepared by the reaction of starch in an alkaline solution (40% aqueous NaOH) with monochloroacetic acid. The degree of substitution (DS) of carboxymethyl starch is defined as the average number of carboxymethyl groups per glucose unit. Adding bulky functional groups like carboxymethyl or carboxyethyl groups reduces the tendency of the starch to recrystallize and makes the starch less prone to be damaged by heat and bacteria. The functionalization influences the properties of the starch with a variety of pharmaceutical applications as a binder, disintegrant, and controlled release polymer (Emeje and Asha, 2012). CM-St has also been reported to be capable of preventing the detrimental influence of hydrophobic lubricants (such as magnesium stearate) on the disintegration time of tablets or capsules.

1.2.1. Carboxymethyl starch as disintegrant

To improve the efficiency of disintegration, a new class of 'superdisintegrants' was developed. These superdisintegrants include products like sodium starch glycolate which is prepared by cross-linking of sodium carboxymethyl starch. Currently few products are commercialized under different trademarks for example: PRIMOJEL® commercialized by DFE Pharma (Bolhuis *et al.*, 1994), EXPLOTAB®, VIVASTAR® commercialized by JRS Pharma (Bolhuis and Anthony A., 2006; Bolhuis *et al.*, 2009) and GLYCOLYS®, which is produced by Roquette Pharma. Disintegration of orally administered solid dosage forms generally takes place in the gastro-intestinal tract. There is no single mechanism which can explain the disintegration behavior of sodium starch glycolate. The important proposed mechanisms of disintegration include liquid wicking, swelling and deformation recovery. Liquid wicking is considered to be a crucial first step for tablet disintegration. For sodium starch glycolate, a larger rate and extent of water uptake has been observed to have resulted in faster disintegration. The fast swelling is the

most important disintegration mechanism for sodium starch glycolate (Bhandari *et al.*, 2013). Sodium starch glycolate is a hygroscopic agent that absorbs gastric and / or intestinal fluids and rapidly swells.

1.2.2. Carboxymethyl starch as an excipient for highly loaded pharmaceutical dosage forms

A carboxymethyl derivative of cross-linked starch was prepared by Mulhbacher *et al*, 2001. This chemical modification on cross-linked starch improved loading capacity when compared with cross-linked, but not ionically modified, starch (Mulhbacher *et al.*, 2001). The cross-linked starch derivative (i.e., carboxymethyl starch cross-linked (CM-St-CL), aminoethyl AE-St-CL, and acetate Ac-St-CL) tablets afford higher loading (40–60% depending on drug solubility) compared with cross-linked nonderivatized starch (20–30% load) (Mulhbacher and Mateescu, 2005; Mateescu *et al.*, 2006). Swelling and diffusion studies (Mulhbacher *et al.*, 2004) with these derivatives allowed the elucidation of mechanisms involved in drug controlled release - hydrogel hydration and erosion were the main phenomena responsible for the good linearity found in dissolution profiles. Another attempt was to improve the loading capacity by functionalization with both cationic (Aminoethyl AE) and anionic (Carboxymethyl CM) groups on starch chain backbone. The obtained ampholytic polymer was able to prolong the release of highly soluble drugs required at high dose i.e. metformin (Sakeer *et al.*, 2017a).

1.2.3. Carboxymethyl starch as pH responsive excipient

CM-St is a pH-responsive tablet excipient that modulates drug release according to the physiological pH values. An oral dosage form can be readily prepared by dry-mixing and directly compressing drugs and CM-St. The carboxyl groups of starch may dimerize by hydrogen bonds enhancing the stability of tablets. When the CM-St tablets were transferred at a higher pH in simulated intestinal fluid (SIF, pH 6.8),

protons of the matrix started to exchange with cations from the intestinal medium enhancing hydration and facilitating the swelling, diffusion and erosion of the matrix, as well as the release of bioactive agents (Assaad *et al.*, 2011). The CM-St is added to tablets as a pH-responsive excipient to protect bioactive agents from being destroyed by strong acidic gastric fluids (Calinescu *et al.*, 2007).

1.2.4. Carboxymethyl starch as pH-independent excipient

A novel pH-independent matrix forming agent based on CM-St was proposed by Le *et al*, in 2017. Calcium carboxymethyl-starch (CaCM-St) complex was prepared starting from sodium carboxymethyl-starch (NaCM-St). This novel excipient possesses a high capacity to uptake biological fluids (gastric and intestinal), hydrating the matrix while maintaining the tablet integrity (due to calcium complexation). Furthermore, this complexation with Ca^{2+} ions allows (i) the removal of the sodium mobile counterions of the obtained NaCM-St excipient and (ii) the reduction of the available of carboxyl groups to protonation during the passage through gastric fluid (pH <2.0) (Le Tien and Mateescu, 2017). The advantage of this system is that it would lessen the variability of drug release kinetics among different subjects. It may also prevent interactions of the matrix with the certain API such as the mesalamine, which was found to strongly interact with CM-St and making thus difficult its release (Friciu *et al.*, 2013).

1.2.5. Carboxymethyl starch microsphere for gastro-retentive formulation

Microspheres, as an oral multi-particulate drug delivery system, tend to be dispersed in regions of the gastrointestinal tract (GIT) ensuring a more reliable and reproducible release profile and thus more uniform drug absorption (Asghar and Chandran, 2006). Cross-linked starch due to its cost-effectiveness, large availability, biocompatibility, biodegradability, non-immunogenicity and mucoadhesive nature (Demirgöz *et al.*, 2000) was considered as an excipient for mucoadhesive microsphere drug delivery systems (Ahuja *et al.*, 1997; Dodou *et al.*, 2005). Cross-

linked starch microspheres were successfully used for nasal or pulmonary drug delivery (Pereswetoff-Morath, 1998; Chaudhari *et al.*, 2010) but their high hydration and swelling in GIT fluids had limited their use for oral drug delivery due to poor mucoadhesivity. Mulhbacher et al. (2006) have shown that mucoadhesivity of cross-linked starch at neutral pH can be improved by grafting small amount of carboxymethyl groups to starch chains. Lemieux *et al.* (2015), prepared carboxymethyl starch mucoadhesive microspheres (CMS-MS) in gastroretentive dosage form. The results of drug release and permeability alongside with mucoadhesive properties of the CMS-MS suggested that CMS with DS between 0.6 and 1.0 could be a suitable excipient for gastroretentive oral delivery dosage forms.

CHAPTER II

COMPLEXES OF STARCH AND CHITOSAN

2.1. Introduction

Recently, a considerable attention has been given to chitosan because of its unique properties and abundant sourcing.

Chitosan (Fig 2.1) is derived from chitin, which is the second most abundant polysaccharide on earth next to cellulose and is available from waste products in the shellfish industry (Wong *et al.*, 1992). Chitosan possesses repeating units of β 1- 4, 2-deoxy-2-aminoglucose. The amino groups can be protonated to NH_3^+ and readily form electrostatic interactions with anionic groups in an acid environment. Different factors can affect the properties of chitosan such as molecular weight (Park *et al.*, 2002), and the degree of deacetylation (Wiles *et al.*, 2000).

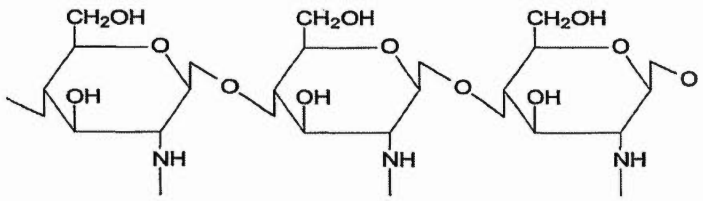


Figure 2.1 Chemical structure of chitosan.

Starch is widely used in food, pharmaceutical and biomedical applications due to its biocompatibility, biodegradability, non-toxicity and abundant sources (Calinescu *et al.*, 2005; Calinescu *et al.*, 2007; Rowe *et al.*, 2009; Sakeer *et al.*, 2017b)

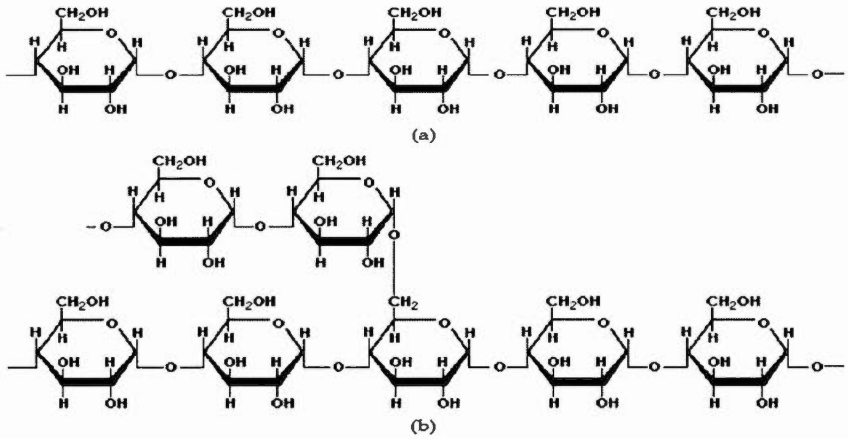


Figure 2.2 Chemical structure of starch presenting (a) amylose and (b) amylopectin.

Starch is a carbohydrate consisting of two different structures, amylose and amylopectin. Amylose is a relatively long, linear α-glucan containing around 99% α-1, 4 - linkages and around 1% α- 1, 6 - linkages, while amylopectin has a highly branched structure containing about 95% α- 1, 4 - linkages and about 5% α- 1, 6 - linkages (Xie *et al.*, 2013; Chen *et al.*, 2015).

2.2. Unmodified starch – chitosan complexes

2.2.1. Unmodified starch – chitosan films

Different attempts were performed to prepare biodegradable blend films from starch and chitosan (Xu *et al.*, 2005c; Bourtoom and Chinnan, 2008; Mathew and Abraham, 2008; Feng *et al.*, 2013; Mei *et al.*, 2013a; Mei *et al.*, 2013b; Aquino *et al.*, 2015; Mei *et al.*, 2015; Ren *et al.*, 2017; Talón *et al.*, 2017). The obtained films were suggested for different application, for example as a carrier for essential oils (Mathew *et al.*, 2006; Pelissari *et al.*, 2009; Avila-S *et al.*, 2010; Avila-Sosa *et al.*, 2012; Mehdizadeh *et al.*, 2012; Shaaban and Mahmoud, 2014; Mei *et al.*, 2015) or for drug delivery systems (Subramanian *et al.*, 2014; Huo *et al.*, 2016). Xu et al (2005b) prepared

composite films starting from unmodified starch and chitosan. The mechanical properties, water resistance, and compatibility of the obtained films were evaluated (Xu *et al.*, 2005c). The results indicated that the composite films had increasing tensile strengths and elongation-at-breaks, and decreasing water vapor transmission rates with increasing starch to chitosan ratios. The crystalline structure of chitosan was depressed with the addition of the gelatinized starch and a broad amorphous peak appeared. The amino group peak in the IR spectrum of chitosan molecule shifted from 1578 to 1584 cm^{-1} with the incorporation of starch. In conclusion, the results suggested that these two film-forming components were compatible and an interaction existed between them (Xu *et al.*, 2005c). Shaaban *et al,* (2014) used a composite film prepared from chitosan and starch incorporated with Thymus vulgaris essential oil, and evaluated their antibacterial, antioxidant and optical properties. The results revealed that the antibacterial and antioxidant properties increased significantly with the incorporation of essential oil (EO). On incorporating EO, there was an increase in the total colour differences, yellowness index and whiteness index, which were significantly higher than those of the control, and the transparency was reduced. Also, the results showed that chitosan edible films incorporated with Thymus vulgaris essential oil could be used as active films because of their excellent antibacterial and antioxidant activities (Shaaban and Mahmoud, 2014). Fig. 2.1 shows the cross-section scanning electron microscope (SEM) micrographs of the films, where remarkable differences can be observed. Chitosan films presented a heterogeneously-fractured surface, probably due to the presence of more ordered and crystalline regions. On the other hand, although starch films exhibited a smoother appearance, the presence of crystalline regions at the top and bottom of the films can also be observed. The starch-chitosan blend presented a more homogenous, smoother structure, indicating a good compatibility between both polymers (Talón *et al.*, 2017). In pure chitosan films, polyphenols (Tannic acid, [TA] or Thyme extract, [TE]) led to a more dense structure with a more regular packaging of polymer chains, which could be related to the interactions between chitosan and

the polyphenols. In the blended films, the microstructure was only modified when using TA and the film structure became less smooth, exhibiting areas with more brittle fracture in agreement with the cross-linking action. The incorporation of TE into starch or chitosan-starch films was observed to have no notable effect on the film microstructure (Talón *et al.*, 2017). The starch – chitosan composite was suggested as a promising alternative carrier for Vero cell culture (Lin *et al.*, 2016). Lin *et al*, prepared carbohydrate – chitosan composites including glucose – chitosan, sucrose – chitosan and starch – chitosan with varied carbohydrate concentrations as carrier for Vero cell culture (are a lineage of cells used in cell cultures). The results revealed that 30 % starch – chitosan composite were the best carriers for the growth of Vero cells (Lin *et al.*, 2016). The initial number of attached cells on the surface of composite carriers did not have any significant effect on subsequent cell production. Vero cells on the starch – chitosan composite were able to convert starch inside the composite carriers into glucose and further utilized the glucose for their growth. Moreover, by crosslinking with serum, the starch – chitosan composite supported a better cell production in the normal medium without adding fetal bovine serum, as well as a good extracellular virus production. Chitosan - starch mixture was used also to prepare a support for drug delivery (Subramanian *et al.*, 2014; Huo *et al.*, 2016). Huo et al 2016, prepared a film consisting of chitosan-microcapsules/starch blend for antofloxacin (a fluoroquinolone antibacterial with activity against Gram-positive and Gram-negative bacteria) controlled release. The results indicated that the release of drug from the prepared film was pH-sensitive (Huo *et al.*, 2016).

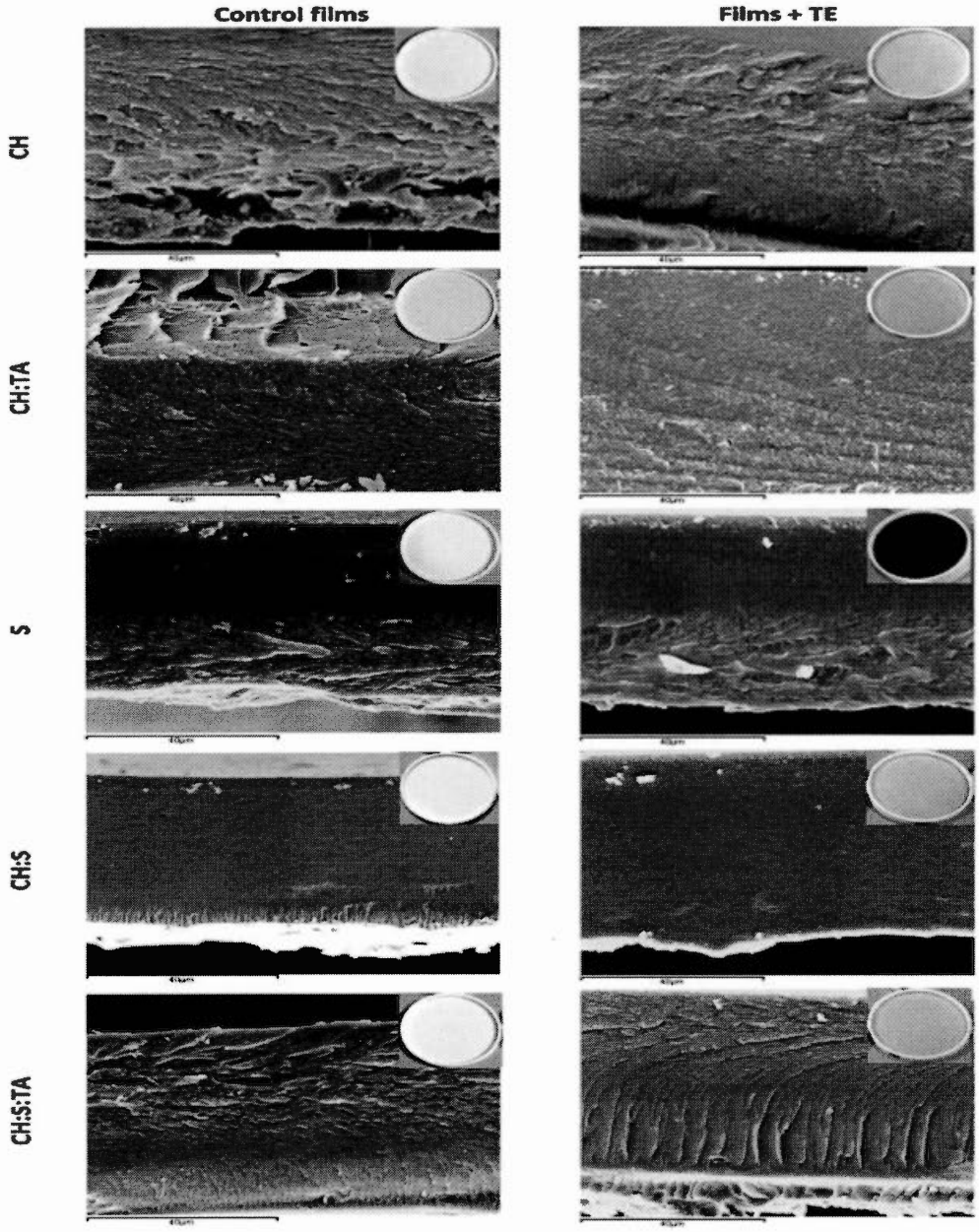


Figure 2.3 SEM micrographs of the cross-sections of the films containing or not thyme extract (TE) (magnification 1500×) and inserts are camera pictures of chitosan (CH), chitosan : tannic acid (CH :TA), starch (S), chitosan : starch (CH :S), chitosan : starch : tannic acid (CH :S :TA) [from(Talón *et al.*, 2017)].

2.2.2. Unmodified starch – chitosan tablet

Unmodified starch – chitosan powders were investigated as an excipient for solid dosage forms. Combination of chitosan and starch powders or chitosan and lactose powders were prepared, characterized and compared with a mixture of chitosan and crystalline cellulose powder (Sawayanagi *et al.*, 1982). The fluidity of combined powder consisting of chitosan and starch was greater than that of chitosan with crystalline cellulose powder. The hardest tablet was obtained with combined chitosan and starch, followed by chitosan with crystalline cellulose. Tablets containing less than 70% of chitosan passed the disintegration test. The results revealed that chitosan could be used with starch as diluent / filler for tablet preparation in direct compression method (Sawayanagi *et al.*, 1982).

2.2.3. Unmodified starch – chitosan scaffolds

Potato, corn, and sweet potato starches and chitosan, as well as blends of these, were used to produce porous structures for tissue engineering scaffolds (Nakamatsu *et al.*, 2006). The techniques used to produce the porous structures included a solvent-exchange phase separation technique and the well-established thermally induced phase separation method. The results showed a wide range of pore structures having a size ranged from 1 to 400 μm. On the other hand, the pore morphology in starch scaffolds was affected by the initial freezing temperature / freezing rate, whereas in chitosan scaffolds, the shape and size of pores were determined by the implemented processing route. The mechanical properties of the scaffolds were assessed by indentation tests. The results revealed that the indentation collapse strength depends on the pore geometry and the material type (Nakamatsu *et al.*, 2006).

2.3. Modified starch – chitosan complexes

2.3.1. Cross-linked starch – chitosan microspheres

Blending of chitosan with other polymers such as starch and crosslinking them are both convenient and effective methods to improve the physical and chemical properties of chitosan for practical applications (Baran *et al.*, 2004; Okolo *et al.*, 2013; Perez and Francois, 2016). Beads consisting of starch and chitosan have been prepared after cross-linking with glutaraldehyde or sodium trimetaphosphate. These beads were utilized as a carrier for metformin hydrochloride drug (N,N-dimethylimidodicarbonimidic diamide hydrochloride) as a model, drug of choice in the treatment of type 2 diabetes, particularly in overweight and obese people. The results indicated that the obtained beads were able to slow the release of metformin over 10 h (Kumari and Rani, 2011; Okolo *et al.*, 2013). Fig 2.2 shows the morphology of dried beads prepared with chitosan or polymeric blends with different crosslinking times. The bead diameters, regardless of crosslinking time, were 3.70 ± 0.03 mm for chitosan beads and 3.00 ± 0.05 mm for matrices prepared with blends (Perez and Francois, 2016). Other general properties revealed that none of the analyzed macrospheres were completely spherical in shape and also crosslinking time does not significantly affect the final size (Perez and Francois, 2016). According to the experimental procedure, the main factor that could affect the size of the beads is the diameter of the plastic tip used in the dripping technique. The micrographs of the surfaces of the cross-sectioned beads showed many cavities of different sizes. It could be due to the consequence of occluded air bubbles incorporated during the preparation of the polymeric material used to manufacture the beads. The observed inner structure could be useful in the preparation of a controlled-release fertilizer because it could enhance the loading of the fertilizer inside the polymeric matrix (Perez and Francois, 2016).

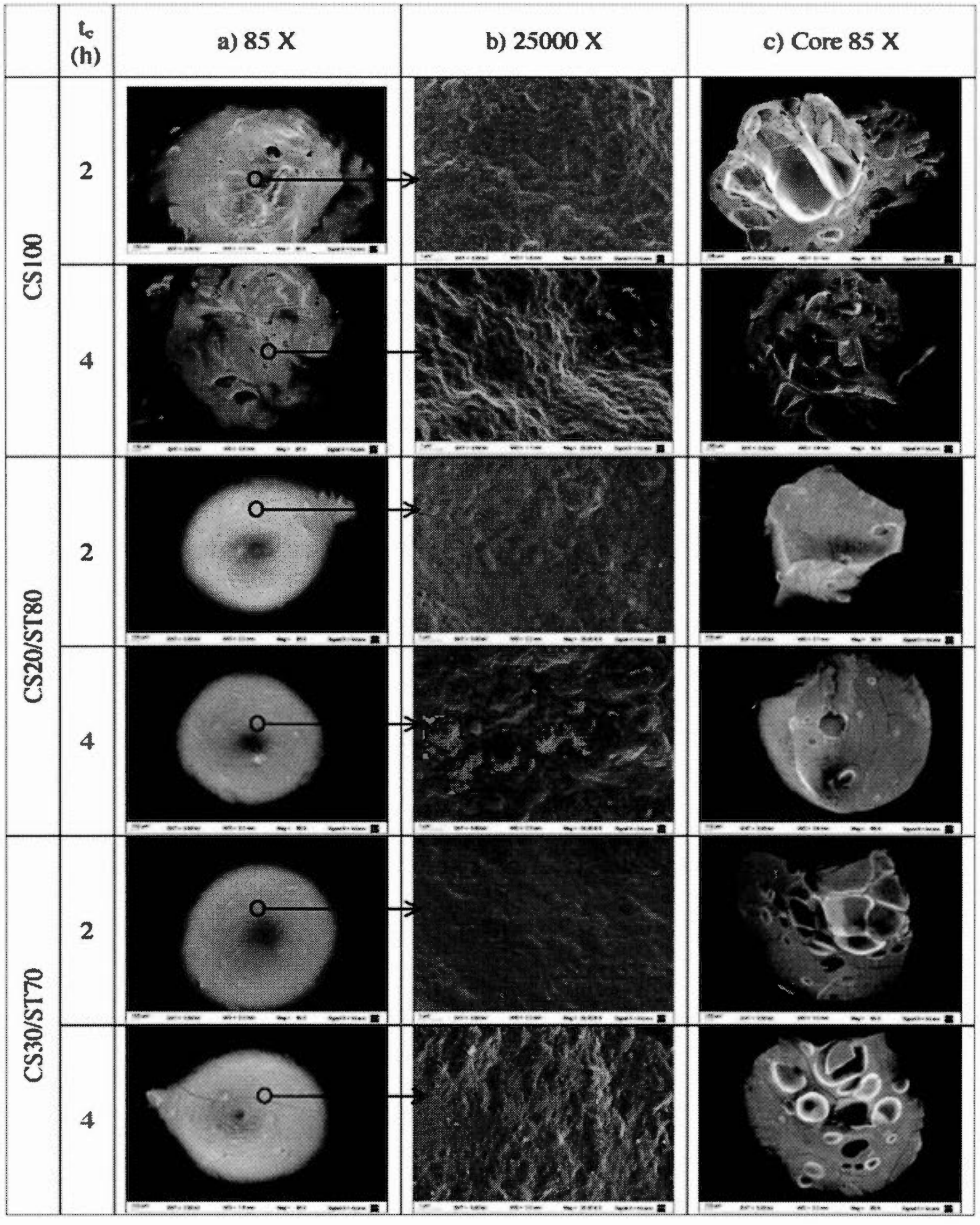


Figure 2.4 Scanning electron micrographs of beads prepared with 2 h or 4 h of crosslinking time: (a) the entire bead, (b) the external surface and (c) a cross-section of pure chitosan (CS 100), chitosan 20% : starch 80% (CS20/ST80) and chitosan 30% : starch 70% (CS30/ST70) [from(Perez and Francois, 2016)].

2.3.2. Carboxymethyl starch – chitosan physical mixture

Starch modification is generally achieved through derivatization such as cross-linking (Lenaerts *et al.*, 1998), etherification, esterification (Mulhbacher *et al.*, 2001; Calinescu *et al.*, 2005; Sakeer *et al.*, 2017b) and grafting (Kaur *et al.*, 2007) of functional groups onto the carbohydrate structure. Carboxymethylation of starch is an etherification achieved by the reaction of hydroxyl groups in starch with sodium monochloroacetate (SMCA) generating carboxymethyl starch (CM-St) which was used as a matrix forming agent for drug delivery either to protect the active pharmaceutical ingredient (API) from gastric medium (Calinescu *et al.*, 2005; Calinescu *et al.*, 2007), as controlled release excipients (Lemieux *et al.*, 2009) or as excipient for chronodelivery medication (Ispas-Szabo *et al.*, 2016). Several attempts have been made to modulate the properties of obtained matrices containing CM-St in simulated intestinal fluid (SIF) by reducing its solubility, since CM-St contains carboxyl groups which can be deprotonated, ionized, and then promote matrix erosion and solubilization. To this end, an approach was proposed by mixing of CM-St with polymers containing insoluble amine groups (chitosan), which could help as a stabilizing agent. A physical mixture of CM-St and chitosan powders was prepared by Calinescu *et al*, (2012). The mixture was used to prepare monolithic tablets to protect diamine oxidase (DAO) and/or catalase therapeutic enzymes against SGF and to control their delivery in SIF. The results indicated that the CM-St:Chitosan (1:1) matrix afforded a good gastric protection to DAO and catalase. Variable amounts of DAO were delivered in SIF containing pancreatin, with maximal release reached at about 8 h, a time convenient for tablets to attain the colon. Up to 50% of the initial enzymatic activity of catalase formulated with CM-St:Chitosan was found after 8 h in SIF (Calinescu *et al.*, 2012). Fig 2. 3 (a, b) presents the surface of the entire tablets based on CM-St:Chitosan excipients with 0%, 50% and 100% enzyme loading. The CM-St:Chitosan formulations with plant diamine oxidase (PSDAO) only were less stable at 50% loading and unstable when formulated without CM-St : Chitosan

(100% PSDAO). Thus, the excipient-free formulation, based on 100% PSDAO only, was rapidly disintegrated and dissolved during the incubation in SGF medium. This lower stability of PSDAO tablet could be related to a certain amount of phosphate in the powder extract remaining after the preparation procedures. On the other hand, the presence of hydrophilic phosphate in PSDAO preparation can be an advantage for formulation, ensuring the tablet hydration and the release of loaded enzyme (Calinescu *et al.*, 2012). Differently, the excipient-free tablets, based on 100% catalase, were compact after the SGF incubation, with no tendency of swelling nor disintegration, but exhibiting an orange external surface of the tablets, showing lesser protection in acidic medium. When the tablets were cross-sectioned, the core of the tablets was dry, with no gastric fluid inside the tablets and, consequently, no solubilization of the bromocresol green particles. Furthermore, a peripheral blue layer (pH greater than 5.4) close to the surface of the tablets was found (Fig 2.3 a, b). Thus, the presence of the proteins in the formulations may also contribute to gastro-protection, as found for catalase at loading of 50% and 100%. These aspects are useful and important for further enzyme formulations, showing that not only the CM-St:Chitosan excipients afforded a certain gastro-protection of bioactive agents, but protein itself can generate an outer protective gel layer keeping dry the core of the tablets (Calinescu *et al.*, 2012).

Physical mixture of CM-St : chitosan was also proposed as an excipient for colon delivery of probiotic bacteria (Calinescu and Mateescu, 2008). A combined mechanism of bacteria release was proposed for CM-ST:chitosan monolithic tablets, involving the swelling of the tablets (due to the CM-St), followed by the erosion and dissolution of CM-St. In addition, a gel-forming barrier of Chitosan in acidic conditions was contributed to the delay of the bacteria delivery (Calinescu and Mateescu, 2008).

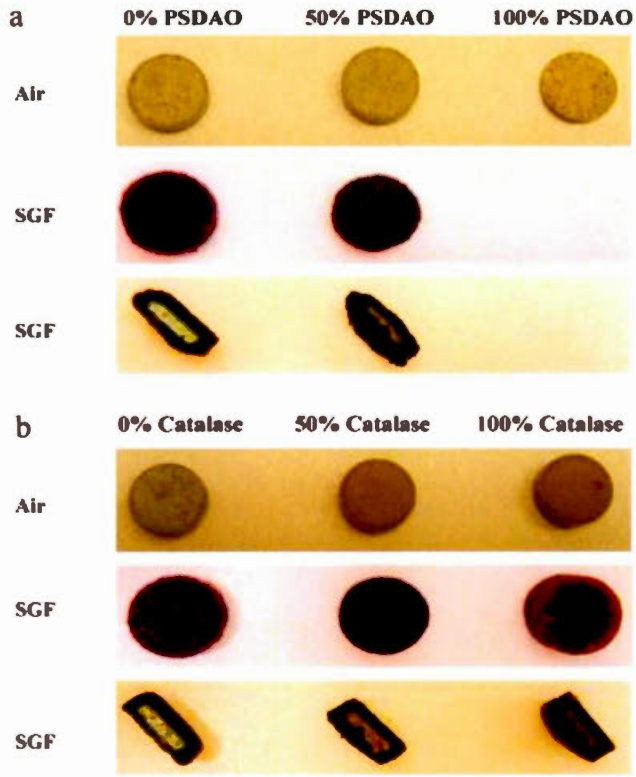


Figure 2. 5 pH stability of enzyme formulations in tablets based on CM-St:Chitosan. Monolithic tablets based on CM-St : Chitosan (1:1) with 0%, 50% enzyme loading and excipient-free 100% (a) plant diamine oxidase (PSDAO) or (b) catalase, containing bromocresol green (pH indicator). Untreated (air) or incubated tablets for 60 min in simulated gastric fluid containing pepsin, at 50 rpm and 37 °C (whole and cross-sections of tablets). The blue color indicates that the CMS:Chitosan matrix forms an outer gel barrier, affording thus a certain protection against gastric acidity, despite the fact that the tablets are not coated with gastro-protective materials. Substitution degree of CM-St: 0.126.

2.3.3. Carboxymethyl starch – chitosan polyelectrolyte complex (PEC)

Polyelectrolyte complex (PEC) refers to a chemical complex of polyelectrolytes. A polyelectrolyte is a polymer whose repeating units, or some of them, bear an electrolyte group. Such groups will dissociate in aqueous solution, making the

polymer charged. These charged polymers are also called polyions, polycations (positively charged polymers), and polyanions (negatively charged polymers) (Assaad and Mateescu, 2012b). A polyelectrolyte complex (PEC) is formed by association of opposite charged polyelectrolytes. More specifically, a polyelectrolyte complex is formed through electrostatic interactions between the positive charges of a polycation (chitosan) and the negative charges of a polyanion (carboxymethyl starch, maleic starch half-ester acid) (Xiao and Fang, 2009; Assaad and Mateescu, 2012b). Hydrogen bonding may also play a role in the formation of the complex. Typically, when a polycation and a polyanion are mixed together in an aqueous solution, a polyelectrolyte complex forms due to the strong interactions between them. These interactions lead to the formation of the complex (in essence a new macromolecular association) where the polyanion and the polycation are bonded together through electrostatic interactions and also possibly by hydrogen bonds (Assaad and Mateescu, 2012b). The CM-St polyanion when mixed with polycation, chitosan, may generate a polyelectrolyte complex that could be easy to assess visually. Indeed, the polyanion is solubilized in a solvent and mixed with the polycation solution yielding a complex. Its formation is often evidenced by a thickening effect, coagulation, gellification and/or PEC precipitation. Thus, starting from two solutions of polyelectrolytes, mixing results in thickening, coagulation, gellification and / or precipitation due to the fact that the formed polyelectrolyte complex is less soluble than the separate polyanion and polycation (Assaad and Mateescu, 2012b). Polyelectrolyte complex formation can be seen as a self-assembly process by which a polysalt is produced. As such, a polyelectrolyte complex is different from a simple mixture of its constituent polyelectrolytes. It is a different chemical entity with different characteristics, such as morphology, density, solubility, and X-ray diffraction (order degree). Assaad *et al*, (2011) prepared PEC of carboxymethyl starch (CM-St) and chitosan. The PEC was characterized and tested *in vitro* as a carrier for oral drug delivery. The results indicated that the PEC which contained 14% (w/w) of chitosan, showed a polymorphism with a lower order degree than those of CM-St and of chitosan alone

(Fig 2. 4.) NMR imaging analysis showed slower fluid diffusion inside PEC monolithic tablets than inside CM-St tablets (Assaad *et al.*, 2011). The PEC could be a more suitable drug carrier for colon targeting than CM-St (Fig 2. 5), since it can prolong acetaminophen release time from 8 h to 11 h and aspirin release time from 13 h to 30 h (Assaad *et al.*, 2011). PEC of CM-St / chitosan was proposed as an excipient for oral administration of ovalbumin (Assaad *et al.*, 2012a). The results showed that when CM-St was used as an excipient, more than 70% of the loaded ovalbumin remained undissolved after 1 h of incubation in SGF with pepsin due to protonation of the polymer in SGF and thus, reducing the liberation of the enzyme from the matrix. The complete dissolution, after transfer of tablets into SIF with pancreatin, occurred within a total time of about 6 h. High protection (more than 90% stability in SGF) and prolonged release (more than 13 h) were obtained with 50% CM-St / 50% chitosan PEC excipients (Assaad *et al.*, 2012a).

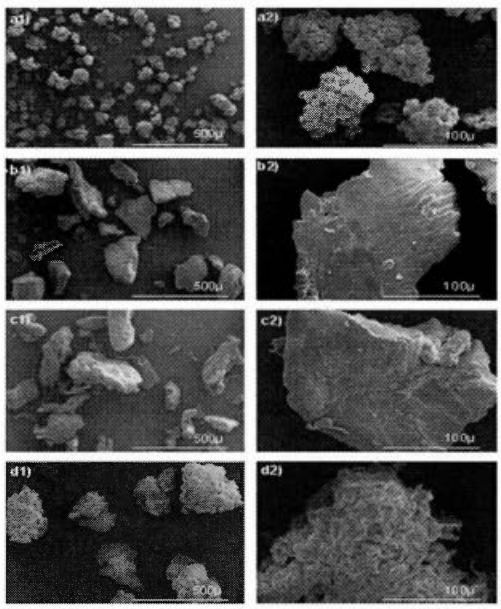


Figure 2. 6 Scanning electron microscopy micrographs of (a) CM-St, (b) chitosan-400, (c) chitosan-700, and (d) PEC at magnifications of 100× and 500× and voltage of 15 kV [from(Assaad *et al.*, 2011)].

Saikia et al (2015) prepared Montmorillonite (MMT) containing magnetic iron oxide nanoparticles coated with polyelectrolyte complex PEC of CM-St and chitosan to prolonge the release of isoniazid (antibiotic used for the treatment of tuberculosis) (Saikia *et al.*, 2015). The result revealed that the coating of PEC showed good stability, biocompatibility and mucoadhesivity of the iron oxide magnetic nanoparticles (Saikia *et al.*, 2015).

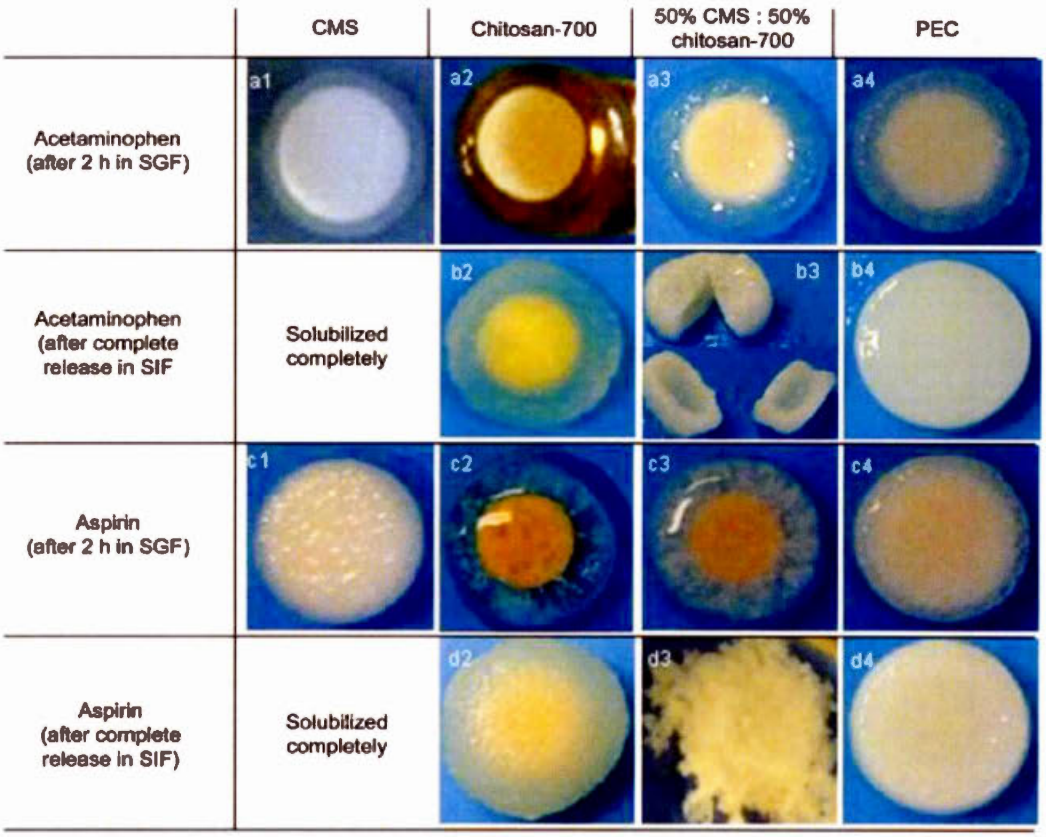


Figure 2. 7 Photographs of CM-St (CMS), chitosan-700, 50% CMS:50% chitosan-700 and PEC tablets (200 mg, 20% loading) during dissolution tests (1 L, 37 °C, 100 rpm). Photographs were taken for the tablets, first after 2 h of incubation in SGF and then after the complete drug (acetaminophen or aspirin) release in SIF. The sizes of tablets were not normalized [from(Assaad *et al.*, 2011)].

2.3.4. Carboxylated starch-chitosan nanoparticles

Chitosan and carboxymethyl starch nanoparticles where prepared and studied as a drug delivery system to the colon. The 5-aminosalicylic acid (anti-inflammatory drug, used to treat inflammatory bowel disease) was chosen as a model drug molecule. The nanoparticles were formulated by a complex coacervation process under mild conditions. The results revealed that the release of 5-aminosalicylic acid from nanoparticle was based on an ion-exchange mechanism (Saboktakin *et al.*, 2011).

2.3.5. Carboxylated starch-chitosan scaffolds

Shahriarpanah et al (2016) proposed new bioactive composite scaffolds from carboxylated starch-chitosan for bone regeneration (Fig 2.6). In order to introduce COOH groups into the scaffolds, chitosan was first dissolved in citric acid and then mixed with different amounts of starch. The results indicated that chitosan scaffolds showed the highest pore size and porosity, while no apatite (crystal consisting of group of phosphate minerals usually hydroxylapatite, fluorapatite and chlorapatite) deposition was observed even after 14 days of soaking in simulated body fluid (Shahriarpanah *et al.*, 2016). For composite samples, the pore size and porosity decreased as the starch content increased. In spite of such decrease, the pore size measurements were in the optimal range for bone regeneration. The bone-like apatite mineralization, compressive strength, carboxyl content, and swelling ratio of the composites increased with additional starch. Cell culture experiments demonstrated that higher starch content can enhance proliferation, alkaline phosphatase assay (ALP, generally considered as an early stage marker of osteoblastic cells, which stimulates hydroxyapatite formation and mineralization) activity, and mineralization of osteoblast-like cells (MG63) (Shahriarpanah *et al.*, 2016).

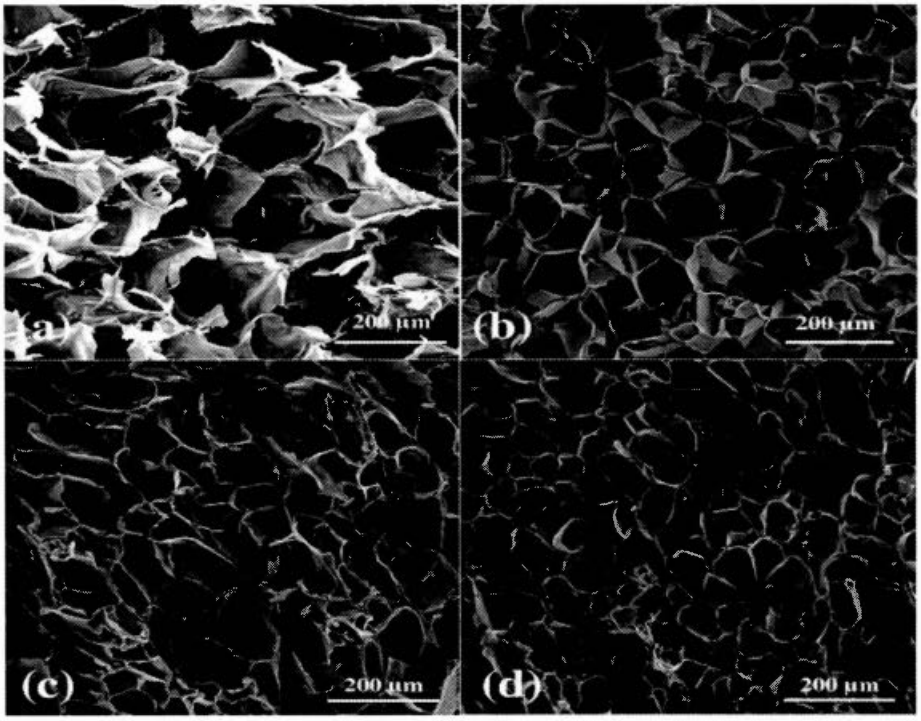
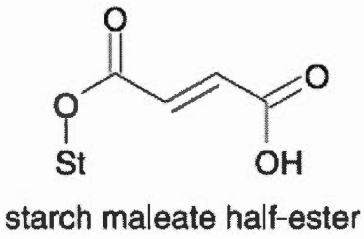


Figure 2.8 The SEM images of composite scaffolds (a) pure chitosan, (b) 70% chitosan and 30% starch, (c) 50% chitosan and 50% starch, and (d) 30% chitosan and 70% starch [from(Shahriarpanah *et al.*, 2016)].

2.3.6. Maleic starch half-ester acid – chitosan polyelectrolyte complex

A polysaccharide based polyelectrolyte complex was formed via ionic self-assembly of a carboxylic derivative of starch, maleic starch half-ester acid (MSA) shown in Fig 2. 7, with chitosan (Xiao and Fang, 2009). FTIR results confirm the formation of a complex between chitosan and MSA. Thermogravimetric analysis (TGA) showed that the thermal resistance of the complex was higher than that of two components and the corresponding blend. X-ray diffraction (XRD) analysis result showed that the complex was amorphous,whereas its components were semi-crystalline. The drug release behavior of the complex that contains 5-fluorouracil was pH-responsive.



starch maleate half-ester

Figure 2. 9 Starch (St) maleate half-ester (MSA) structure.

All the experimental results showed that the complex consisted of MSA and chitosan, and also indicated that the driving force for the self-assembly of the complex was predominantly electrostatic interactions between two oppositely charged polyelectrolytes, cationic chitosan, and the anionic MSA (Xiao and Fang, 2009).

CHAPTER III

MACROPHAGES IN BIOMEDICAL SCIENCE

3.1. Origin and role of macrophages

Monocytes are generated by myeloid progenitor cells that give rise to monoblasts, pro-monocytes and finally to monocytes (Fig 3.1). Under homeostatic conditions, tissue macrophages comprise Langerhans cells in the epidermis, osteoclast in the bone, alveolar macrophages in the lung, red-pulp, white-pulp, marginal-zone and metallophilic macrophages in the spleen, Kupffer cells in the liver and microglial cells in the central nervous system (Gordon and Taylor, 2005). Monocytes circulate in blood for 1–3 days under homeostatic conditions (van Furth and Cohn, 1968; Gombozhapova *et al.*, 2017).

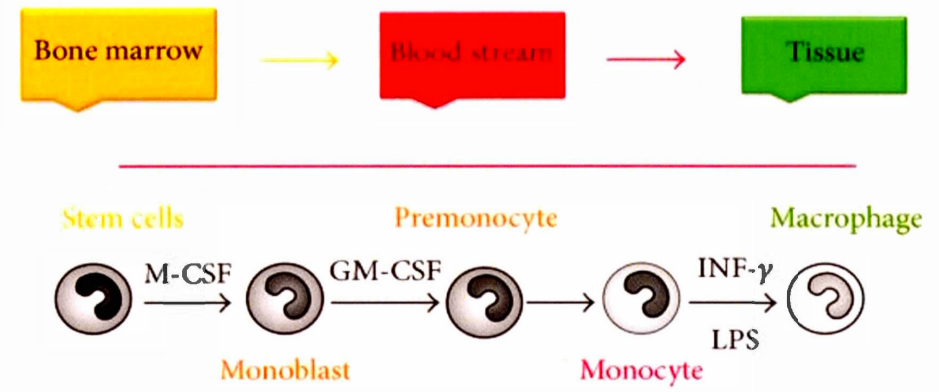


Fig 3.1. Monocyte and macrophage differentiation. Immature monocytes, released from bone marrow, migrate through the bloodstream under the influence of different cytokines and chemokines into tissues (lung, liver, spleen peritoneal cavity, and brain) where they differentiate into resident macrophages under the influence of IFN-γ and LPS [from(Almatroodi *et al.*, 2014)].

The induction of this cell differentiation occurs with the presence of colony-stimulating factors (CSFs) that are secreted by stromal cells in the blood and tissues

(Hamilton, 2008; Sheikh *et al.*, 2015). Granulocyte-macrophages, granulocytes, and macrophage CSFs are thought to be involved in spreading, motility, and cytoskeletal reorganization (Insogna *et al.*, 1997). Signalization by these cytokines allows monocytes to leave the bone marrow and enter the bloodstream where they remain until being signaled to enter tissues by chemo-attractants. With respect to their phenotype and functions, macrophages are very heterogeneous, a characteristic which is determined by signaling molecules and the cellular environment. Stimuli that produce an early macrophage response are typically from innate immune cells. Additionally, macrophages can respond to signals from cells that are antigen-specific (Gordon, 2007). Macrophages are also capable to produce factors that alter their own phenotype through autocrine signaling. Macrophages can be classified as: i) classically activated, ii) wound healing, and iii) regulatory.

i) Classically activated macrophages, also called M1 macrophages, are produced during a cell-mediated immune response (Martinez and Gordon, 2014; Mills *et al.*, 2015). The presence of interferon-γ (INFγ) and tumor necrosis factor (TNF), or lipopolysaccharide (LPS) creates a macrophage population that secretes pro-inflammatory cytokines (Mackaness, 1977; O'Shea and Murray, 2008). Natural killer cells produce INFγ, in response to infections in order to activate macrophages, causing them to secrete their own pro-inflammatory cytokines. This creates a group of macrophages with microbicidal and tumoricidal functions.

ii) Wound healing or reparative macrophages make up a group of cells which, like classically activated macrophages, arise due to innate or adaptive signaling mechanisms. These cells were formerly called alternatively activated macrophages, or M2s. However, the cells are more associated with wound repair than defense (Das *et al.*, 2015).

iii) Regulatory macrophages occur as a result of either an innate or adaptive immune response.

Macrophages display an interesting characteristic known as macrophage plasticity. Upon differentiation into one of the various macrophage subtypes, these cells, are not

terminally differentiated, and can respond to local microenvironment signals (Bertrand *et al.*, 2005; Davies *et al.*, 2013; Ginhoux and Jung, 2014). Many different types of signals exist to promote phenotype switching, and vary from cytokines to the presence of a foreign body.

3.2. Macrophages for biomedical applications

Macrophages are currently investigated in various biotechnological and biomedical fields for certain therapeutic applications. Macrophages with a possible role in inflammatory processes and in malignancy were reported as a new therapeutic target. Nowadays, functions of monocytes/macrophages and their subsets are actively investigated in cancer (Ostuni *et al.*, 2015), infectious, autoimmune, liver (You *et al.*, 2013), kidney and cardiovascular diseases (Kwan *et al.*, 2014).

Consequently, there is a growing interest for techniques of macrophage separation, particularly aimed to investigate anti-macrophage novel strategies against cancer. Whitworth *et al*, (1990) investigated the possible role of macrophages as a carrier for immune-modulator agents. In fact, macrophages can be activated to become tumoricidal by interaction with phospholipid vesicles (liposomes) containing immunomodulators. Tumoricidal macrophages can recognize and destroy neoplastic cells *in vitro* and *in vivo*, leaving nonneoplastic cells uninjured(Whitworth *et al.*, 1990). The exact mechanism(s) by which macrophages discriminate between tumorigenic and normal cells is unknown, but it is independent of tumor cell characteristics such as immunogenicity, metastatic potential, and sensitivity to cytotoxic drugs. Moreover, macrophage destruction of tumor cells apparently is not associated with the development of tumor cell resistance (Fidler, 1985a).

Macrophages are found in association with malignant tumors in a definable pattern, suggesting that the most direct way to achieve macrophage-mediated tumor regression is *in situ* macrophage activation. Intravenously administered liposomes are

cleared from the circulation by phagocytic cells, including macrophages, so when liposomes containing immunomodulators are endocytosed, cytotoxic macrophages are generated in situ. The administration of such liposomes in certain protocols has been shown to bring about eradication of cancer metastases (Kleinerman *et al.*, 1983a; Kleinerman *et al.*, 1985; Fidler *et al.*, 1985b). Macrophage destruction of metastases *in vivo* is significant, provided that the total tumor burden at the start of treatment is minimal. For this reason, various methods have been investigated to achieve maximal cytoreduction in metastases by modalities such as chemotherapy or radiotherapy prior to macrophage-directed therapy (Whitworth *et al.*, 1990).

The ability of tumoricidal macrophages to distinguish neoplastic from bystander nonneoplastic cells presents an attractive possibility for treatment of the few tumor cells which escape destruction by conventional chemotherapy, radiotherapy and surgery treatments. Macrophage-directed therapy has been studied in several human protocols, yielding important biological information about the use of liposome-encapsulated macrophage activators in cancer patients (Fidler and Schroit, 1988).

Han *et al,*(2016) used macrophage recruitment in tumors. They developed active, transportable, cancer theragnostic macrophage-based microrobots as vectors to deliver therapeutic agents to tumor regions. The macrophage-based microrobots contained docetaxel (DTX)-loaded poly-lactic-co-glycolic-acid (PLGA) nanoparticles (NPs) for chemotherapy and Fe_3O_4 magnetic NPs (MNPs) for active targeting using an electromagnetic actuation (EMA) system. The macrophage-based microrobots were obtained through the phagocytosis of the drug NPs and MNPs into the macrophages (Figure 3.1). The anticancer effects of the microrobots on tumor cell lines (CT-26 and 4T1) were evaluated *in vitro* by cytotoxic assay (Han *et al.*, 2016),. In addition, the active tumor targeting by the EMA system and macrophage recruitment, and the chemotherapeutic effect of the microrobots were evaluated using three-dimensional (3D) tumor spheroids. The microrobots exhibited clear cytotoxicity toward tumor cells, with a low survivability rate (<50%). The 3D tumor spheroid

assay showed that the microrobots demonstrated hybrid actuation through active tumor targeting by the EMA system and infiltration into the tumor spheroid by macrophage recruitment, resulting in tumor cell death caused by the delivered antitumor drug. Thus, the active, transportable, macrophage-based theragnostic microrobots can be considered to be biocompatible vectors for cancer therapy (Han *et al.*, 2016).

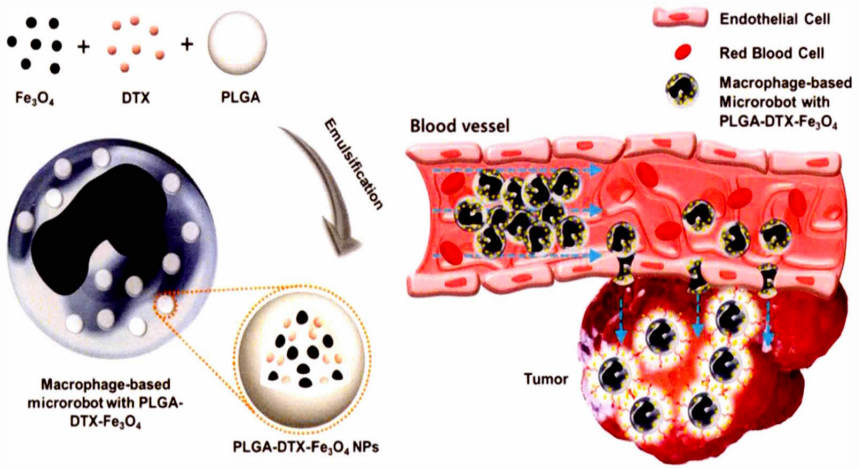


Figure 3.2 Preparation of macrophage-based microrobots [from(Han *et al.*, 2016)] Docetaxel (DTX) and Fe_3O_4 have been encapsulated in poly-lactic-co-glycolic-acid (PLGA) nanoparticles (NPs) that were phagocytized by macrophage cells, vectorized and uptaken by tumor tissue.

3.3. Techniques of macrophage separation

Macrophages can be obtained in a relatively pure form as primary cultures for analytical and biochemical manipulations but they do not generally replicate in culture. Furthermore, because of their relatively short lives, it may be difficult to obtain enough amounts of macrophages for large scale applications. Moreover, they are very sensitive to small changes in their environment and may be damaged considerably, even when delicately handled after cell culture and addition on Petri

dishes (Adams, 1979; Féréol *et al.*, 2006). Detaching adherent macrophages from a culture dish is difficult, since these cells adhere avidly to plastic surfaces of cell culture devices (i.e. Petri dishes, microplates).

The adhesion of macrophages on cross-linked starch microspheres, not modified with ionic groups, was studied previously (Desmangles *et al.*, 1992). The results showed good adhesion and recovery of macrophage cells after amylolysis of starch microspheres (Desmangles *et al.*, 1992).

Godek *et al*, studied the retention of macrophages on hydrophobic fluorinated surfaces (Godek *et al.*, 2009). The results indicated a possible compatibility between macrophages and hydrophobic surfaces. Another study showed that hydrophilic and anionic polyethylene terephthalate modified surfaces inhibit adhesion of monocyte and macrophage cells (Brodbeck *et al.*, 2002).

Several procedures are currently applied to recover macrophages such as mechanical detachment , vortex or by gentle scraping of macrophages with a rubber policeman (Fleit *et al.*, 1984; Porcheray *et al.*, 2005; Jaguin *et al.*, 2013) or pre-treatment with scandicain K, proteinases, or pronase (Malorny *et al.*, 1981), which is limited as it has mitogenic effects on macrophages. By mechanical detachment, only about half of cells often may remain viable (Adams, 1979). Consequently, high variability and significant loss of viable cells are major limitations for existing procedures.

An interesting application of starch was used for enriching macrophage cell populations by adhesion on cross-linked starch microspheres followed by liquefaction of microbeads with alpha-amylase (Desmangles *et al.*, 1992).

One of a major objective of the present project was to study and understand the critical role of surface properties of starch materials on the attachment of macrophages and consequently the influences on their viability aimed to ameliorate the preparative procedures macrophage cells.

CHAPTER IV

PROJECT PRESENTATION AND RESEARCH ACHIEVEMENTS

Polysaccharides are the most abundant macromolecules in nature and present several major advantageous characteristics for pharmaceutical applications. They may be stable, non-toxic, hydrophilic and biodegradable. One of the most available natural polymers is starch which is composed of amylose (unbranched-chain) and amylopectin (branched-chain). The α-1,4 links between glucose units can be hydrolyzed by alpha-amylase, which is an endo-amylase. The presence of hydroxyl groups in starch allows for chemical derivatization and crosslinking, and thus conducting to new derivatives with improved physico-chemical properties.

The present project proposes

A. **Modified starch as biomaterials to retain macrophages and a procedure to obtain macrophage cells**. The starch materials were used as support - substrates and based on the fact that, even modified starch still maintains, at least in part, it is still susceptible to amylolysis, we are proposing a different procedure to detach macrophages without damaging them. Macrophages are currently studied *in vitro* in various biomedical fields including their use as targets in cancer therapy. The mononuclear phagocyte system, which includes macrophages, is a host-wide system of phagocytic cells with similar properties. Enhance viability of macrophages after incubation is still a challenge. Macrophages can adhere avidly to plastic surfaces of cell culture devices (i.e., Petri dishes, micro well plates, and multi - well plates). In the current procedure of macrophage preparation (mechanical detachment) the viability of cells is only about 50% (Adams, 1979) One of the main objectives

of this doctoral project was to introduce a new procedure for macrophage cells recovery with a higher viability and improved yields.

B. **Ampholytic starch as a new pharmaceutical excipient**. The CarboxyMethyl AminoEthyl Starch (CM-AE-St), represents the basis for a new class of materials with self-assembling properties able to generate stable structures for biomedical and pharmaceutical applications (Sakeer *et al.*, 2017a). Following functionalization with carboxymethyl and with aminoethyl groups, starch could exhibit ampholytic properties. These derivatives are multifunctional and can be used in various applications for instance as excipients for drug delivery. The new materials will be able to stabilize the monolithic tablet forms and to control the release of various bioactive molecules mainly through physical associations, i.e. hydrogen bonding and ionic interactions.

Thesis Project Objectives:

I. **To provide an alternative procedure for preparation of macrophage cells with improved viability.**

II. **To elaborate and study of a new class of ampholytic starch derivatives.** The present project will explore various synthesis routes and applicability of ampholytic starch derivatives.

Experimental issues related to the main objectives are:

I.1. Synthesis of various starch derivatives with filmogenic properties and their use to coat insert devices for macrophage culture.

I.2. Selection of starch materials affording highest viability and best yields for macrophage recovery.

II.1. Synthesis of different variants of ampholytic starch with different degrees of substitution in terms of amounts of grafted CM and AE groups on starch backbone.

II.2. Select appropriate ampholytic starch grade for controlled drug delivery (especially for highly soluble, short half-life molecules) and compare their dissolution patterns with those of commercial products.

Project hypothesis:

1. Starch derivatives are expected to present filmogenic properties. The concept for the novel macrophage separation procedure is that formed films would still be susceptible to amylolysis. They can be used as substrate/support for macrophage separation by mild enzymatic amylolysis, providing a better viability than the current methods.
2. The presence of anionic and cationic groups on starch will afford an enhanced stability via hydrogen association and via ionic interaction. The concept consists of the simultaneous presence of anionic and cationic groups on polysaccharidic chains which will ensure an ampholytic character. The ratio between these charged groups can be variable and generate different specific properties (i.e. hydration) which can be useful for biomedical applications. The presence of CM groups on the polysaccharidic chains may offer gastro-resistance, whereas AE groups will reduce the polymer solubility in SIF.

The intermolecular polyelectrolytic complexation (PEC) of anionic polymers (i.e. CM-St) and cationic polymers (i.e. chitosan) was already described (Assaad *et al.*, 2011) allowing an improved control of colon delivery of drugs. This doctoral project proposes an intramolecular polyelectrolyte complexation if the conformational structure allows, and / or polyelectrolyte complexation between the anionic CM and cationic AE groups located on neighboring chains that may also contribute to CM-AE-St stabilization. In gastric fluid, the ampholytic starch

undergoes protonation of both carboxylic groups (generating a compaction of the outer layer around the tablet) and of aminoethyl groups which produce an outer hydrogel able to release the active agent (i.e. metformin) in the early stage of gastric residence. In the intestinal fluid, the protonated carboxyl groups of the ampholytic CM-AE-St, located at the outer gel layer of the monolithic tablets, will gradually change the protons for Na^+ cations mostly arriving with the $NaHCO_3$ secretion of the pancreas. This will facilitate hydration, fluid uptake, erosion, and polymeric material dissolution in the simulated intestinal medium.

The major advantage of these novel starch ampholytic excipients is that, the starch backbone can still be degraded by pancreatic alpha-amylase, favoring the release of drug, if needed, in the upper intestine (which is not the case of PEC involving chitosan) and better controlling the delivery over larger intestinal segments, than that allowed by CM-starch only.

A schematic presentation is showed below (Fig 1. 4), to easily follow up and summarize the main steps in each investigated topic of this project.

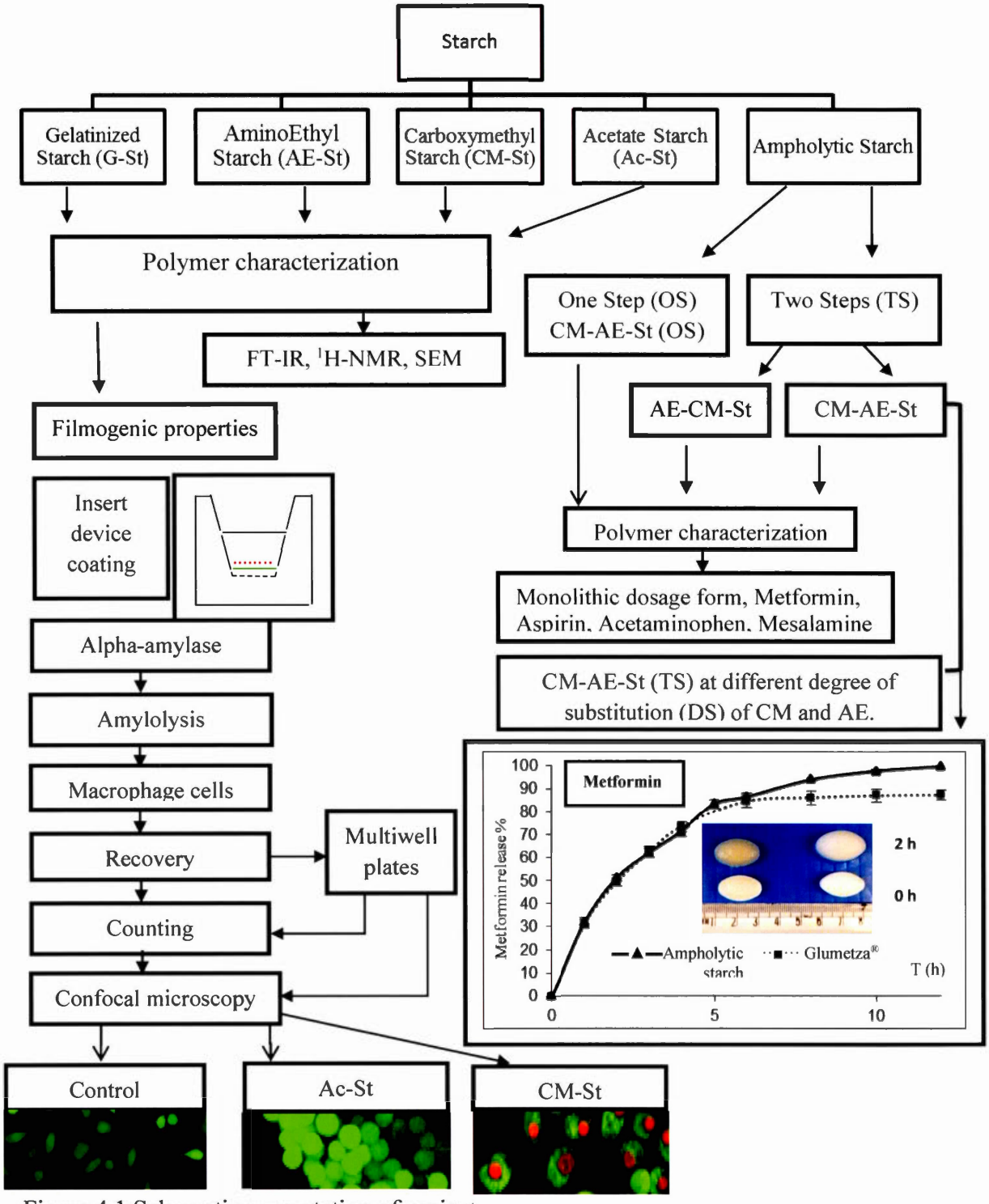


Figure.4.1 Schematic presentation of project

PART II - EXPERIMENTAL CONTRIBUTIONS

CHAPTER V

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STARCH MATERIALS AS BIOCOMPATIBLE SUPPORTS AND PROCEDURE FOR FAST SEPARATION OF MACROPHAGES

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Contribution of the main author and the co-authors:

The main author Mr. Khaleel SAKEER was responsible for all major parts of the article, in writing the manuscript body, and all experimental parts, and editing the figures and reviewing the references.

First Co-author: Dr. Tatiana Scorza, from department of biological Sciences (UQAM). Her main contribution was related to *in vitro* experiments mainly through providing the macrophage cells.

Second co-author: Mr. Hugo Romero was a master's student in Dr. Tatiana Scorza's research group and his major contribution was preparing macrophages and ELISA tests.

Third co-author: Dr. Pompilia Ispas-Szabo was involved in some editorial parts and manuscript revision.

Fifth co-author: Prof. Mircea Alexandru Mateescu is the project director, responsible for supervision of all activities and providing the required directions about all experimental parts and interpretations of the obtained results.

Abstract

Different starch derivatives were evaluated as supports for attachment and recovery of macrophages (RAW 264.7 line). Gelatinized starch (G-St), acetate starch (Ac-St), carboxymethyl starch and aminoethyl starch were synthesized and characterized by FTIR, ^{1}H NMR, SEM and static water contact angle. These polymers are filmogenic and may coat the holder devices, used for macrophage adhesion, well. They also present a susceptibility to mild hydrolysis with alpha-amylase, liberating the adhered macrophages. Cell counts, percentage of dead cells and level of tumor necrosis factor (TNF-α) were used to evaluate the possible interaction between macrophages and starch films. The high percentage of cell adhesion (90-95 % on G-St and on Ac-St) associated with enzymatic detachment of macrophages from film-coated inserts, resulted in higher viabilities compared with those obtained with cells detached by current methods: scrapping or vortex. This novel method allows a fast macrophage separation, with excellent yields and high viability of recovered cells.

Keywords:

Macrophage separation, alpha-amylase, atarch derivatives, acetate starch, gelatinized starch, tumor necrosis factor (TNF-α).

1. Introduction

Starch is widely used in food, pharmaceutical and biomedical applications due to its biocompatibility, biodegradability, non-toxicity and abundant sources (Rowe *et al.*, 2009). Starch modification is generally achieved through derivatization such as cross-linking (Lenaerts *et al.*, 1998), etherification, esterification (Mulhbacher *et al.*, 2001; Calinescu *et al.*, 2005) and grafting (Kaur *et al.*, 2007) of functional groups onto the carbohydrate structure. Such modifications can profoundly alter the physicochemical and morphological properties of starch, its enzymatic digestibility and can consequently modulate its current use as an excipient in drug delivery dosage forms (Mulhbacher *et al.*, 2004; Massicotte *et al.*, 2008). An interesting reported application of starch was its use for enrichment of macrophage cell populations by adhesion on cross-linked starch microspheres followed by liquefaction of microbeads with alpha-amylase (Desmangles *et al.*, 1992). Macrophages are currently investigated in various biochemical and biomedical fields as well as for therapeutic applications (You *et al.*, 2013; Kwan *et al.*, 2014; Wooden and Ciborowski, 2014; Ostuni *et al.*, 2015). Macrophages with a possible role in inflammatory processes and malignancy have been reported as a new therapeutic target. There is a growing interest for techniques of macrophage separation, particularly to investigate anti-macrophages novel strategies against cancer. Macrophages can be obtained in a relatively pure form as primary cultures for analytical and biochemical manipulations but they do not generally replicate in culture, have relatively short-lives, and may be difficult to obtain enough amounts for large scale. They are very sensitive to small changes in their environment and may be damaged considerably, even when delicately handled after cell culture (Adams, 1979; Féréol *et al.*, 2006). Detaching adherent macrophages from a culture dish is difficult, since these cells adhere avidly to plastic surfaces of cell culture devices (i.e. Petri dishes, microplates). Several procedures are currently applied to regain macrophages such as mechanical detachment by gentle scraping of macrophages with a rubber policeman (Fleit *et al.*, 1984; Porcheray *et al.*, 2005;

Jaguin *et al.*, 2013) or pre-treatment with scandicain K, proteinase, or pronase (Malorny *et al.*, 1981), which is not ideal as it has mitogenic effects on macrophages. Frequently by mechanical detachment, about half of cells may remain viable (Adams, 1979). Consequently, high variability and significant loss of viable cells are major limitations for existing procedures.

Based on our previous separation of macrophages by retention on a cross-linked starch column and further detachment by enzymatic hydrolysis of the chromatographic support (Desmangles *et al.*, 1992), four starch materials namely gelatinized starch (G-St), acetate starch (Ac-St), carboxymethyl starch (CM-St) and aminoethyl starch (AE-St) were investigated for their ability to form films susceptible to amylolysis to be used as substrate/support for macrophage separation by mild enzymatic amylolysis. This approach is different to the previous reported method (Desmangles *et al.*, 1992) to separate macrophages using cross-linked starch as a chromatographic support. In general, cross-linked materials are adequate to form microspheres but present lower filmogenic ability than the uncross-linked materials (Krumova *et al.*, 2000; Berezkin and Kudryavtsev, 2015). A major objective of this study was to understand the critical role of surface properties of starch materials on the attachment of macrophages and consequently the influences on their viability.

2. MATERIALS AND METHODS

2.1. Materials

High amylose starch (Hylon VII) was supplied by National Starch (Bridgewater, NJ, USA). Sodium monochloroacetic acid, 3,5-dinitrosalicylic acid, sodium potassium tartrate tetrahydrate (Sigma-Aldrich, Germany), D-(+)-maltose monohydrate (Sigma-Aldrich, Japan), amyloglucosidase (EC 3.2.1.3) from *Aspergillus niger* $\geq$ 300 U/mL (Sigma-Aldrich, Denmark), acetic anhydride (Anachemia, Montreal, Canada), α-

amylase (EC 3.2.1.1) from *Bacillus subtilis* 402 U/mg (Fluka, Switzerland), 2-chloroethylamine hydrochloride (Fluka, Switzerland) were all used as received without further purification. CellTracker™ Green CMFDA (5-chloromethylfluorescein diacetate) and propidium iodide (Invitrogen, UK), lipopolysaccharide (LPS, L3012, Sigma-Aldrich), TNF ELISA kits from Biolegend (San Diego, CA) were used for macrophage cell characterization. The RAW macrophage cells (ATCC TIB-71) were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and antibiotics (Penicillin and Streptomycin). Subcultures were prepared by gentle scrapping and aspiration prior to testing in starch coated supports.

2.2. Preparation of starch filmogenic materials

An amount of 12.50 g of Hylon VII was suspended for hydration in 50 mL of distilled water at 60-70 °C under continuous vertical stirring (Servodyne Mixer, 50000-40, IL, USA). A volume of 75 mL of 5 M NaOH was added to the starch suspension, continuing the stirring for 60 min at 60-70 °C. Then the solution was cooled down and neutralized with glacial acetic acid (until pH 6.8) to get gelatinized starch (G-St). The gelatinized starch was further derivatized either by direct addition of 18.75 mL acetic anhydride, or by addition of 18.75 g sodium monochloroacetate or 2-chloroethylamine hydrochloride (each solubilized in a minimal water volume) with stirring and continuing the reaction for 1h at 60-70 °C to obtain acetate (Ac-St), carboxymethyl (CM-St), or aminoethyl (AE-St) starch derivatives, respectively. Then, each solution was cooled down and neutralized with glacial acetic acid (to reach pH 6.8). The derivatized starch powders were obtained by precipitation from the reaction solution with an equivalent volume of methanol/water (70:30) v/v solution. For all starch materials, the process was repeated until a final conductivity of filtrate decreased at about 50 µS/cm. Then, 200 mL of methanol 100 % were used, followed by 200 mL of acetone 100 % for final drying. The collected powders were

left at room temperature for complete air drying overnight and sieved to obtain particles of less than 300 μm.

2.3. Evaluation of substitution degree of derivatives

For the CM-St and the AE-St: the degree of substitution (DS) was determined by back-titration as previously described (Stojanović *et al.*, 2005; Assaad *et al.*, 2011). Briefly, 100 mg of polymer were solubilized in 10 mL of 0.05 M NaOH and then the excess of NaOH was titrated (n = 3) with 0.05 M HCl using phenolphthalein as indicator. The blank (20 mL of 0.05 M NaOH) was also titrated by the same method. The degree of substitution of Ac-St was determined titrimetrically, following the method of Sodhi and Singh (2005) with minor modifications (Sodhi and Singh, 2005). Acetylated starch (0.1 g) was placed in a 25 mL flask and 6 mL of dimethyl sulfoxide (DMSO) was added. The loosely stoppered flask was agitated, warmed to 50°C for 30 min, cooled down and then 4 mL of 0.05 M KOH was added. The alkali excess was back-titrated with 0.05 M HCl using phenolphthalein as an indicator. The amounts of –COOH, $–NH_2$ and $–COCH_3$ groups and the DS were calculated (Stojanović *et al.*, 2005) using the following equations:

$$n = (V_b - V) * C_{HCl} \quad (1)$$

$$DS = \frac{162 * n}{m - W * n} \quad (2)$$

where Vb (mL) is the volume of HCl used for the titration of the blank; V (mL) is the volume of HCl used for the titration of the sample; C_{HCl} is the concentration of HCl; 162 (g/mol) is the molecular mass of glucose unit; W = (58 or 44 or 43) (g/mol) is the increase in the mass of glucose unit by substitution with one carboxymethyl, aminoethyl and acetyl group respectively, and m (g) is the mass of dry sample.

2.4. Fourier transform infrared (FT-IR) analysis

The FT-IR spectra of samples as powders were recorded (64 scans at a 4 cm^{-1} resolution) using a Thermo-Nicolet 6700 (Madison, WI, USA) FT-IR spectrometer equipped with a deuterated triglycine sulfate-KBr (DTGS-KBr) detector and a diamond smart ATR (attenuated total reflection) platform.

2.5. ^{1}H NMR measurements

The ^{1}H NMR spectra were collected using a high-field 600 MHz Bruker Avance III HD spectrometer running TopSpin 3.2 software and equipped with a 5 mm TCI cryoprobe. The temperature of samples was maintained at 27°C. The samples were dissolved in deuterated dimethyl sulfoxide-d6 (DMSO-d6), then heated at 65 °C for 30 min, and kept at 4 °C for 2h.

2.6. Scanning electron microscopy (SEM)

The morphology of the particles and film surface were examined by a Hitachi (S-4300SE/N) scanning electron microscope with variable pressure (Hitachi High Technologies America, Pleasanton, CA, USA) at 5-7 kV and magnifications of 100 and 1000X for powders and of 500x and 1000x for film surface. Samples were mounted on metal stubs and sputter-coated with gold.

2.7. Film casting and macrophage culture

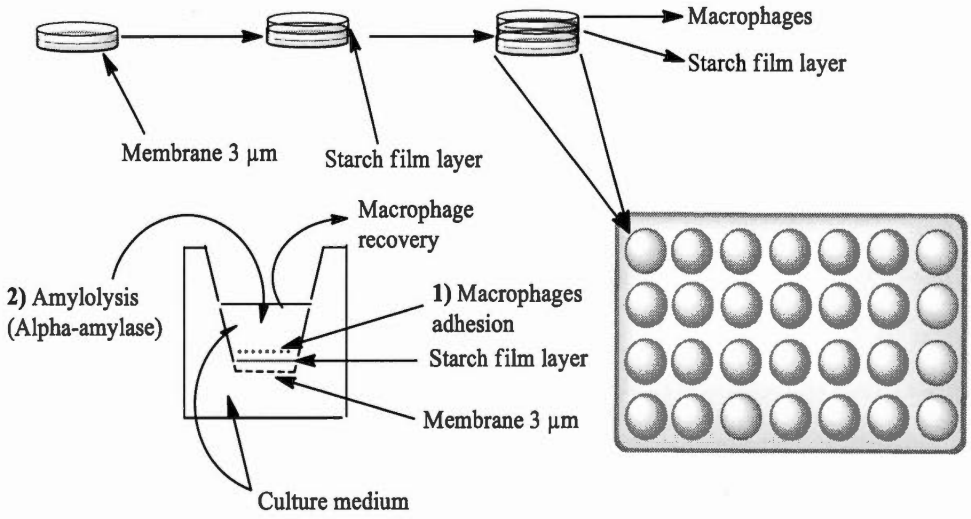
2.7.1. Preparation of film-forming solutions of starch materials

Gelatinized starch (G-St), acetate starch (Ac-St), carboxymethyl starch (CM-St) and aminoethyl starch (AE-St) have been dispersed at 0.5% (w/v) in purified water and heated to 95 °C. Then the solutions were cooled down to room temperature and centrifuged at 5000 rpm for 2 min. For each film forming material, the supernatant was cast on a cell culture insert device with a base filter of polyethylene terephthalate (PET) having 3.0 microns pore aperture (BD Falcon Cell Culture Inserts, 353092,

USA). The solution was evaporated at 40 °C for 12 h to form the film coating of the insert device.

2.7.2. Macrophage incubation

Before incubation, the insert and plates (Costar® 3516 6 well plate, USA) were sterilized by UV-ray for 15 min. Then, macrophage suspensions, in a RPMI-1640 culture medium containing FBS 10 % and Penicillin/Streptomycin (1x), were incubated for 48 h in a humidified atmosphere of air and 5% CO_2 at 37°C. The culture medium was introduced from the outside of cell culture insert (Scheme 1).



Scheme 1: Design of device and procedure with adhesion (1) and amylolysis (2) steps for fast recovery of macrophages.

2.7.3. Microscopy

The morphology of macrophage cells was investigated after incubation for 48 h onto the cell culture insert coated with G-St, CM-St, Ac-St or AE-St. Macrophages were labeled with fluorescent staining CellTracker™ Green CMFDA and propidium iodide following manufacturer instructions. Cells were visualized using a Nikon Eclipse Ti microscope (Nikon Canada, Mississauga, ON) equipped with phase

contrast and epifluorescence optics. Photomicrographs were acquired using a Digital Sight DS-Qi1Mc camera and NIS-Elements 3.0 software (Nikon Canada).

2.7.4. Susceptibility to enzymatic hydrolysis of starch films

The film hydrolysis was done in three steps: (a) **Hydration step**: Culture medium was replaced by 40 mM phosphate buffer pH 7.4 at 37 °C inside and outside of each cell culture insert; (b) **Liquefaction step**: A solution of an alpha-amylase (EC 3.2.1.1 from *Bacillus subtilis*) in 40 mM phosphate buffer pH 7.4 (1000 U/ mL) was used for liquefaction of film layer. (c) **Saccharification step**: A 40 mM phosphate buffer (pH 7.4) was used to dilute amyloglucosidase from *Aspergillus niger* to 100 U/ mL and then used for saccharification of the starch film spices resulting from partial hydrolysis with alpha-amylase under gentle shaking followed by incubation in a humidified atmosphere of air and 5% CO_2 at 37°C (Aneja, 2009; Lareo *et al.*, 2013).

2.7.5. Determination of enzymatic activity on the starch filmogenic supports

Enzymatic activity of alpha-amylase was measured on the same film amylolysis conditions using the dinitrosalicylic (DNS) method (Bernfeld, 1955) to measure the reducing sugar groups released as result of alpha 1,4 glycosidic group hydrolysis. At different time points, a hydrolyzed solution volume of 0.5 mL was withdrawn immediately 0.5 mL of DNS reagent was added to stop the hydrolysis reaction. Then, the reaction media were boiled for 5 min to develop the color of reduced 3-amino-5-nitro salicylic acid. Subsequently, after 5 min, the solutions were cooled in an ice-bath to room temperature and 1 mL of each cooled solution was diluted with 4 mL of distilled water. The absorbance of the final solution after filtration was measured against a blank solution without filmogenic material at 540 nm. Maltose solutions were used (as standard reducing sugar) to generate a standard curve. The required time for film hydrolysis was observed visually.

2.7.6. Macrophage cell recovery and counting

The current recovery approach for macrophages is the scratching procedure (used as control) and the recovery by the novel direct collection from starch coated inserts devices after the mild enzymatic film hydrolysis were compared by counting done with a hemacytometer (Nikon TMS-F), and using Trypan blue as staining agent.

2.7.7. Macrophage activation

Following 48 h incubation an amount of 50 ng/50 µL LPS per 1 mL of culture medium was added and the cells re-incubated for additional 72 h.

2.7.8. Quantitation of tumor necrosis factor (TNF-α)

After the 72 h incubation, the culture medium over and under of macrophage layer was gently removed and centrifuged at 12000 rpm for 10 min. The amount of TNF-α was quantified by the ELISA kit (Catalogue No 430904, Biolegend, Canada). TNF-α level in samples were determined according to the manufacturer's instructions. A standard curve in concentrations from 7.8 pg/mL to 125 pg/mL was done in duplicate and the level of TNF-α in the supernatants was evaluated by use of the standard curve as reference. The optical density at 450 nm was measured with a microplate reader.

2.8. Statistical analysis

All tests were performed in triplicate and data are reported as means ± SD. Statistical analysis of data was performed using one way ANOVA, followed by Fisher's post hoc tests with a minimum confidence level ($P < 0.05$) for statistical significance.

3. RESULTS AND DISCUSSIONS

3.1. Polymer and film characterization

The degree of substitution of starch derivatives CM-St, Ac-St and AE-St, as determined by back-titration were about 0.018, 0.022 and 0.024, respectively. These values represent the average number of carboxymethyl, acetate or aminoethyl groups per glucose unit, respectively. The grafting of each functional group on the starch chains was confirmed by structural analysis, FT-IR and ^{1}H NMR.

The Fourier transform infrared (FT-IR) spectra of the obtained starch materials **(Fig. 1)** present a broad band at 3200-3300 cm^{-1} due to the stretching vibrations of –OH. Small bands at 2927 cm^{-1} and at 2323 cm^{-1} attributed to the -CH stretching vibration and a band at 1079 cm^{-1} ascribed to $-CH_2-O-CH_2$ stretching vibrations (Ispas-Szabo *et al.*, 1999). In case of CM-St, there are additional bands at 1589 cm^{-1} and at 1323 cm^{-1} ascribed to COO^- group (Friciu *et al.*, 2013). The high intensity of the band at 999 cm^{-1} for AE-St could be ascribed to C-N stretching vibrations, whereas the weak shoulder at around 1735 cm^{-1} could be assigned to $-NH_3^+$ group (Deng *et al.*, 2006; Assaad *et al.*, 2011). In the case of Ac-St, the weak shoulder at around 1556 cm^{-1} corresponds specifically to the -C-O stretching of acetyl groups (Colthup *et al.*, 1990; Bello *et al.*, 2010).

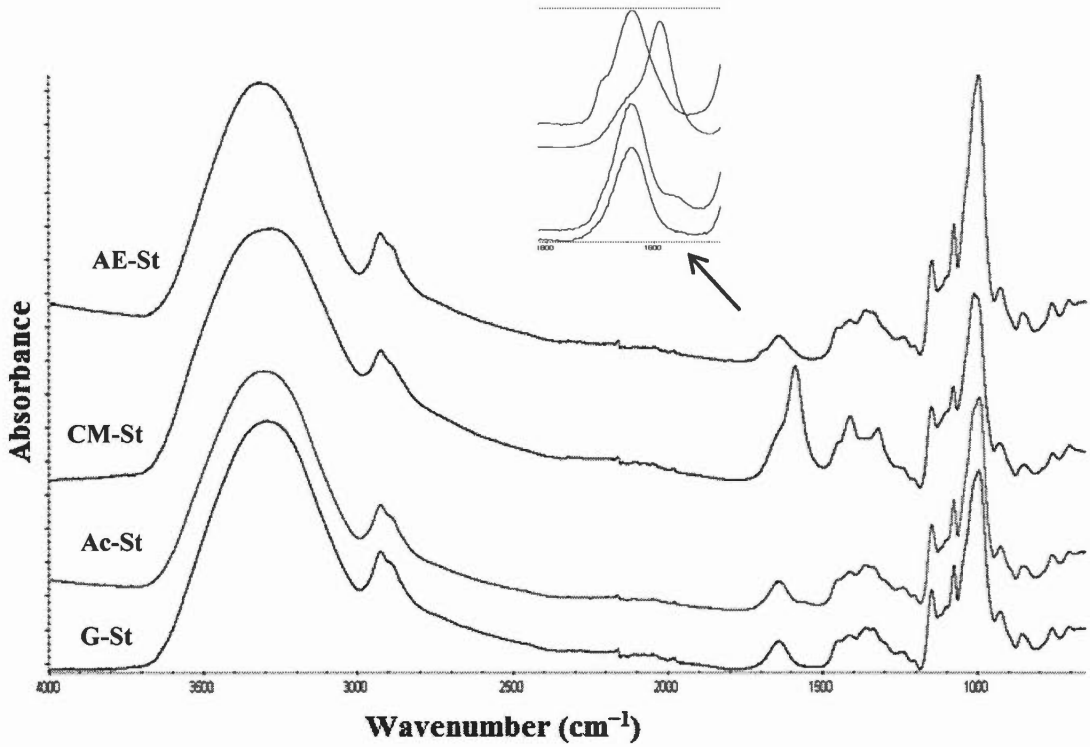


Fig 1: FT-IR spectra of Gelatinized starch (G-St), Acetate starch (Ac-St), Carboxymethyl starch (CM-St) and Amino-Ethyl starch (AE-St).

The ^{1}H NMR spectra of the starch materials **(Fig. 2)** present proton signals at 5.3 ppm for H1 and at 3.3–3.9 ppm for H2-6 on the starch backbone (Yang *et al.*, 2014b) while the peak at 5.6 ppm can be assigned to OH3. The most significant peaks for AE-St are at δ = 4.15 - 4.25, δ = 3.16 - 3.18, which belong to the hydrogens of aminoethyl group. In case of Ac-St the peaks at δ =1.9 - 2.1 and at δ =3.5 ppm are ascribed to methyl protons of acetate groups (Xu and Hanna, 2005b). In case of CM-St, peak broadening may be due to the limited solubility of CM-St in DMSO.

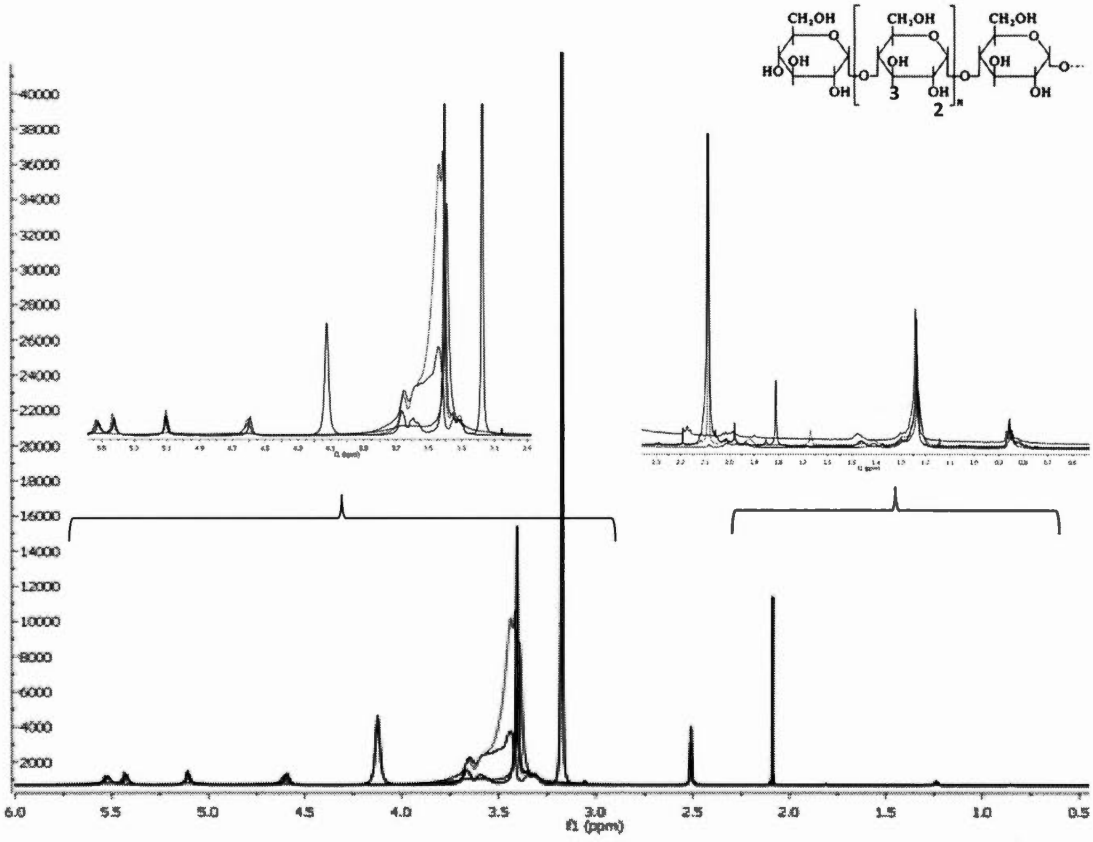


Fig 2: ^{1}H NMR spectra of Gelatinized starch (Red), Acetate starch (Green), Carboxymethyl starch (Blue) and Amino-Ethyl starch (Black).

The obtained zeta potential (ζ) charge values in solution were -32 mV for G-St and -38 mV for CM-St. These values are consistent with the chemical modification of starch by carboxymethyl groups providing a stronger negative charge (Wongsagonsup *et al.*, 2005a; Wongsagonsup *et al.*, 2005b). Grafting starch with acetate groups reduced the value of zeta potential for acetate starch to -26 mV and this can be explained by a decreased polarity in comparison with G-St. The positive zeta potential value for AE-St +10 mV is related to cationic groups grafted on starch molecules.

Static water contact angle **(Fig. 3)** allowed the evaluation of the wettability/hydrophilicity of the films for coating the insert surfaces. The CM-St and AE-St films presented a lower angle (67° and 78° respectively) in comparison to G-St (89°) and Ac-St (105°), meaning that G-St and Ac-St are less polar and more hydrophobic.

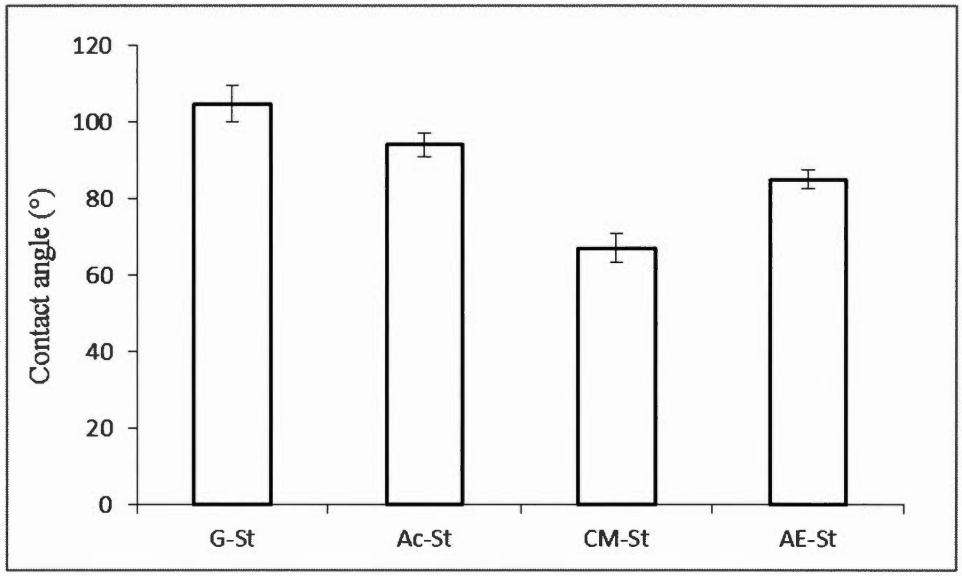


Fig 3: Water contact angle measurement for insert coating films of Gelatinized starch (G-St), Acetate starch (Ac-St), Carboxymethyl starch (CM-St) and Amino-Ethyl starch (AE-St) (n = 3).

Scanning electron microscopy (SEM) of starch materials as powders and films are presented in **Fig. 4**. The native starch (Hylon VII) has is granular predominantly round or oval in shape **(Fig. 4)**, with a smooth surface and uniform range of size distribution (5–10 μm). The granular aspect fits well with the known crystalline structure of native starch (Friciu *et al.*, 2013), stabilized by hydrogen bonds between the hydroxyl groups of glucopyranose units. The aspect of the four materials: G-St, CM-St, AE-St and of Ac-St is different, depending on the modification of the starch structure.

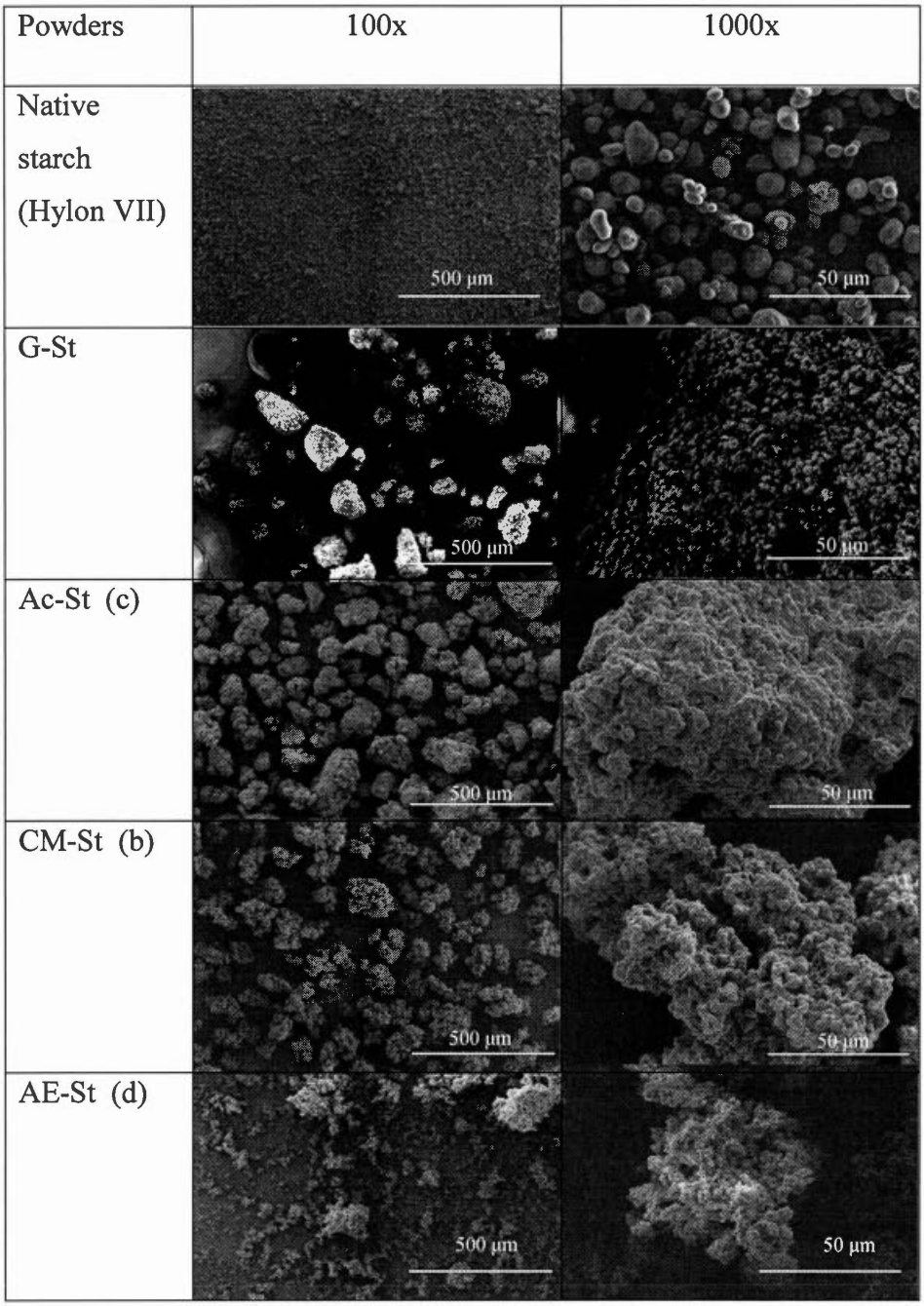


Fig 4. Scanning electron microscopy micrographs of Native starch (Hylon VII), (a) Gelatinized starch (G-St), (b) Acetate starch (Ac-St), (c) Carboxymethyl starch (CM-St) and (d) Amino-Ethyl starch (AE-St) powders at magnifications of 100x and 1000x.

The G-St (**Fig. 4a**) showed a round and sponge-like shape which is due to the physical modification (gelatinization) of native starch. Differently, the CM-St (**Fig. 4b**) presented an irregular shape with an uneven surface likely due to the association of numerous small particles forming larger granules. These were similar shapes as obtained by Friciu *et al.*,2013. The carboxylic groups may reduce the network self-assembling by hydrogen association between hydroxyl groups and promote repulsion effects loading to a structural reorganization (Lemieux *et al.*, 2010). The acetylation (**Fig. 4c**) generated a slightly rough surface of granules which appeared fused in a kind of aggregate. The acetyl groups can also decrease the starch stabilization by hydrogen bonding and, at the same time, the glucose units with polar hydroxyl groups and non-polar (acetate) functions, may favor starch macromolecules to coalesce together resulting in a kind of fusion of granules (Singh *et al.*, 2004; Bello *et al.*, 2010). The AE-St (**Fig. 4d**) grains showed a porous irregular shape, where amine groups may promote hydrogen bonding resulting in a reorganization of the AE-St network. As far as films are concerned the SEM micrographs of G-St and CM-St films at magnifications of 500x and 1000x **(Fig. 5a, b)** showed a homogeneous and smooth surface, whereas Ac-St and AE-St films **(Fig. 5c, d)** showed continuous matrices, with small cracks and less smooth surface.

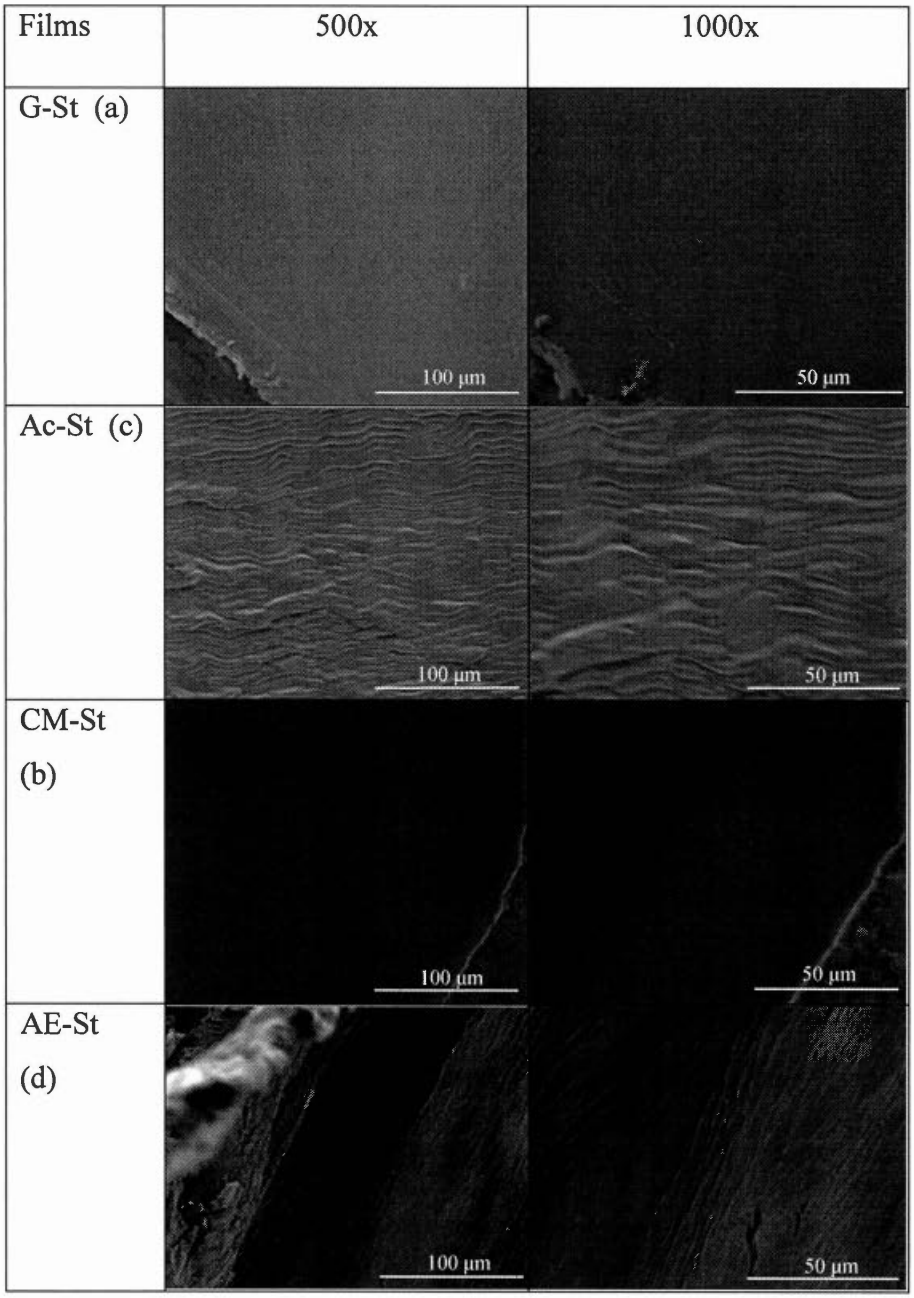


Fig 5. Scanning electron microscopy micrographs of films: Gelatinized starch (G-St), (b) Acetate starch, (Ac-St), (c) Carboxymethyl starch (CM-St) and (d) Amino-Ethyl starch (AE-St) at magnifications of 500× and 1000×.

3.2. Macrophage cell attachment and recovery by film amylolysis

Morphology of macrophage cells. Intact macrophage cultures were treated with two staining agents: CMFDA to show live cells (green) and propidium iodide to stain dead cells with altered membrane permeability (red).

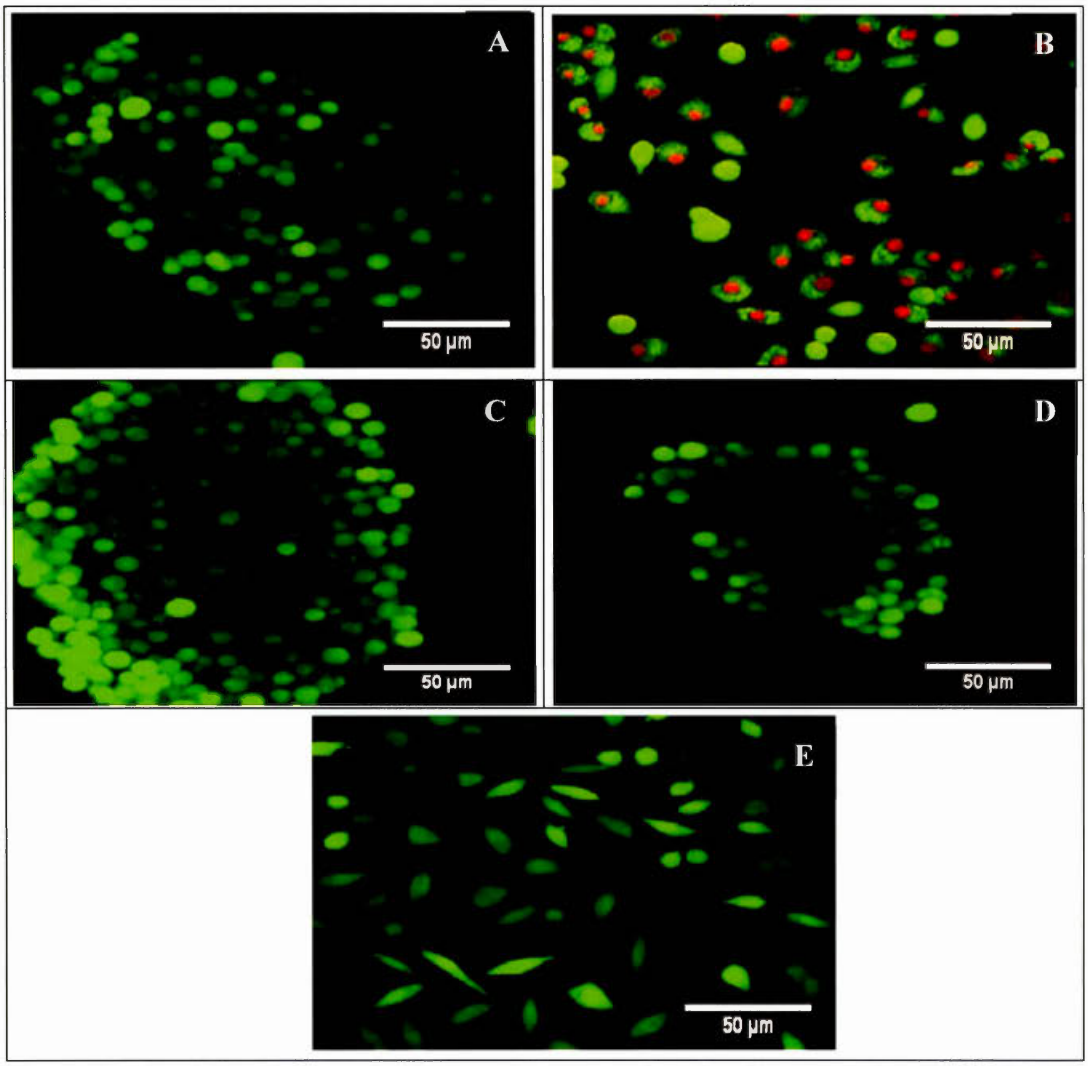


Fig 6: Confocal fluorescence microscopy images showing live cells (green) and dead macrophage cells (red) after incubation 48 h on cell inserts coated with Amino-Ethyl starch (A), Carboxymethyl starch (B), Acetate starch (C), Gelatinized starch (D), and control (uncoated insert) (E), scale bar 50 µm.

Control cultures on uncoated insert devices appear as plump or stellate, monolayers rounded and spindle-like with a majority of live cells. Macrophages incubated on insert devices coated with G-St, Ac-St and AE-St showed round, compact and mostly live cells **Fig. 6**. Differently, prevalently dead cells were observed when incubated in insert coated by CM-St film, owning round, spindle-like and translucent cytoplasm. This behaviour suggests that the carboxymethyl functionalized film may cause membrane disruption and cell apoptosis. Similar damaged membranes and apoptosis have been observed with certain agents such as carboxy- silicalite (Petushkov *et al.*, 2009).

Determination of enzymatic activity with starch filmogenic supports as substrates: The film amylolysis process was investigated by measuring the enzymatic activity of alpha-amylase with various films as substrate (**Fig 7-A**). It was found that G-St, AC-St and AE-St showed similar film hydrolysis rate over the first 40 min. Then, the G-St hydrolysis was faster than that of AC-St and AE-St. This behavior was considered as normal because there is no chemical modification of the G-St. The lowest enzymatic activity was observed with CM-St film, where the released amount of maltose after 75 min was almost half of that liberated from G-St. The film hydrolysis was also followed visually. Even without complete amylolysis, the CM-St film was dissolved in less than 10 min, because CM-St is soluble in alkaline medium. Differently, G-St film was partially hydrolyzed in 30 min, AC-St and AE-St in 40 min. Macrophages adhere on adequate surfaces and floating cells are characteristically dying cells. Macrophage counting suggested good adhesion on G-St, on Ac-St and on AE-St materials. **Fig. 7-B** presents the non-adherent (floating) fraction of macrophages after incubation of cell culture on cell-holder devices (insert) coated with CM-St, AE-St, Ac-St or G-St. the higher percentages of dead macrophages (floating) were observed at inserts coated with anionic CM-St (about 32±5%) or with the cationic AE-St (about 32±9%), whereas a low percentage of dead cells was observed with inserts coated with non-ionic and neutral polymers Ac-St (5±2%) and G-St (9±3%) respectively, suggesting higher percentage of living cells

from these films. These adhesion data on non-ionic Ac-St and G-St are in agreement with our previous report showing good adhesion and recovery by amylolysis of macrophage cells on cross-linked starch microspheres, not modified with ionic groups (Desmangles *et al.*, 1992). The best retention on AC-St fits well with a study from Godek *et al*, (2009), showing that macrophages adhere preferentially to highly hydrophobic fluorinated surfaces (Godek *et al.*, 2009). Similar results, but not on carbohydrate materials, were observed by Brodbeck showing that the hydrophilic and anionic polyethylene terephthalate modified surfaces inhibit adhesion of monocyte and macrophage cells (Brodbeck *et al.*, 2002).

Due to membrane disruption and cell inducing apoptosis along with low macrophage viability on CM-St, this support was excluded from further investigation and cell harvesting and counting was continued with control inserts (uncoated) and with G-St, Ac-St and AE-St coated inserts. Cell harvesting was done by scrapping for control cells (cultured on uncoated insert devices) or by enzymatic hydrolysis for inserts coated with starch materials. After incubation for 48 h, cell numbers increased about 3.2 times for control uncoated inserts, 4.2 times for Ac-St and 5.3 times for G-St whereas only 1.5 times was observed for AE-St coated insert **(Fig. 7-C)**. Furthermore, 129 % and 164 % more cells were recovered from inserts devices coated with G-St and Ac-St when compared to controls (un-coated inserts), whereas a 53% drop of the yield was obtained for AE-St coated inserts. This inhibitory effect could be explained by a too strong interaction of cationic aminoethyl groups of starch film with membrane phospholipids of macrophage cells (Kurtz-Chalot *et al.*, 2014). Therefore the AE-St was not retained for further investigation.

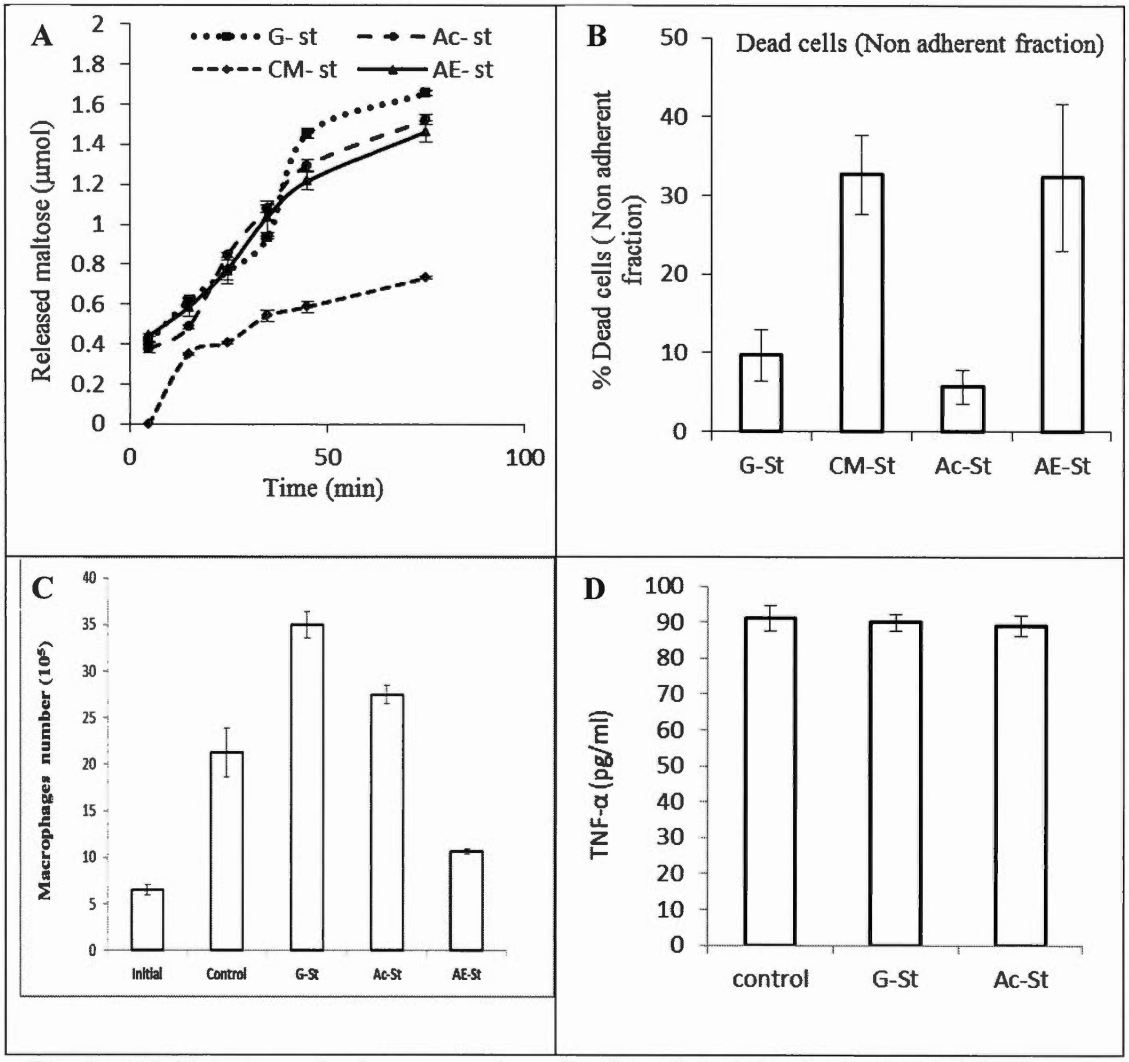


Fig 7: (A) Release reducing sugar (µmol) after Gelatinized starch (G-St), Acetate starch (Ac-St), Carboxymethyl starch (CM-St), and Amino-Ethyl starch (AE-St) film hydrolysis by alpha-amylase; (B) Percentage of dead cells (%) incubated on cell insert coated with G-St, Ac-St, CM-St and AE-St; (C) Macrophage count with an initial number ($6.5x10^5$) and incubated 48 h on inserts coated with film of G-St, Ac-St, AE-St or Control (uncoated); (D) Tumor necrosis factor TNF-α (pg/mL) from recovered macrophage activated by lipopolysaccharide (LPS) 50 ng/mL.

Macrophage activation by Lipopolysaccharide (LPS) and quantitation of induced tumor necrosis factor (TNF-a) allowed the investigation of the possible effect of starch derivatives with macrophage activities. The cells were stimulated with LPS, a component of the outer membrane of Gram negative bacteria, which is a potent activator of monocytes and macrophages (Mace *et al.*, 1988). LPS triggers the abundant secretion of cytokines by macrophages including tumor necrosis factor (TNF-a), interleukin (IL)-1, and IL-6 (Meng and Lowell, 1997). In our study, the amount of TNF-α secreted by macrophages in response to LPS was in the same range as a similar study (Lichtman *et al.*, 1998). Moreover, there were no differences **(Fig 7-D)** in TNF-α produced by control cells harvested from uncoated inserts (91±3.5 pg/mL) or by macrophages harvested from G-St (90±2.3 pg/mL) and Ac-St (89 ±2.9 pg/mL) coated inserts. The functional groups grafted on polysaccharide chains not only have a direct effect on viability of cells, but they can impact macrophage adhesion. For instance, the non-derivatized starch (G-St) and the Ac-St with hydrophobic acetate groups oriented toward culture medium, are better supports for adhesion of macrophage cells than the anionic (CM-St) and cationic (AE-St) starch derivatives, which are less compatible. The minimal percentage of dead cells (non-adherent fraction) was observed with inserts coated with G-St and Ac-St. Therefore, these Gelatinized starch and Acetate starch materials affording a best viability, could be a good choice as support material for macrophage culture due to the high compatibility with cells and also for their susceptibility to mild enzymatic amylolysis. These features of G-St and Ac-St allow the recovery of macrophage cells with better viability and high yields. Furthermore, the activation by LPS indicated that macrophage cells cultured on G-St and on the starch acetate derivative are producing almost the same level of TNF-α as the control (uncoated insert). This result together with the low percentage of dead cells could be an evidence of biocompatibility of G-St and Ac-St supports as materials for macrophage preparation by this novel mild enzymatic procedure.

4. CONCLUSION

The present study is proposing a new type of application for modified starch based on its film-forming capacity. The proposed approach, focused on adhesion of macrophage cells on Ac-St or G-St films followed by their detachment by enzymatic amylolysis, is faster and the mild conditions afford a better viability of macrophage cells in comparison with the classical procedure (mechanical detachment). Starch films are easy to apply on the inserts and their biocompatibility is an important characteristic for cell viability. This study opens new perspectives to obtain macrophage cells with a high viability, avoiding significant loss of viable cells which still limits the current scratching procedures. Further studies will be conducted in order to evaluate the impact of the substitution degree of Ac-St on the attachment and activity of macrophages.

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CHAPTER VI

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AMPHOLYTIC STARCH EXCIPIENTS FOR HIGH LOADED DRUG FORMULATIONS: MECHANISTIC INSIGHTS

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Contribution of the main author and the co-authors:

The main author Mr.Khaleel SAKEER was responsible for all major parts of the article, in writing the manuscript body, and all experimental parts, and editing the figure alongside with gathering information and reviewing the references.

First co-author: Dr. Pompilia Ispas-Szabo she was involved in some editorial parts and manuscript revision.

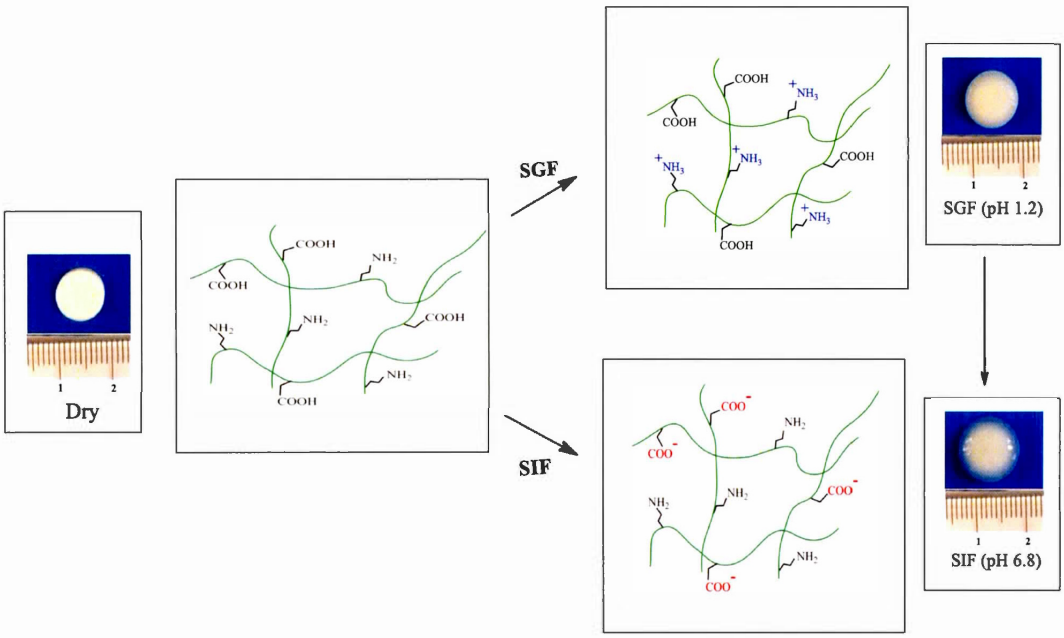
Second co-author: Mr. Nassim Benyerbah was responsible for some dissolution tests and editorial parts.

Third co-author: Prof. Mircea Alexandru Mateescu is the project director, responsible for supervision of all activities and providing the required directions about all experimental parts and interpretations of the obtained results.

Abstract

Ampholytic starch derivatives are proposed as a new class of excipients carrying simultaneously anionic carboxymethyl (CM) and cationic aminoethyl (AE) groups on starch (St) polymeric chains. Three different types of derivatives were obtained by using the same reagents and varying only the order of their addition in the reaction medium: in one step method (OS) the two reactants were added simultaneously, whereas in two steps method (TS) either CMSt or AESt were prepared separately in the first step, followed by subsequent addition of the second reactant. It was found that all ampholytic derivatives were able to generate monolithic tablets by direct compression and allowed 60% loading of acidic (Acetylsalicylic acid), basic (Metformin), zwitterionic (Mesalamine) or neutral (Acetaminophen) compounds as drug models. The *in vitro* dissolution tests followed for 2 h in SGF and then in SIF, showed that the mentioned starch derivatives were stabilized by self-assembly and generated matrices were able to control the release of drugs for about 24 h. The addition order of reagents has an impact on ampholytic starch properties offering thus a high versatility of this new class of starch excipients that can be tailored for challenging formulations with high dosages of several drugs.

Keywords: Ampholytic starch, controlled release, monolithic tablet, self-assembling, high loading formulation



Graphical abstract

1. Introduction

The few past decades have been marked by innovative concepts of new drug delivery systems focused on improved effectiveness of medication and patient compliance. Molecular self-assembly based on noncovalent, weak interactions established between macromolecules represents a simple way to generate various supramolecular structures with a wide range of properties (Mateescu *et al.*, 2006). This versatility is related to the possibility to tailor the polymeric structure in order to obtain properties suiting a specific application (Rattanakit *et al.*, 2012; Hayashi *et al.*, 2017). Hydrogels are largely used in controlled delivery of therapeutic agents and, despite the variety of their structures, there are two main features that can be modulated: hydrophilicity/hydrophobicity ratio and the ionic charges. Many research studies were dedicated to natural sourced such as cellulose (Hiremath and Saha, 2008; Vanhoorne *et al.*, 2016), starch (Ispas-Szabo *et al.*, 1999; Ispas-Szabo *et al.*, 2016), and chitosan derivatives (Polk *et al.*, 1994; Souza *et al.*, 2014; Santos Menegucci *et al.*, 2015), or synthetic polymers i.e. methacrylates (Okor and Obi, 1990), polyethylene glycol (Okor and Obi, 1990) and derivatives, in order to better control the drugs release. Most self-assembled molecules present hydrophobic and /or hydrophilic domains where the hydrophilic zones can carry charges (anionic, cationic or zwitterionic) or may be uncharged but polar (Okor and Obi, 1990; Aji *et al.*, 2017; Yan *et al.*, 2017). Intramolecular interactions may be responsible for the conformation of the molecules and conduct to specific secondary structures (helix, coils, sheet) whereas the intermolecular non-covalent interactions may be involved in self-assembled macromolecular structures like nanogels (Lamprecht *et al.*, 2014), polyelectrolyte capsules (Szczepanowicz *et al.*, 2014; Szczepanowicz *et al.*, 2016), cubosomes (Yang *et al.*, 2014a), etc. Due to its biocompatibility and abundant source, starch is widely used in pharmaceutical and biomedical applications (Calinescu *et al.*, 2005; Calinescu *et al.*, 2007; Sakeer *et al.*, 2017b) The availability of three hydroxyl groups per glucose unit offers the possibility for chemical

modification enriching thus the panel of starch properties and applications. Starch modification was achieved through various reactions such as cross-linking (Lenaerts *et al.*, 1998), esterification, etherification (Mulhbacher *et al.*, 2001; Calinescu *et al.*, 2005; Sakeer *et al.*, 2017b), and grafting (Kaur *et al.*, 2007) of functional groups onto the carbohydrate backbone. The esterification (i.e. by derivatization with acetic anhydride) can reduce starch hydrophilicity generating matrices for drug release (Mulhbacher *et al.*, 2001) or provide interesting film-forming properties (Sakeer *et al.*, 2017b). Differently, starch etherification with sodium monochloroacetate produced carboxymethyl starch (CMSt): an ionic matrix forming excipient for drug delivery with multiple applications such as protection of the active pharmaceutical ingredient (API) from gastric acidity (Calinescu *et al.*, 2005; Calinescu *et al.*, 2007), drug controlled release (Lemieux *et al.*, 2009) or chronodelivery medication (Ispas-Szabo *et al.*, 2017). Very limited studies investigated aminoethyl starch (AESt) as a biomedical material (Mell *et al.*, 1968; Sakeer *et al.*, 2017b). AESt can be obtained by aminoethylation of starch by etherification with chloroethylamine hydrochloride. Some previous studies illustrated amphoteric starch carrying quaternary ammonium (QA) and phosphate groups (Lin *et al.*, 2012; Peng *et al.*, 2016) or QA and CM groups (Shimei *et al.*, 2006; Yang *et al.*, 2014c) or QA and succinate groups (Lekniute *et al.*, 2013) but the majority of these ampholytic derivatives were used as flocculants in environmental processes (Xu *et al.*, 2005a; Shimei *et al.*, 2006; Lekniute *et al.*, 2013; Yang *et al.*, 2014b; Yang *et al.*, 2014c; Peng *et al.*, 2016). Another approach consisted in a physical dry mixture of CMSt with polymers containing amine groups (i.e. chitosan) or the preparation of polyelectrolyte complex (PEC) obtained by co-processing which are able to sustain the release of neutral and acidic APIs in SIF at 20% drug loading (Assaad *et al.*, 2011).

In the present study, we are investigating a new class of ampholytic starch derivatives carrying both anionic CM and cationic AE groups on the starch backbone chains. In a previous study we investigated only one type of starch derivative as matrix for metformin delivery (Sakeer *et al.*, 2017a) while now we are exploring three types of

ampholytic starch obtained by three different methods using the same reagents. Considering the simultaneous presence of CM and AE functional groups and their eventual pH-dependent ionization on different dissolution media, it was of interest to understand the capacity of these excipients to control the liberation of various drugs. Thus neutral (acetaminophen), acidic (acetyl salicylic acid – ASA), basic (metformin) and zwitterionic (mesalamine) active molecules were used as tracers in monolithic tablets with the aim to evaluate the usefulness of the new class of excipients for drug formulation mainly for controlled delivery of high loaded dosage forms.

2. Materials and methods

2.1. Materials

High amylose starch (Hylon VII) was supplied by National Starch/Ingredion (Bridgewater, NJ, USA). Mesalamine (pharmaceutical grade) was a product of PharmaZell (Raubling, Germany). Metformin (1,1-dimethylbiguanide hydrochloride) was from MP Biomedicals (Solon, OH, USA) and acetylsalicylic acid (ASA) was from Fisher Scientific (Hampton, NH , USA). Acetaminophen and sodium monochloroacetate (SMCA) were from Sigma-Aldrich (Germany) and 2-chloroethylamine hydrochloride (CEAHC) was from Fluka (Switzerland). The other reagents were chemical grade and used without further purification.

2.2. Preparation of starch derivatives

To investigate the effect of ionic charges on the control of drug delivery, various starch derivatives were synthesized by two different procedures (Fig. 1):

A) One step (OS) approach: the two reagents sodium monochloroacetate (SMCA) and 2-chloroethylamine hydrochloride (CEAHC) were rapidly dissolved in water and then added simultaneously to gelatinized starch.

B) Two steps (TS) approach: the polymers were prepared in two ways by changing the order to introduce the functional groups: 1) the anionic (CM) groups were grafted first and then followed by cationic (AE) ones. 2) the cationic groups were first introduced on polysaccharidic chains followed by anionic ones. Thus AESt was prepared first by dissolving CEAHC in water and added to gelatinized starch. The obtained polymer was precipitated and then re-dissolved and reacted with SMCA in order to obtain AECMSt.

Practically, in OS method an amount of 12.50 g of starch was dispersed in 50 mL of distilled water at 60 -70 °C and then gelatinized with 75 mL of 5 M NaOH under continuous stirring for 1 h at 60 -70 °C. An amount of 9.37 g of SMCA was rapidly solubilized in a minimal volume of water. Separately, an amount of 9.37 g CEAHC was dissolved in a minimal water volume and rapidly added to gelatinized starch simultaneously with the SMCA continuing the stirring for 1 h maintaining the pH 9-10. Then, the preparation was cooled down and neutralized with glacial acetic acid. The carboxymethyl aminoethyl starch CMAESt (OS) powder was separated by precipitation from the slurry with an equivalent volume of methanol:water (70:30 v/v). The derivative was washed with methanol:water (70:30 v/v) until a final conductivity (Fisher Scientific Accumet Research AR20, San Diego, CA, USA) of filtrate decreased to less than 75 µS/cm. Pure methanol and acetone were used for final drying; the collected powder was left overnight at room temperature for complete air drying and sieved to obtain particles of less than 300 µm.

For the two steps (TS) method, the starch was first gelatinized as previously described for the OS procedure. To produce Carboxymethyl starch (CMSt), the proper amount of SMCA was quickly solubilized in a minimal water volume and added to the gelatinized starch maintaining the stirring for 1h at 60-70 °C at pH 9-10. Separately, the Aminoethyl starch (AEST) was obtained by rapidly solubilising the proper mass of CEAHC in water and then added to the gelatinized starch continuing

the stirring for 1h at 60-70 °C and pH 9-10. Then, the solution was cooled down and neutralized with glacial acetic acid. The polymer powders were obtained by precipitation with methanol and dried as described previously. The CMSt or AESt were each re-dispersed in 50 mL of distilled water followed by addition of 75 mL of 5 M NaOH and heated at 60-70 °C for 1 h under continuous stirring for gelatinization. Amounts of 9.37 g CEAHC (in case of CMSt), to obtain CMAESt (TS) or 9.37 g SMCA (in case of AESt), to obtain AECMSt (TS) were solubilized in a minimal water volume and added to gelatinized starch keeping the stirring for 1 h at 60-70 °C and pH 9-10. Then, the solutions were cooled down and neutralized with glacial acetic acid. The CMAESt (TS) and AECMSt (TS) powders were each obtained by precipitation with methanol and drying as described above.

2.3. Evaluation of the degree of substitution

A) The degree of substitution (DS) with CM groups was determined by back-titration as previously described by Stojanovic et al., 2005 (Stojanović *et al.*, 2005) with small modification. Briefly, 100 mg of polymer (n = 3) were solubilized in 10 mL of 0.05 M NaOH, and phenolphthalein was added as indicator. The excess of NaOH was titrated with 0.05 M HCl. The blank (10 mL of 0.05 M NaOH) was also titrated by the same method. The amount of –COOH groups and the DS were calculated **(Stojanović *et al.*, 2005)** using the equations (1, 2):

$$n = (V_b - V) \times C_{HCl} \qquad (1)$$

$$DS = \frac{162 \times n}{m - W \times n} \qquad (2)$$

where Vb (mL) is the volume of HCl used for the titration of the blank; V (mL) is the volume of HCl used for the titration of the sample; C_{HCl} (mol/L) is the concentration of HCl; 162 (g/mol) is the molar mass of glucose unit; W= (58) (g/mol) is the

increase in the mass of glucose unit by substitution with a CM- group, and m (g) is the mass of dry sample.

B) The degree of substitution (DS) expressed in terms of amino groups on the final derivative was determined with the ninhydrin reagent (2 g ninhydrin dissolved in 75 mL dimethylsulfoxide under nitrogen flushing and completed with 25 mL of 4 M lithium acetate buffer, pH 5.2). A volume of 0.5 mL of the ninhydrin reagent was added to a volume of 0.25 mL of ampholytic starch solution (15 mg/mL) in deionized water (in triplicate). The vials were immediately capped, vortexed, and heated in a covered boiling water bath for 30 min. Vials were then cooled below 30 °C in a cold water bath. The absorbance at 570 nm was measured on a UV/VIS spectrophotometer with zero set against a similarly treated blank of water. Glycine (Sigma) solutions (0.12 - 0.51 mg/mL) were used to generate a standard curve (Le Tien *et al.*, 2003; Le Dévédec *et al.*, 2008).

2.4. Zeta potential (ζ) and pH determination

Measurements of surface charge of various polymer solutions were performed using ZetaPlus/Bl-PALS (Brookhaven Instrument Corp, Holtsville, NY, USA). The measurements were carried-out at 25 °C. Analysis of the particle surface charge was done in triplicates. The pH of polymer solution 1% (w/v) in water at room temperature was measured by a Fisher Scientific Accumet Research AR20 pH-meter.

2.5. Viscosity determination

The viscosity of starch derivatives at 1 % (w/v) in water and in phosphate buffer pH 6.8 at 25 °C was measured on a Brookfield viscometer (DV-II pro viscometer, Middleboro, MA, USA). The experiment was done with a spindal CPE 40 at 10 rpm after 4 h and after 48 h.

2.6. Fourier transform infrared (FT-IR) analysis

The FT-IR spectra of native starch (Hylon VII) and of its derivatives as powders were recorded (64 scans at a resolution of 4 cm^{-1}) using a Thermo-Nicolet 6700 FT-IR spectrometer (Madison, WI, USA) equipped with a deuterated triglycine sulfate-KBr (DTGS-KBr) detector and a diamond smart attenuated total reflection (ATR) platform.

2.7. ^{1}H NMR measurements

To determine the starch functionalization pattern, ^{1}H NMR spectra were collected using a high-field 600 MHz, Bruker Avance III HD spectrometer running TopSpin 3.2 software and equipped with a 5 mm TCI cryoprobe. The temperature of samples was regulated at 27 °C. The samples were dissolved either in deuterium oxide (D_2O) or in deuterated dimethyl sulfoxide-d6 (DMSO-*d6*) with both methyl groups deuterated, then heated at 65 °C for 30 min, and kept at 4 °C for 2h.

2.8. Thermogravimetric analysis

Thermogravimetric analysis (TG) of the samples was carried out in platinum crucible at a heating rate of 10 °C/min between 25 and 900 °C under nitrogen atmosphere (flow rate 100 mL/min) using a TA® Instruments incorporated high-resolution thermo gravimetric analyzer Seiko TG/DTA 6200 (Japan).

2.9. Scanning electron microscopy

The microstructure of the sample particles was examined by a Hitachi (S-4300SE/N) scanning electron microscopy with variable pressure (Hitachi High Technologies America, Pleasanton, CA, USA) at voltage of 3-15 kV and magnifications of 100× and 1000×. Samples were mounted on metal stubs and sputter-coated with gold.

2.10. X-ray diffraction

X-ray diffractometry of polymers was performed using a Siemens D-5000 (Munich, Germany) device. The samples were exposed to X-ray radiation (Cu Kα at a wavelength of 1.789 Å) and a scanning rate of 0.05 °/min. Samples (ground into powders with an agate mortar and pestle) were loaded on a low background quartz plate in an aluminium holder and analyzed through a 2θ range of 5-50 degrees. The XRD spectra were treated using DiffracPlus software.

2.11. Micromeritics properties of starch derivatives powders

Micromeritics study a number of characteristics, including particle size, size distribution, particle shape, angle of repose, porosity, true volume, apparent density and bulkiness. The USP compendial methods were applied to investigate the flow properties by measuring the angle of repose according to < 1174 > USP powder flow procedure, whereas the bulk and tapped densities of the polymer powders were determined by calculating Carr's index and Hausner's ratio according to the < 616 > USP method, using a Vankel tapped density tester (Varian, NC, USA). The angles of repose of ampholytic starch powders were measured by using the fixed funnel method. Briefly, the ampholytic starch powder was poured into a funnel which was fixed at a position so that the outlet orifice of the funnel was 2 cm above a black surface. The powder was flowed down from the outlet orifice to form a cone on the surface and the angle of repose was then calculated by measuring the height of the cone (h) and the radius of its base (r) with the help of calibrated scale (Patil-Gadhe and Pokharkar, 2014). The angle of repose (Θ) was calculated in triplicate according to the equation (3)

$$\theta = tan^{-1}\left(\frac{h}{r}\right) \qquad (3)$$

To measure tapped density of the obtained powder a known mass of ampholytic starch powder was poured into a calibrated measuring cylinder and the volume occupied by this powder was recorded (n=3). The tapped density ρ_{tap} was determined by volume measurement of the tapped mass until no further changes in the powder volume were observed (Shah *et al.*, 2008). The Carr's index (Carr, 1965) and Hausner's ratio (Hausner, 1967) were calculated according to equations (4) and (5):

$$CI = \frac{\rho_{tab} - \rho_{bulk}}{\rho_{tab}} \times 100 \qquad (4)$$

$$HR = \frac{\rho_{tab}}{\rho_{bulk}} \qquad (5)$$

Where CI is Carr's compressibility index; HR is Hausner's ratio; ρ_{tap} is the tap density; and ρ_{bulk} is the bulk density.

2.12. Preparation of tablets

Monolithic tablets (with 60% drug loading) were obtained by direct compression (2.5 T/cm^2) of a homogenous mixture of excipient and drug powder with a total tablet weight of 833 mg for acetaminophen, metformin or aspirin and 667 mg in case of mesalamine using flat-faced punches with 12.95 mm diameter and a Carver hydraulic press Model C 3912 Hydraulic Cylinder (Wabash, IN, USA). The above loadings were chosen in line with commercial products for a better comparison.

2.13. Determination of the fluid uptake and erosion

Fluid uptake and erosion properties have been investigated with placebo tablets obtained by direct compression (2.5 T/cm^2) using flat-faced punches with 9.50 mm diameter. The choice of diameter of 12.9 mm for tablets with medication was to better fit with the commercial formulations. The diameter 9.50 mm was chosen for placebo tablets in order to better understand the role of polymeric excipients only.

The studies were carried out by immersing the weighed dry tablet (W1) in 40 mL of either simulated gastric fluid (SGF, pH 1.2) or simulated intestinal fluid (SIF, pH 6.8, phosphate buffer). The immersed tablets were incubated in 40 mL fluid and submitted to rotation at 50 rpm (Glas-Col rotator, Terre Haute, IN, USA). After 2, 4, 6, 8 h, tablets were withdrawn and carefully weighed (recorded as W_2). The recovered tablets were then placed into an oven at 35–40 °C for two days until a constant weight was obtained. The final weight of the tablets was measured (W_3) after complete drying at constant mass (Sakeer *et al.*, 2010a; Sakeer *et al.*, 2010b). Fluid uptake was determined according to Equation (6):

$$\%\, Weight\ change = \frac{W_2 - W_1}{W_1} X100 \qquad (6)$$

The degree of erosion was determined according to equation (7):

$$\%\, Erosion = \frac{W_1 - W_3}{W_1} X100 \qquad (7)$$

2.14. *In vitro* dissolution tests

The dissolution tests were carried out for all tablets either separately in (SGF) and in (SIF) dissolution media, or after incubation of tablets for 2 h in SGF followed by SIF. An USP paddle apparatus 2 was used. The dissolution volume (mL), the paddle speed (rpm) and wavelength (λ nm) were selected for each drug as per pharmacopeial requirements (USP, 2015): for mesalamine in SGF (500 mL, 100 rpm, λ_{300} nm) and in SIF (900 mL, 50 rpm, λ_{330} nm), for acetaminophen in SGF and SIF (900 mL, 50 rpm, λ_{243} nm), for metformin (1000 mL, 100 rpm, for SGF λ_{218} nm and for SIF λ_{232} nm), and finally for ASA in SGF (900 mL, 100 rpm, λ_{280} nm) and in SIF (λ_{227} nm). The concentrations of the released active molecules at different times were calculated based on a standard curve of each drug in SGF and in SIF.

2.15. Release kinetic patterns

The drug release kinetic parameters were evaluated according to: zero-order kinetics (Costa *et al.*, 2001), first-order kinetics (Costa *et al.*, 2001), Higuchi's model (Higuchi, 1961; Higuchi, 1963), Hixson–Crowell's model (Hixson and Crowell, 1931) and Korsmeyer–Peppas model (Korsmeyer *et al.*, 1983).

2.16. Statistical analysis

All tests were done in triplicate and data are reported as means ± SD. For statistical analysis, the one way ANOVA was followed by Fisher's post hoc tests with a minimum confidence level ($P < 0.05$) for statistical significance.

A.3. Results and discussions

A1 Structural insights

The hydroxyl groups on the glucopyranose ring are susceptible to react with the proposed reagents SMCA and / or CEAHC in the order C_2, C_6 and C_3. In ampholytic starch, the hydroxyl group can be involved in substitution with CM (CH_2COONa) groups after reaction with SMCA or substitution with AE ($CH_2CH_2NH_2$) groups by reaction with CEAHC, whereas secondary interactions between CEAHC and SMCA may produce ethylamino carboxymethyl (EACM) ($CH_2CH_2NHCH_2COONa$) or ethylamino dicarboxymethyl (EADCM) $CH_2CH_2N(CH_2COONa)_2$ groups. In the case of one step polymer preparation (Fig. 1) at least three different types of structures can theoretically occur: 1) CM and AE starch derivatives; 2) CM and secondary and/or tertiary amines, but exhibiting terminal aminoethyl (AE) groups; 3) ethylamine groups substituted with CM groups exhibiting AECM or $AE(CM)_2$ (EADCM). In two steps method, when CM groups are grafted firstly, followed by introduction of AE groups, one type of starch derivative is expected presenting CM and AE groups. Differently, for AECMSt, when AE groups are grafted first and then followed by CM

groups, there is a good probability of amine groups to be substituted with CM groups and to generate also EACM and EADCM groups (Fig. 1).

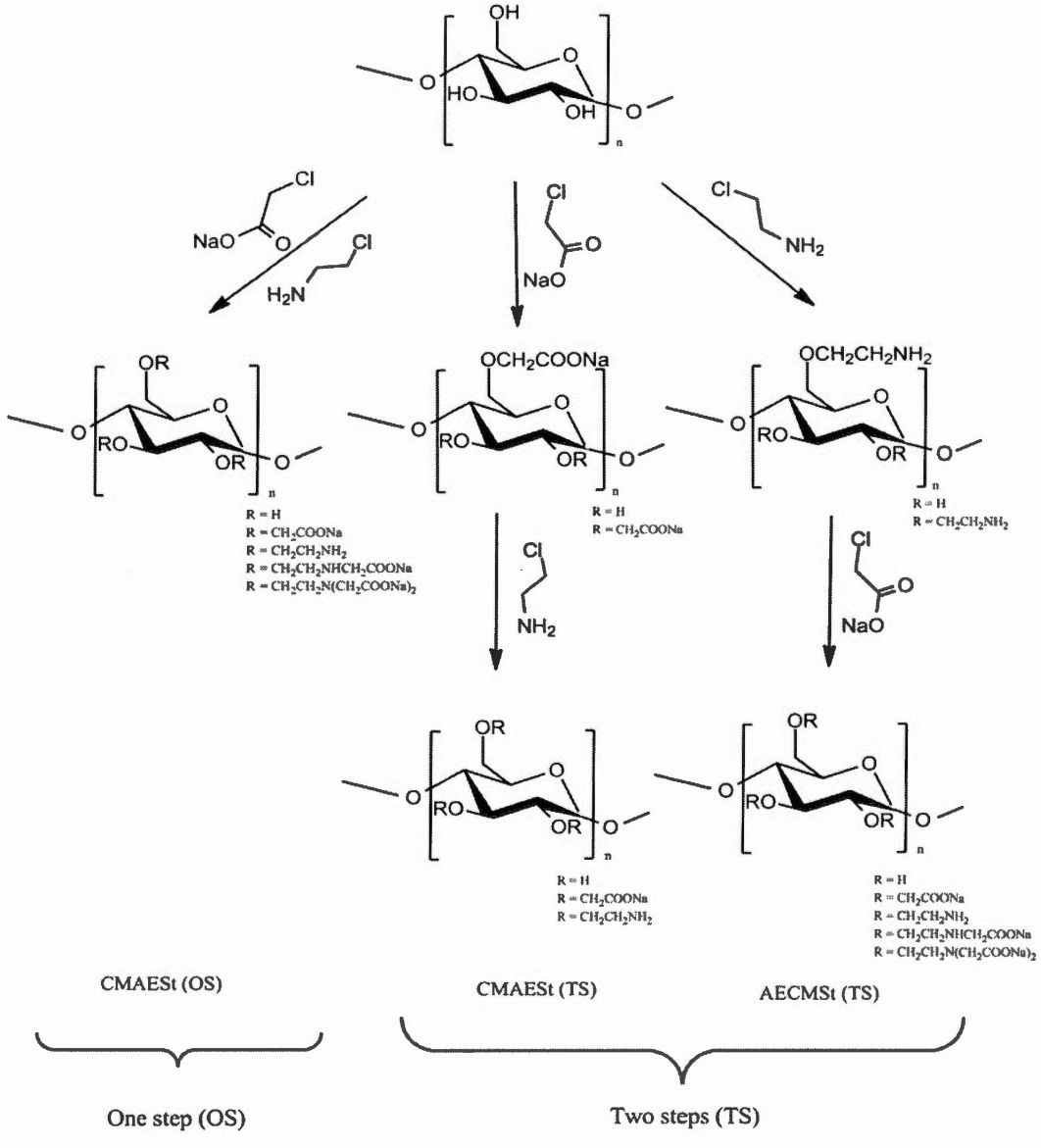


Fig. 1. Schematical presentation of starch derivatization with sodium monochloroacetate and with chloroethylamine hydrochloride to generate ampholytic starch carrying carboxymethyl (CM) and aminoethyl (AE) functions obtained by one step (OS) or two steps (TS) preparation methods.

The degrees of substitution (DS) with CM determined by back-titration of the ampholytic samples were 0.024, 0.041 and 0.028 for CMAESt (OS), CMAESt (TS) and AECMSt (TS), respectively. These DS values represented the average number of carboxymethyl groups per glucose unit. The DS in terms of primary amine (AE) groups were 0.012, 0.012 and 0.015 mmol/g for CMAESt (OS), CMAESt (TS) and AECMSt (TS), respectively. The ionic charge of the aqueous solutions of ampholytic starches calculated as zeta potential (ζ) was -25.9 mV for CMAESt (OS), whereas ζ values of -42.98 mV were found for CMAESt TS and -52.5 mV for AECMSt (TS). These values are consistent with the chemical modification of starch by CM, AE, EACM or EADCM groups. The highest value for AECMSt (TS) could be ascribed due to the higher amount of EACM and / or EADCM providing a stronger negative charge (Wongsagonsup *et al.*, 2005a; Wongsagonsup *et al.*, 2005b). For the 1% (w/v) polymer solution in water the pH values were 6.42, 6.2 and 6.9 for CMAESt (OS), CMAESt (TS), and AECMSt (TS) respectively, indicating that almost all ampholytic starch were neutral. The viscosity of CMAESt (TS) after 4 h in water was 8.34 cP, higher than that of AECMSt (TS) and of CMAESt (OS), whereas after 48 h all polymers showed almost similar viscosity. These values indicate that all polymers are hydrated almost at the same extent in water but with different rates.

Table 1: Viscosity (cP) of starch derivatives at 1 % w/v in distilled water (W) and in SIF measured after 4 h and respectively after 48 h, at 25°C.

	CMAESt (OS)		CMAESt (TS)		AECMSt (TS)	
Time/Media	W	SIF	W	SIF	W	SIF
4 h	7.94	7.57	8.34	2.82	7.75	2.88
48 h	8.95	5.82	8.77	3.00	8.41	3.00

On the other hand, CMAESt (OS) showed higher viscosity after 4 h and after 48 h in phosphate buffer pH 6.8 in comparison with CMAESt (TS) and AECMSt (TS) probably due to the higher solubility of both CMAESt TS and AECMSt in phosphate

buffer, whereas CMAESt (OS) undergo swelling rather than solubilization in SIF (Table 1).

A2 **Fourier transform infrared (FT-IR) analysis**

For starch and its derivatives (Fig. 2), a broad band (3200-3400 cm^{-1}) with a maximum at 3296 $cm^{-1}$is due to the stretching vibrations of -OH, and a small band at 2927 cm^{-1} is attributed to the -CH stretching vibration. The band at 1079 cm^{-1} was ascribed to CH_2-O-CH_2 stretching vibrations. The bands at 1589 cm^{-1} and at 1417 cm^{-1} were ascribed to $-COO^-$ group (Stojanović *et al.*, 2005; Ispas-Szabo *et al.*, 2017). The higher intensity in case of CMAESt (TS) and AECMSt (TS) could be due to higher amount of carboxylate groups. The high intensity of the band at 999 cm^{-1} could be ascribed to C-N stretching vibrations, whereas the weak shoulder between 1630-1735 cm^{-1} could be assigned to –NH bend (Deng *et al.*, 2006; Assaad *et al.*, 2011). The presence of these additional bands confirms the grafting of CM, AE, EACM or EADCM onto the starch backbone.

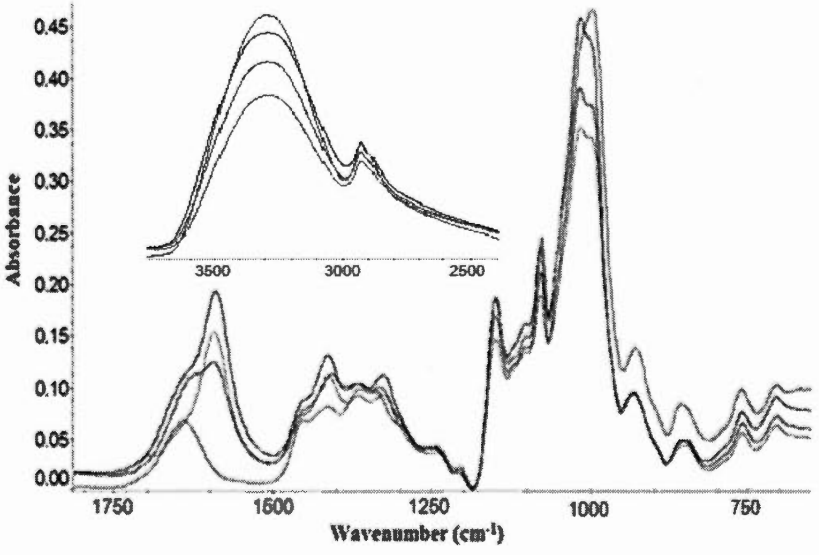


Fig. 2. FT-IR spectra of Native Starch (red), CMAESt (OS) (blue), CMAESt (TS) (black) and AECMSt (TS) (green).

A3 ^{1}H NMR measurements

Fig. 3 (I, II) present the ^{1}H NMR spectra of the native starch (Hylon VII) and its ampholytic derivatives in D_2O and in DMSO-d6, respectively.

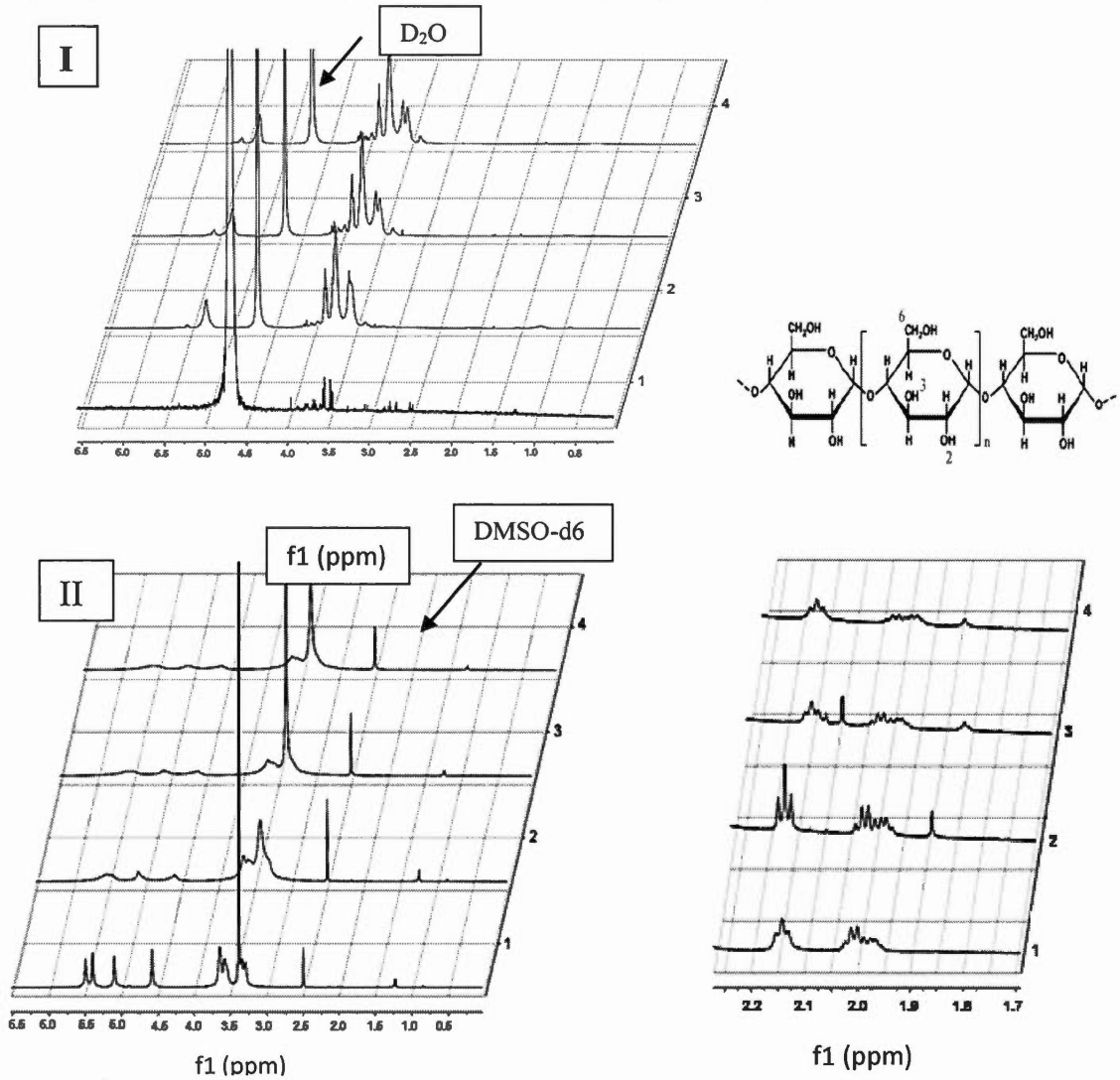


Fig. 3. ^{1}H NMR spectra in D_2O (I) and in DMSO-d6 (II) for Native Starch (1), CMAESt (OS) (2), CMAESt (TS) (3) and AECMSt (TS) (4).

When D_2O was used as a solvent, the proton signals at 5.3 ppm were ascribed to H1 and those at 3.3–3.9 ppm were assigned to H2-6 (Yang *et al.*, 2014b). The most significant peaks at δ = 2.17-2.20, δ = 3.16-3.18 were found for ampholytic starch

derivatives and not for native starch. They belong to the hydrogens of aminoethyl groups (Noga *et al.*, 2012; Amar-Lewis *et al.*, 2014). The lower intensity of starch pattern is due to the limited solubility of starch in D_2O. With DMSO-d6 as solvent the peaks were sharp. The peak at 3.31 ppm was ascribed to H2, and those at 3.36 ppm to H4, at 3.57 ppm to H5 and at 3.64 ppm to H3. The chemical shifts of –OH(2), –OH(3), and –OH(6) were possibly at origin of peaks between 4.58 and 5.50 ppm, whereas the proton signals at 5.3 ppm were assigned to H1(Namazi *et al.*, 2011; Yang *et al.*, 2014b). The most significant peaks are at $\delta = 3.16 - 3.18$, which belong to the hydrogens of aminoethyl group. The peak at $\delta = 1.6$ is related to the methylene group of CM (Namazi *et al.*, 2011).

A4 **Thermogravimetric analysis**

The thermogravimetric pattern (TGA and DTG curves) of native starch and of its derivatives investigated in a temperature range of 25 °C to 600 °C are presented in Fig. 4. All samples showed a two-steps weight loss below 600°C. The first one was minor and seems to correspond to the loss of intramolecular and intermolecular water around 50–110 °C. The second one may be related to material decomposition (Li *et al.*, 2010; Zhang *et al.*, 2014). Water is considered one of the main products of decomposition at temperatures below 270 °C. Further heating up to 600 °C resulted in carbonization and ash formation. The maximal decomposition temperature (MDT) was 291 °C for AECMSt (TS), whereas MDT for CMAESt (OS) was 294 °C and MDT for CMAESt (TS) was 293 °C. All values are lower than the MDT of native Starch (324 °C). The reason seems to be the substitution of hydroxyl groups of starches with CM and / or AE and / or EACM or EADCM groups. By calculating the area under the curve (AUC) for the main stage of polymer degradation, the decomposed component could be qualitatively determined. The AUC of the main decomposition stage of native starch was 80.6 and it is higher than AUC of other derivatives, which means that native starch is decomposed as a function of

temperature at a different pattern than other ampholytic starch derivatives, due to their functionalization starch with CM, AE, EACM or EADCM groups. The AUC for CMAESt (TS) and AECMSt (TS) are very close each other, suggesting that similar amounts of resembling components are decomposed as a function of temperature. (Tiwari and Hihara, 2009; Tiwari and Hihara, 2012).

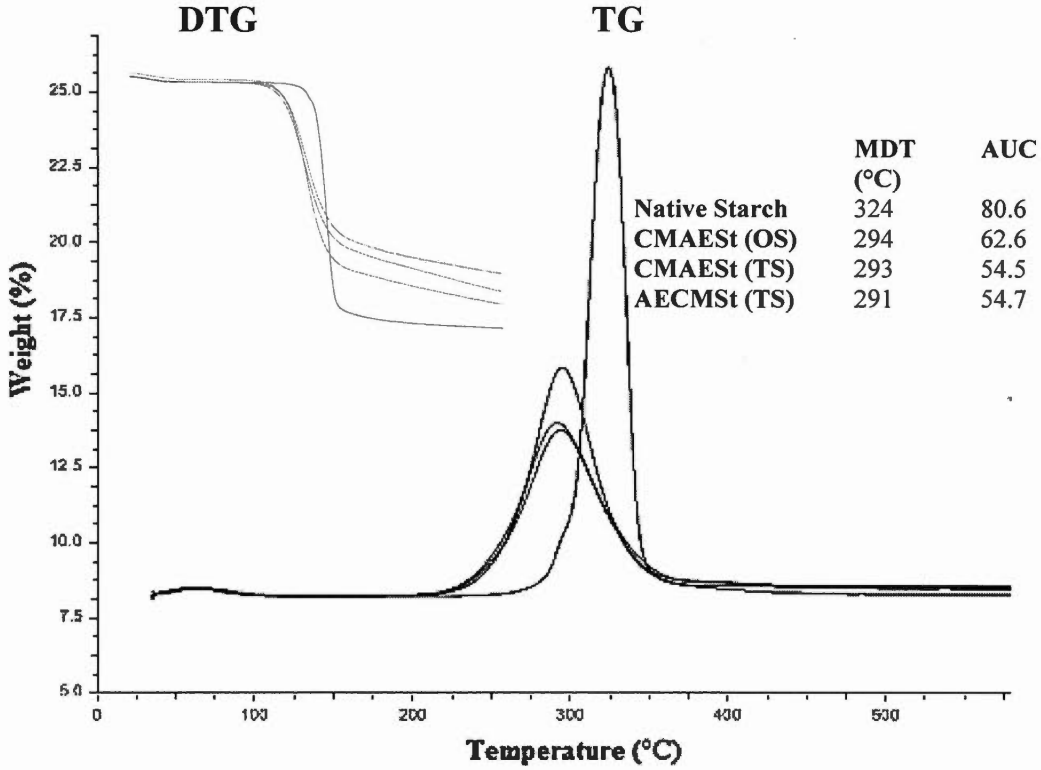


Fig. 4. Thermogravimetric (TG) and its derivative (DTG) diagrams of Native Starch (black), CMAESt (OS) (blue), CMAESt (TS) (green) and AECMSt (TS) (red).

A5 **X-ray diffraction**

X-ray diffraction of native starch (Hylon VII) powder and of its derivatives (Fig. 5) showed the starch as the most ordered organization with diffraction peaks at 2θ = 16.78°, 17.48°, 19.84°, 25.88° corresponding to a type B structure and at 2θ = 23.02°, 13.38° corresponding to a V type structure. By derivatization, a pronounced reduction

in order degree was observed with peaks decreased or disappeared (those at 2θ = 19.84°, 25.88°), suggesting the loss of the B-type double helix, possibly due to the effect of derivatization of the hydroxyl groups with either CM groups (Wang *et al.*, 2010; Gao *et al.*, 2011) or AE groups (Kuo and Lai, 2007; Pi-xin *et al.*, 2009; Chang *et al.*, 2014) or EACM or EADCM groups. However, hydrogen bonds contributed to maintain the starch order in a certain extent. The CMAESt (OS) pattern presented sharper peaks at 2θ = 13°-16° and 2θ = 21°-25° than CMAESt (TS) or AECMSt (TS).

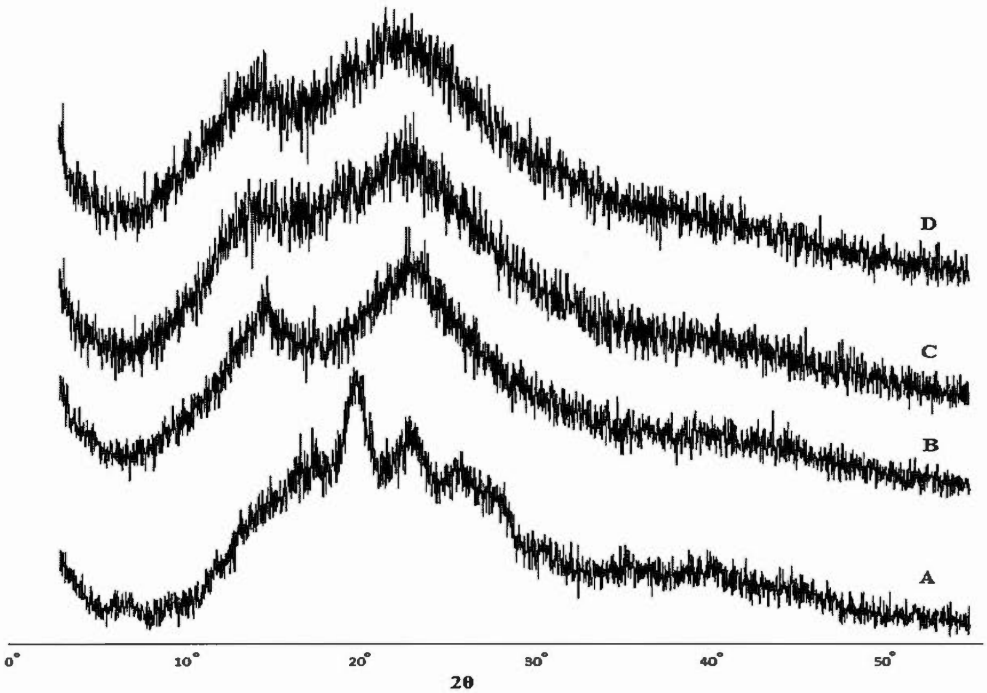


Fig. 5. X-ray diffraction patterns of Native Starch (A), CMAESt (OS) (B), CMAESt (TS) (C) and AECMSt (TS) (D) powders.

A6 **Scanning electron microscopy**

The aspects of native starch (Hylon VII) and of CMAESt (OS) of CMAESt (TS) and of AECMSt (TS) derivatives are different (Fig 6) depending on modifications operated on the starch structure.

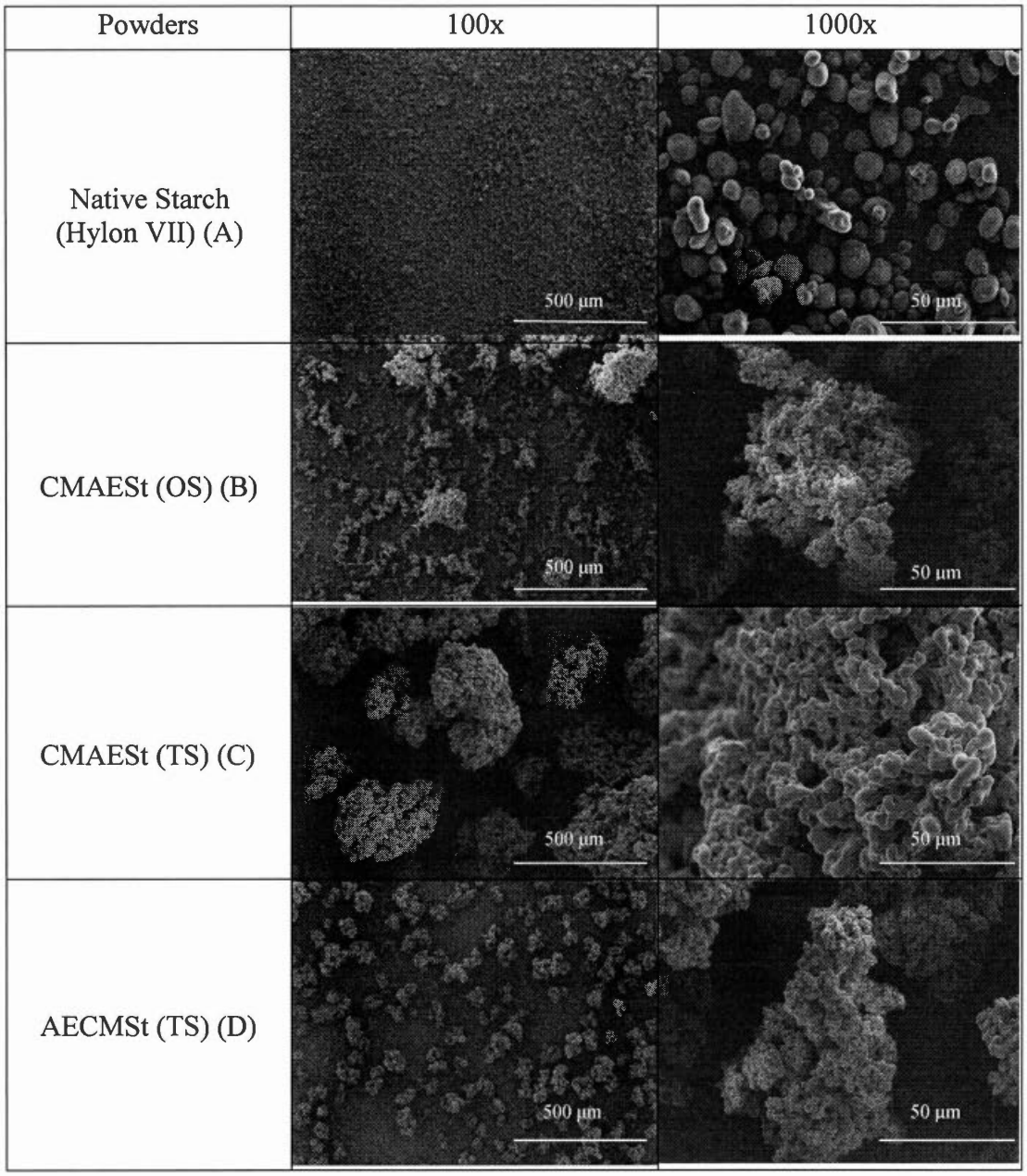


Fig. 6. Scanning electron microscopy micrographs of Native Starch (A), CMAESt (OS) (B), CMAESt (TS) (C) and AECMSt (TS) (D) powders at magnifications of 100x and 1000x.

The native starch (Hylon VII) granules (Fig. 6 A) were predominantly round or oval in shape, with a smooth surface. This seems due to the high content of amylose (more than 70%), which is more crystalline, known to exhibit double helix-B form and to be strongly stabilized by hydrogen associations between the hydroxyl groups of glucopyranose units (Friciu *et al.*, 2013). The CMAESt (OS) is characterized by an irregular shape with an uneven surface likely due to the association of numerous small particles forming larger granules (Fig. 6 B). The presence of carboxylic groups probably reduced the network self-assembling by hydrogen associations between hydroxyl groups and promoted repulsion effects leading to the reorganization of the polymeric matrix (Lemieux *et al.*, 2010). Smoother surfaces were observed Fig. 6 C, D. in case of CMAESt (TS) and of AECMSt (TS) which might be due to having higher DS of CM (Sakeer *et al.*, 2017a).

A7 **Micromeritic properties of starch derivatives powders**

The flow properties of the powder formulations are important parameters from industrial perspective because improper flowability may lead to problems in storage of excipients, handling and processing operations. The micromeritic properties were assessed by determining angle of repose (Θ), Carr's index (CI) and Hausner's ratio (HR) and the results are summarized in Table 2.

Table 2: Micromeritic properties of starch derivatives powders

	θ (°)	**CI (%)**	**HR**
CMAESt (OS)	38.30	38	1.6
CMAESt (TS)	33.02	26	1.35
AECMSt (TS)	27.92	17	1.2

The angles of repose for all the obtained starch derivatives powders were found to be in the range of 27–38 °, whereas CI and HR were in the range of 17–38% and 1.2–1.6, respectively. The angle of repose less than 30° indicates excellent flow

properties, whereas a value of 56° indicates 'very poor' flow. Particles with high internal friction or cohesion present an increased angle of repose. Moreover, CI and HR should be less than 20% and 1.20, respectively, to ensure optimal flowability for powders (Singh *et al.*, 2013). AECMSt (TS) showed the lowest angle of repose (27 °), Carr's index (17%) and Hausner's ratio (1.2) providing the best micromeritic properties among all derivatives.

A8 **Determination of the fluid uptake and erosion**

Fluid uptake and erosion tests in SGF and in SIF separately, are shown in Fig. 7 I, II. It was noticed that AECMSt (TS) has a better ability to absorb SGF fluid and generates a higher swelling compared to CMAESt (OS) and CMAESt (TS). In SIF, CMAEST (OS) showed higher fluid uptake and hence maintain the integrity of the tablet. On the contrary, AECMSt (TS) tablets were totally solubilized after 6 h. The erosion of tablets formulated with AECMSt (TS) in SGF was lower than that of CMAESt (OS) and of CMAESt (TS). On other hand, in SIF, the solubility of CMAESt (OS) was limited and thus its erosion was slower than that of CMAESt (TS) and of AECMSt (TS). The radial swelling (dimension changes, mm) of tablets was also observed in SGF and SIF (Fig. 7 III,IV). It appeared that CMAESt (OS) and CMAESt (TS) have almost same swelling in SGF. On the contrary, AECMSt (TS) showed the lower swelling in SIF, due to its simultaneous higher solubility in SIF. Figure. 8 (I,II,III) presents the fluid uptake, erosion and diameter changes of tablets containing only the ampholytic starch derivatives (excipient-free) first incubated 2 h in SGF and then moved to SIF. The CMAESt (OS) showed the highest ability of fluid uptake (Fig 8 I) with lower erosion pattern (Fig 8 II) due to its limited solubility in SGF and in SIF. Differently, AECMSt (TS) showed the lower tendency to swell and higher erosion behaviour due to its solubility in SIF.

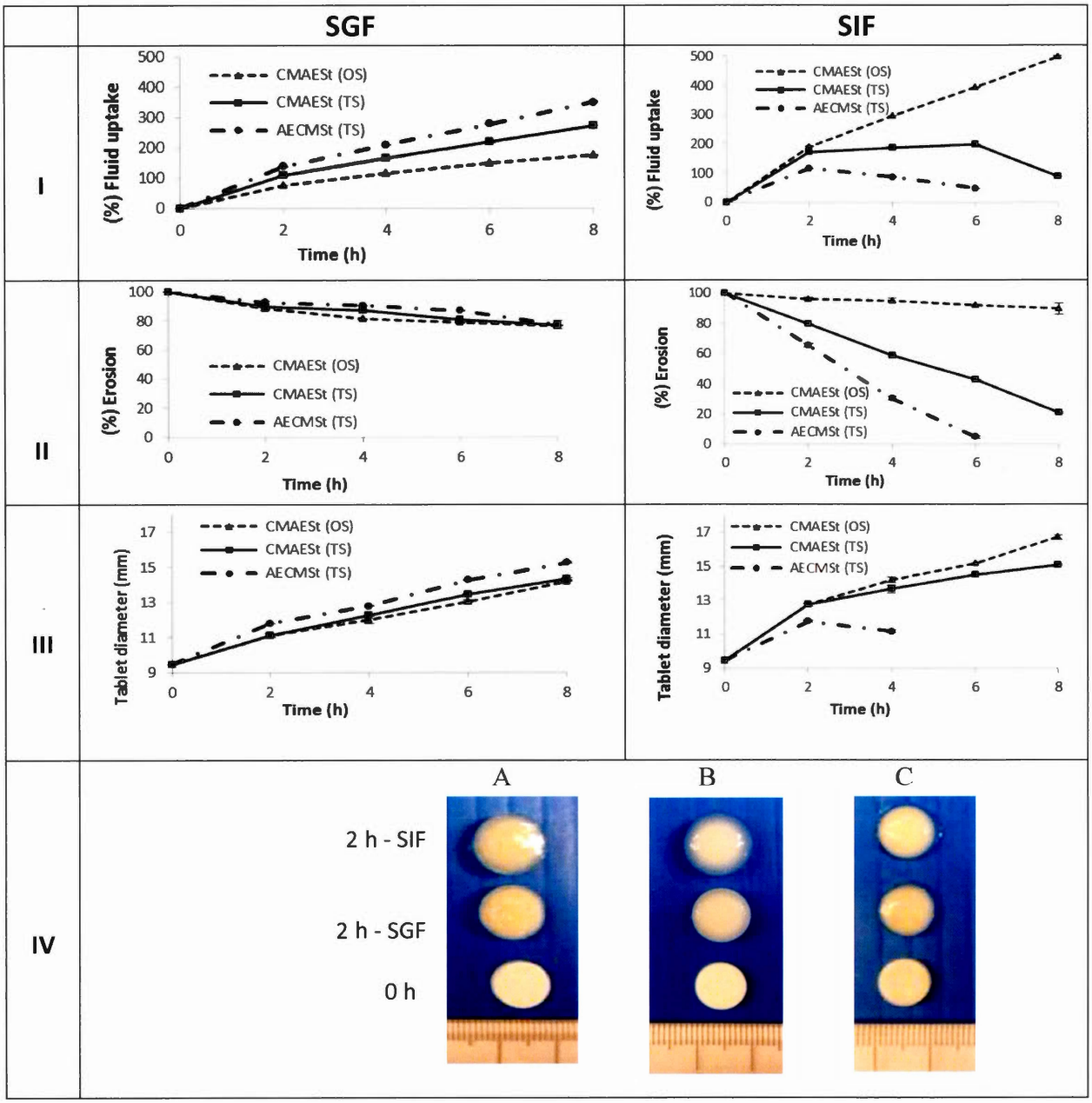


Fig. 7. Fluid uptake (**I**), erosion (**II**) and radial tablet swelling (diameter changes) (**III**) of drug-free tablets formulated with CMAESt (OS), CMAESt (TS) and AECMSt (TS) as matrix forming excipients in SGF or in SIF. **(IV)** Photographs of tablets based on CMAESt (OS) (**A**), CMAESt (TS) (**B**) and AECMSt (TS) (**C**) at time zero (0 h, dry phase), after 2 h incubation in SGF and after 2 h in SIF.

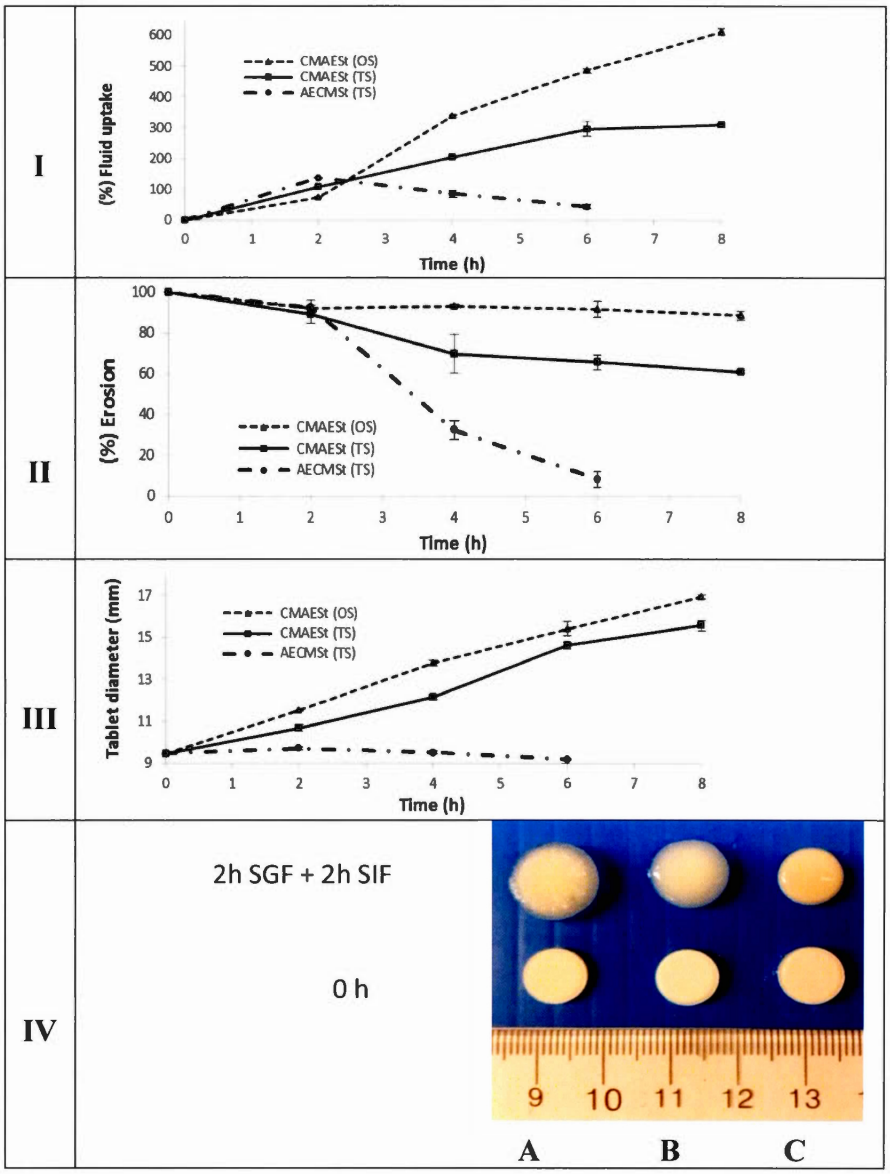


Fig. 8. Fluid uptake (**I**), erosion (**II**) and radial tablet swelling (**III**) of a drug-free tablet formulated with CMAESt (OS), CMAESt (TS) and AECMSt (TS) as a matrix forming excipients incubated 2 h in SGF and then in SIF for 6 h. **(IV)** Photographs of tablets formulated with CMAESt (OS) (**A**), CMAESt (TS) (**B**) and AECMSt (TS) (**C**) after incubation for 2 h in SGF and then for another 2 h in SIF.

The highest fluid uptake and limited erosion for CMAEST (OS) was in accordance with the highest dimension changes (Fig 8 III). CMAESt (OS) presented limited erosion in SGF but when moved to SIF a gel layer was formed on the outer surface of the tablet. Differently, the CMAESt (TS) tablets formed a compact gel in SGF which contributed to keep the integrity of the tablets and prevented further fluid penetration and dissolution of the hydrated gel layer formed on the outer surface of the tablet (Fig 8, IV) when the tablet moved to SIF. The fluid uptake and erosion reflected well the supposed structures of the ampholytic matrices in function of the processing of derivatization. For instance, the better absorption of SGF by AECMST (TS) suggests a preferential carboxymethylation (step II) of the AE-groups already grafted in step I to give AECM or $AE(CM)_2$ groups, rather than of initial hydroxylic groups of starch. Similarly, for the higher substituted AECMSt (TS), the high SIF uptake can be explained by the extern location of carboxylic groups in structures type ACEM and $AE(CM)_2$, in detriment of amine groups (which would generate immiscibility in SIF). Thus, for this ampholytic derivative, due to carboxymethylation (step II), there are less primary amine groups but more carboxylic groups, when compared with the CMAEST (OS) and with the CMAEST (TS). This fits the higher reactivity of primary amine groups than that of hydroxylic groups in nucleophilic substitution type SN1. Furthermore, it is also expected during the first derivatization with chloroethyl amine, to have a continuation of substitution at level of primary amine groups to generate secondary and tertiary aminations, with a pattern of limited dendrimer which may be controlled by the ratio of chloroethylamine: starch. Moreover, irrespective of the type of amination, each graft will present primary amine groups that are susceptible to the first carboxymethylation step. Details of this process will be dealt with in another report.

A9 *In vitro* dissolution tests

Ampholytic starch excipients were tested in terms of controlled drug release from high loaded formulations. Monolithic tablets with various drug loading (60%) were prepared and the dissolution patterns were first followed in SGF and SIF, separately (Fig. 9). To find out the effect of drug properties on the behavior of ampholytic starch excipients, four tracers with different characteristics: ASA (acidic drug), metformin (basic drug), mesalamine (amphoteric drug), or acetaminophen (neutral drug) were selected to be formulated by direct compression of mixed powders directly with each ampholytic starch excipients and further evaluated for dissolution behaviour. Aspirin is an acetyl derivative of salicylic acid, with an acid dissociation constant pKa 3.5 at 25 °C (Dressman *et al.*, 2012; Suwalsky *et al.*, 2013). Metformin (**Table 3)** has acid dissociation constant values pKa of 2.8 and 11.5 and exists very largely as cationic species (hydrophilic chlorohydrate) at physiological pH values. The metformin pKa values make metformin a stronger base than most other basic drugs (Desai *et al.*, 2014a). Mesalamine (5-ASA) exhibits amphoteric properties (**Table 3**; its solubility increases at acidic pH values (pH < 3) in the stomach and at more alkaline values (pH > 5.5) in the lower part of the small intestine (French and Mauger, 1993). Acetaminophen, an extensively conjugated system, consists of a benzene ring substituted by one hydroxyl group and an amide group in the para position (1,4). The conjugation greatly reduces the basicity of the nitrogen, while making the hydroxyl acidic through delocalisation of charge developed on the phenoxide anion (Kääriäinen *et al.*, 2017) **Table 3**. The cumulative ASA release from tablets in SGF and in SIF are shown in Fig. 9, I. Both CMAESt (TS) and AECMSt (TS) derivatives were able to control the release of ASA in SGF, where 55 %, of ASA was released within 24 h by tablets of CMAESt (TS) and 47 % of ASA released by AECMSt (TS). In SIF only CMAESt (TS) was able to prolong the release of ASA for more than 12 h. Fig. 9, II shows the release of metformin in SGF and in SIF.

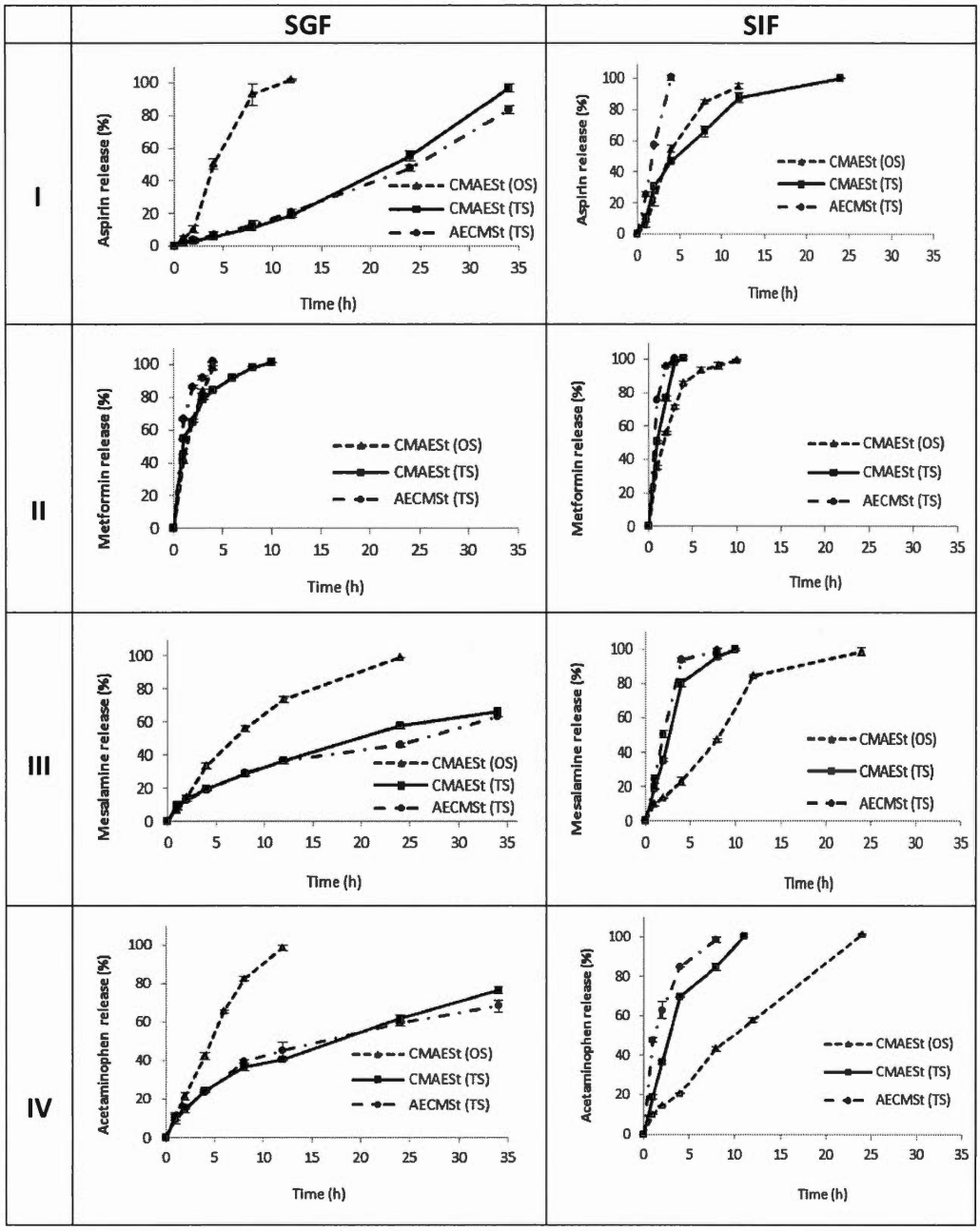
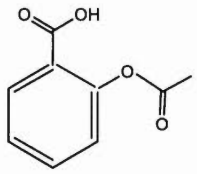
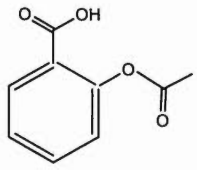
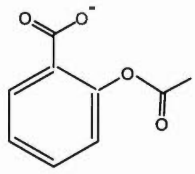
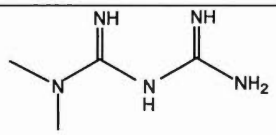
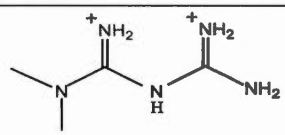
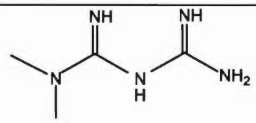
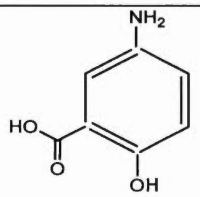
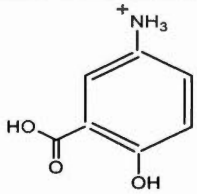
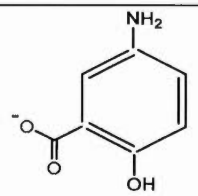
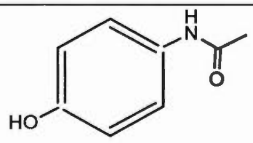
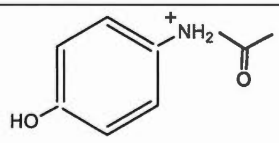
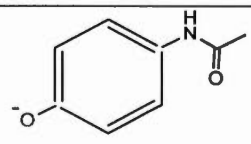


Fig. 9. Release profiles of Aspirin (I), Metformin (II), Mesalamine (III) and Acetaminophen (IV) in SGF or in SIF from tablets (60% loading) formulated with CMAESt (OS), CMAESt (TS) and AECMSt (TS) as a matrix forming polymers.

Controlling the release of metformin is still a challenge for formulator due to its high solubility and aqueous solution pH 1.2 to 6.8 (Desai *et al.*, 2014a). Because of its short half-life (< 3 h), frequent administrations of high dosages (up to 2.5 g daily) are needed to maintain its required plasma concentration (Garber *et al.*, 1997; Stepensky *et al.*, 2001; Qin *et al.*, 2014). It was found with our ampholytic starch that 92 % of metformin was released within 6 h in SGF from tablets contained CMAESt (TS) as matrix forming excipients, whereas 93 % was liberated within 6 h SIF from CMAESt (OS).

Table 3: Structures of bioactive molecules in neutral, SGF, and SIF media.

	Structure	SGF	SIF
Aspirin			
Solubility		7.01 mg/mL	43.6 mg/mL
Metformin			
Solubility		300 mg/mL	300 mg/mL
Mesalamine			
Solubility		18.2 mg/mL	8.4 mg/mL
Acetaminophen			
Solubility		20.3 mg/mL	20.3 mg/mL

The release of mesalamine in SGF and SIF is presented in Fig. 9, III. In SGF, all ampholytic starch polymers were able to prolong the release of mesalamine over 12 h. In SIF only CMAESt (OS) and CMAESt (TS) prolonged the release of mesalamine to 10 h. Fig. 9, IV shows the release of acetaminophen in both SGF and SIF. In SGF, CMAESt (TS) and AECMSt (TS) sustained the release of acetaminophen with 61.69 % liberated from tablets of CMAESt (TS) and 59.3 % acetaminophen liberated from tablets of AECMSt (TS) within 24 h. In SIF CMAESt (OS) released 57.75 % of acetaminophen within 12 h, while CMAESt (TS) released 84.5 % of acetaminophen within 8 h. In conclusion CMAESt (OS) was able to control the release of amphoteric or neutral drugs only in SIF. From the polymers prepared in two steps procedure either CMAESt (TS) or AECMSt (TS) was able to sustain the release of acidic, amphoteric, or neutral drugs in SGF. Differently in SIF, CMAESt (TS) were able to better control the release of medication in comparison with AECMSt (TS). Furthermore, to find out the impact of whole gastrointestinal tract (GIT) on the polymeric matrices, the tablets were first exposed to SGF for 2 h and then transferred to SIF at the same condition of dissolution test Fig. 10. The release profiles showed that all ampholytic starch excipients were able to control the release of acidic, basic and neutral drugs at high loading (60 %). In case of ASA and acetaminophen, all ampholytic starch derivatives showed similar behaviour in controlling of the release of active principles (Fig 10, Table 4). For metformin only the CMAESt (TS) was able to slower the release for a duration up to 6 h. The ability of CMAESt (TS) to prolong the release of metformin could be correlated with the higher DS of CM groups, 0.041 compared with DS 0.024 for CM groups in CMAESt (OS) and DS 0.028 of AECMSt (TS).

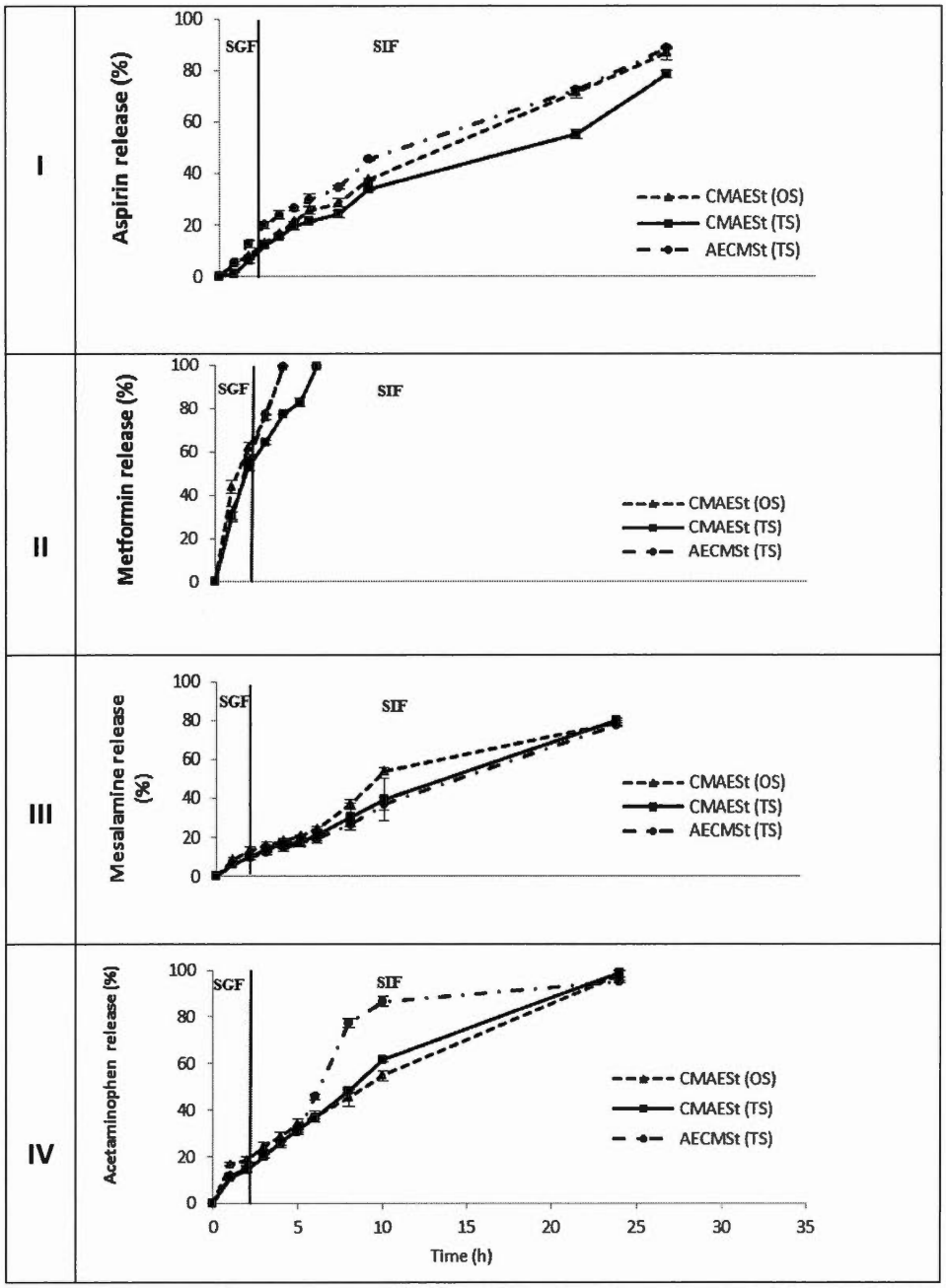


Fig. 10. Release profiles of Aspirin (I), Metformin (II), Mesalamine (III) and Acetaminophen (IV) from tablet (60% loading) formulated with CMAESt (OS), CMAESt (TS) and AECMSt (TS) as matrix forming polymers followed for 2 h in SGF and then transferred to SIF.

Another explanation may be the formation of a kind of internal stabilization between carboxylic groups and the amine groups of ampholytic matrix. This is less probable in term of AECMST (TS), where carboxyl groups are probably lower in peripheral layer and less accessible to form complex. Evaluation of the release profile of metformin from tablets loaded on CMAESt (TS) prepared at different DS of CM or AE groups recently published(Sakeer *et al.*, 2017a), showed that CMAESt (TS) at higher DS of both CM and AE can modulate the release of metformin up to 12 h (Sakeer *et al.*, 2017a).

Table 4: Time (h) of 90% drug release from monolithic tablet contained ampholytic starch derivatives as matrix forming agents in SGF, SIF and SGF followed by SIF.

Active molecule	CMAESt (OS)			CMAESt (TS)			AECMSt (TS)		
	SGF	SIF	SGF +SIF	SGF	SIF	SGF +SIF	SGF	SIF	SGF +SIF
Aspirin	8h	12h	30h	33h	17h	33h	> 35h	3.8h	30h
Metformin	3.5h	8h	3.5h	5.8h	2h	5.5h	3h	1.5h	3.5h
Mesalamine	19.5h	20h	28h	> 35h (60%)	6h	28h	> 35h (60%)	3h	28h
Acetaminophen	10h	21h	22h	> 35h (70%)	10h	22h	> 35h (70%)	7h	16h

In case of Mesalamine, CMAESt (OS) exhibits less variability with media changes compared with the other two derivatives synthesized via TS procedure. Knowing that Mesalamine is a zwitterion molecule carrying amino and carboxylic groups (Table 3) that will be ionized in SGF and SIF and potential drug-polymer ionic interactions can occur either with CM or AE ionized groups. This could explain similar release profiles irrespective to the media changes (Fig 10, Table 4). Same rationale seems not be applicable for CMAESt (TS) and AECMSt (TS) derivatives obtained by the two steps procedure. It is worth to note that both TS derivatives were more substituted (one for CM and the other for AE) compared with OS derivative where the DS for the two groups were close each other and this is reflected in higher zeta potential values.

Less control observed when tablets were exposed to SIF only (3 h and respectively 6 h) could be explained by repulsive forces between mesalamine and charged derivatives. The 90 % drug release times were in line with the solubility data of the active principles. For instance the shortest release time was found for metformin which presents the highest solubility (table 3), whereas long release times (28-30 h) were found for mesalamine and aspirin which both present relatively low solubility. For acetaminophen, intermediate release time 16-22 h fitted well its solubility in gastric and in intestinal fluids. Although relatively fast in comparison with the release of acetaminophen, aspirin and mesalamine, the release time of 5.5 h for metformin seems adequate for this medication to be liberated in the intestinal tract including the colon from monolithic tablets obtained with CMAESt (TS). The release kinetics of the four tracers in SGF and in SIF was evaluated based on the equations presented in Table 1 S, where Q_t in equations A, B, C and E is the amount of the drug released at time t, Q_t in equation D is the amount of drug remaining in pharmaceutical dosage form at time t and Q_0 is the initial amount of drug in the pharmaceutical dosage forms. The K_0, K_1, K_H, Ks, K_P are respectively the zero-order, first-order, Higuchi's, Hixson–Crowell's, Korsmeyer–Peppas release rate constants and n is the release exponent. If the n value is 0.5 or less, the release mechanism follows a Fickian diffusion, whereas at higher values for mass transfer in the range $0.5 < n < 1$, the system will follow a non-Fickian model (anomalous transport). The system follows a zero-order drug release and a case-II transport if the n value is equal to 1. Here the relaxation process of the macromolecules occurring upon water imbibition into the system is the rate- controlling step. For the values of n higher than 1, the mechanism of drug release is regarded as super case-II transport (Korsmeyer *et al.*, 1983).

The kinetic release profiles of ASA in SGF and in SIF for all polymers was fitting zero order model and first order model respectively, with the Korsmeyer's release exponent $n>0.5$ which can be related to case-II transport Table 1 S. Due to the high loading (60%) and solubility of metformin, the proposed polymeric excipients were

not able to prolong the release of metformin for a duration longer than the commercial products (Sakeer *et al.*, 2017a).

Table 5: Kinetic parameters of Aspirin (i), Metformin (ii), Mesalamine (iii), and acetaminophen (iv) from tablets (60% loading) formulated with CMAESt (OS) (I), CMAESt (TS) (II) and AECMSt (TS) (III) as a matrix forming excipients in SGF then transferred to SIF.

Dosage forms		Kinetic analysis										
		A		B		C		D		E		
		$Q_t = Q_0 + K_0 t$		$\log Q_t = \log Q_0 + \frac{K_1 t}{2.303}$		$Q_t = K_H t^{1/2}$		$Q_0^{1/3} - Q_t^{1/3} = K_s t$		$Q_t = K_p t^n$		
		K_0	r^2	K_1	r^2	K_H	r^2	Ks	r^2	n	KP	r^2
I	i	0.06	**0.993**	-0.008	0.990	1.69	0.96	0.004	0.992	0.95	0.14	0.97
	ii	-	-	-	-	-	-	-	-	-	-	-
	iii	0.05	0.93	0.006	0.992	0.018	**0.993**	0.002	0.96	0.85	0.60	0.98
	iv	0.05	**0.990**	-0.001	0.98	1.9	0.96	0.002	0.98	0.59	1.81	0.95
II	i	0.078	0.989	-0.009	0.993	1.9	**0.995**	0.006	0.897	1.15	0.5	0.96
	ii	0.21	0.95	-0.005	**0.99**	5.5	0.98	0.005	0.90	0.55	5.8	0.95
	iii	0.053	**0.993**	-0.006	0.979	1.28	0.98	0.002	0.93	0.79	0.65	0.98
	iv	0.06	0.95	-0.002	0.990	2.3	0.96	0.003	**0.992**	0.81	0.58	0.96
III	i	0.044	0.97	-0.009	0.95	2.2	**0.98**	0.005	0.89	0.79	0.78	0.97
	ii	-	-	-	-	-	-	-	-	-	-	-
	iii	0.052	0.994	-0.005	0.997	1.14	**0.997**	0.002	0.96	0.79	0.41	0.97
	iv	0.06	0.71	-0.001	0.885	2.7	0.86	0.004	**0.97**	0.98	1.13	0.98

The kinetic equation was applied only for CMAESt (OS) in SIF and for CMAESt (TS) in SGF. In both cases the drug release was fitting the first order model with the Korsmeyer's release exponent n close to 0.5 suggesting that the release is matching non-Fickian model (anomalous transport). The release of mesalamine was following different models in different dissolution media. The Korsmeyer's release exponent n was close to 0.5 in SGF indicating that the system fits well to Fickian diffusion. In SIF, n values were close to 1 (Table 1 S) matching to non-Fickian model (anomalous transport). CMAESt (TS) was able to control the release of acetaminophen in SGF and in SIF and the release was fitting first order model in both media.

Table 1 S: Kinetic parameters of Aspirin (i), Metformin (ii), Mesalamine (iii), and Acetaminophe (iv) released in SGF and in SIF from tablets (60% loading) formulated

with CM-AE-St (OS) (I), CM-AE-St (TS) (II) and AE-CM-St (TS) (III) as matrix forming excipients.

Dosage forms			Kinetic analysis										
			A		B		C		D		E		
			$Q_t = Q_0 + K_0 t$		$\log Q_t = \log Q_0 + \frac{K_1 t}{2.303}$		$Q_t = K_H t^{1/2}$		$Q_0^{1/3} - Q_t^{1/3} = K_s t$		$Q_t = K_p t^n$		
			K_0	r^2	K_1	r^2	K_H	r^2	Ks	r^2	n	KP	r^2
I	i	SGF	0.198	**0.995**	-0.006	0.96	5.9	0.994	0.005	0.92	0.88	0.38	0.98
		SIF	0.222	0.991	-0.011	0.92	6.7	**0.996**	0.006	0.90	0.87	0.46	0.75
	ii	SGF	-	-	-	-	-	-	-	-	-	-	-
		SIF	0.194	0.906	-0.008	**0.990**	5.39	0.965	0.004	0.85	0.53	8.71	0.88
	iii	SGF	0.089	**0.994**	-0.001	0.992	3.12	0.993	0.002	0.95	0.67	0.91	0.91
		SIF	0.112	0.978	-0.002	0.885	3.82	0.92	0.003	**0.99**	0.93	0.49	0.92
	iv	SGF	-	-	-	-	-	-	-	-	-	-	-
		SIF	0.066	**0.994**	-0.001	0.973	3.05	0.976	0.002	0.976	0.70	0.73	0.83
II	i	SGF	0.025	**0.993**	-0.003	0.989	0.86	0.95	0.002	0.98	0.94	0.04	0.96
		SIF	0.107	0.953	-0.002	0.972	3.87	**0.98**	0.002	0.83	0.70	1.3	0.76
	ii	SGF	0.125	0.925	-0.059	**0.994**	3.45	0.97	0.002	0.90	0.74	18.6	0.83
		SIF	-	-	-	-	-	-	-	-	-	-	-
	iii	SGF	0.040	0.991	-0.005	**0.997**	1.41	0.996	0.001	0.95	0.55	1.02	0.99
		SIF	0.146	0.849	-0.008	**0.986**	4.99	0.921	0.003	0.79	0.64	11.8	0.53
	iv	SGF	0.030	0.980	-0.006	**0.992**	1.57	0.991	0.001	0.93	0.56	3.96	0.62
		SIF	0.115	0.885	-0.004	**0.970**	4.21	0.955	0.002	0.796	0.64	5.36	0.66
III	i	SGF	0.028	**0.998**	-0.003	0.995	0.977	0.967	0.002	0.97	0.93	0.03	0.97
		SIF	0.40	0.988	-0.025	0.955	9.645	**0.999**	0.009	0.94	0.80	0.24	0.99
	ii	SGF	-	-	-	-	-	-	-	-	-	-	-
		SIF	-	-	-	-	-	-	-	-	-	-	-
	iii	SGF	0.041	0.98	-0.005	0.991	1.47	**0.999**	0.001	0.92	0.52	0.81	0.84
		SIF	0.168	0.756	-0.013	**0.974**	5.41	0.857	0.003	0.71	0.90	0.67	0.80
	iv	SGF	0.028	0.905	-0.006	0.956	1.67	**0.982**	0.002	0.925	0.56	1.69	0.79
		SIF	-	-	-	-	-	-	-	-	-	-	-

CMAESt (OS) was able to prolong the release of acetaminophen only in SIF. Differently, AECMSt (TS) sustained the release of acetaminophen in SGF. The value of release exponent n was greater than 0.5 fitting non-Fickian models (anomalous transport). When the dissolution was conducted in simulating GIT transit (SGF for 2 h then followed by SIF), the ASA showed different release kinetics, with n between 0.5 - 1 (Table 5) indicating a non-Fickian model (anomalous transport). Metformin and mesalamine followed first order kinetics and zero order respectively n between 0.5 - 1 indicating anomalous transport which means that both swelling and diffusion are going together (Table 5). Acetaminophen followed a zero order for CMAEST

(OS) and Hixson–Crowell's order for both CMAESt (TS) and AECMSt (TS) with between 0.5 – 1 indicating to anomalous transport of active ingredient. Therefore, interesting to note is that using the same reagents and changing only the order of their addition in the reaction, produced compounds with different properties. Thus, the ampholytic starch from one step CMAESt (OS) was able to control the release of acidic, amphoteric and neutral medication in SIF, whereas the polymers obtained from two steps method CMAESt (TS) and AECMSt (TS) were able to sustain the release of same tracers also in SGF. When the dissolution was done first in SGF then followed by SIF, all ampholytic starch showed an ability to prolong the release of high loading medications (60%) for up to 24 h. Self-stabilization of ampholytic starch CMAESt (OS) could be explained by the limited solubility of the polymer in SGF. The carboxylic groups are compacted following protonation and amino groups may form stable macromolecular gels, lowering the solubility. In SIF the higher swelling ability and lower chain flexibility (as supported by viscosity measurement) and limited solubility of CMAEST (OS) are key factors of controlling the release of medications. Moreover, a polyelectrolyte complexation between the anionic CM- and cationic AE- groups located on neighboring chains, may also contribute to CMAESt (OS) gel formation and stabilization. On other hand, self-stabilization of ampholytic starch CMAESt (TS) could be explained by protonation of CM groups and generating an outer gel layer by hydration of AE groups in SGF. In SIF, the compacted gel layer generated in SGF alongside with limited solubility of AE, forming a barrier toward further penetration of intestinal fluid inside the gel layer, lowered the erosion and solubilization of outer gelled layer surface. Consequently, the tablet integrity is maintained prolonging the release of the active ingredients from the dosage form. Another aspect which could contribute to starch ampholytic derivatives behavior is the repartition of ionic charges on the starch backbone which would be more uniform in case of OS synthesis compared with TS procedure. It should be noticed that for TS an intermediate precipitation of the first derivative was done prior to introduction of the second functional group. This might influence the arrangements of chains which

will be stabilized by self-assembling (polymer-polymer ionic interactions). This may impact the surface exposure for further interactions (introduction of the second ionic group, polymer-drug interactions, etc).

4. Conclusion

The presence of the two groups on the same macromolecular backbone will generate a beneficial effect for the usage of such ampholytic starch polymers as excipients for the challenging formulation of drugs with high loading affording the controlled release of APIs during the transit along the gastrointestinal tract. The proposed new starch derivatives offer a wide range of applications where the polymer-polymer or polymer-drug self-assembling via electrostatic interactions are able to control the drug release at high loading of actives molecules, even for particularly challenging APIs as mesalamine and as metformin. Further studies are going on CMAESt (OS), at different DS of CM or AE groups in order to determine the position of CM, AE, EACM or EADCM on starch backbone chain and for elucidation of contribution of starch morphological aspects.

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CHAPTER VII

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SELF-STABILIZING AMPHOLYTIC STARCH EXCIPIENTS FOR SUSTAINED RELEASE OF HIGHLY SOLUBLE DRUGS: THE CASE STUDY OF METFORMIN

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Contribution of the main author and the co-authors:

The main author Mr. Khaleel SAKEER was responsible for all major parts of the article, in writing the manuscript body, and all experimental parts, and editing the figure alongside with gathering information and reviewing the references.

First Co-author: Dr. Pompilia Ispas-Szabo was involved in some editorial parts and manuscript revision.

Second co-author: Prof. Mircea Alexandru Mateescu, is the project director, responsible for supervision of all activities and providing the required directions about all experimental parts and interpretations of the obtained results.

Abstract

A new class of starch derivatives carrying cationic and anionic functional groups was developed to provide an alternative for the formulation of highly soluble drugs. The new ampholytic starch derivatives were synthesized in two steps; first the CarboxyMethyl (CM) groups were grafted on starch chains followed by the introduction of AminoEthyl (AE) groups. The final product: CarboxyMethyl-AminoEthyl-Starch (CM-AE-St) could be obtained with different degrees of substitution by varying the number of CM and AE groups. It was hypothesized that the simultaneous presence of anionic and cationic groups will generate a stronger self-stabilization of starch matrices and an improved control of drug release. Metformin (biopharmaceutical classification system: BCS, class I) was selected as model drug and monolithic tablets with 50% and 60% loading were prepared by direct compression of the active molecule with various CM-AE-St derivatives. The *in vitro* drug dissolution tests have shown that higher degrees of substitution for both CM- and AE- groups favor the ability of ampholytic CM-AE-St to control the drug release in simulated gastric fluid and in simulated intestinal fluid. Tablets based on CM-AE-St derivatives were compared to the commercial Glumetza® (50% loading). The drug release was controlled for 12h exhibiting a similar Higuchi's model dissolution profile for the two dosage forms. Structural studies (FT-IR, ^{1}H NMR, SEM, TG, X-ray diffraction) run on CM-AE-St derivatives put in evidence derivatization and self-stabilization phenomena. These new ampholytic starch derivatives offer a simple and convenient alternative to formulate and manufacture highly soluble drugs in a single step process.

Keywords: Ampholytic starch, CarboxyMethyl-AminoEthyl-Starch, Metformin, Controlled release, Monolithic tablet.

1. INTRODUCTION

In the context of a growing interest for sustained drug release devices and for high loaded dosage forms there is a need for new excipients, particularly for the formulation of highly soluble drugs such as metformin, metoprolol, metronidazole, chloroquine. To improve these challenging formulations, an adequate carrier is expected to be able to keep the tablet size small enough to allow ease of swallowing alongside with a simple method of tablet preparation. Current procedures for formulation of such highly soluble molecules consist of embedding them in release-modifying polymers, often followed by the application of additional functional coatings. The large majority of dosage forms are using cellulose derivatives, polyethylene oxides or other gel-forming polymers and frequently using multi-step processes that may include wet granulation and hot melt methods. Polymeric self-assembling excipients may improve the formulation of such highly soluble drugs and facilitate the manufacturing processes. Excipients based on cross-linked high amylose starch functionalized with different groups: anionic (carboxymethyl: CM), cationic (aminoethyl: AE) or neutral (acetate: Ac) allowed preparation of monolithic tablets with acetaminophen as model drug with 40 and 60 % loading (Mulhbacher *et al.*, 2001). Cross-linked CM-Starch with degree of substitution (DS) of 0.10 showed an improvement of controlled acetaminophen release with 90% of release within 16.5 h and 14.2 h for drug loading 40% and 60%, respectively (Mulhbacher *et al.*, 2001). A non-crosslinked CM-starch (CM-St) was then developed as a pH-sensitive excipient able to protect active principle ingredients (API) against gastric acidity (Calinescu *et al.*, 2005; Calinescu *et al.*, 2007) and to form a pH responsive matrix for controlled drug release in intestinal medium (Lemieux *et al.*, 2009). CM-St may allow resistance to gastric fluid due to the protonation of the carboxylate groups in gastric acidity by forming an outer gel layer. Thus, an *in situ* formed layer is able to ensure local buffering properties and to maintain a compact shape of the tablets during the passage through the stomach. Protonated carboxylic groups from

neighboring chains can be dimerized by dipole-dipole interactions and associated by hydrogen bonding, stabilizing thus the polymeric network (Ispas-Szabo *et al.*, 2016). In intestinal medium, protons are changed with sodium ions and tablets swell, releasing the active agent (Calinescu *et al.*, 2005). Several attempts have been made to prolong drug release in simulated intestinal fluid (SIF) from matrices containing CM-St with a low degree of substitution DS (0.09-0.11) presenting a better controlled release of drugs (Assaad and Mateescu, 2010). In order to further delay the release, a physical mixture of carboxymethyl starch and chitosan was proposed to deliver diamine oxidase and catalase to the low intestine (Calinescu *et al.*, 2012) and a co-processing procedure was proposed to obtain a polyelectrolyte complex (PEC) of carboxymethyl starch chitosan as an excipient for colon delivery (Assaad *et al.*, 2011). However, it is difficult to formulate sustained release forms of cationic drugs such as chlorohydrate salts, which are highly soluble and required in large amount (high loading). Metformin (dimethylbiguanide) hydrochloride is an orally administered drug used to lower blood glucose concentrations in patients with non-insulin-dependent diabetes mellitus (Bailey, 1992; Bailey and Turner, 1996). Metformin has properties of a strong base (pK_a 2.8 and 11.5) and, as the hydrochloride salt, it is highly water-soluble and in solutions of pH 1.2 to 6.8 (Desai *et al.*, 2014a). Because of its short half-life (< 3 h), frequent administrations at high doses (daily dose up to 2.5 g) are needed to maintain its required plasma concentration (Garber *et al.*, 1997; Stepensky *et al.*, 2001; Qin *et al.*, 2014). Metformin is considered mostly absorbed in the duodenum (Song *et al.*, 2006). Some adverse effects (*i.e.* diarrhea, abdominal discomfort, nausea) caused by immediate release dosage forms of metformin (Glucophage®) remain unsolved. Metformin was formulated for controlled release systems in various dosage forms i.e. mucoadhesive beads (Nayak and Pal, 2014), gastro-floating bilayer tablets (He *et al.*, 2014) combination with triacetyl-β-cyclodextrin (Corti *et al.*, 2008), iron (III) cross-linked alginate–carboxymethyl cellulose hydrogel beads (Swamy and Yun, 2015) and, osmotic pump tablet (Qin *et al.*, 2014). The commercially available controlled release

product Glumetza® tablets (metformin hydrochloride: Depomed Inc.) (Laustsen, 2005; Laustsen, 2006) and the majority of sustained release devices are designed as gastroretentive forms based on a prolonged gastric residence controlled by swelling or by floating or by mucoadhesion to retain the dosage form releasing the active pharmaceutical principle in the stomach. With Glumetza®, too much metformin release in the stomach and to the upper intestine may generate tissue saturation (Proctor *et al.*, 2008) and thus a lower absorption with decreased efficiency. Furthermore, metformin was found to be absorbed not only at the level of the upper intestine, but over the whole intestinal tract (Bailey, 1992; Song *et al.*, 2006). Gastrointestinal absorption of metformin is incomplete, possibly related to a saturable absorption process and about 20%-30% of an oral dose was considered to be recovered in the faeces (Tucker *et al.*, 1981). Absorption is estimated to be complete within 6 h after administration and is presumably confined to the upper intestine (Tucker *et al.*, 1981; Vidon *et al.*, 1988). Metformin is also poorly absorbed from the stomach and with a rate-limiting absorption from the duodenum. In fact, the whole intestine is necessary for sufficient absorption of the drug (Vidon *et al.*, 1988). A too high absorption with saturation at the upper intestine may generate side effects and a lesser efficiency by reducing the amount of drug available for lower intestine absorption. Consequently, a formulation of metformin with continuous release over the entire gastrointestinal tract could reduce adverse gastric side effects. In this context, we are proposing a new carrier: an ampholytic starch that has the ability to control the release of high-doses of highly soluble drugs and to keep the tablet size small enough with a single step procedure of tablet preparation. An ampholytic starch can be defined as a starch derivative exhibiting both anionic (i.e. carboxylic) and cationic (i.e. aminoethyl) groups. An ampholytic starch has been previously used as a flocculating agent (Yang *et al.*, 2014b; Yang *et al.*, 2014c) in the textile industry as a warp sizing agent (Zhu *et al.*, 2012) and also as a papermaking agent (Solarek *et al.*, 1996). The aim of the present study was to prepare and characterize Carboxymethyl AminoEthyl Starch (CM-AE-St) as a novel excipient. Due to its ampholytic

character; this CM-AE-St is expected to modulate the release of high loaded soluble drugs. To the best of our knowledge, this is the first investigation of an ampholytic starch as a carrier for sustained delivery of active ingredients along the gastrointestinal tract.

2. MATERIALS AND METHODES

2.1. Materials

High amylose starch (Hylon VII) was supplied by National Starch (Bridgewater, NJ, USA). Metformin (1,1-dimethylbiguanide hydrochloride) was from MP Biomedicals (Solon, OH, USA), sodium monochloroacetate was from Sigma-Aldrich (Germany), and 2-chloroethylamine hydrochloride from Fluka (Switzerland).

2.2. Preparation of ampholytic starch derivatives

The polymers were prepared in two steps: the first step consisted in introducing the carboxymethyl anionic group and the second one was the grafting of the CM-starch derivative with cationic aminoethyl (AE) groups. Sodium monochloroacetate was dissolved in water and rapidly added to gelatinized starch suspension to obtain carboxymethyl starch. Briefly, an amount of 50 g of starch was suspended for hydration for 5 min in 200 mL of distilled water at 60-70 °C under continuous vertical stirring and 300 mL of 5 M NaOH were added under stirring maintaining the reaction medium for 1h at 60-70 °C for gelatinization. In different experiments, various quantities (18.75, 56.25) g of sodium monochloroacetate solubilized in a minimal water volume were added rapidly under stirring which was continued for 1h at 60-70 °C (to introduce carboxylic groups). Then, the solution was cooled down and neutralized with glacial acetic acid. The CM-St was precipitated from slurry with an equivalent volume of methanol/water (70:30) v/v followed decantation. The process was repeated until a final conductivity (Fisher Scientific Accumet Research AR20, San Diego, CA, USA) of filtrate decreased to less than 75 µS/cm. Finally, pure

methanol and then acetone were used for drying. The collected CM-St was left overnight at room temperature for complete drying and the powder sieved to obtain particles of less than 300 µm. The polymer was re-dissolved for amination which was done by reaction with 2-chloroethylamine hydrochloride. To prepare CM-AE-St, 12.5 g of CM-St from the previous step were redissolved in 50 mL of distilled water followed by addition of 75 mL of 5 M NaOH and heated at 60-70 °C for 1 h under continuous stirring. Different quantities (4.68, 9.37, 14.06) g of 2-chloroethylamine hydrochloride solubilized in a minimal water volume were rapidly added under stirring continued for 1h at 60-70 °C (to graft cationic groups). Finally, the suspension was cooled down and neutralized with glacial acetic acid. The CM-AE-St powder was obtained by precipitation with methanol and drying as described above.

2.3. Evaluation of the degree of substitution

A) The degree of substitution (DS) of CM-St was determined by back-titration as previously described by Stojanovic et al., 2005 (Stojanović *et al.*, 2005). Briefly, 300 mg of polymer (n = 3) were solubilized in 20 mL of 0.05 M NaOH and the excess of NaOH was titrated with 0.05 M HCl using phenolphthalein as indicator. The blank (20 mL of NaOH) was also titrated by the same method. The amount of –COOH groups (*n*) and the DS were calculated (Stojanović *et al.*, 2005) using the equations (1, 2) :

$$n = (V_b - V)XC_{HCl} \quad (1)$$

$$DS = \frac{162Xn}{m - WXn} \quad (2)$$

where V_b (mL) is the volume of HCl used for the titration of the blank; V (mL) is the volume of HCl used for the titration of the sample; C_{HCl} (mol/L) is the concentration of HCl; 162 (g/mol) is the molar mass of glucose unit; W= (58) (g/mol) is the

increase in the mass of glucose unit by substitution with a CM- group, and m (g) is the mass of dry sample.

B) The degree of substitution (DS) expressed in terms of amino groups on the final derivative was determined with the ninhydrin reagent (2 g ninhydrin dissolved in 75 mL dimethylsulfoxide under nitrogen flushing and completed with 25 mL of 4M lithium acetate buffer, pH 5.2). A volume of 0.5 mL of the ninhydrin reagent was added to a volume of 0.25 mL of ampholytic starch solution (15 mg/mL) in deionized water (in triplicate). The tubes were immediately capped, vortexed, and heated in a covered boiling water bath for 30 min. Tubes were then cooled below 30 °C in a cold water bath. The absorbance at 570 nm was measured on a UV/VIS spectrophotometer with zero set against a similarly treated blank of water. Glycine (Sigma) solutions (0.12 - 0.51 mg/mL) were used to generate a standard curve (Le Tien *et al.*, 2003; Le Dévédec *et al.*, 2008).

2.4. Preparation of tablets

Monolithic tablets (50% and 60 % (w/w) drug loading) were obtained by direct compression (2.5 tons) of a homogenous mixture of excipient and drug powders. Flat-faced punches with 12.95 mm diameter and oval shape punches with 17.5 mm length and width of 10.30 mm were used with a Carver hydraulic press (Model C 3912 Hydraulic Cylinder, Wabash, IN, USA).

2.5. Determination of the fluid uptake and erosion

Fluid uptake and erosion studies were carried out by immersing the weighed dry tablet (W_1) in 1000 mL of either simulated gastric fluid (SGF) (pH 1.2 obtained by first dissolving 2 g of sodium chloride in 500 mL of water; then the pH was adjusted at 1.2 ± 0.1 with concentrated HCl 37%; the final volume was completed with purified water to 1000 mL) or simulated intestinal fluid (SIF) (pH 6.8 phosphate buffer obtained by first dissolving 6.8 g of monobasic potassium phosphate in 500

mL of water; then the pH was adjust at 6.8 ± 0.1 with 0.2 M sodium hydroxide and the final volume was completed with purified water to 1000 mL) using the same conditions as those for the *in vitro* drug release studies (i.e. 37°C and stirring rate 100 rpm). After 2 and 10 h, the tablets were withdrawn and carefully weighed (recorded as W_2). The recovered tablets were then placed into an oven at 35–40°C for two days until a constant weight was obtained. The final weight of the tablets was measured (W_3) after complete drying at constant mass (Sakeer *et al.*, 2010a; Sakeer *et al.*, 2010b). Fluid uptake was determined according to Equation (3):

$$\%\ Weight\ change = \frac{W_2 - W_1}{W_1} x100 \qquad (3)$$

The degree of erosion was determined according to equation (4):

$$\%\ Erosion = \frac{W_1 - W_3}{W_1} x\ 100 \qquad (4)$$

2.6. Fourier transform infrared (FT-IR) analysis

The FT-IR spectra of powder samples were recorded (64 scans at a resolution of 4 cm^{-1}) using a Thermo-Nicolet 6700 (Madison, WI, USA) FT-IR spectrometer equipped with a deuterated triglycine sulfate-KBr (DTGS-KBr) detector and a diamond smart attenuated total reflection platform.

2.7. ^{1}H NMR measurements

In order to determine the CM-AE-St functionalization pattern, high-field 600 MHz, ^{1}H NMR spectra were collected using a Bruker Avance III HD spectrometer running a TopSpin 3.2 software and equipped with a 5 mm TCI cryoprobe. The temperature of samples was regulated at 27 °C. The samples were dissolved in deuterium oxide D_2O, heated at 65 °C for 30 min, and kept at 4 °C for 2h prior to analysis.

2.8. Thermogravimetric analysis

Thermogravimetric analysis (TG) for each powder sample was carried out in platinum crucible at a heating rate of 10 °C/min between 25 and 600 °C under nitrogen atmosphere (flow rate 100 mL/min). TG patterns were established with a TA® Instruments incorporated high-resolution thermo gravimetric analyzer Seiko TG/DTA 6200 (Japan).

2.9. Scanning electron microscopy (SEM)

The morphology of the sample particles was examined by a Hitachi (S-4300SE/N) scanning electron microscopy with variable pressure (Hitachi High Technologies America, Pleasanton, CA, USA) at 5-7 kV and magnifications of and 1000×. Samples were mounted on metal stubs and sputter-coated with gold.

2.10. X-ray diffraction

X-ray diffractometry of polymers was performed using a Siemens D-5000, (Munich, Germany) device. The samples were exposed to X-ray radiation (Cu Kα) with wavelength of 1.789 Å and a scanning rate of 0.05 °/min. Samples (ground into powders with an agate mortar and pestle) were measured on a low background quartz plate in an aluminium holder and analyzed through a 2θ range of 5-50 degrees. The XRD spectra were treated using a DiffracPlus software.

2.11. *In vitro* dissolution tests

Metformin dissolution tests were carried out: a) for tablets loaded with 60% of metformin, in SGF and SIF dissolution media, separately; b) for tablets loaded with 50% of drug, in SGF for 2h followed by SIF for up to 12h; c) Glumetza® dissolution was conducted only in SGF since it is a gastroretentive designed formulation. An USP paddle apparatus 2 was used with a dissolution volume of 1000 mL and the

paddle speed at 100 rpm. The dissolved drug was detected at λ_{232} nm for SIF whereas for SGF the sample was diluted with phosphate buffer and detected at the same wavelength.

2.12. Release kinetic patterns

The drug release kinetic parameters were evaluated according to zero-order kinetics (Costa *et al.*, 2001), first-order kinetics (Costa *et al.*, 2001), Higuchi's model (Higuchi, 1961; Higuchi, 1963), Hixson–Crowell's model (Hixson and Crowell, 1931) and Korsmeyer–Peppas model (Korsmeyer *et al.*, 1983) Table1.

2.13. Statistical analysis

All tests were done in triplicate and data are reported as means ± SD. For statistical analysis the one way ANOVA was followed by Fisher's post hoc tests with a minimum confidence level ($P < 0.05$) for statistical significance.

3. RESULTS

3.1. Ampholytic starch characterization and structural insights:

In the starch structure, hydroxyl groups are particularly susceptible to react through nucleophilic attacks located at the glucopyranose ring in the order C_2, C_6 and C_3. For our ampholytic starch, hydroxyl group can be involved in substitution of the hydrogen atom with carboxymethyl in a first step and with aminoethyl in a second step. The degree of substitution (DS) of the two series of obtained CM-St samples determined by back-titration were 0.02 and 0.06 representing the average number of carboxymethyl groups per glucose unit corresponding to two series of CM-St. The CM-St (DS 0.02) was aminoethylated in three variants with different quantities of 2-chloroethylamine hydrochloride obtaining a series of CM-AE-St with amination DS of (0.064-0.049-0.024 mmol/g). The CM-St (DS 0.06) generated a CM-AE-St series

with amination DS of (0.059-0.045-0.019 mmol/g). **Fig. 1, A** shows the preparation procedure for the CM-AE-St derivatives.

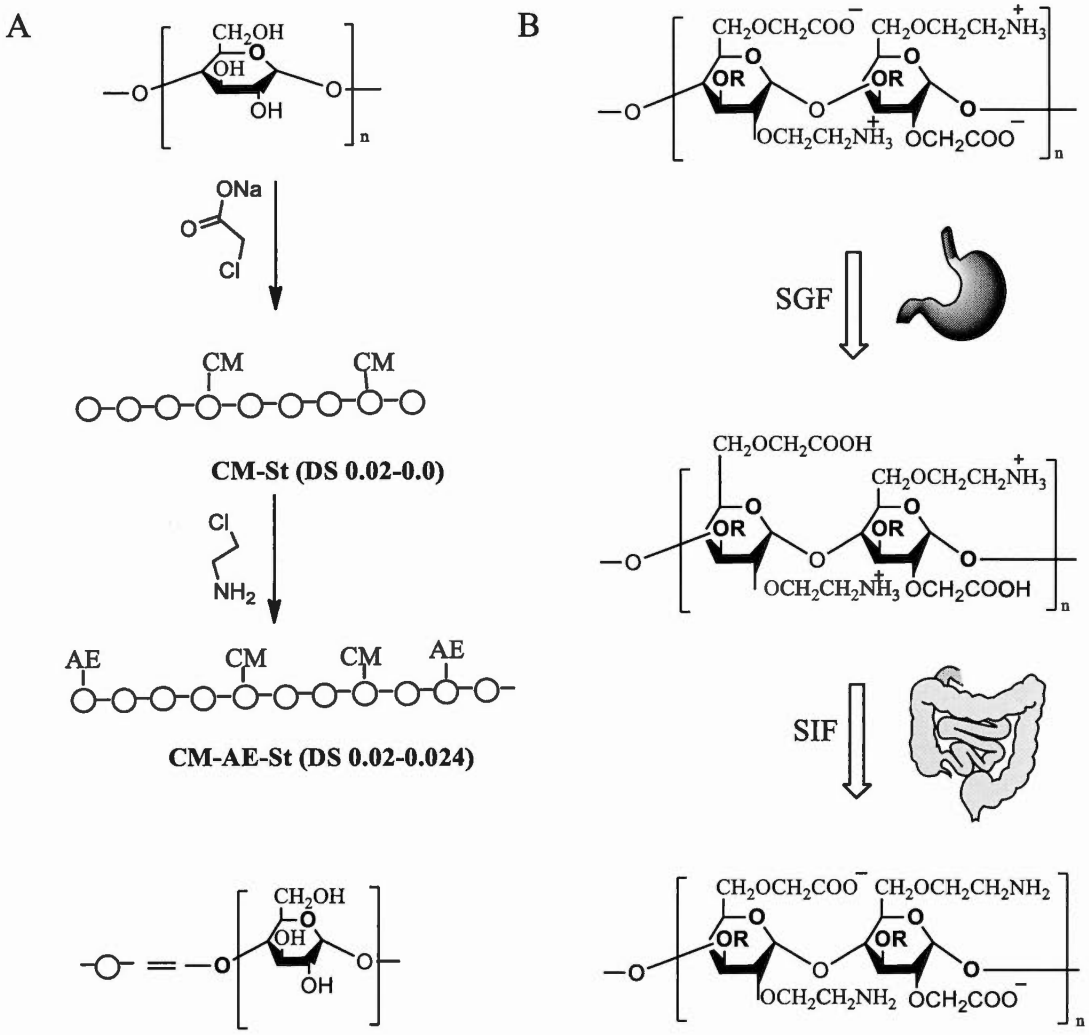


Fig. 1. Hypothetical presentation of (A) starch derivatization with sodium monochloroacetate and with chloroethylamine hydrochloride to generate ampholytic starch carrying carboxymethyl (CM) and aminoethyl (AE) functions and schematical representation (B) of ampholytic starch in simulated gastric (SGF) and intestinal (SIF) fluid where R can be either CM or AE or H. In DS 0.02-0.024: 0.02 is ascribed to DS of CM and 0.024 ascribed to DS of AE.

3.2. Scanning electron microscopy (SEM)

The aspects of starch and of CM-St and CM-AE-St derivatives are different depending on modifications on the starch structure. The native starch (Hylon VII) granules **(Fig. 2 A)** were predominantly round or oval in shape, with a smooth surface. This seems due to the high content of amylose (more than 70%), which is more crystalline, known to exhibit double helix-B form and to be strongly stabilized by hydrogen associations between the hydroxyl groups of glucopyranose units (Friciu *et al.*, 2013). The CM-St **(Fig. 2, B, B')** is characterized by an irregular shape with an uneven surface likely due to the association of numerous small particles forming larger granules (Nattapulwat *et al.*, 2009; Friciu *et al.*, 2013). The presence of carboxylic groups probably reduced the network self-assembling by hydrogen associations between hydroxyl groups and promoted the repulsion effects leading to the reorganization of the network (Lemieux *et al.*, 2010). The amination treatment generated a slightly smoother surface for the CM-AE-St granules **(Fig. 2. C-E, C'-E')**.

3.3. Fourier transform infrared (FT-IR) analysis

For starch and its derivatives **(Fig. 3, I, II)** a broad band with a maximum at 3296 cm^{-1} is due to the stretching vibrations of –OH, whereas the small band at 2927 cm^{-1} was attributed to the -CH stretching vibration. The band at 1079 cm^{-1} was ascribed to CH_2-O-CH_2 stretching vibrations for CM-St and CM-AE-St. In case of CM-St, there are few additional bands at 1589 cm^{-1} and at 1417 cm^{-1} ascribed to COO- group (Assaad and Mateescu, 2010; Friciu *et al.*, 2013). The higher intensity in case of CM-St (0.06) is due to its higher DS. In the case of CM-AE-St, a band at 999 cm^{-1} can be assigned to C-N stretching vibrations whereas a peak at 1366 cm^{-1} was ascribed to -CH_2 group of AE (Deng *et al.*, 2006; Assaad *et al.*, 2011). The presence of these additional bands confirms the grafting of CM and AE onto the starch backbone.

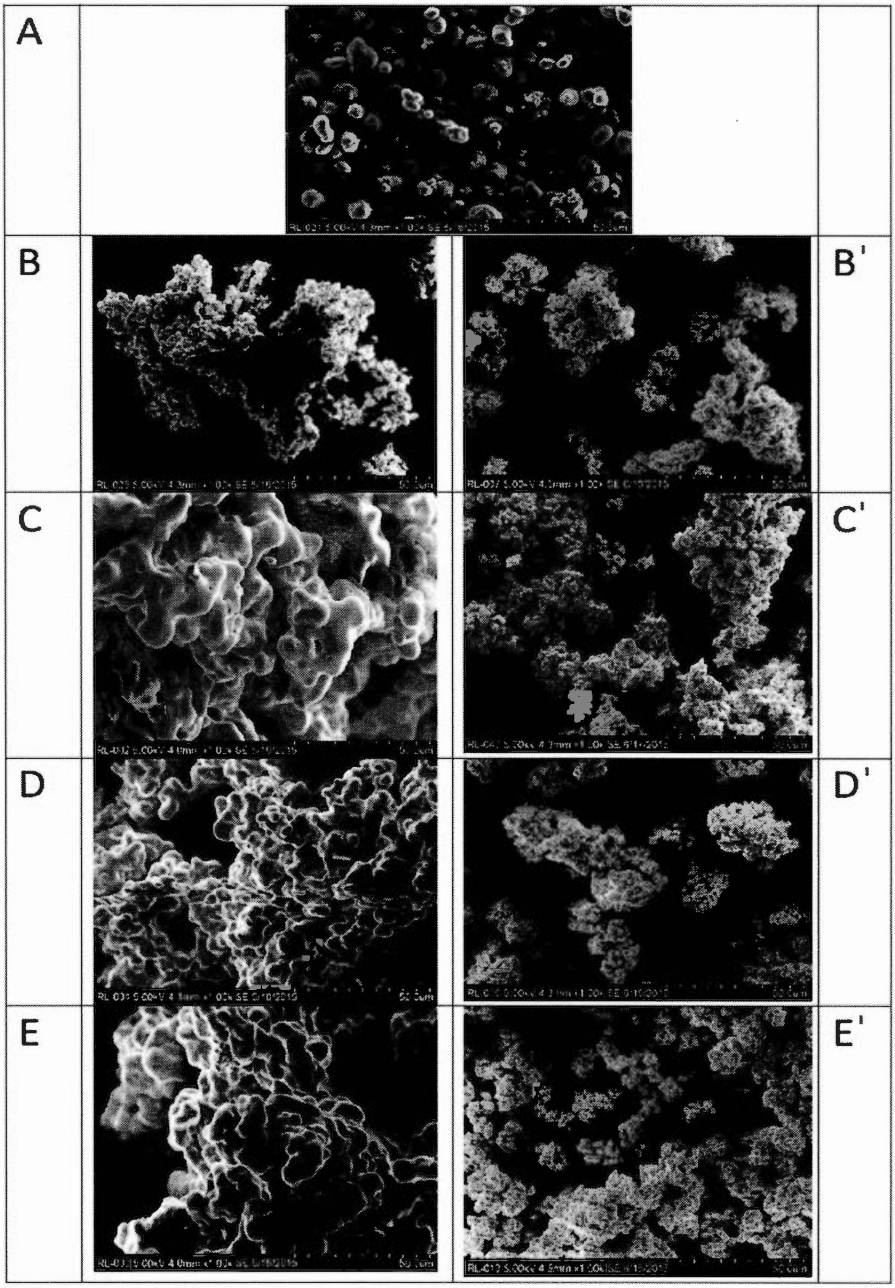


Fig. 2. Scanning electron microscopy micrographs of (A) Native Starch, (B) CM- St (DS 0.02), (B') CM- St (DS 0.06), (C) CM-AE-St (DS 0.02-0.024), (C') CM-AE-St (DS 0.06-0.019), (D) CM-AE-St (DS 0.02-0.049), (D') CM-AE-St (DS 0.06-0.045), (E) CM-AE-St (DS 0.02-0.064) and (E') CM-AE-St (DS 0.06-0.059) at magnification of 1000.

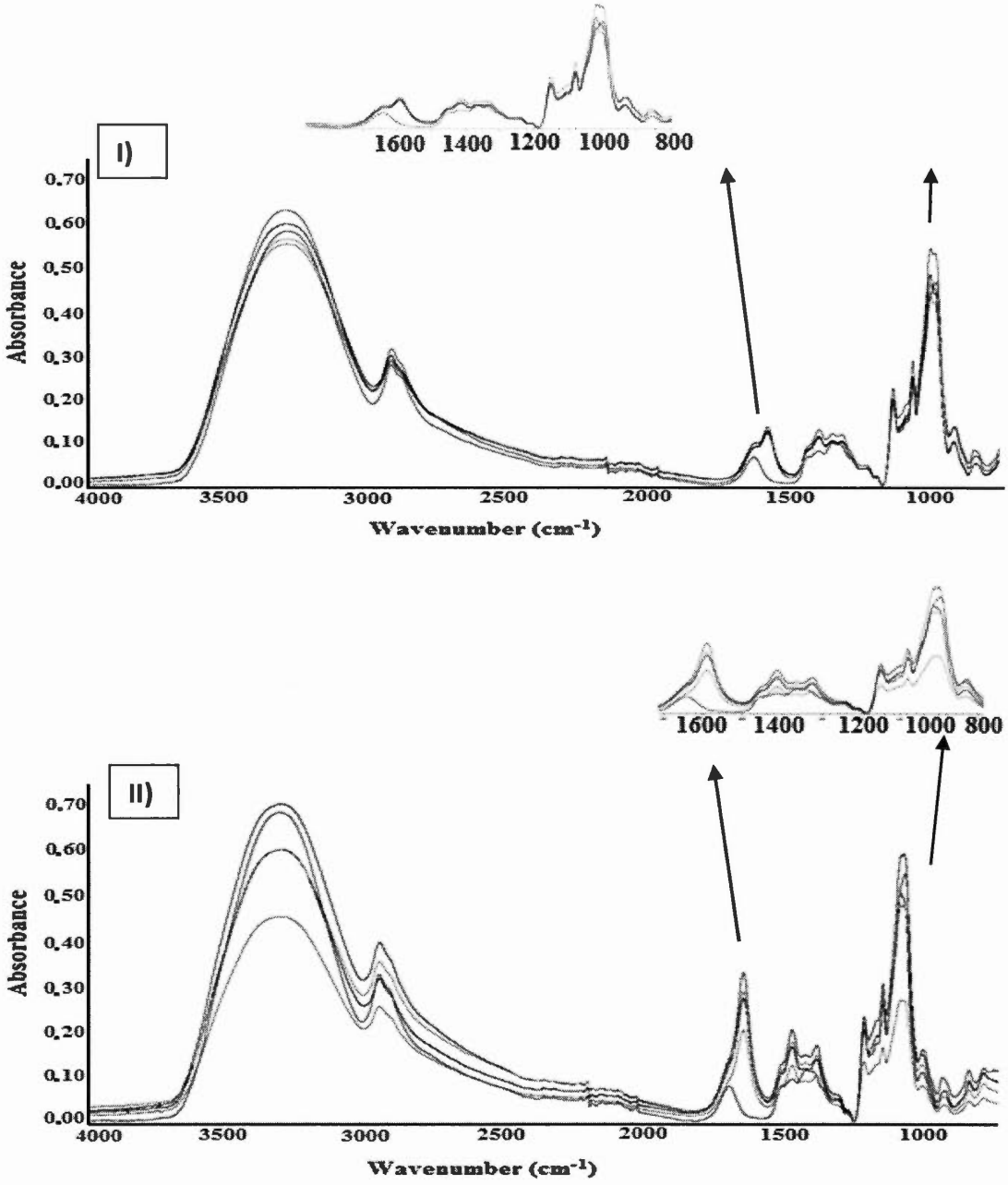
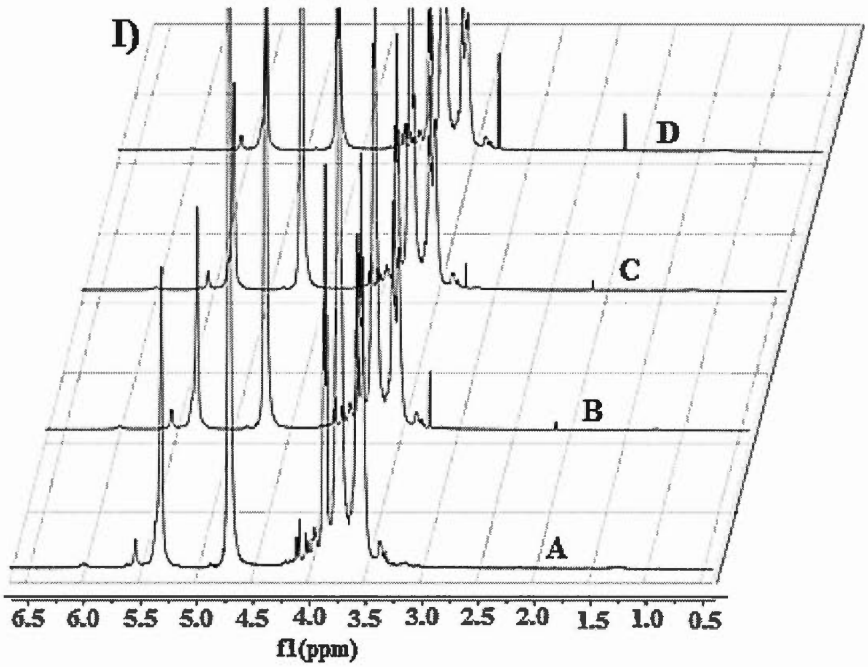


Fig. 3. FT-IR spectra of (Red) Native Starch and of (**I**): (Blue) CM- St (DS 0.02), (Black) CM-AE-St (DS 0.02-0.024), (Green) CM-AE-St (DS 0.02-0.049), and (Brown) CM-AE-St (DS 0.02-0.064), and of (**II**) (Blue) CM- St (DS 0.06), (Black) CM-AE-St (DS 0.06-0.019, (Green) CM-AE-St (DS 0.06-0.045), and (Brown) CM-AE-St (DS 0.06-0.059).

3.4. ^{1}H NMR measurements

Fig. 4, I, II shows ^{1}H NMR spectra of the CM-St and CM-AE-St, with proton signals at 5.3 ppm for H1 and at 3.3–3.9 ppm for H2-6 (Yang *et al.*, 2014b). The most significant peaks at δ = 2.17-2.20, δ = 3.16-3.18 were found for CM-AE-St only and not for CM-St. They belong to the hydrogens of aminoethyl groups (Noga *et al.*, 2012; Amar-Lewis *et al.*, 2014) and presented intensities proportional to the amount of AE groups.



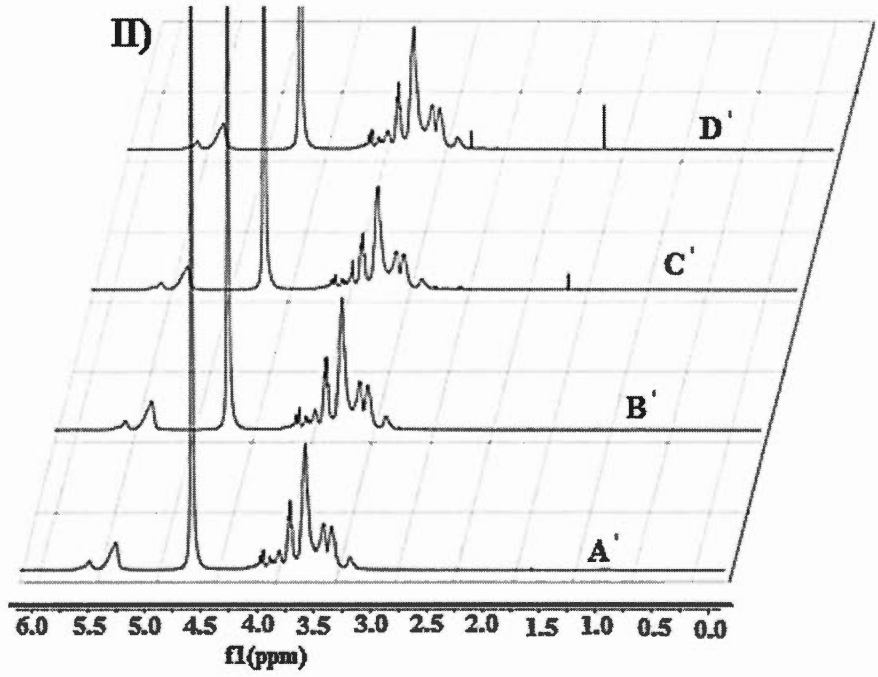


Fig. 4. ^{1}H NMR spectra **I):** (A) CM- St (DS 0.02), (B) CM-AE-St (DS 0.02-0.024), (C) CM-AE-St (DS 0.02-0.049), and (D) CM-AE-St (DS 0.02-0.064). and **II)** of (A') CM- St (DS 0.06), (B') CM-AE-St (DS 0.06-0.019, (C') CM-AE-St (DS 0.06-0.045), and (D') CM-AE-St (DS 0.06-0.059).

3.5. Thermogravimetric analysis

The thermogravimetric pattern (TGA and DTG curves) of Starch, of CM-St and of CM-AE-St investigated in a temperature range of 25 °C to 600 °C, are presented in **Fig. 5 I, II**. All samples showed a two-steps weight loss below 600°C. The first one is minor and seems to correspond to the loss of intramolecular and intermolecular water around 50–110 °C. The second one may be related to material decomposition (Li *et al.*, 2010; Zhang *et al.*, 2014). The decomposition of starch may include molecular chain breakdown with hydrolysis of α-1,4 glucosidic linkages in the amylose and amylopectin of the starch. The scission of α-1,6 linkages may occur, but α-1,4 linkages are more susceptible to hydrolysis than α-1,6 linkages (Liu *et al.*, 2010).

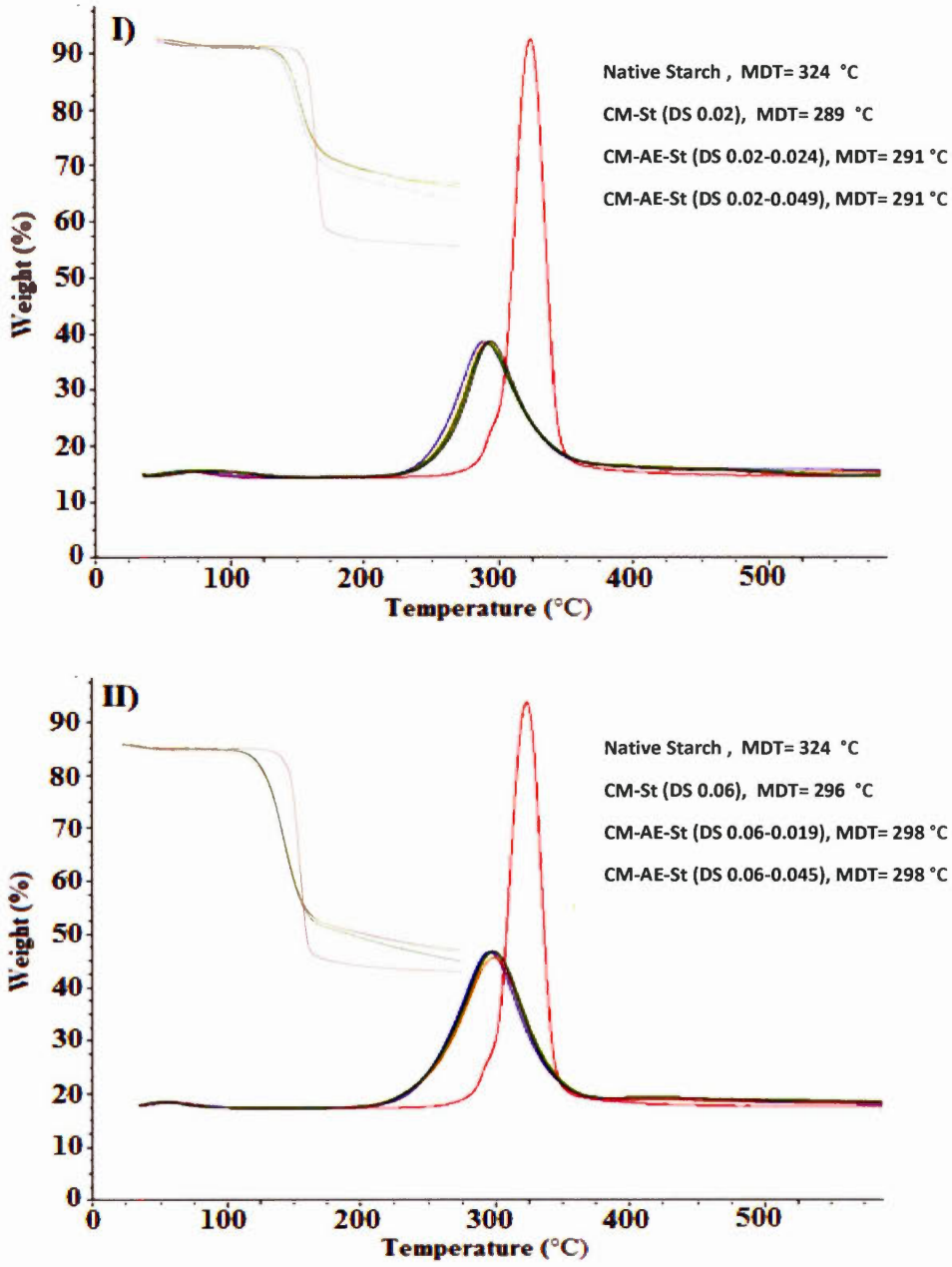


Fig. 5. Thermogravimetric (TG) and its derivative (DTG) diagram of Native Starch (Red) and of **I)** (Blue) CM- St (DS 0.02), (Black) CM-AE-St (DS 0.02-0.024), (Green) CM-AE-St (DS 0.02-0.049), and (Brown) CM-AE-St (DS 0.02-0.064). and of **II)** (Blue) CM- St (DS 0.06), (Black) CM-AE-St (DS 0.06-0.019, (Green) CM-AE-St (DS 0.06-0.045), and (Brown) CM-AE-St (DS 0.06-0.059).

Water is considered one of the main products of decomposition at temperatures below 270 °C. Further heating up to 600 °C resulted in carbonization and ash formation. The maximal decomposition temperature (MDT) was 289 °C for CM-St (DS 0.02), whereas MDT for CM-St (DS 0.06) was 296 °C. Both values are lower than the MDT of native starch (324 °C). The reason seems to be the substitution of hydroxyl groups of native starch with CM groups. The results also indicate that CM-St was decomposed earlier but slower than native starch.

3.6. X-ray powder diffraction

X-ray diffraction of starch, CM and CM-AE-St powders (**Fig. 6, I, II**) showed for starch the most ordered organization with diffraction peaks at 2θ = 16.78°, 17.48°, 19.84°, 25.88° corresponding to a B-type morphology (characterized by a double helix (Immel and Lichtenthaler, 2000). Peaks at 2θ = 23.02°, 13.38° correspond to a V-type morphology (a single helix having hydrophobic channel [48]). Following derivatization, a pronounced reduction in order degree was observed with disappeared peaks at 2θ = 19.84°, 25.88°, suggesting the loss of the B-type double helix. This loss in crystallinity and disappearance of starch B-type could be attributed to the effect of derivatization by substitution of the hydroxyl groups with either CM groups (Wang *et al.*, 2010; Gao *et al.*, 2011) or AE groups (Kuo and Lai, 2007; Pi-xin *et al.*, 2009; Chang *et al.*, 2014). However, hydrogen bonds contributed to maintaining in a certain extent the starch order. The CM-AE-St pattern differed from that of CM-St (i.e. peaks at 2θ = 13°-16° and 2θ = 21°-25°). For CM-St, the impact of grafting on the structure morphology was more pronounced for DS (0.06) than for CM-St (0.02), probably because of a stronger stabilization for DS 0.02 due to carboxyl dimerization by hydrogen bonding.

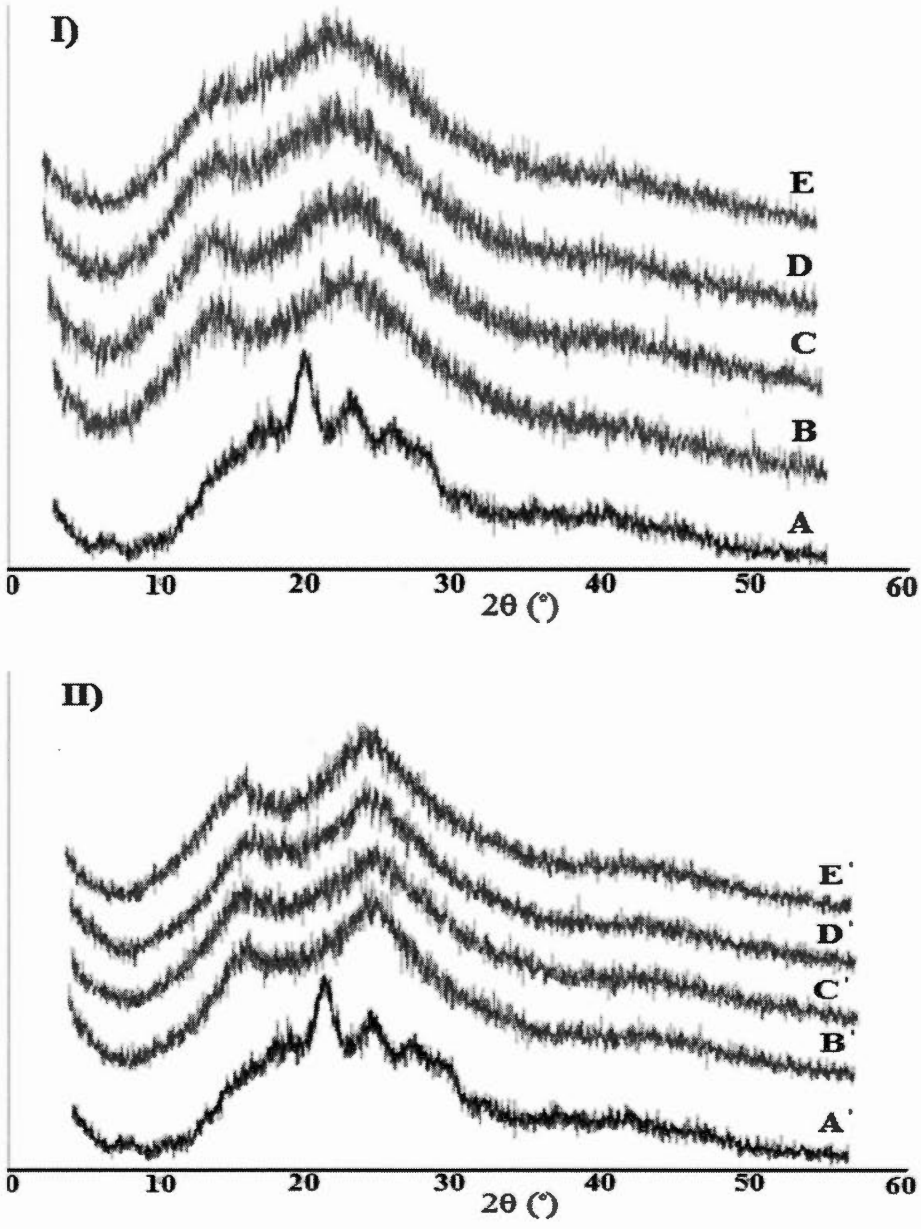


Fig. 6. X-ray diffraction patterns of (A) Native Starch and of (**I**): (B) CM- St (DS 0.02), (C) CM-AE-St (DS 0.02-0.024), (D) CM-AE-St (DS 0.02-0.049), and (E) CM-AE-St (DS 0.02-0.064). and (**II**): (B') CM- St (DS 0.06), (C') CM-AE-St (DS 0.06-0.019, (D') CM-AE-St (DS 0.06-0.045), and (E') CM-AE-St (DS 0.06-0.059).

With increasing numbers of grafted AE groups, the band at 2θ = 13°-16° was reduced and almost disappeared in the case of CM-AE-St (0.02-0.064) in comparison with CM-St (0.02). Differently, the band at 2θ = 21°-25° was of higher intensity and had shifted to a lower value.

3.7. *In vitro* dissolution tests

The behavior of starch derivatives in SGF and in SIF, dissolution tests *in vitro* were first investigated in each medium separately. Since metformin is freely soluble and because it is required in high doses, modulating its release in the gastrointestinal tract is a challenging task. The presence of two functional groups CM and AE on the same chain can promote self-stabilization of the matrix in acidic and in neutral-alkaline media. It was previously shown (Mateescu *et al.*, 2006; Lemieux *et al.*, 2010) that the optimal degree of substitution for CM-St to exert controlled release was in the range (0.07-0.11). In the present study, CM-St was prepared with two degrees of substitution (0.02 and 0.06), and then each of them substituted with different amounts of AE groups. Similarly to the previous study (Assaad and Mateescu, 2010), the release of metformin from CM-St with lower DS (0.02) was faster than with higher DS (0.06). Also the release in SGF was slower than in SIF since CM-St is compacted in gastric acidity due to protonation of carboxyl groups in SGF **(Fig. 7).** The same CM-St (DS 0.02) after grafting with different amounts of AE groups was able to control the release of metformin **(Fig. 7. I).** By increasing the amount of AE groups, the release of metformin was prolonged in both SGF and in SIF. For instance, CM-AE-St (0.02-0.024) in 2h released 95% of metformin in acidic medium while CM-AE-St (0.02-0.064) released 55% of metformin. Similar results were observed with CM-St (DS 0.06) after grafting with increasing amounts of AE groups **(Fig. 7. II)**

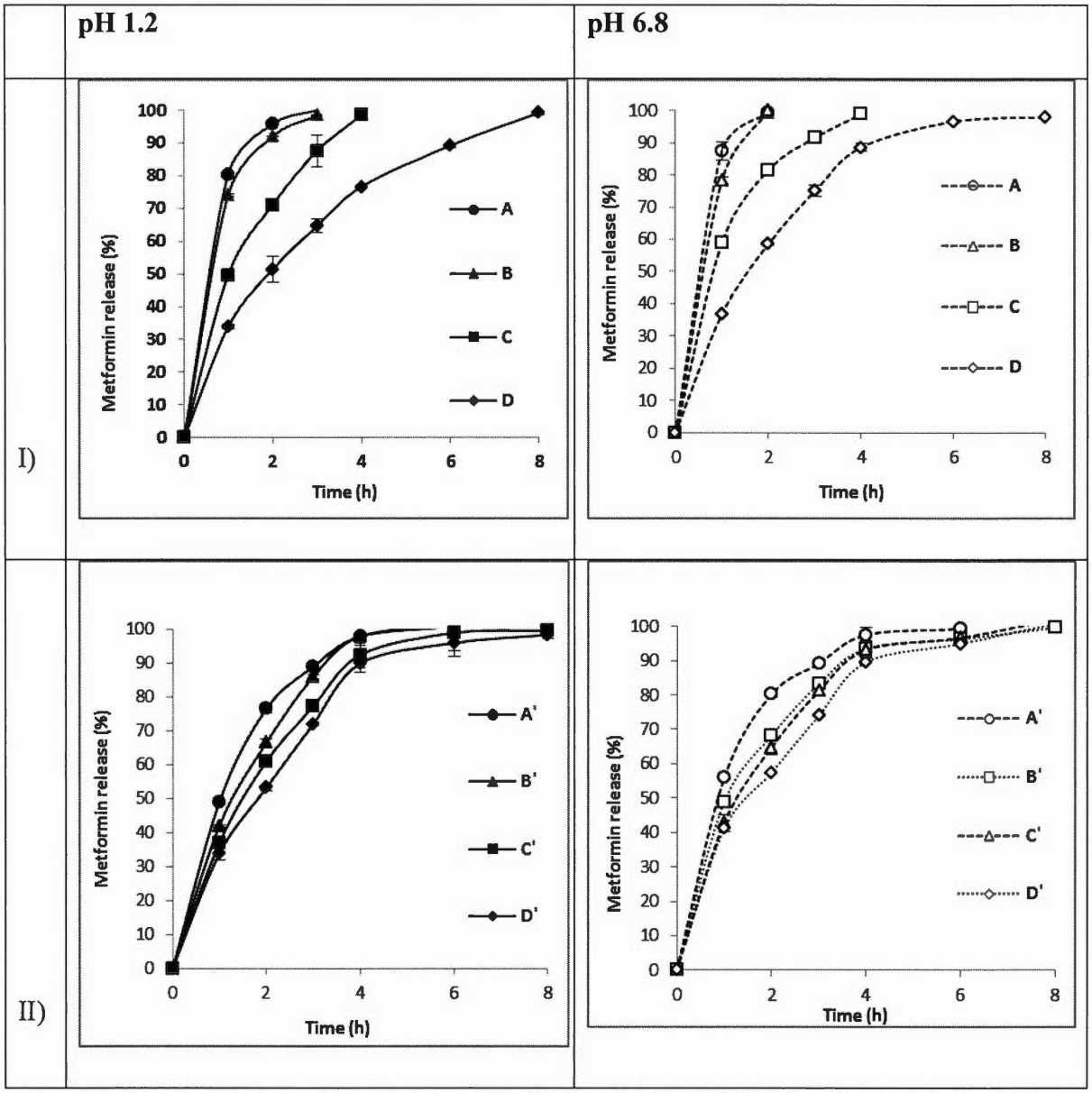


Fig. 7. Release profiles of metformin from 1000 mg tablet (60% loading) of **I**): (A) CM- St (DS 0.02), (B) CM-AE-St (DS 0.02-0.024), (C) CM-AE-St (DS 0.02-0.049), and (D) CM-AE-St (DS 0.02-0.064). **II**) and of (A') CM- St (DS 0.06), (B') CM-AE-St (DS 0.06-0.019, (C') CM-AE-St (DS 0.06-0.045), and (D') CM-AE-St (DS 0.06-0.059) at pH 1.2 (left) and pH 6.8 (right) side.

The release kinetics of metformin in SGF and in SIF was evaluated based on the equations presented in **Table 1**, where Q_t in equations A, B, C and E is the amount of the drug released at time t, whereas Q_t in equation D is the amount of drug remaining in pharmaceutical dosage form at time t. Q_0 is the initial amount of drug in the pharmaceutical dosage form and K_0, K_1, K_H, Ks, K_P are respectively the zero-order, first-order, Higuchi's, Hixson–Crowell's, Korsmeyer–Peppas release rate constants and n is the release exponent. If the n value is 0.5 or less, the release mechanism follows Fickian diffusion, whereas at higher values for mass transfer in the range $0.5 < n < 1$, the system will follow a non-Fickian model (anomalous transport). The system follows a zero-order drug release and a case-II transport if the n value is equal to 1. Here the relaxation process of the macromolecules occurring upon water uptake by the system is the rate-controlling step. Water acts as a plasticizer and decreases the glass transition temperature of the polymer (Siepmann and Peppas, 2001). For the values of n higher than 1, the mechanism of drug release is regarded as super case-II transport (Korsmeyer *et al.*, 1983). The kinetic release profiles of metformin in SGF and SIF from tablets based on CM-AE-St with different degrees of substitution respectively DS 0.02-0.064 and DS 0.06-0.059 **(Table 1)** appear to fit well ($r^2 > 0.99$) the first order model with the Korsmeyer's release exponent $n<0.5$, which can be related to a diffusion controlled release of the drug associated with a small contribution of polymer relaxation.

Table 1: Kinetic equations and corresponding values of metformin release from tablets (60% loading) based on (A) CM-AE-St (DS 0.02-0.064), (B) CM-AE-St (DS 0.06-0.059) at pH 1.2 and pH 6.8, and from tablets (50% loading) based on (C) CM-AE-St (DS 0.06-0.059) compared to Glumetza®.

	Dosage forms		**Ampholytic starch**				**Ampholytic starch C (500 mg)**	**Glumetza (500 mg)**
			A		**B**			
	Kinetic analysis		**pH 1.2**	**pH 6.8**	**pH 1.2**	**pH 6.8**		
A	$Q_t = Q_0 + K_0 t$	K_0	0.338	0.301	0.326	0.306	0.383	0.386
		r^2	0.949	0.946	0.918	0.961	0.983	0.986
B	$\log Q_t = \log Q_0 + \frac{K_1 t}{2.303}$	K_1	-0.006	-0.008	-0.007	-0.008	0.0059	0.0058
		r^2	**0.998**	0.968	**0.997**	**0.993**	0.947	0.984
C	$Q_t = K_H t^{1/2}$	K_H	4.971	5.41	5.138	5.36	4.603	4.573
		r^2	0.991	**0.988**	0.973	0.972	**0.991**	**0.999**
D	$Q_0^{1/3} - Q_t^{1/3} = K_s t$	Ks	0.006	0.005	0.006	0.005	0.0077	0.0078
		r^2	0.909	0.907	0.877	0.876	0.9548	0.955
E	$Q_t = K_p t^n$	n	0.455	0.443	0.472	0.461	0.624	0.603
		KP	4.58	3.631	4.38	4.47	0.943	2.781
		r^2	0.961	0.969	0.950	0.992	0.957	0.977
F	$f_1 = \frac{\sum_{j=1}^{n} \lvert R_j - T_j \rvert}{\sum_{j=1}^{n} R_j} \times 100$						2	
G	$f_2 = 50\log\left\{\left[1 + \left(\frac{1}{n}\right)\sum_{j=1}^{n} \lvert R_j - T_j \rvert^2\right]^{-0.5} \times 100\right\}$						89	

Based on these results the CM-AE-St (DS 0.06-0.059) derivative was selected to undergo further investigations and was compared to the commercial product Glumetza®. To better understand the effect of gastric acidity on the ampholytic starch matrix. Tablets have been incubated 2h in SGF and then transferred in SIF to follow the metformin release for a period of 12h. This study was conducted with ampholytic starch loaded with 50% metformin (similarly to 1000 mg Glumetza tablets with 500 mg metformin loading) for a better comparison of the two dosage forms.

An oblong punch was used to prepare CM-AE-St tablets of 1g (50% metformin loading), similar to Glumetza® in shape and weight **Figs. 8. I, insert**.

Figs. 8. I, shows the dissolution profiles of metformin released from the commercial tablet Glumetza® and from tablets based on ampholytic starch CM-AE-St (DS 0.06-0.059), both with 50% metformin loading. The insert present photographs of Glumetza® (G) and of tablet based on CM-AE-St (DS 0.06-0.059) at time 0 h and after 2 h incubated in SGF at pH 1.2. **Table 1** illustrates drug release kinetic data with similarity factor (f2) and difference factor (f1), where, "n" in equations (F) and (G) in **Table 1**, is the number of sampling points, Rj and Tj are percent of the dissolved reference (R) and tested (T) tablets at each time point (j) respectively. For curves to be considered similar, f_1 values should be close to 0, and f 2 values should be close to 100. Generally, f_1 values up to 15 (0-15) and f_2 values greater than 50 (50-100) ensure sameness or equivalence of the two curves and, thus, of the performance of the test (T) and reference (R) products (Moore and Flanner, 1996; FDA, August 1997 May 5. 2011.). Up to 6h, the dissolution profile of CM-AE-St tablet was similar to that of Glumetza®, with about 85% release of metformin. The kinetic analysis of the release profiles of metformin from all tested tablets (**Table 1**) shows a good fit to the Higuchi's model ($r^2 > 0.99$), with the Korsmeyer's diffusional exponent $n \approx 0.5$ which can be related to diffusion with a small amount of relaxational controlled release of metformin.

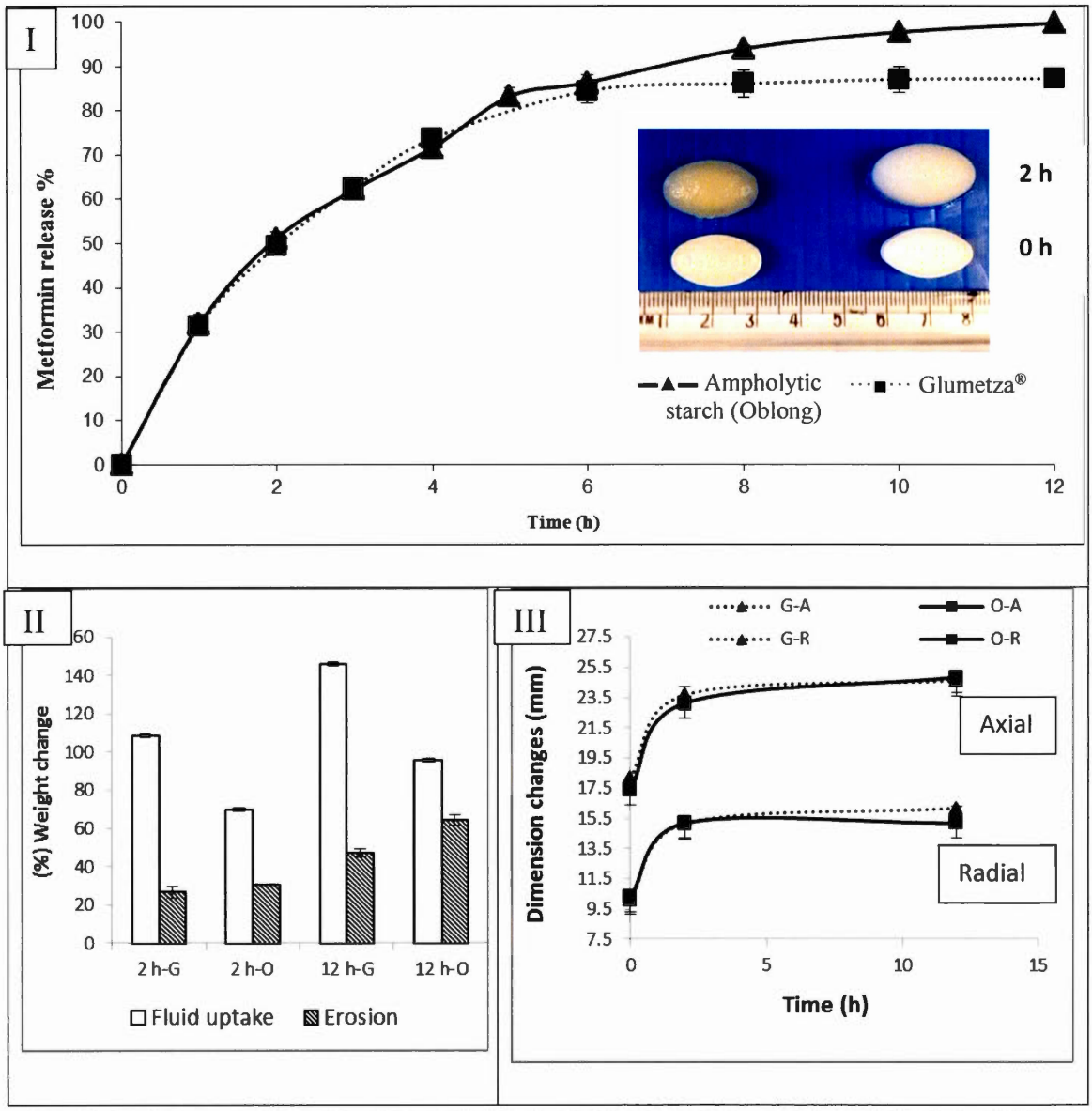


Fig. 8. Release profiles (**I**), Fluid uptake and Erosion (**II**), Axial and Radial tablet dimension changes (**III**) of Glumetza® (G) and tablet based on CM-AE-St(DS 0.06-0.059) oblong (O) of 1 g (50% loading). Oblong tablet was incubated: 2 h in SGF at pH 1.2 and up to 12 h in SIF at pH 6.8, whereas Glumetza® 500 mg incubated only in SGF at pH 1.2 up to 12 h. Insert in **(I)** Photographs of Glumetza® (G) and of tablet based on CM-AE-St (DS 0.06-0.059) at time 0 h and after 2 h incubated in SGF at pH 1.2.

3.8. Determination of the fluid uptake and erosion

Fluid uptake and erosion tests are shown in **Fig. 8. II**. It is noticed that Glumetza® tablets have more ability to absorb fluid, and therefore generate a higher swelling compared to oblong tablets based on CM-AE-St (DS 0.06-0.059). Furthermore, Glumetza® tablets showed a similar degree of erosion in comparison with the CM-AE-St oblong ones after 2 h in SGF, while after 12 h the tablets based on CM-AE-St (DS 0.06-0.059) showed higher erosion due to the greater polymer solubility in SIF in comparison to the limited solubility of the carrier used for Glumetza®. Axial and radial dimension changes (mm) of tablets were also observed at 2 and 12h (**Fig 8. III).** It appeared that the axial and radial sizes of both CM-AE-St (DS 0.06-0.059) tablet and Glumetza® were almost similar after 2 h. After 12h of incubation in acidic SGF, Glumetza® tablets showed a slightly greater radial increase from the initial tablet width of 10.13 mm up to 16.20 mm, when compared CM-AE-St (DS 0.06-0.059) with an initial width of 10.31 mm and 15.20 mm after 12 h of incubation in SIF.

4. DISCUSSIONS

Many attempts have been made to sustain the release of metformin, either by changing the metformin salts from hydrochloride to succinate (Kim and Park, 2015), or by selecting longer and heat involving methods of formulation like twin screw melt granulation/compression, hot melt extrusion and injection molding (Verstraete *et al.*, 2016; Nart *et al.*, 2017). Also floating (He *et al.*, 2014) or gastro-retentive tablets (Priyadarshini *et al.*, 2016), and coated pellets (Hu *et al.*, 2006; Xu *et al.*, 2015) have been used. All these forms are using either complicated methods of preparation, or lower concentration of metformin, and none of them can be compared in terms of loading and dissolution profile with the commercial available product Glumetza®. Between pharmaceutical excipients currently used for controlled drug delivery systems, modified starches emerged as multitasking materials able to fulfill multiple

roles: binders, fillers, matrix-formers, etc (Mateescu *et al.*, 2015). This multifunctionality associated with easiness of chemical modification was valorized for a wide range of applications. It was previously shown that the carboxylic groups on starch chains have a major role in the stabilization of the CM-St matrix in gastric fluid (Calinescu *et al.*, 2005; Calinescu *et al.*, 2007), having also a marked impact on matrix swelling, erosion, and dissolution, thus contributing to the mechanisms of drug release. Previous studies (Assaad *et al.*, 2011) reported the release of metformin from monolithic dry-coated and uncoated tablets of CM-St, CM-St and chitosan physical mixture or polyelectrolyte complex (PEC) of CM-St and chitosan, with metformin 20% loading. All these dry-coated or uncoated formulations released more than 80% of metformin within 3 h, except dry-coated formulations containing a physical mixture of CM-St and chitosan of high molecular weight which prolonged the release of 80% metformin to about 4 h. Based on the previous study (Assaad *et al.*, 2011), the ampholytic starch was proposed with both amino and carboxyl groups on the same starch backbone chain **Fig 1, A**. Our concept is that self-stabilization of ampholytic starch may occur in both acidic (gastric) and intestinal (neutral) media **Fig 1, B**. This self-stabilization seems to be proven by structural analysis. The fusion of CM-AE-St granules after amination observed in SEM could be attributed to the introduction of aminoethyl groups on the CM-St backbone, which could increase the hydrogen bonding and also inducing an ionic stabilization between the carboxymethyl and amine groups. It was also interesting to note that a low degree of substitution, the carboxymethylation has no impact on polymer stability (Rudnik *et al.*, 2006). By comparing the MDT values of CM-AE-St derivatives to those of CM-St for both DS (0.020 and 0.06) it appears that after amination, the MDT was raised to 294 °C for CM-AE-St (DS 0.02) and to 299 °C for CM-AE-St (DS 0.06), suggesting an enhanced stability of CM-AE-St compared to CM-St. This may be related to additional hydrogen association and ionic stabilization in the case of the ampholytic CM-AE-St. When the hydroxyl groups were modified, the hydrogen bonds were mostly broken and consequently starch lost its crystallinity. The most ordered

ampholytic starch excipient was that obtained with the highest DS (0.06-0.059) due to an additional ionic stabilization involving the CM- and AE-groups (**Fig. 6.II**). The presence of CM groups on the polysaccharidic chains may offer gastro-resistance, whereas AE groups will reduce the polymer solubility in SIF. Moreover, a polyelectrolyte complexation between the anionic CM- and cationic AE- groups located on neighboring chains, may also contribute to CM-AE-St stabilization. In gastric fluid, the ampholytic starch undergoes protonation of both carboxylic (generating a compaction of the outer layer around the tablet) and aminoethyl groups which produce an outer hydrogel able to release metformin in the early stage of gastric residence. Due to the ionic stabilization involving carboxylic and ethylamine groups in case of CM-AE-St based tablets, the fluid uptake was moderate (69% after 2 h and 95% after 12 h), whereas in the case of Glumetza® it was higher (108% after 2h and 146% after 12 h). The pill might be able to continue the intestinal transit through the pylorus, with the gastric emptying waves differing thus from gastroretentive dosage forms (i.e Glumetza®). In the intestinal fluid, the protonated carboxyl groups of the ampholytic CM-AE-St, located at the outer gel layer of the monolithic tablets, will gradually change the protons for Na^+ cations mostly arriving with the $NaHCO_3$ secretion of the pancreas. This will facilitate hydration, fluid uptake, erosion, and polymeric material dissolution in the simulated intestinal medium. In the case of CM-AE-St based tablets the erosion increased from 30% after 2h in SGF to 64% after 12h in SIF. In comparison with CM-St alone, CM-AE-St polymer solubility was lowered due to lesser solubility of amine groups in neutral media. The metformin release from Glumetza® is controlled by hypromellose (HPMC) (insoluble) as a matrix forming agent. About 85-88% of metformin was released in the first 6 h, without further liberation in SGF, which may be due to a tight gel formed around the tablets. It is considered that when HPMC is hydrated in aqueous media, more than one state of water exists in the surface gel layer of a HPMC matrix (Ford and Mitchell, 1995; McCrystal *et al.*, 1999). It was postulated that water may exist as (a) tightly bound water that interacts with polymer chains and

is nonfreezable, (b) free water which is freezable and (c) water that exists in bound states between these two extremes (Ford and Mitchell, 1995; McCrystal *et al.*, 1999). The high fluid uptake of Glumetza® (108% after 2h and 146% after 12 h) is required to get a tablet ensuring gastro-retentive releasing medication. Taking into consideration the tight bond between HPMC and water, other factors relating to the drug itself can alter the mechanism of drug liberation. Highly soluble drugs are thought to be released, from tablet formulated with HPMC, principally but not exclusively, by diffusion (the case of Glumetza® having n = 0.60 in Korsmeyer–Peppas equation) whilst poorly soluble drugs are released primarily by erosion. In addition, highly soluble drugs may act as pore formers that may make the pathways within gel structures less tortuous (Ford, 2014). With regard to HPMC, another factor to be taken into account is that ionically charged drugs are less mobile due to their potential interaction with the gel. This may increase the time taken for such drugs to diffuse through the gel structure (Sung *et al.*, 1996; Fyfe and Blazek-Welsh, 2000). Both tablet types, CM-AE-St and Glumetza®, are in compliance with USP requirements (USP, 2015) for the dissolution of metformin in the controlled release dosage form. The f1=2 and f2=89 values confirm also that *in vitro* drug release of CM-AE-St tablets is similar to that of commercial product Glumetza®. It is noteworthy that the remaining unabsorbed metformin accumulates in the gut mucosa prior to be ultimately eliminated with feces. Buse, Fineman et al (Buse *et al.*, 2016) provided clinical evidence suggesting the primary hypoglycemic effect of metformin resides in the human gut and they described a novel delayed-release metformin formulation. It was hypothesized that gut exposure of metformin, but not circulation, accounts for most of its antihyperglycemic effect (Buse *et al.*, 2016). This is why our ampholytic starch may be an excipient of interest for gut delivery.

5. CONCLUSION

The ampholytic CM-AE-St excipient was able to control the release of metformin in SGF and SIF for 8h. The new proposed polymeric excipient can be self-stabilized and compacted in acidic medium due to protonation of CM groups and generating an outer gel by hydration of AE groups. Furthermore, the AE groups become insoluble in neutral intestinal medium. Thus, the presence of the two groups on the same macromolecular backbone will generate a beneficial effect for the usage of such polymer as a carrier for the formulation of highly soluble drugs helping to control the release of drug during the transit along the gastrointestinal tract. In comparison with commercial product, the tablets of CM-AE-St (DS 0.06-0.059) with 50% drug loading have a similar dissolution profile to Glumetza® but releasing the drug over the whole intestinal tract and follow USP requirements for sustained release tablets of metformin. This opens a new perspective of ampholytic starch derivatives as novel pharmaceutical excipients for the challenging formulation of highly soluble drugs with required high loading. Further studies are going to investigate the pharmacokinetic performance of CM-AE-St (DS 0.06-0.059) in comparison with commercial products.

Acknowledgments

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CHAPTER VIII

DISCUSSION AND CONCLUSIONS

The premise of the first part of the presented project was based on a study on the separation of macrophages by retention on a cross-linked starch column and further detachment by enzymatic hydrolysis of the chromatographic support (Desmangles *et al.*, 1992). Four starch materials namely: gelatinized starch (G-St), acetate starch (Ac-St), carboxymethyl starch (CM-St) and aminoethyl starch (AE-St) were investigated for their ability to form films susceptible to amylolysis to be used as substrate/support for macrophage separation by mild enzymatic amylolysis. This approach is different to the previous reported method (Desmangles *et al.*, 1992) to separate macrophages using cross-linked starch as a chromatographic support. In general, cross-linked materials are adequate to form microspheres but present lower filmogenic ability than the uncross-linked materials (Krumova *et al.*, 2000; Berezkin and Kudryavtsev, 2015). A major objective of this study was to understand the critical role of surface properties of starch materials on the attachment of macrophages and consequently the influences on their viability.

Starch materials were obtained either by gelatinization or chemical modification. Such modifications on starch can profoundly alter the physicochemical and morphological properties of starch, its enzymatic digestibility and can consequently expand its current use as an excipient in drug delivery dosage forms (Mulhbacher *et al.*, 2004; Massicotte *et al.*, 2008). The grafting of each functional group on the starch chains was confirmed by structural analysis using FT-IR, ^{1}H NMR, and SEM. Marked differences between the starch derivatives on FT-IR spectra were observed at level of bands in the range 1000-2000 cm^{-1}. In case of CM-St, the bands at 1589 cm^{-1} and at 1323 cm^{-1} have been ascribed to COO^{-} group (Friciu *et al.*, 2013). The high intensity of the band at 999 cm^{-1} for AE-St could be ascribed to C-N stretching

vibrations, whereas the weak shoulder at around 1735 cm^{-1} was assigned to $-NH_3^+$ group (Deng *et al.*, 2006; Assaad *et al.*, 2011). In the case of Ac-St, the weak shoulder at around 1556 cm^{-1} corresponds specifically to the -C-O stretching of acetyl groups (Colthup *et al.*, 1990; Bello *et al.*, 2010). On the other hand, ^1H NMR results showed that the most significant peaks for AE-St were at δ = 4.15 - 4.25, δ = 3.16 - 3.18 ppm, which belong to the hydrogens of aminoethyl group, whereas in the case of Ac-St, the peaks at δ =1.9 - 2.1 ppm and at δ =3.5 ppm were ascribed to methyl protons of acetate groups (Xu and Hanna, 2005b). SEM of native starch (Hylon VII) showed a granular aspect predominantly round or oval in shape with a smooth surface and uniform range of size distribution (5–10 μm). The granular aspect fits well with the known crystalline structure of native starch (Friciu *et al.*, 2013) stabilized by hydrogen bonds between the hydroxyl groups of glucopyranose units. The G-St showed a round and sponge-like shape which is due to the physical modification (gelatinization) of native starch. Differently, the CM-St presented an irregular shape with an uneven surface likely due to the association of numerous small particles forming larger granules of similar shapes as that obtained by Friciu *et al.,* 2013. The acetyl groups can decrease the starch stabilization by hydrogen bonding. At the same time, the glucose units carrying polar hydroxylic groups and non-polar (acetate) functions, may favor starch macromolecules to coalesce together resulting in a kind of fusion of granules (Singh *et al.*, 2004; Bello *et al.*, 2010). The carboxylic groups may reduce the network self-assembling by hydrogen association between hydroxyl groups and promote repulsion effects leading to a structural reorganization (Lemieux *et al.*, 2010). The acetylation generated a slightly rough surface of granules which appeared fused in a kind of aggregate. The AE-St grains showed a porous irregular shape, whereas amine groups may promote additional and stronger hydrogen bonding resulting in a reorganization of the AE-St network.

These starch polymers are filmogenic and were used to coat holder devices for macrophage adhesion. The contact angle was measured for the prepared films of

starch derivatives. The CM-St and AE-St films presented a lower angle (67° and 78° respectively) in comparison to G-St (89°) and Ac-St (105°), meaning that G-St and Ac-St were less polar as expected and even more hydrophobic. The obtained films from starch derivatives presented a susceptibility to mild hydrolysis with alpha-amylase, which was used to liberate of the adhered macrophages. The enzymatic activity of alpha-amylase with starch filmogenic supports was determined. It was found that G-St, AC-St and AE-St showed similar film hydrolysis rate over the first 40 min. Then, the G-St hydrolysis was faster than that of AC-St and AE-St. This behavior was considered as normal because there is no chemical modification of the G-St. The lowest enzymatic activity was observed with CM-St film, where the released amount of maltose after 75 min was almost half of that liberated from G-St. The film hydrolysis was also followed visually. Even without complete amylolysis, the CM-St film was dissolved in less than 10 min, because CM-St is soluble in alkaline medium. Differently, G-St film was partially hydrolyzed in 30 min, AC-St and AE-St in 40 min.

The possible interaction between macrophages and starch films was evaluated by cell counts, percentage of dead cells and level of tumor necrosis factor (TNF-α). Control cultures on uncoated insert devices appear as plump or stellate, monolayers rounded and spindle-like with majority of live cells. Macrophages incubated on insert devices coated with G-St, Ac-St and AE-St showed round, compact and mostly live cells. Differently, prevalently dead cells were observed when incubated in insert coated by CM-St film, owning round, spindle-like and translucent cytoplasm.

Macrophages adhere on adequate surfaces whereas floating cells are characteristically dying cells. Macrophage counting suggested good adhesion on G-St, on Ac-St and on AE-St materials. The higher percentages of dead macrophage (floating) were observed at inserts coated with anionic CM-St (about 32±5%) or with the cationic AE-St (about 32±9%), whereas a low percentage of dead cell was observed with

inserts coated with non-ionic polymers Ac-St (5±2%) or G-St (9±3%) respectively, suggesting higher percentage of living cells from these films.

Macrophages can be obtained in a relatively pure form as primary cultures for analytical and biochemical manipulations but they do not generally replicate in culture, have relatively short-lives, and may be difficult to obtain enough amounts for large scale experiments. They are very sensitive to small changes in their environment and may be damaged considerably, even when delicately handled after cell culture (Adams, 1979; Féréol *et al.*, 2006). Detaching adherent macrophages from a culture dish is difficult, since these cells adhere avidly to plastic surfaces of cell culture devices (i.e. Petri dishes, microplates). Several procedures are currently applied to regain macrophage cells such as mechanical detachment by gentle scraping of macrophages with a rubber policeman (Fleit *et al.*, 1984; Porcheray *et al.*, 2005; Jaguin *et al.*, 2013) or pre-treatment with scandicain K, proteinase, or pronase (Malorny *et al.*, 1981). A procedure which was limitative as it has mitogenic effects on macrophages. Frequently by mechanical detachment, about half of cells may remain viable (Adams, 1979). Consequently, high variability and significant loss of viable cells represent major limitations for existing methods.

In the presented project, the cell harvesting was done by scrapping of cells cultured on uncoated insert devices (control procedure) or by enzymatic (alpha-amylase) hydrolysis of films from inserts coated with different starch materials. After incubation for 48 h, cell numbers increased about 3.2 times for control uncoated inserts, 4.2 times for Ac-St and 5.3 times for G-St whereas only 1.5 times was observed for AE-St coated insert. Furthermore, 129 % and 164 % more cells were recovered from inserts devices coated with G-St and Ac-St when compared to controls (un-coated inserts), whereas a 53% drop of the yield was obtained for AE-St coated inserts. This inhibitory effect could be explained by a strong interaction of cationic aminoethyl groups of starch film with membrane phospholipids of macrophage cells (Kurtz-Chalot *et al.*, 2014).

The effect of starch derivatives on macrophage activity was investigated by activation of macrophage by lipopolysaccharide (LPS) and quantitation of induced tumor necrosis factor (TNF-α). LPS triggers the abundant secretion of cytokines by macrophages including tumor necrosis factor (TNF-α), interleukin (IL)-1, and IL-6 (Meng and Lowell, 1997). In our study, the amount of TNF-α secreted by macrophages in response to LPS was in the same range as reported in similar studies (Lichtman *et al.*, 1998). Moreover, there were no differences in TNF-α produced by control cells harvested from uncoated inserts (91±3.5 pg/mL) or by macrophages harvested from G-St (90±2.3 pg/mL) and Ac-St (89 ±2.9 pg/mL) coated inserts.

The functional groups grafted on polysaccharide chains not only have had a direct effect on the viability of cells, but they can impact macrophage adhesion. For instance, the non-derivatized starch (G-St) and the Ac-St with nonpolar acetate groups oriented toward culture medium, are better supports for adhesion of macrophage cells than the anionic (CM-St) and cationic (AE-St) starch derivatives which are less compatible. The lowest percentage of dead cells (non-adherent fraction) was observed with inserts coated with G-St and Ac-St. Therefore, these gelatinized starch and acetate starch materials affording a best viability, could be a good choice as support material for macrophage culture due to the high compatibility with cells and also for their susceptibility to mild enzymatic amylolysis. These features of G-St and Ac-St allowed the recovery of macrophage cells with better viability and high yields. This result together with the low percentage of dead cells could be an evidence of biocompatibility of G-St and Ac-St supports as materials for macrophage preparation by this novel mild enzymatic procedure.

This study opens up new perspectives to obtain macrophage cells with a high viability, avoiding significant loss of viable cells which still limits the current scratching procedures.

As far as the synthesis and characterization of ampholytic starch is concerned, to the best of our knowledge, the results presented in chapter VI and in chapter VII may be considered as novel considering the different methods of preparation.

In fact, we are probably the first group proposing ampholytic starch having CM and AE groups on the same backbone chain for use as pharmaceutical excipients or as carriers of bioactive agents (Sakeer *et al.*, 2017a). Previous studies described amphoteric starch carrying quaternary ammonium (QA) and phosphate groups (Lin *et al.*, 2012; Peng *et al.*, 2016), or QA and CM groups (Shimei *et al.*, 2006; Yang *et al.*, 2014c), or QA and starch-graft-poly(acrylamide-co-acrylic acid) (Yang *et al.*, 2014b), or QA and succinic anhydride (Lekniute *et al.*, 2013). The majority of these ampholytic derivatives have been proposed for applications as flocculants (Xu *et al.*, 2005a; Shimei *et al.*, 2006; Lekniute *et al.*, 2013; Yang *et al.*, 2014b; Yang *et al.*, 2014c; Peng *et al.*, 2016). Etherification of starch by the reaction of hydroxyl groups of starch with sodium monochloroacetic acid (SMCAA) produced carboxymethyl starch (CM-St) which was used as a matrix - forming agent for drug delivery, either to protect the active pharmaceutical ingredient (API) from gastric medium (Calinescu *et al.*, 2005; Calinescu *et al.*, 2007), or as controlled release excipients (Lemieux *et al.*, 2009) or even as excipient for chronodelivery medication (Ispas-Szabo *et al.*, 2016). Only limited studies investigated aminoethyl starch (AE-St) as biomedical material (Mell *et al.*, 1968; Sakeer *et al.*, 2017b). AE-St was obtained by aminoethylation of starch after etherification with chloroethylamine hydrochloride (CEAHC).

The hydroxyl groups on glucopyranose ring are susceptible to react with the proposed reagents SMCA and / or CEAHC in the order C_2, C_6 and C_3. In ampholytic starch, the hydroxyl group can be involved in substitution with CM (CH_2COONa) groups after reaction with SMCA or substitution with AE ($CH_2CH_2NH_2$) groups by reaction with CEAHC, whereas secondary interactions between CEAHC and SMCA may produce ethylamino carboxymethyl (EACM) ($CH_2CH_2NHCH_2COONa$) or ethylamino dicarboxymethyl (EADCM) $CH_2CH_2N(CH_2COONa)_2$ groups. In the case of one step polymer preparation (Fig. 6.2) at least three different types of structures can theoretically occur: 1) CM and AE starch derivatives; 2) CM and secondary and/or

tertiary amines, but exhibiting terminal aminoethyl (AE) groups; 3) ethylamine groups substituted with CM groups exhibiting AECM or $AE(CM)_2$ (EADCM). In two steps method, when CM groups are grafted firstly, followed by introduction of AE groups, one type of starch derivative is expected presenting CM and AE groups. Differently, for AECMSt, when AE groups are grafted first and then followed by CM groups, there is a good probability of amine groups to be substituted with CM groups and to generate also EACM and EADCM groups (Fig. 6.2). The degrees of substitution (DS) with CM determined by back-titration of the ampholytic samples were 0.024, 0.041 and 0.028 for CMAESt (OS), CMAESt (TS) and AECMSt (TS), respectively. These DS values represented the average number of carboxymethyl groups per glucose unit. The DS in terms of primary amine (AE) groups were 0.012, 0.012 and 0.015 mmol/g for CMAESt (OS), CMAESt (TS) and AECMSt (TS), respectively. The ionic charge of the aqueous solutions of ampholytic starches calculated as zeta potential (ζ) was -25.9 mV for CMAESt (OS), whereas ζ values of -42.98 mV were found for CMAESt TS and -52.5 mV for AECMSt (TS). These values are consistent with the chemical modification of starch by CM, AE, EACM or EADCM groups. The highest value for AECMSt (TS) could be ascribed due to the higher amount of EACM and / or EADCM providing a stronger negative charge (Wongsagonsup *et al.*, 2005a; Wongsagonsup *et al.*, 2005b). For the 1% (w/v) polymer solution in water the pH values were 6.42, 6.2 and 6.9 for CMAESt (OS), CMAESt (TS), and AECMSt (TS) respectively, indicating that almost all ampholytic starch were neutral. The viscosity of CMAESt (TS) after 4 h in water was 8.34 cP, higher than that of AECMSt (TS) and of CMAESt (OS), whereas after 48 h all polymers showed almost similar viscosity. These values indicate that all polymers are hydrated almost at the same extent in water but with different rates. On the other hand, CMAESt (OS) showed higher viscosity after 4 h and after 48 h in phosphate buffer pH 6.8 in comparison with CMAESt (TS) and AECMSt (TS) probably due to the higher solubility of both CMAESt TS and AECMSt in phosphate buffer, whereas CMAESt (OS) undergo swelling rather than solubilization in SIF (Table 6.1).

The obtained polymer was characterized structurally by FT-IR and by ^{1}H NMR. The presence of new bands was in accordance with either CM and / or AE groups grafting on starch backbone. Moreover, TGA results indicate that the maximal decomposition temperature (MDT) was 291 °C for AECMSt (TS), whereas MDT for CMAESt (OS) was 294 °C and MDT for CMAESt (TS) was 293 °C. All values are lower than the MDT of native Starch (324 °C). The reason seems to be the substitution of hydroxyl groups of starches with CM and / or AE and / or EACM or EADCM groups. By calculating the area under the curve (AUC) for the main stage of polymer degradation, the decomposed component could be qualitatively determined. The AUC of the main decomposition stage of native starch was 80.6 and it is higher than AUC of other derivatives, which means that native starch is decomposed as a function of temperature at a different pattern than other ampholytic starch derivatives, due to their functionalization starch with CM, AE, EACM or EADCM groups. The AUC for CMAESt (TS) and AECMSt (TS) are very close each other, suggesting that similar amounts of resembling components are decomposed as a function of temperature. (Tiwari and Hihara, 2009; Tiwari and Hihara, 2012).

X-ray diffraction analysis showed that by derivatization, a pronounced reduction in order degree was observed with peaks decreased or disappeared (those at $2\theta = 19.84^{\circ}$, 25.88°), suggesting the loss of the B-type double helix, possibly due to the effect of derivatization of the hydroxyl groups with either CM groups (Wang *et al.*, 2010; Gao *et al.*, 2011) or AE groups (Kuo and Lai, 2007; Pi-xin *et al.*, 2009; Chang *et al.*, 2014) or EACM or EADCM groups. However, hydrogen bonds contributed to maintain the starch order in a certain extent. The CMAESt (OS) pattern presented sharper peaks at $2\theta = 13^{\circ}$-16° and $2\theta = 21^{\circ}$-25° than CMAESt (TS) or AECMSt (TS).

In order to understand the behaviour of ampholytic starch derivatives in simulated gastro-intestinal tract, the fluid uptake, erosion and diameter changes of tablets containing only the ampholytic starch derivatives incubated 2 h in SGF and then

moved to SIF, were investigated. It was clear that CMAESt (OS) showed the highest ability of fluid uptake with lowest erosion pattern due to its limited solubility in SGF and in SIF. Differently, AECMSt (TS) showed a lower tendency to swell and higher erosion due to its solubility in SIF. The higher fluid uptake and limited erosion for CMAEST (OS) was in accordance with the higher dimension changes. CM-AE-St (OS) presented erosion in SGF but when moved to SIF a gel layer was formed on the outer surface of the tablet. On contrary, CMAESt (TS) formed a compact gel in SGF. The compact gel kept the integrity of the tablet and lowered further fluid penetration and dissolution of the hydrated layer on the outer surface of the tablet when the tablet moved to SIF.

Few approaches were suggested to overcome on the shortage of high solubility associated with CM-St in simulated intestinal fluid (SIF), since the carboxyl groups are deprotonated, ionized and then solubilized. For instance, 50% protonation of CM-St could slower the solubility of polymer in SIF (Assaad and Mateescu, 2010). It was also proposed a mixture of CM-St with polymers containing amine groups insoluble in SIF, which could help as electrolytic complexation agent i.e. chitosan. The association of chitosan with CM-St was done either by physical mixture of these two polymers (Calinescu *et al.*, 2012) or by polyelectrolyte (PE) complexation (Assaad *et al.*, 2011) processing. The PE polymer was able to sustain the release of neutral and acidic API in SIF at 20% drug loading (Assaad *et al.*, 2011).

In the present project, monolithic tablets consisting of 60% loading of Acetylsalicylic acid (ASA) or Metformin or Mesalamine or Acetaminophen as drug models were prepared by direct compression of the active molecule with various ampholytic starch derivatives. The dissolution studies were conducted in simulated gastric fluid (SGF) and in simulated intestinal fluid (SIF). The results showed that the addition order of reagents can modulate on polymer properties. The ampholytic starch obtained from one step: CMAESt (OS) was able to control the release of acidic, amphoteric and neutral medications in SIF. Differently, the polymers obtained by two steps method

CMAESt (TS) and AECMSt (TS) were able to sustain the release of these tracers in SGF. Thus starting from the same reagents, but with different order of addition, it was possible to obtain polymers having different characteristics.

When the *in vitro* dissolution test was followed for 2 h in SGF and then in SIF, all ampholytic starch derivatives were able to modulate the release of acidic, amphoteric, and neutral drugs for about 24 h.

Self-stabilization of ampholytic starch CMAESt (OS) could be explained by the limited solubility of the polymer in SGF. In SIF a higher swelling ability and lower chain flexibility (as observed from viscosity measurement) with a limited solubility of CMAEST (OS) are the key factors of controlling the release pattern of medications. Moreover, a polyelectrolyte complexation between the anionic CM- and cationic AE- groups located on neighboring chains, may also contribute to CM-AE-St (OS) gel formation and stabilization. On other hand, self-stabilization of ampholytic starch CMAESt (TS) could be explained by protonation of CM groups and formation of an outer gel layer by hydration of AE groups in SGF. In SIF, the compacted gel layer generated in SGF alongside with limited solubility of AE, formed a barrier toward further penetration of intestinal fluid inside the pills matrix and thus, lowering the erosion and solubilzation of the tablet, particularly of outer gelled layer surface. Consequently, maintaining the tablet integrity the release of active ingredient from the dosage form was prolonged. Thus, the presence of the two groups on the same macromolecular backbone will generate a beneficial effect for the usage of such polymer as excipients for challenging formulation of drugs with high loading and with control of the APIs release during the transit along the gastrointestinal tract.

CMAESt (TS) underwent further investigation, since it could be obtained in different degrees of substitution by varying the number of CM and AE groups. It was hypothesized that the simultaneous presence of anionic and cationic groups will

generate a stronger self-stabilization of starch matrices and an improved control of drug release.

In the context of a growing interest for sustained drug release devices and for high loaded dosage forms there is a need for new excipients, particularly for the formulation of highly soluble drugs such as metformin. Known as one of the most recommended drug for diabetes type II (Bailey, 1992; Bailey and Turner, 1996), metformin is also highly hydrosoluble and for this reason difficult to formulate as sustained release (SR). Metformin (biopharmaceutical classification system: BCS, class I) was approached as model drug and monolithic tablets with 50% and 60% loading were prepared by direct compression of the active molecule with various CM-AE-St derivatives. Drug classes are defined as table 8.1: class 1. High solubility-high permeability drugs, class 2. Low solubility-high permeability drugs, class 3. High solubility-low permeability drugs and class 4. Low solubility-low permeability drugs (Amidon *et al.*, 1995).

Table 8.1: Biopharmaceutical classification system (BCS).

Class	**Solubility**	**Permeability**
I	High	High
II	Low	High
III	High	Low
IV	Low	Low

Metformin has properties of a strong base (pK_a 2.8 and 11.5) and, as hydrochloride, it is highly hydro-soluble and in aqueous solutions of pH 1.2 to 6.8 (Desai *et al.*, 2014a). Because of its short half-life (< 3 h), frequent administrations at high daily

doses (up to 2.5 g) are needed to maintain its required plasma concentration (Garber *et al.*, 1997; Stepensky *et al.*, 2001; Qin *et al.*, 2014). Metformin is considered mostly absorbed in the duodenum (Song *et al.*, 2006). Some adverse effects (i.e. diarrhea, abdominal discomfort, nausea) caused by immediate release dosage forms of metformin (Glucophage®) remain unsolved. Different approaches were previously implemented for formulations of such highly soluble molecules such as embedding them in release-modifying polymers, often followed by the application of additional functional coatings. The large majority of commercial dosage forms are based on cellulose derivatives, polyethylene oxides or other gel-forming polymers. For production, frequently using a multi-step processes may include wet granulation and hot melt methods. However, it is generally considered as difficult to formulate sustained release forms of cationic drugs such as chlorohydrate salts, which are highly soluble and required in large amount (high loading). Metformin is also poorly absorbed from the stomach and with a rate-limiting absorption from the duodenum. The commercially available controlled release product Glumetza® tablets (metformin hydrochloride: Depomed Inc.) (Laustsen, 2005; Laustsen, 2006) and the majority of sustained release devices are designed as gastroretentive forms based on a prolonged gastric residence controlled by swelling or by floating or by mucoadhesion to retain the dosage form releasing the active pharmaceutical principle in the stomach. With Glumetza®, too much metformin release in the stomach and to the upper intestine may generate a tissue saturation (Proctor *et al.*, 2008) and thus a lower absorption with a certain decrease of efficiency. Furthermore, metformin was found to be absorbed not only at the level of the upper intestine, but over the whole intestinal tract (Bailey, 1992; Song *et al.*, 2006). Thus, the gastrointestinal absorption of metformin is incomplete, possibly related to the saturable absorption process. Furthermore, about 20%-30% of an oral dose was considered to be recovered in the faeces (Tucker *et al.*, 1981). Absorption is estimated to be completed within 6 h after administration and is presumably confined to the upper intestine (Tucker *et al.*, 1981; Vidon *et al.*, 1988). In fact, the whole intestine is necessary for sufficient absorption of the drug

(Vidon *et al.*, 1988). A too high absorption with saturation at the upper intestine may generate side effects and a lesser efficiency by reducing the amount of drug available for the lower intestine absorption. Consequently, a formulation of metformin with continuous release over the entire gastrointestinal tract could reduce adverse gastric side effects is still needed. In this context, we have proposed as a new carrier the ampholytic starch expected to control the release of high-dose of highly soluble drugs and to keep the tablet size small enough alongside with a single step procedure of tablet preparation.

The *in vitro* drug dissolution tests from tables prepared from ampholytic starch and metformin (Sakeer *et al.*, 2017a), showed that higher degrees of substitution for both CM- and AE- groups favor the ability of ampholytic CM-AE-St to control the drug release in simulated gastric fluid and in simulated intestinal fluid. The presence of CM groups on the polysaccharidic chains may offer gastro-resistance, whereas AE groups can reduce the polymer solubility in SIF. Moreover, a polyelectrolyte complexation between the anionic CM- and cationic AE- groups located on neighboring chains, may also contribute to CM-AE-St polyelectrolytes stabilization. In gastric fluid the ampholytic starch undergoes protonation of both carboxylic (generating a compaction of the outer layer around the tablet) and aminoethyl groups which produce an outer hydrogel able to release metformin in the early stage of gastric residence.

Tablets based on CM-AE-St derivatives were compared to the commercial Glumetza® (both at 50% loading). Due to the ionic stabilization involving carboxylic and ethylamine groups in the case of CM-AE-St based tablets, the fluid uptake was moderate (69% after 2 h and 95% after 12 h), whereas in the case of Glumetza® it was higher (108% after 2h and 146% after 12 h). The CM-AE-St pill might be able to continue the intestinal transit through the pylorus, with the gastric emptying waves, differing thus from gastroretentive dosage forms (i.e Glumetza®). In the intestinal fluid, the protonated carboxyl groups of the ampholytic CM-AE-St, located at the

outer gel layer of the monolithic tablets, will gradually change the protons for Na^+ cations mostly arriving with the $NaHCO_3$ secretion of the pancreas. This will facilitate hydration, fluid uptake, erosion, and polymeric material gradual dissolution in the simulated intestinal medium. In the case of CM-AE-St (TS) based tablets the erosion increased from 30% after 2h in SGF to 64% after 12h in SIF. In comparison with CM-St alone, CM-AE-St polymer solubility was markedly lowered due to lesser solubility of amine groups in neutral media.

The metformin release was controlled for 12 h exhibiting a similar Higuchi's model dissolution profile for the two dosage forms (our tablets formulated with CM-AE-St (TS) and Glumetza®). Both tablet types, CM-AE-St and Glumetza®, are in compliance with USP requirements (USP, 2015) for the dissolution of metformin in the controlled release dosage form. The f1=2 and f2=89 values confirm also that *in vitro* drug release of CM-AE-St tablets is similar to that of commercial product Glumetza®. It is noteworthy that the remaining unabsorbed metformin accumulates in the gut mucosa prior to be ultimately eliminated with feces. Buse, Fineman et al (Buse *et al.*, 2016) provided clinical evidence suggesting the primary hypoglycemic effect of metformin resides in the human gut and they described a novel delayed-release metformin formulation. It was hypothesized that gut exposure of metformin, rather than circulation, accounts for most of its anti-hyperglycemic effect (Buse *et al.*, 2016). This is why our ampholytic starch may be an excipient of interest for gut delivery.

CHAPTER IX

ANNEXES: Published Articles, Abstracts and Poster presentations.

A.1. Published Articles:

A.1.1 Starch materials as biocompatible supports and procedure for fast separation of macrophages. Published in Carbohydrate Polymers, 2017, vol. 163, 108-117. 159

A.1.2 Self-stabilizing ampholytic starch excipients for sustained release of highly soluble drugs: the case study of metformin. Published in AAPS PharmSciTech, 2017, DOI: 10.1208/s12249-017-0723-3.....169

A.1.3 Ampholytic starch - excipients for high loaded drug formulations: Mechanistic insights. Published in International Journal of Pharmaceutics, 2017, 535, 201-216.....184

A.2. Oral presentation

A self-stabilizing ampholytic starch excipients for sustained release of highly soluble drugs: the case of metformin. 19th Annual CBGRC, November 18th, 2016, Concordia University. Montreal, Quebec, Canada. 200

A.3. Extended abstract:

New class of self-stabilizing ampholytic starch as excipients for drug sustained release. Control Release Society annual meeting, July 16-19, 2017. Boston, Massachusetts, USA. 201

A.4. Posters

A.4.1. Ampholytic starch excipients for high loaded drug formulations. 7e Colloque Pharmaquam (Université du Québec à Montréal). 31 August, 2017. Montréal, Québec, Canada. 204

A.4.2. Novel ampholytic starch derivatives as excipients for high loaded drug formulations. 7e Colloque RQRM (Réseau québécois de recherche sur les médicaments). 30 Sept – 1 Oct, 2017. Québec, Canada. 205

A.1. Published Articles

A.1.1. Starch materials as biocompatible supports and procedure for fast separation of macrophages. Published in Carbohydrate Polymers, 2017, vol. 163, 108-117.

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Starch materials as biocompatible supports and procedure for fast separation of macrophages

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ABSTRACT

Different starch derivatives were evaluated as supports for attachment and recovery of macrophages (RAW 264.7 line). Gelatinized starch (G-St), acetate starch (Ac-St), carboxymethyl starch and aminoethyl starch were synthesized and characterized by FTIR, ^{1}H NMR, SEM and static water contact angle. These polymers are filmogenic and may coat well the holder devices used for macrophage adhesion. They also present a susceptibility to mild hydrolysis with alpha-amylase, liberating the adhered macrophages. Cell counts, percentage of dead cells and level of tumor necrosis factor (TNF-α) were used to evaluate the possible interaction between macrophages and starch films. The high percentage of cell adhesion (90–95% on G-St and on Ac-St) associated with enzymatic detachment of macrophages from film-coated inserts, resulted in higher viabilities compared with those obtained with cells detached by current methods scrapping or vortex. This novel method allows a fast macrophage separation, with excellent yields and high viability of recovered cells.

1. Introduction

Starch is widely used in food, pharmaceutical and biomedical applications due to its biocompatibility, biodegradability, non-toxicity and abundant sources (Rowe, Sheskey, Cook, & Fenton, 2009). Starch modification is generally achieved through derivatization such as cross-linking (Lenaerts et al., 1998), etherification, esterification (Calinescu, Mulhbacher, Nadeau, Fairbrother, & Mateescu, 2005; Mulhbacher, Ispas-Szabo, Lenaerts, & Mateescu, 2001) and grafting (Kaur, Singh, & Liu, 2007) of functional groups onto the carbohydrate structure. Such modifications can profoundly alter the physicochemical and morphological properties of starch, its enzymatic digestibility and can consequently modulate its current use as excipient in drug delivery dosage forms (Mulhbacher, Ispas-Szabo, & Mateescu, 2004; Massicotte, Baille, & Mateescu, 2008). An interesting reported application of starch was its use for enrichment of macrophage cell populations by adhesion on cross-linked starch microspheres followed by liquefaction of microbeads with alpha-amylase (Desmangles, Flipo, Fournier, & Mateescu, 1992). Macrophages are currently investigated in various biochemical and biomedical fields as well as for therapeutic applications (Kwan, Wu, & Chadban, 2014; Ostuni, Kratochvill, Murray, & Natoli, 2015; Wooden & Ciborowski, 2014; You et al., 2013). Macrophages with a possible role in inflammatory processes and malignancy were reported as a new therapeutic target. There is a growing interest for techniques of macrophage separation, particularly to investigate anti-macrophages novel strategies against cancer. Macrophages can be obtained in a relatively pure form as primary cultures for analytical and biochemical manipulations but they do not generally replicate in culture, have relatively short-lives, and may be difficult to obtain enough amounts for large scale. They are very sensitive to small changes in their environment and may be damaged considerably, even when delicately handled after cell culture (Adams, 1979; Féréol et al., 2006). Detaching adherent macrophages from a culture dish is difficult, since these cells adhere avidly to plastic surfaces of cell culture devices (i.e. Petri dishes, microplates). Several procedures are currently applied to regain macrophage such as mechanical detachment by gentle scraping of macrophages with a rubber policeman (Fleit, Fleit, & Zolla-Pazner, 1984; Jaguin, Houlbert, Fardel, & Lecureur, 2013; Porcheray et al., 2005) or pre-treatment with scandicain K, proteinase, or pronase (Malorny, Neumann, & Sorg, 1981), which is limitative as it has mitogenic effects on macrophages. Frequently by mechanical detachment, about half of cells may remain viable

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(Adams, 1979). Consequently, high variability and significant loss of viable cells are major limitations for existing procedures.

Based on our previous separation of macrophages by retention on a cross-linked starch column and further detachment by enzymatic hydrolysis of the chromatographic support (Desmangles et al., 1992), four starch materials namely gelatinized starch (G-St), acetate starch (Ac-St), carboxymethyl starch (CM-St) and aminoethyl starch (AE-St) were investigated for their ability to form films susceptible to amylolysis to be used as substrate/support for macrophage separation by mild enzymatic amylolysis. This approach is different to the previous reported method (Desmangles et al., 1992) to separate macrophages using cross-linked starch as a chromatographic support. In general, cross-linked materials are adequate to form microspheres but present lower filmogenic ability than the uncross-linked materials (Berezkin & Kudryavtsev, 2015; Krumova, López, Benavente, Mijangos, & Pereña, 2000). A major objective of this study was to understand the critical role of surface properties of starch materials on the attachment of macrophages and consequently the influences on their viability.

2. Materials and methods

2.1. Materials

High amylose starch (Hylon VII) was supplied by National Starch (Bridgewater, NJ, USA). Sodium monochloroacetic acid, 3,5-Dinitrosalicylic acid, sodium potassium tartrate tetrahydrate (Sigma-Aldrich, Germany), D-(+)-Maltose monohydrate (Sigma-Aldrich, Japan), amyloglucosidase (EC 3.2.1.3) from *Aspergillus niger* ≥300 U/mL (Sigma-Aldrich, Denmark), acetic anhydride (Anachemia, Montreal, Canada), α-amylase (EC 3.2.1.1) from *Bacillus subtilis* 402 U/mg (Fluka, Switzerland), 2-chloroethylamine hydrochloride (Fluka, Switzerland) were all used as received without further purification. CellTracker™ Green CMFDA (5-chloromethylfluorescein diacetate) and propidium iodide (Invitrogen, UK), lipopolysaccharide (LPS, L3012, Sigma-Aldrich), TNF ELISA kits from Biolegend (San Diego, CA) were used for macrophage cells characterization. The RAW macrophage cells (ATCC TIB-71) were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and antibiotics (Penicillin and Streptomycin). Subcultures were prepared by gentle scrapping and aspiration prior to testing in starch coated supports.

2.2. Preparation of starch filmogenic materials

An amount of 12.50 g of Hylon VII was suspended for hydration in 50 mL of distilled water at 60–70 °C under continuous vertical stirring (Servodyne Mixer, 50000-40, IL, USA). A volume of 75 mL of 5 M NaOH was added to the starch suspension, continuing the stirring for 60 min at 60–70 °C. Then the solution was cooled down and neutralized with glacial acetic acid (until pH 6.8) to get gelatinized starch (G-St). The gelatinized starch was further derivatized either by direct addition of 18.75 mL acetic anhydride, or by addition of 18.75 g sodium monochloroacetate or 2-chloroethylamine hydrochloride (each solubilized in a minimal water volume) under stirring and continuing the reaction for 1 h at 60–70 °C to obtain acetate (Ac-St), carboxymethyl (CM-St), or aminoethyl (AE-St) starch derivatives, respectively. Then, each solution was cooled down and neutralized with glacial acetic acid (to reach pH 6.8). The derivatized starch powders were obtained by precipitation from the reaction solution with an equivalent volume of methanol/water (70:30) v/v solution. For all starch materials, the process was repeated until a final conductivity of filtrate decreased at about 50 μS/cm. Then, 200 mL of methanol 100% were used, followed by 200 mL of acetone 100% for final drying. The collected powders were left at room temperature for complete air drying overnight and sieved to obtain particles of less than 300 μm.

2.3. Evaluation of substitution degree of derivatives

For the CM-St and the AE-St: the degree of substitution (DS) was determined by back-titration as previously described (Assaad, Wang, Zhu, & Mateescu, 2011; Stojanović, Jeremić, Jovanović, & Lechner, 2005). Briefly, 100 mg of polymer were solubilized in 10 mL of 0.05 M NaOH and then the excess of NaOH was titrated ($n = 3$) with 0.05 M HCl using phenolphthalein as indicator. The blank (20 mL of 0.05 M NaOH) was also titrated by the same method. The degree of substitution of Ac-St was determined titrimetrically, following the method of Sodhi and Singh (2005) with minor modifications. Acetylated starch (0.1 g) was placed in a 25 mL flask and 6 mL of Dimethyl sulfoxide (DMSO) were added. The loosely stopper flask was agitated, warmed to 50 °C for 30 min, cooled down and then 4 mL of 0.05 M KOH were added. The alkali excess was back-titrated with 0.05 M HCl using phenolphthalein as an indicator. The amounts of —COOH, —NH_2 and —$COCH_3$ groups and the DS were calculated (Stojanović et al., 2005) using the following equations:

$$n = (V_b - V) * C_{HCl} \quad (1)$$

$$DS = \frac{162 * n}{m - W * n} \quad (2)$$

where Vb (mL) is the volume of HCl used for the titration of the blank; V (mL) is the volume of HCl used for the titration of the sample; C_{HCl} is the concentration of HCl; 162 (g/mol) is the molecular mass of glucose unit; W = (58 or 44 or 43) (g/mol) is the increase in the mass of glucose unit by substitution with one carboxymethyl, aminoethyl and acetyl group respectively, and m (g) is the mass of dry sample.

2.4. Fourier transform infrared (FT-IR) analysis

The FT-IR spectra of samples as powders were recorded (64 scans at a 4 cm^{-1} resolution) using a Thermo-Nicolet 6700 (Madison, WI, USA) FT-IR spectrometer equipped with a deuterated triglycine sulfate-KBr (DTGS-KBr) detector and a diamond smart ATR (attenuated total reflection) platform.

2.5. ^1H NMR measurements

The ^1H NMR spectra were collected using a high-field 600 MHz Bruker Avance III HD spectrometer running TopSpin 3.2 software and equipped with a 5 mm TCI cryoprobe. The temperature of samples was maintained at 27 °C. The samples were dissolved in deuterated dimethyl sulfoxide-d6 (DMSO-*d*6) with both methyl groups deuterated, then heated at 65 °C for 30 min, and kept at 4 °C for 2 h.

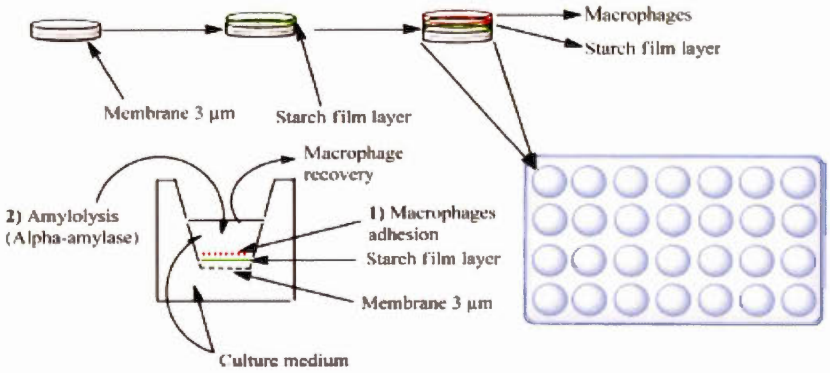
2.6. Scanning electron microscopy (SEM)

The morphology of the particles and film surface were examined by a Hitachi (S-4300SE/N) scanning electron microscope with variable pressure (Hitachi High Technologies America, Pleasanton, CA, USA) at 5–7 kV and magnifications of 100 and 1000× for powders and of 500× and 1000× for film surface. Samples were mounted on metal stubs and sputter-coated with gold.

2.7. Film casting and macrophage culture

2.7.1. Preparation of film-forming solutions of starch materials

Gelatinized starch (G-St), acetate starch (Ac-St), carboxymethyl starch (CM-St) and aminoethyl starch (AE-St) have been dispersed



Scheme 1. Design of device and procedure with adhesion (1) and amylolysis (2) steps for fast recovery of macrophages.

at 0.5% (w/v) in purified water and heated to 95 °C. Then the solutions were cooled down to room temperature and centrifuged at 5000 rpm for 2 min. For each film forming material the supernatant was cast on a cell culture insert device with a base filter of polyethylene terephthalate (PET) having 3.0 µm pore aperture (BD Falcon Cell Culture Inserts, 353092, USA). The solution was evaporated at 40 °C for 12 h to form the film coating of the insert device.

2.7.2. Macrophage incubation

Before incubation the insert and plates (Costar® 3516 6 well plate, USA) were sterilized by UV-ray for 15 min. Then, macrophage suspensions in a RPMI-1640 culture medium containing FBS 10% and Penicillin/Streptomycin 1x, were incubated for 48 h in a humidified atmosphere of air and 5% CO_2 at 37 °C. The culture medium was introduced from the outside of cell culture insert (Scheme 1).

2.7.3. Microscopy

The morphology of macrophage cells was investigated after incubation for 48 h onto the cell culture insert coated with G-St, CM-St, Ac-St or AE-St. Macrophages were labeled with fluorescent staining CellTracker™ Green CMFDA and propidium iodide following manufacturer instructions. Cells were visualized using a Nikon Eclipse Ti microscope (Nikon Canada, Mississauga, ON) equipped with phase contrast and epifluorescence optics. Photomicrographs were acquired using a Digital Sight DS-Qi1Mc camera and NIS-Elements 3.0 software (Nikon Canada).

2.7.4. Susceptibility to enzymatic hydrolysis of starch films

The film hydrolysis was done in three steps: (a) **Hydration step**: Culture medium was replaced by 40 mM phosphate buffer pH 7.4 at 37 °C inside and outside of each cell culture insert: (b) **Liquefaction step**: A solution of an alpha-amylase (EC 3.2.1.1 from *Bacillus subtilis*) in 40 mM phosphate buffer pH 7.4 (1000 U/ mL) was used for liquefaction of film layer. (c) **Saccharification step**: A 40 mM phosphate buffer pH 7.4 was used to dilute amyloglucosidase from *Aspergillus niger* up to (100 U/ mL) and then used for saccharification of the starch film spices resulted from partial hydrolysis with alpha-amylase under gentle shaking followed by incubation in a humidified atmosphere of air and 5% CO_2 at 37 °C (Aneja, 2009; Lareo et al., 2013).

2.7.5. Determination of enzymatic activity on the starch filmogenic supports

Enzymatic activity of alpha-amylase was measured on the same film amylolysis conditions using the dinitrosalicylic (DNS) method (Bernfeld, 1955) to measure the reducing sugar groups released as

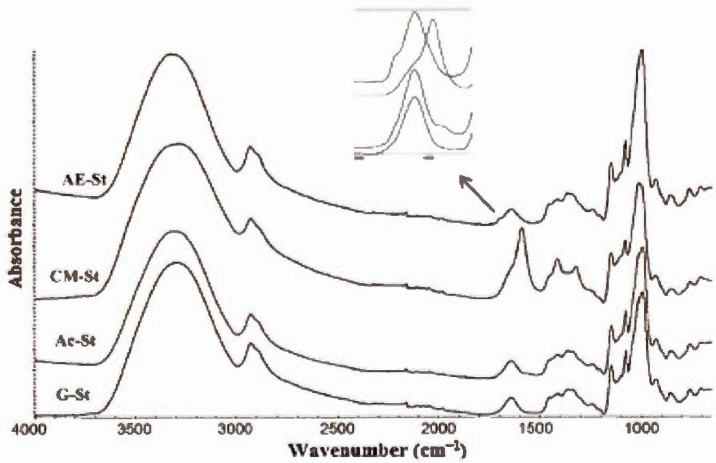


Fig. 1. FT-IR spectra of Gelatinized starch (G-St), Acetate starch (Ac-St), Carboxymethyl starch (CM-St) and Amino-Ethyl starch (AE-St).

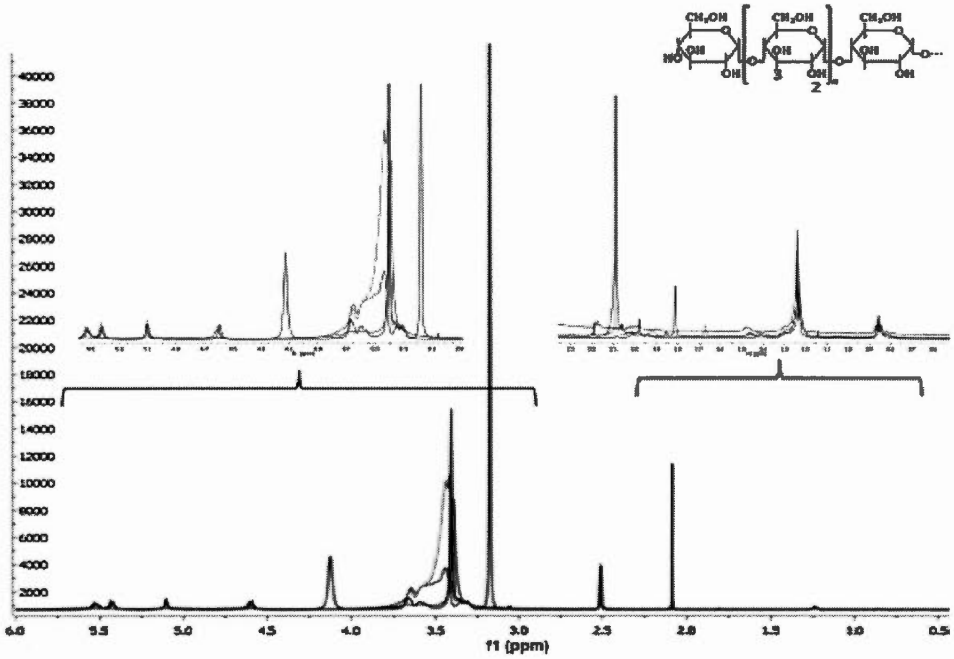


Fig. 2. 1H NMR spectra of Gelatinized starch (Red), Acetate starch (Green), Carboxymethyl starch (Blue) and Amino-Ethyl starch (Black).

result of alpha 1,4 glycosidic group hydrolysis. At different time points a hydrolyzed solution volume of 0.5 mL was withdrawn immediately 0.5 mL of DNS reagent was added to stop the hydrolysis reaction. Then, the reaction media were boiled for 5 min to develop the color of reduced 3-amino-5-nitro salicylic acid. Subsequently, after 5 min precisely the solutions were cooled in an ice-bath to room temperature and 1 mL of each cooled solution was diluted with 4 mL of distilled water. The absorbance of the final solution after filtration was measured against a blank solution without filmogenic material at 540 nm. Maltose solutions were used (as standard reducing sugar) to generate a standard curve. The required time for film hydrolysis was observed visually.

2.7.6. Macrophage cell recovery and counting

Macrophages current recovery approach was the scratching procedure (used as control) and the recovery by the novel direct collection from starch coated inserts devices after the mild enzymatic film hydrolysis were compared by counting done with a hemacytometer (Nikon TMS-F), and using Trypan blue as staining agent.

2.7.7. Macrophage activation

Follwoing 48 h incubation an amount of 50 ng/50 μL LPS per 1 mL of culture medium was added and the cells re-incubated for additional 72 h.

2.7.8. Quantitation of tumor necrosis factor (TNF-α)

After 72 h incubation, the culture medium over and under of macrophage layer was gently removed and centrifuged at 12000 rpm for 10 min. The amount of TNF-α was quantified by the ELISA kit (Catalogue No 430904, Biolegend, Canada). TNF-α level in samples were determined according to the manufacturer's instructions. A standard curve in concentrations from 7.8 pg/mL to 125 pg/mL was done in duplicate and the level of TNF-α in the supernatants was evaluated by use of the standard curve as reference. The optical density at 450 nm was measured with a microplate reader.

2.8. Statistical analysis

All tests were performed in triplicate and data are reported as means ± SD. Statistical analysis of data was performed using one way ANOVA, followed by Fisher's post hoc tests with a minimum confidence level ($P < 0.05$) for statistical significance.

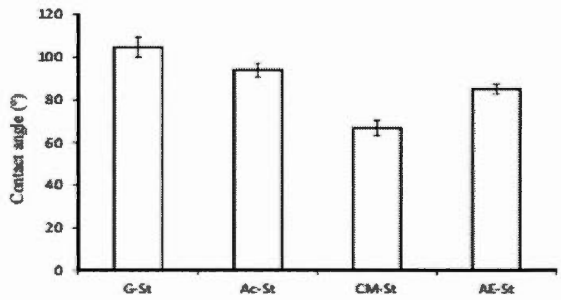


Fig. 3. Water contact angle measurement for insert coating films of Gelatinized starch (G-St), Acetate starch (Ac-St), Carboxymethyl starch (CM-St) and Amino-Ethyl starch (AE-St) (n = 3).

3. Results and discussions

3.1. Polymer and film characterization

The degree of substitution of starch derivatives CM-St, Ac-St and AE-St, as determined by back-titration were about 0.018, 0.022 and 0.024, respectively. These values represent the average number of carboxymethyl, acetate or aminoethyl groups per glucose unit, respectively. The grafting of each functional group on the starch chains was confirmed by structural analysis, FT-IR and ^{1}H NMR.

The Fourier transform infrared (FT-IR) spectra of the obtained starch materials (Fig. 1) present a broad band at 3200–3300 cm^{-1} due to the stretching vibrations of —OH. Small bands at 2927 cm^{-1} and at 2323 cm^{-1} attributed to the —CH stretching vibration and a band at 1079 cm^{-1} ascribed to —CH_2—O—CH_2 stretching vibrations (Ispas-Szabo, Ravenelle, Hassan, Preda, & Mateescu, 1999). In case of CM-St, there are additional bands at 1589 cm^{-1} and at 1323 cm^{-1} ascribed to COO^- group (Friciu, Tien Le, Ispas-Szabo, & Mateescu, 2013). The high intensity of the band at 999 cm^{-1} for AE-St could be ascribed to C—N stretching vibrations, whereas the weak shoulder at around 1735 cm^{-1} could be assigned to —NH_3^+ group (Assaad et al., 2011; Deng, Jia, Zhang, Yan, & Hou, 2006). In the case of Ac-St, the weak shoulder at around 1556 cm^{-1} corresponds specifically to the —C—O stretching of acetyl groups (Bello-Pérez, Agama-Acevedo, Zamudio-Flores, Mendez-Montealvo, & Rodriguez-Ambriz, 2010; Colthup, Daly, & Wiberley, 1990).

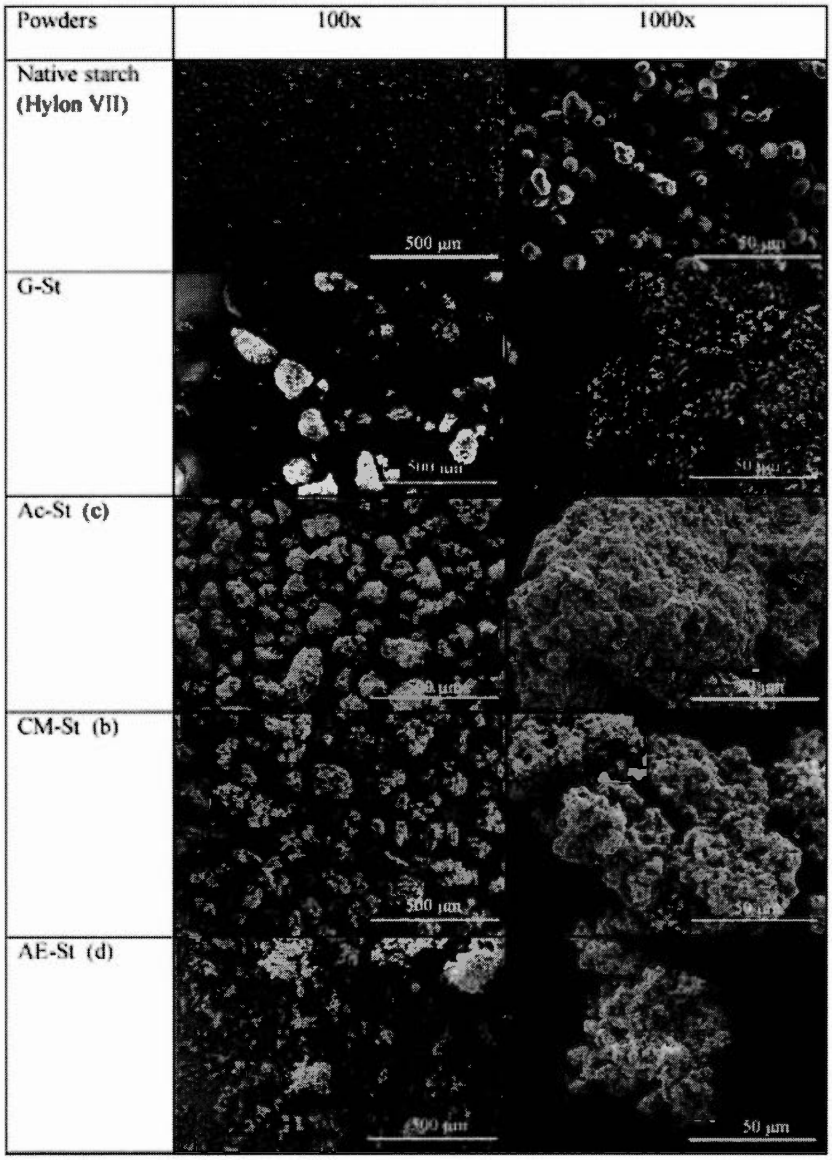


Fig. 4. Scanning electron microscopy micrographs of Native starch (Hylon VII), (a) Gelatinized starch (G-St), (b) Acetate starch (Ac-St), (c) Carboxymethyl starch (CM-St) and (d) Amino-Ethyl starch (AE-St) powders at magnifications of 100x and 1000x.

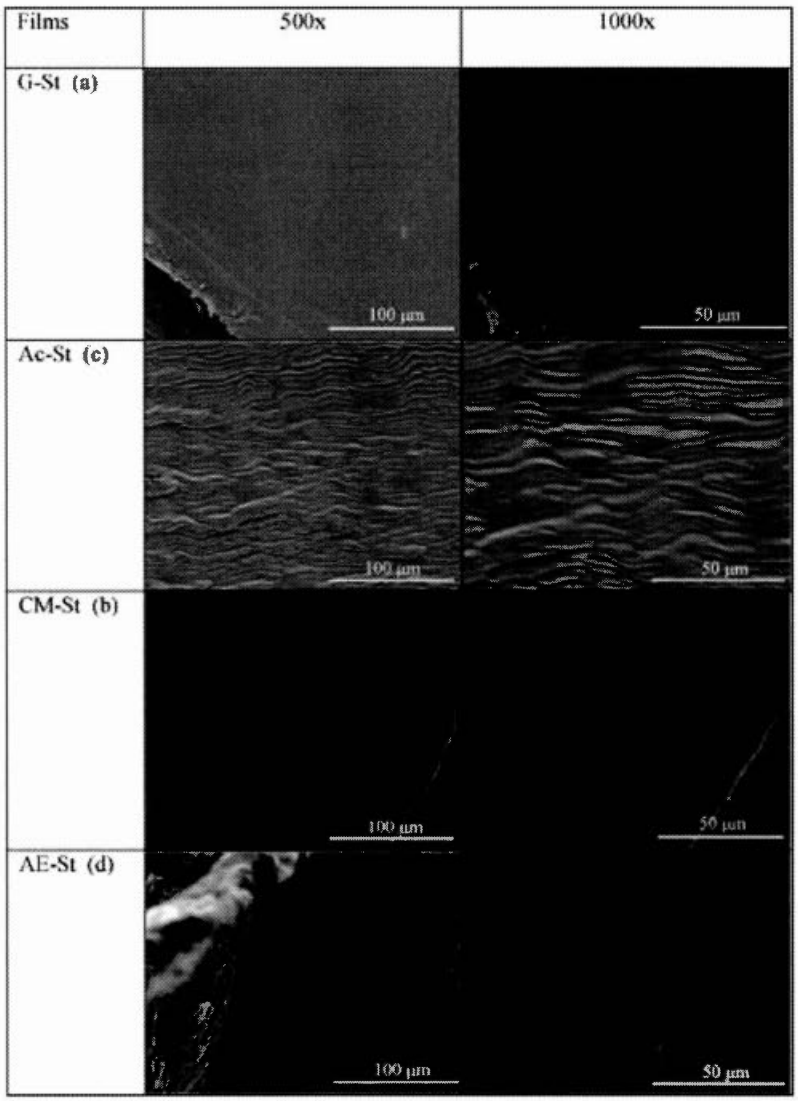


Fig. 5. Scanning electron microscopy micrographs of films: Gelatinized starch (G-St), (b) Acetate starch, (Ac-St), (c) Carboxymethyl starch (CM-St) and (d) Amino-Ethyl starch (AE-St) at magnifications of 500× and 1000×.

The ^1H NMR spectra of the starch materials (Fig. 2) present proton signals at 5.3 ppm for H1 and at 3.3–3.9 ppm for H2-6 on the starch backbone (Yang et al., 2014) while the peak at 5.6 ppm can be assigned to OH3. The most significant peaks for AE-St are at δ = 4.15–4.25, δ = 3.16–3.18, which belong to the hydrogens of aminoethyl group. In case of Ac-St the peaks at δ = 1.9–2.1 and at δ = 3.5 ppm are ascribed to methyl protons of acetate groups (Xu and Hanna, 2005). In case of CM-St sharpless peaks may be due to the limited solubility of CM-St in DMSO.

The obtained zeta potential (ζ) charges values in solution were −32 mV for G-St and −38 mV for CM-St. These values are consistent with the chemical modification of starch by carboxymethyl groups providing a stronger negative charge (Wongsagonsup, Shobsngob, Oonkhanond, & Varavinit, 2005a; Wongsagonsup, Shobsngob, Oonkhanond, & Varavinit, 2005b). Grafting starch with acetate groups reduced the value of zeta potential for acetate starch to −26 mV and this can be explained by a decreased polarity in comparison with G-St. The positive zeta potential value for AE-St +10 mV is related to cationic groups grafted on starch molecules.

Static water contact angle Fig. 3 allowed the evaluation of the wettability/hydrophilicity of the films for coating of the insert surfaces. The CM-St and AE-St films presented a lower angle (67° and 78° respectively) in comparison to G-St (89°) and Ac-St (105°), meaning that G-St and Ac-St are less polar and even more hydrophobic.

Scanning electron microscopy (SEM) of starch materials as powders and films are presented in Fig. 4. The native starch (Hylon VII) has a granular aspect predominantly round or oval in shape (Fig. 4), with smooth surface and uniform range of size distribution (5–10 μm). The granular aspect fits well with the known crystalline

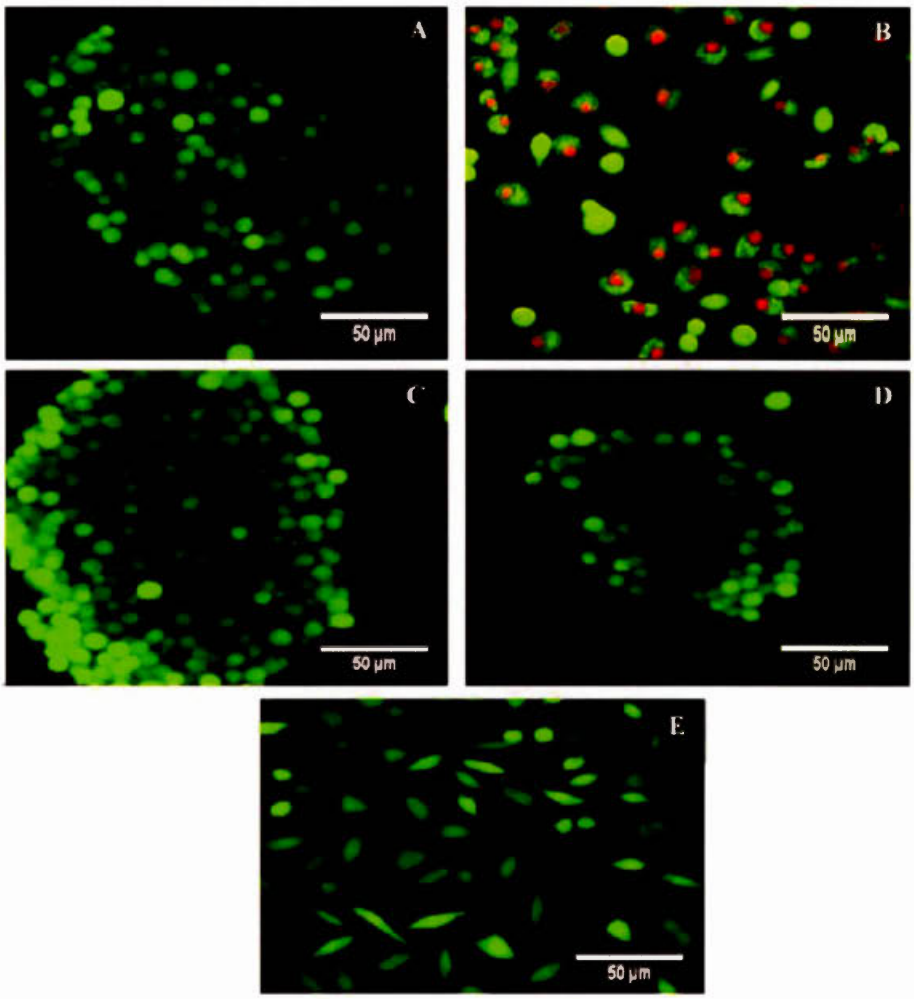


Fig. 6. Confocal fluorescence microscopy images showing live cells (green) and dead macrophage cells (red) after incubation 48 h on cell inserts coated with Amino-Ethyl starch (A), Carboxymethyl starch (B), Acetate starch (C), Gelatinized starch (D), and control (uncoated insert) (E), scale bar 50 µm.

structure of native starch (Friciu et al., 2013) stabilized by hydrogen bonds between the hydroxyl groups of glucopyranose units. The aspect of the four materials: G-St, CM-St, AE-St and of Ac-St is different, depending on modification operated on starch structure.

The G-St (Fig. 4a) showed a round and sponge-like shape which is due to the physical modification (gelatinization) of native starch. Differently, the CM-St (Fig. 4b) presented an irregular shape with an uneven surface likely due to the association of numerous small particles forming larger granules similar shapes were obtained by Friciu et al. (2013). The carboxylic groups may reduce the network self-assembling by hydrogen association between hydroxyl groups and promote repulsion effects loading to a structural reorganization (Lemieux, Gosselin, & Mateescu, 2010). The acetylation (Fig. 4c) generated a slightly rough surface of granules which appeared fused in a kind of aggregate. The acetyl groups can also decrease the starch stabilization by hydrogen bonding and, at the same time, the glucose units with polar hydroxylic groups and non-polar (acetate) functions, may favor starch macromolecules to coalesce together resulting in a kind of fusion of granules (Bello-Pérez et al., 2010; Singh, Kaur, & Singh, 2004). The AE-St (Fig. 4d) grains showed a porous irregular shape, where amine groups may promote hydrogen bonding resulting to a reorganization of the AE-St network. As far as films are concerned the SEM micrographs of G-St and CM-St films at magnifications of 500× and 1000× (Fig. 5a and b) showed a homogeneous and smooth surface, whereas Ac-St and AE-St films (Fig. 5c and d) showed continuous matrices, with small cracks and less smooth surface.

3.2. Macrophage cells attachment and recovery by film amylolysis

3.2.1. Morphology of macrophage cells

Intact macrophage cultures were treated with two staining agents: CMFDA to show live cells (green) and propidium iodide to stain dead cells with altered membrane permeability (red).

Control cultures on uncoated insert devices appear as plump or stellate, monolayers rounded and spindle-like with majority of

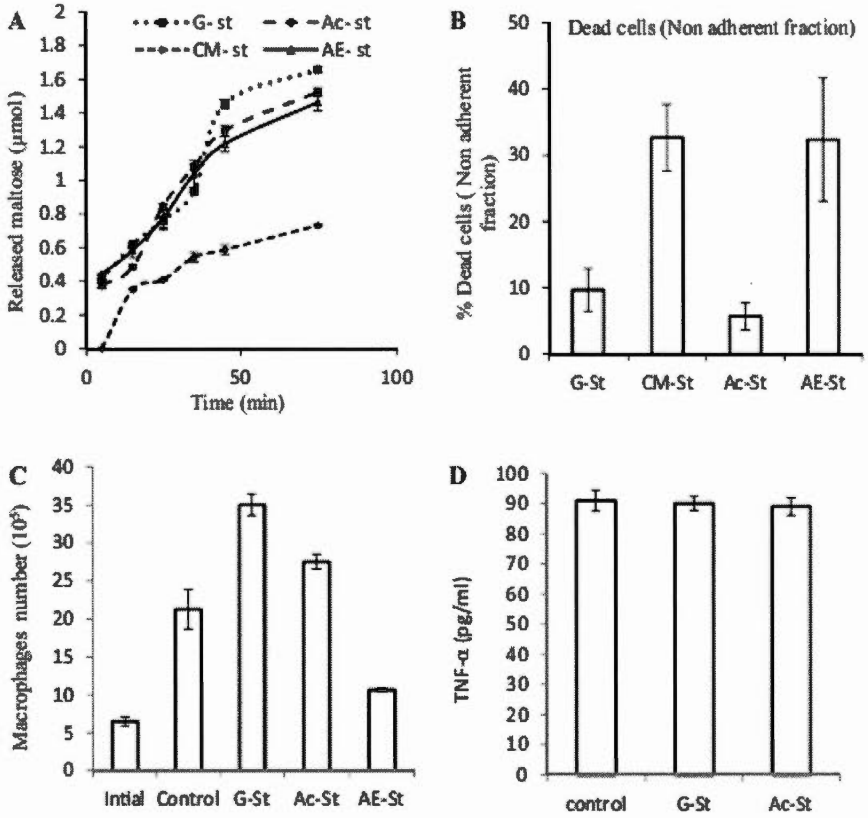


Fig. 7. (A) Release reducing sugar (µmol) after Gelatinized starch (G-St), Acetate starch (Ac-St), Carboxymethyl starch (CM-St), and Amino-Ethyl starch (AE-St) film hydrolysis by alpha-amylase; (B) Percentage of dead cells (%) incubated on cell insert coated with G-St, Ac-St, CM-St and AE-St; (C) Macrophage count with an initial number (6.5×10^5) and incubated 48 h on inserts coated with film of G-St, Ac-St, AE-St or Control (uncoated); (D) Tumor necrosis factor TNF-α (pg/mL) from recovered macrophage activated by lipopolysaccharide (LPS) 50 ng/mL.

live cells. Macrophages incubated on insert devices coated with G-St, Ac-St and AE-St showed round, compact and mostly live cells Fig. 6. Differently, prevalently dead cells were observed when incubated in insert coated by CM-St film, owning round, spindle-like and translucent cytoplasm. This behaviour suggests that the carboxymethyl functionalized film may cause membrane disruption and cell apoptosis. Similar damaged membranes and apoptosis have been observed with certain agents such as carboxy- silicalite (Petushkov, Intra, Graham, Larsen, & Salem, 2009).

3.2.2. *Determination of enzymatic activity with starch filmogenic supports as substrates*

The film amylolysis process was investigated by measuring the enzymatic activity of alpha-amylase with various films as substrate (Fig. 7A). It was found that G-St, AC-St and AE-St showed similar film hydrolysis rate over the first 40 min. Then, the G-st hydrolysis was faster than that of AC-St and AE-St. This behaviour was considered as normal because there is no chemical modification of the G-St. The lowest enzymatic activity was observed with CM-St film, where the released amount of maltose after 75 min was almost half of that liberated from G-St. The film hydrolysis was also followed visually. Even without complete amylolysis, the CM-St film was dissolved in less than 10 min, because CM-St is soluble in alkaline medium. Differently, G-St film was partially hydrolyzed in 30 min, AC-St and AE-St in 40 min. Macrophages adhere on adequate surfaces and floating cells are characteristically dying cells. Macrophage counting suggested good adhesion on G-St, on Ac-St and on AE-St materials. Fig. 7B presents the non-adherent (floating) fraction of macrophages after incubation of cell culture on cell-holder devices (insert) coated with CM-St, AE-St, Ac-St or G-St. the higher percentages of dead macrophage (floating) were observed at inserts coated with anionic CM-St (about $32 \pm 5\%$) or with the cationic AE-St (about $32 \pm 9\%$), whereas a low percentage of dead cell was observed with insert coated with non-ionic and neutral polymers Ac-St ($5 \pm 2\%$) and G-St ($9 \pm 3\%$) respectively, suggesting higher percentage of living cells from this films. These adhesion data on non-ionic Ac-St and G-St are in agreement with our previous report showing good adhesion and recovery by amylolysis of macrophage cells on cross-linked starch microspheres, not modified with ionic groups (Desmangles et al., 1992). The best retention on AC-St fits well with a study of Godek, Michel, Chamberlain, Castner, and Grainger (2009), showing that macrophages adhere preferentially to highly hydrophobic fluorinated surfaces (Godek et al., 2009). Similar results, but not on carbohydrate materials, were observed by Brodbeck et al. (2002) showing that the hydrophilic and anionic polyethylene terephthalate modified surfaces inhibit adhesion of monocyte and macrophage cells (Brodbeck et al., 2002).

Due to membrane disruption and cell inducing apoptosis along with low macrophage viability on CM-St, this support was excluded from further investigation and cell harvesting and counting was

continued with control insert (uncoated) and with G-St, Ac-St and AE-St coated inserts. Cell harvesting was done by scrapping for control cells (cultured on uncoated insert devices) or by enzymatic hydrolysis for inserts coated with starch materials. After incubation for 48 h, cell numbers increased about 3.2 times for control uncoated inserts, 4.2 times for Ac-St and 5.3 times for G-St whereas only 1.5 times was observed for AE-St coated insert (Fig. 7C). Furthermore, 129% and 164% more cells were recovered from inserts devices coated with G-St and Ac-St when compared to controls (un-coated inserts), whereas a 53% drop of the yield was obtained for AE-St coated inserts. This inhibitory effect could be explained by a too strong interaction of cationic aminoethyl groups of starch film with membrane phospholipids of macrophage cells (Kurtz-Chalot et al., 2014). Therefore the AE-St was not retained for further investigation.

Macrophage activation by Lipopolysaccharide (LPS) and quantitation of induced tumor necrosis factor (TNF-α) allowed the investigation of the possible effect of starch derivatives with macrophage activities. The cells were stimulated with LPS, a component of the outer membrane of Gram negative bacteria, which is a potent activator of monocytes and macrophages (Mace, Ehrke, Hori, Maccubbin, & Mihich, 1988). LPS triggers the abundant secretion of cytokines by macrophages including tumor necrosis factor (TNF-a), interleukin (IL)-1, and IL-6 (Meng & Lowell, 1997). In our study, the amount of TNF-α secreted by macrophages in response to LPS was in the same range as reported in a similar study (Lichtman, Wang, & Lemasters, 1998). Moreover, there were no differences (Fig. 7D) in TNF-α produced by control cells harvested from uncoated inserts (91 ± 3.5 pg/mL) or by macrophages harvested from G-St (90 ± 2.3 pg/mL) and Ac-St (89 ± 2.9 pg/mL) coated inserts. The functional groups grafted on polysaccharide chains not only have a direct effect on viability of cells, but they can impact macrophage adhesion. For instance the non-derivatized starch (G-St) and the Ac-St with hydrophobic acetate groups oriented toward culture medium, are better supports for adhesion of macrophage cells than the anionic (CM-St) and cationic (AE-St) starch derivatives which are less compatible. The minimal percentage of dead cells (non-adherent fraction) was observed with inserts coated with G-St and Ac-St. Therefore, these Gelatinized starch and Acetate starch materials affording a best viability, could be a good choice as support material for macrophage culture due to the high compatibility with cells and also for their susceptibility to mild enzymatic amylolysis. These features of G-St and Ac-St allow the recovery of macrophage cells with better viability and high yields. Furthermore, the activation by LPS indicated that macrophage cells cultured on G-St and on the starch acetate derivative are producing almost the same level of TNF-α as the control (uncoated insert). This result together with the low percentage of dead cells could be an evidence of biocompatibility of G-St and Ac-St supports as materials for macrophage preparation by this novel mild enzymatic procedure.

4. Conclusion

The present study is proposing a new type of application for modified starch based on its film-forming capacity. The proposed approach, focused on adhesion of macrophage cells on Ac-St or G-St films followed by their detachment by enzymatic amylolysis, is faster and the mild condition affords a better viability of macrophage cells in comparison with the classical procedure (mechanical detachment). Starch films are easy to apply on the inserts and their biocompatibility is an important characteristic for cell viability. This study opens new perspectives to obtain macrophage cells with a high viability, avoiding significant loss of viable cells which still limits the current scratching procedures. Further studies will be conducted in order to evaluate the impact of the substitution degree of Ac-St on the attachment and activity of macrophages.

Acknowledgments

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Research Article

Self-Stabilizing Ampholytic Starch Excipients for Sustained Release of Highly Soluble Drugs: the Case Study of Metformin

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ABSTRACT. A new class of starch derivatives carrying cationic and anionic functional groups was developed aiming to provide an alternative for the formulation of highly soluble drugs. The new ampholytic starch derivatives were synthesized in two steps; first the CarboxyMethyl (CM) groups were grafted on starch chains followed by introduction of AminoEthyl (AE) groups. The final product, CarboxyMethyl-AminoEthyl-Starch (CM-AE-St), could be obtained in different degrees of substitution by varying the number of CM and AE groups. It was hypothesized that the simultaneous presence of anionic and cationic groups will generate a stronger self-stabilization of starch matrices and an improved control of drug release. Metformin (biopharmaceutical classification system—BCS, class I) was selected as model drug and monolithic tablets with 50 and 60% loading were prepared by direct compression of the active molecule with various CM-AE-St derivatives. The *in vitro* drug dissolution tests have shown that higher degrees of substitution for both CM and AE groups favor the ability of ampholytic CM-AE-St to control the drug release in simulated gastric fluid and in simulated intestinal fluid. Tablets based on CM-AE-St derivatives were compared to the commercial Glumetza® (50% loading). The drug release was controlled for 12 h exhibiting a similar Higuchi's model dissolution profile for the two dosage forms. Structural studies (FT-IR, ^{1}H NMR, SEM, TG, X-ray diffraction) run on CM-AE-St derivatives put in evidence derivatization and self-stabilization phenomena. These new ampholytic starch derivatives offer a simple and convenient alternative to formulate and manufacture highly soluble drugs in a single step process.

KEY WORDS: ampholytic starch; CarboxyMethyl-AminoEthyl-Starch; controlled release; metformin; monolithic tablet.

INTRODUCTION

In the context of a growing interest for sustained drug release devices and for high loaded dosage forms, there is a need for new excipients, particularly for the formulation of highly soluble drugs such as metformin, metoprolol, metronidazole, and chloroquine. To improve these challenging formulations, an adequate carrier is expected to be able to keep the tablet size small enough to allow ease of swallowing alongside with a simple method of tablets preparation. Current procedures for formulation of such highly soluble molecules consist by embedding them in release-modifying polymers, often followed by application of additional functional coatings. The large majority of dosage forms are using cellulose derivatives, polyethylene oxides, or other gel-forming polymers and frequently using multi-step processes that may include wet granulation and hot melt methods. Polymeric self-assembling excipients may improve the formulation of such highly soluble drugs and facilitate the manufacturing processes. Excipients based on cross-linked high amylose starch functionalized with different groups-anionic (carboxymethyl, CM), cationic (aminoethyl, AE), or acetate (Ac)-allowed preparation of monolithic tablets with acetaminophen as model drug with 40 and 60% loading (1). Cross-linked CM-Starch with degree of substitution (DS) of 0.10 showed an improvement of controlled acetaminophen release with 90% of release within 16.5 and 14.2 h for drug loading 40 and 60%, respectively (1). A non-cross-linked CM-starch (CM-St) was then developed as a pH-sensitive excipient able to protect active principle ingredients (API) against gastric acidity (2,3) and to form a pH responsive matrix for controlled drug release in intestinal medium (4). CM-St may afford resistance to gastric fluid due to the protonation of the carboxylate groups in gastric acidity by forming an outer gel layer. Thus, an *in situ* formed layer is able to ensure local buffering properties and to maintain a compact shape of the tablets during the passage through the

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stomach. Protonated carboxylic groups from neighboring chains can be dimerized by dipole-dipole interactions and associated by hydrogen bonding, stabilizing thus the polymeric network (5). In intestinal medium, protons are changed with sodium ions and tablets swell, releasing the active agent (2). Several attempts have been made to prolong drug release in simulated intestinal fluid (SIF) from matrices containing CM-St with a low degree of substitution DS (0.09–0.11) which presented a better controlled release of drugs (6). In order to further delay the release, a physical mixture of carboxymethyl starch and chitosan was proposed to deliver diamine oxidase and catalase to the low intestine (7) and a co-processing procedure was proposed to obtain a polyelectrolyte complex (PEC) of carboxymethyl starch chitosan as excipient for colon delivery (8). However, it is difficult to formulate sustained release forms of cationic drugs such as chlorohydrate salts, which are highly soluble and required in large amount (high loading). Metformin (dimethylbiguanide) hydrochloride is an orally administered drug used to lower blood glucose concentrations in patients with non-insulin-dependent diabetes mellitus (9,10). Metformin has properties of a strong base (pK_a 2.8 and 11.5) and, as hydrochloride, it is highly water-soluble and in solutions of pH 1.2 to 6.8 (11). Because of its short half-life (<3 h), frequent administrations at high doses (daily dose up to 2.5 g) are needed to maintain its required plasma concentration (12–14). Metformin is considered mostly absorbed in the duodenum (15). Some adverse effects (i.e., diarrhea, abdominal discomfort, nausea) caused by immediate release dosage forms of metformin (Glucophage®) remain unsolved. Metformin was formulated for controlled release systems in various dosage forms, i.e., mucoadhesive beads (16), gastro-floating bilayer tablets (17) combination with triacetyl-β-cyclodextrin (18), iron(III) cross-linked alginate–carboxymethyl cellulose hydrogel beads (19), osmotic pump tablet (12). The commercially available controlled release product Glumetza® tablets (metformin hydrochloride: Depomed Inc.) (20,21) and the majority of sustained release devices are designed as gastro-retentive forms based on a prolonged gastric residence controlled by swelling or by floating or by mucoadhesion to retain the dosage form releasing the active pharmaceutical principle in the stomach. With Glumetza®, too much metformin release in the stomach and to the upper intestine may generate a tissue saturation (22) and thus a lower absorption with a certain decrease of efficiency. Furthermore, metformin was found to be absorbed not only at the level of the upper intestine, but over the whole intestinal tract (9,15). Gastrointestinal absorption of metformin is incomplete, possibly related to a saturable absorption process and about 20–30% of an oral dose was considered to be recovered in the feces (23). Absorption is estimated to be completed within 6 h after administration and is presumably confined to the upper intestine (23,24). Metformin is also poorly absorbed from the stomach and with a rate-limiting absorption from the duodenum. In fact, the whole intestine is necessary for sufficient absorption of the drug (24). A too high absorption with saturation at the upper intestine may generate side effects and a lesser efficiency by reducing the amount of drug available for the lower intestine absorption. Consequently, a formulation of metformin with continuous release over the entire gastrointestinal tract could reduce adverse gastric side effects. In this context, we are proposing a new carrier-an ampholytic starch that has the ability to control the release of high-dose of highly soluble drugs and to keep the tablet size small enough alongside with a single step procedure of tablet preparation. An ampholytic starch can be defined as a starch derivative exhibiting both anionic (i.e., carboxylic) and cationic (i.e., aminoethyl) groups. An ampholytic starch has been previously used as flocculating agent (25,26) in textile industry as warp sizing agent (27) and also as papermaking agent (28). The aim of the present study was to prepare and characterize Carboxymethyl AminoEthyl Starch (CM-AE-St) as a novel excipient. Due to its ampholytic character, this CM-AE-St is expected to modulate the release of high loaded soluble drugs. To the best of our knowledge, this is the first investigation of an ampholytic starch as carrier for sustained delivery of active ingredients along the gastrointestinal tract.

MATERIALS AND METHODS

Materials

High amylose starch (Hylon VII) was supplied by National Starch (Bridgewater, NJ, USA). Metformin (1,1-dimethylbiguanide hydrochloride) was from MP Biomedicals (Solon, OH, USA), sodium monochloroacetate was from Sigma-Aldrich (Germany), and 2-chloroethylamine hydrochloride from Fluka (Switzerland).

Preparation of Ampholytic Starch Derivatives

The polymers were prepared in two steps: the first step consisted in introducing the carboxymethyl anionic group and the second one was the grafting of the CM-starch derivative with cationic aminoethyl (AE) groups. Sodium monochloroacetate was dissolved in water and rapidly added to gelatinized starch suspension to obtain carboxymethyl starch. Briefly, an amount of 50 g of starch was suspended for hydration for 5 min in 200 mL of distilled water at 60–70°C under continuous vertical stirring and 300 mL of 5 M NaOH was added under stirring maintaining the reaction medium for 1 h at 60–70°C for gelatinization. In different experiments, various quantities (18.75, 56.25) g of sodium monochloroacetate solubilized in a minimal water volume were added rapidly under stirring which was continued for 1 h at 60–70°C (to introduce carboxylic groups). Then, the solution was cooled down and neutralized with glacial acetic acid. The CM-St was precipitated from slurry with an equivalent volume of methanol/water (70:30, *v/v*) followed decantation. The process was repeated until a final conductivity (Fisher Scientific Accumet Research AR20, San Diego, CA, USA) of filtrate decreased to less than 75 µS/cm. Finally, pure methanol and then acetone were used for drying. The collected CM-St was left overnight at room temperature for complete drying and the powder sieved to obtain particles of less than 300 µm. The polymer was re-dissolved for amination which was done by reaction with 2-chloroethylamine hydrochloride. To prepare CM-AE-St, 12.5 g of CM-St from the previous step was re-dissolved in 50 mL of distilled water followed by addition of 75 mL of 5 M NaOH and heated at

60–70°C for 1 h under continuous stirring. Different quantities (4.68, 9.37, 14.06) g of 2-chloroethylamine hydrochloride solubilized in a minimal water volume were rapidly added under stirring continued for 1 h at 60–70°C (to graft cationic groups). Finally, the suspension was cooled down and neutralized with glacial acetic acid. The CM-AE-St powder was obtained by precipitation with methanol and drying as described above.

Evaluation of the Degree of Substitution

A. The degree of substitution (DS) of CM-St was determined by back-titration as previously described by Stojanovic et al., 2005 (29). Briefly, 300 mg of polymer ($n = 3$) was solubilized in 20 mL of 0.05 M NaOH and the excess of NaOH was titrated with 0.05 M HCl using phenolphthalein as indicator. The blank (20 mL of 0.05 M NaOH) was also titrated by the same method. The amount of –COOH groups (n) and the DS were calculated (29) using the Eqs. (1, 2):

$$n = (V_b - V) \times C_{HCl} \quad (1)$$

$$DS = \frac{162 \times n}{m - W \times n} \quad (2)$$

where V_b (mL) is the volume of HCl used for the titration of the blank, V (mL) is the volume of HCl used for the titration of the sample, C_{HCl} (mol/L) is the concentration of HCl, 162 (g/mol) is the molar mass of glucose unit, $W = (58)$ (g/mol) is the increase in the mass of glucose unit by substitution with a CM group, and m (g) is the mass of dry sample.

B. The degree of substitution (DS) expressed in terms of amino groups on the final derivative was determined with the ninhydrin reagent (2 g ninhydrin dissolved in 75 mL dimethylsulfoxide under nitrogen flushing and completed with 25 mL of 4 M lithium acetate buffer, pH 5.2). A volume of 0.5 mL of the ninhydrin reagent was added to a volume of 0.25 mL of ampholytic starch solution (15 mg/mL) in deionized water (in triplicate). The tubes were immediately capped, vortexed, and heated in a covered boiling water bath for 30 min. Tubes were then cooled below 30°C in a cold water bath. The absorbance at 570 nm was measured on a UV/VIS spectrophotometer with zero set against a similarly treated blank of water. Glycine (Sigma) solutions (0.12–0.51 mg/mL) were used to generate a standard curve (31,32).

Preparation of Tablets

Monolithic tablets (50 and 60% (*w/w*) drug loading) were obtained by direct compression (2.5 tons) of a homogenous mixture of excipient and drug powders. Flat-faced punches with 12.95 mm diameter and oval shape punches with 17.5 mm length and width of 10.30 mm were used with a Carver hydraulic press (Model C 3912 Hydraulic Cylinder, Wabash, IN, USA).

Determination of the Fluid Uptake and Erosion

Fluid uptake and erosion studies were carried out by immersing the weighed dry tablet (W_1) in 1000 mL of either simulated gastric fluid (SGF) (pH 1.2 obtained by first dissolving 2 g of sodium chloride in 500 mL of water; then the pH was adjusted at 1.2 ± 0.1 with concentrated HCl 37%; the final volume was completed with purified water to 1000 mL) or SIF (pH 6.8 phosphate buffer obtained by first dissolving 6.8 g of monobasic potassium phosphate in 500 mL of water; then the pH was adjust at 6.8 ± 0.1 with 0.2 M sodium hydroxide and the final volume was completed with purified water to 1000 mL) using the same conditions as those for the *in vitro* drug release studies (i.e., 37°C and stirring rate 100 rpm). After 2 and 10 h, the tablets were withdrawn and carefully weighed (recorded as W_2). The recovered tablets were then placed into an oven at 35–40°C for 2 days until a constant weight was obtained. The final weight of the tablets was measured (W_3) after complete drying at constant mass (33,34). Fluid uptake was determined according to Eq. (3):

$$\% \text{ Weight change} = \frac{W_2 - W_1}{W_1} \times 100 \quad (3)$$

The degree of erosion was determined according to Eq. (4):

$$\% \text{ Erosion} = \frac{W_1 - W_3}{W_1} \times 100 \quad (4)$$

Fourier Transform Infrared (FT-IR) Analysis

The FT-IR spectra of powder samples were recorded (64 scans at a resolution of 4 cm^{-1}) using a Thermo-Nicolet 6700 (Madison, WI, USA) FT-IR spectrometer equipped with a deuterated triglycine sulfate-KBr (DTGS-KBr) detector and a diamond smart attenuated total reflection platform.

^{1}H NMR Measurements

In order to determine the CM-AE-St functionalization pattern, high-field 600 MHz, ^{1}H NMR spectra were collected using a Bruker Avance III HD spectrometer running a TopSpin 3.2 software and equipped with a 5-mm TCI cryoprobe. The temperature of samples was regulated at 27°C. The samples were dissolved in deuterium oxide D_2O, heated at 65°C for 30 min, and kept at 4°C for 2 h prior to analysis.

Thermogravimetric Analysis

Thermogravimetric analysis (TG) for each powder sample was carried out in platinum crucible at a heating rate of 10°C/min between 25 and 600°C under nitrogen

Table I. Kinetic Equations and Corresponding Values of Metformin Release from Tablets (60% Loading) Based on (A) CM-AE-St (DS 0.02-0.064), (B) CM-AE-St (DS 0.06-0.059) at pH 1.2 and pH 6.8, and from Tablets (50% Loading) Based on (C) CM-AE-St (DS 0.06-0.059) Compared to Glumetza®

	Dosage forms		Ampholytic starch				Ampholytic starch C (500 mg)	Glumetza (500 mg)
			A		B			
	Kinetic analysis		pH 1.2	pH 6.8	pH 1.2	pH 6.8		
A	$Q_t = Q_0 + K_0 t$	K_0	0.338	0.301	0.326	0.306	0.383	0.386
		r^2	0.949	0.946	0.918	0.961	0.983	0.986
B	$\log Q_t = \log Q_0 + \frac{K_1 t}{2.303}$	K_1	−0.006	−0.008	−0.007	−0.008	0.0059	0.0058
		r^2	0.998	0.968	0.997	0.993	0.947	0.984
C	$Q_t = K_H t^{1/2}$	K_H	4.971	5.41	5.138	5.36	4.603	4.573
		r^2	0.991	0.988	0.973	0.972	0.991	0.999
D	$Q_0^{1/3} - Q_t^{1/3} = K_x t$	K_x	0.006	0.005	0.006	0.005	0.0077	0.0078
		r^2	0.909	0.907	0.877	0.876	0.9548	0.955
E	$Q_t = K_p t^n$	n	0.455	0.443	0.472	0.461	0.624	0.603
		KP	4.58	3.631	4.38	4.47	0.943	2.781
		r^2	0.961	0.969	0.950	0.992	0.957	0.977
F	$f_1 = \frac{\sum_{j=1}^{n} \lvert R_j - T_j \rvert}{\sum_{j=1}^{n} R_j} \times 100$						2	
G	$f_2 = 50 \log\left\{ \left[1 + \left(\frac{1}{n}\right) \sum_{j=1}^{n} \lvert R_j - T_j \rvert^2 \right]^{-0.5} \times 100 \right\}$						89	

atmosphere (flow rate 100 mL/min). TG patterns were established with a TA® Instruments incorporated high-resolution thermo gravimetric analyzer Seiko TG/DTA 6200 (Japan).

Scanning Electron Microscopy (SEM)

The morphology of the sample particles was examined by a Hitachi (S-4300SE/N) scanning electron microscopy with variable pressure (Hitachi High Technologies America, Pleasanton, CA, USA) at 5–7 kV and magnifications of and ×1000. Samples were mounted on metal stubs and sputter-coated with gold.

X-Ray Diffraction

X-ray diffractometry of polymers was performed using a Siemens D-5000 (Munich, Germany) device. The samples were exposed to X-ray radiation (Cu $K\alpha$) with wavelength of 1.789 Å and a scanning rate of 0.05°/min. Samples (ground into powders with an agate mortar and pestle) were measured on a low background quartz plate in an aluminum holder and analyzed through a 2θ range of 5–50°. The XRD spectra were treated using a DiffracPlus software.

In Vitro Dissolution Tests

Metformin dissolution tests were carried out as follows: (a) for tablets loaded with 60% of metformin, in SGF and SIF dissolution media, separately; (b) for tablets loaded with 50% of drug, in SGF for 2 h followed by SIF for up to 12 h; (c) Glumetza® dissolution was conducted only in SGF since it is a gastro-retentive designed formulation. An USP paddle apparatus 2 was used with a dissolution volume of 1000 mL and the paddle speed at 100 rpm. The dissolved drug was detected at $\lambda_{232\ nm}$ for SIF whereas for SGF the sample was diluted with phosphate buffer and detected at the same wavelength.

Release Kinetic Patterns

The drug release kinetic parameters were evaluated according to zero-order kinetics (35), first-order kinetics (35), Higuchi's model (36,37), Hixson–Crowell's model (38), and Korsmeyer–Peppas model (39) (Table I).

Statistical Analysis

All tests were done in triplicate and data are reported as means ± SD. For statistical analysis, the one way ANOVA was followed by Fisher's post hoc tests with a minimum confidence level ($P < 0.05$) for statistical significance.

RESULTS

Ampholytic Starch Characterization and Structural Insights

In the starch structure, hydroxyl groups are particularly susceptible to react through nucleophilic attacks located at the glucopyranose ring in the order C_2, C_6, and C_3. For our ampholytic starch, hydroxyl group can be involved in substitution of the hydrogen atom with carboxymethyl in a first step and with aminoethyl in a second step. The degree of substitution (DS) of the two series of obtained CM-St samples determined by back-titration were 0.02 and 0.06 representing the average number of carboxymethyl groups per glucose unit

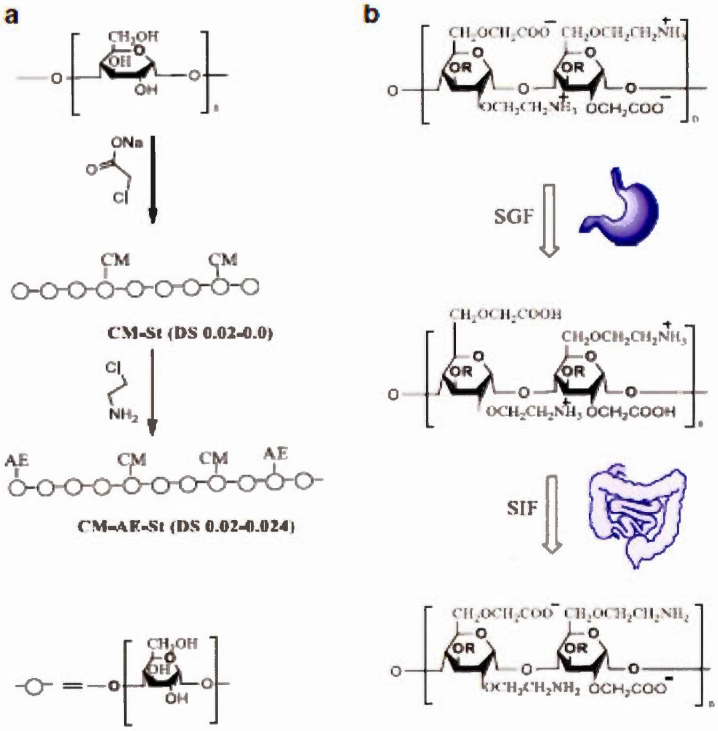

Fig. 1. Hypothetical presentation of a starch derivatization with sodium monochloroacetate and with chloroethylamine hydrochloride to generate ampholytic starch carrying carboxymethyl (*CM*) and aminoethyl (*AE*) functions and schematical representation **b** of ampholytic starch in simulated gastric (*SGF*) and intestinal (*SIF*) fluid where R can be either CM or AE or H. In DS 0.02–0.024, 0.02 is ascribed to DS of CM and 0.024 ascribed to DS of AE

corresponding to two series of CM-St. The CM-St (DS 0.02) was aminoethylated in three variants with different quantities of 2-chloroethylamine hydrochloride obtaining a series of CM-AE-St with amination DS of (0.064-0.049-0.024 mmol/g). The CM-St (DS 0.06) generated a CM-AE-St series with amination DS of (0.059-0.045-0.019 mmol/g). Figure 1a shows the preparation procedure for the CM-AE-St derivatives.

Scanning Electron Microscopy (SEM)

The aspects of starch and of CM-St and CM-AE-St derivatives are different depending on modifications operated on the starch structure. The native starch (Hylon VII) granules (Fig. 2A) were predominantly round or oval in shape, with a smooth surface. This seems due to the high content of amylose (more than 70%), which is more crystalline, known to exhibit double helix-B form and to be strongly stabilized by hydrogen associations between the hydroxyl groups of glucopyranose units (40). The CM-St (Fig. 2B, B') is characterized by an irregular shape with an uneven surface likely due to the association of numerous small particles forming larger granules (40,41). The presence of carboxylic groups probably reduced the network self-assembling by hydrogen associations between hydroxyl groups and promoted the repulsion effects leading to the reorganization of the network (42). The amination treatment generated a slightly smoother surface for the CM-AE-St granules (Fig. 2C–E, C'–E').

Fourier Transform Infrared (FT-IR) Analysis

For starch and its derivatives (Fig. 3I, II), a broad band with a maximum at 3296 cm^{-1} is due to the stretching vibrations of –OH, whereas the small band at 2927 cm^{-1} was attributed to the -CH stretching vibration. The band at 1079 cm^{-1} was ascribed to CH_2-O-CH_2 stretching vibrations for CM-St and CM-AE-St. In case of CM-St, there are few additional bands at 1589 cm^{-1} and at 1417 cm^{-1} ascribed to COO- group (6,40). The higher intensity in case of CM-St (0.06) is due to its higher DS. In the case of CM-AE-St, a band at 999 cm^{-1} can be assigned to C-N stretching vibrations whereas a peak at 1366 cm^{-1} was ascribed to -CH_2 group of AE (8,43). The presence of these additional bands confirms the grafting of CM and AE onto the starch backbone.

^{1}H NMR Measurements

Figure 4I, II shows ^{1}H NMR spectra of the CM-St and CM-AE-St, with proton signals at 5.3 ppm for H1 and at 3.3–

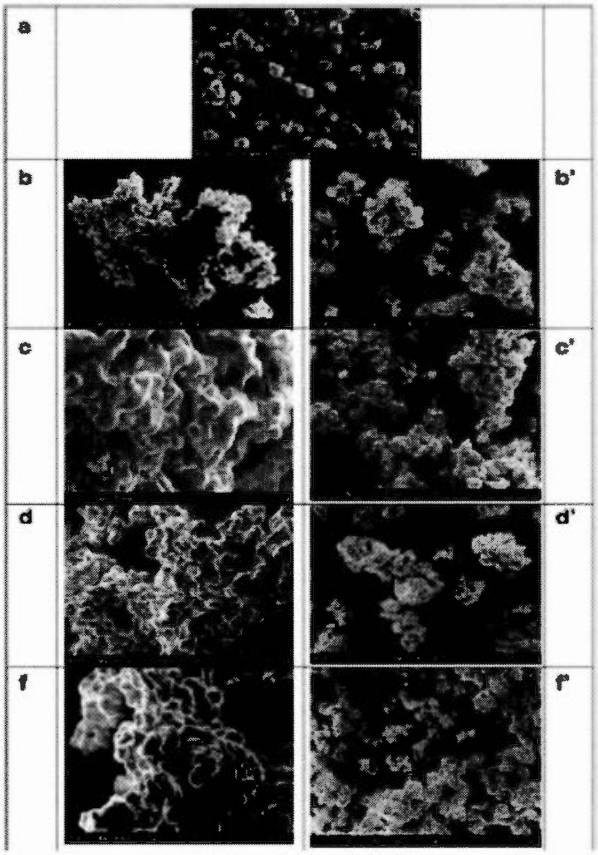

Fig. 2. Scanning electron microscopy micrographs of a Native Starch, (*B*) CM-St (DS 0.02), b' CM- St (DS 0.06), c CM-AE-St (DS 0.02-0.024), c' CM-AE-St (DS 0.06-0.019), d CM-AE-St (DS 0.02-0.049), d' CM-AE-St (DS 0.06-0.045), e CM-AE-St (DS 0.02-0.064), and e' CM-AE-St (DS 0.06-0.059) at magnification of ×1000

3.9 ppm for H2-6 (25). The most significant peaks at $\delta = 2.17–2.20$, $\delta = 3.16–3.18$ were found for CM-AE-St only and not for CM-St. They belong to the hydrogens of aminoethyl groups (44,45) and presented intensities proportional to the amount of AE groups.

Thermogravimetric Analysis

The thermogravimetric pattern (TGA and DTG curves) of starch, of CM-St, and of CM-AE-St investigated in a temperature range of 25 to 600°C is presented in Fig. 5I, II. All samples showed a two-step weight loss below 600°C. The first one is minor and seems to correspond to the loss of intramolecular and intermolecular water around 50–110°C. The second one may be related to material decomposition (46,47). The decomposition of starch may include molecular chain breakdown with hydrolysis of α-1,4 glucosidic linkages in the amylose and amylopectin of the starch. The scission of α-1,6 linkages may occur, but α-1,4 linkages are more susceptible to hydrolysis than α-1,6 linkages (48). Water is considered one of the main products of decomposition at temperatures below 270°C. Further heating up to 600°C resulted in carbonization and ash formation. The maximal decomposition temperature (MDT) was 289°C for CM-St (DS 0.02), whereas MDT for CM-St (DS 0.06) was 296°C. Both values are lower than the MDT of native starch (324°C). The reason seems to be the substitution of hydroxyl groups of native starch with CM groups. The results also indicate that CM-St was decomposed earlier but slower than native starch.

X-Ray Powder Diffraction

X-ray diffraction of starch, CM, and CM-AE-St powders (Fig. 6I, II) showed for starch the most ordered organization with diffraction peaks at $2\theta = 16.78°$, 17.48°, 19.84°, and 25.88° corresponding to a B-type morphology (characterized by a

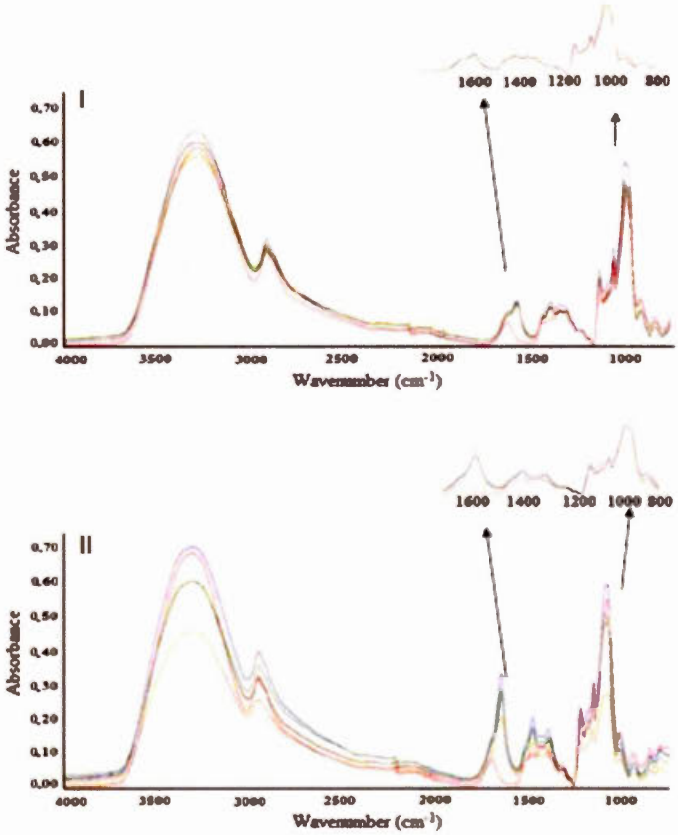

Fig. 3. FT-IR spectra of (*red*) native starch and of (*I*) (*blue*) CM-St (DS 0.02), (*black*) CM-AE-St (DS 0.02-0.024), (*green*) CM-AE-St (DS 0.02-0.049), and (*brown*) CM-AE-St (DS 0.02-0.064), and of (*II*) (*blue*) CM-St (DS 0.06), (*black*) CM-AE-St (DS 0.06-0.019), (*green*) CM-AE-St (DS 0.06-0.045), and (*brown*) CM-AE-St (DS 0.06-0.059)

double helix) (49). Peaks at $2\theta = 23.02°$, 13.38° correspond to a V-type morphology (a single helix having hydrophobic channel [48]). By derivatization, a pronounced reduction in order degree was observed with disappeared peaks at $2\theta = 19.84°$, 25.88°, suggesting the loss of the B-type double helix. This loss in crystallinity and disappearance of starch B-type could be attributed to the effect of derivatization by substitution of the hydroxyl groups with either CM groups (50,51) or AE groups (52–54). However, hydrogen bonds contributed to maintaining in a certain extent the starch order. The CM-AE-St pattern differed from that of CM-St (i.e., peaks at $2\theta = 13–16°$ and $2\theta = 21–25°$). For CM-St, the impact of grafting on the structure morphology was more pronounced for DS (0.06) than for CM-St (0.02), probably because of a stronger stabilization for DS 0.02 due to carboxyl dimerization by hydrogen bonding. With increasing numbers of grafted AE groups, the band at $2\theta = 13–16°$ was reduced and almost disappeared in the case of CM-AE-St (0.02–0.064) in comparison with CM-St (0.02). Differently, the band at $2\theta = 21–25°$ was of higher intensity and had shifted to a lower value.

In Vitro Dissolution Tests

The behavior of starch derivatives in SGF and in SIF, dissolution tests *in vitro* was first investigated in each medium separately. Since metformin is freely soluble and because it is required in high doses, modulating its release in the gastrointestinal tract is a challenging task. The presence of two functional groups CM and AE on the same chain can promote self-stabilization of the matrix in acidic and in neutral-alkaline media. It was previously shown (6,42) that the optimal degree of substitution for CM-St to exert controlled release was in the range (0.07–0.11). In the present study, CM-St was prepared with two degrees of substitution (0.02 and 0.06), and then each of them substituted with different amounts of AE groups. Similarly to the previous study (6), the release of metformin from CM-St with lower DS (0.02) was faster than with higher DS (0.06). Also the release in SGF was slower than in SIF since CM-St is compacted in gastric acidity due to protonation of carboxyl

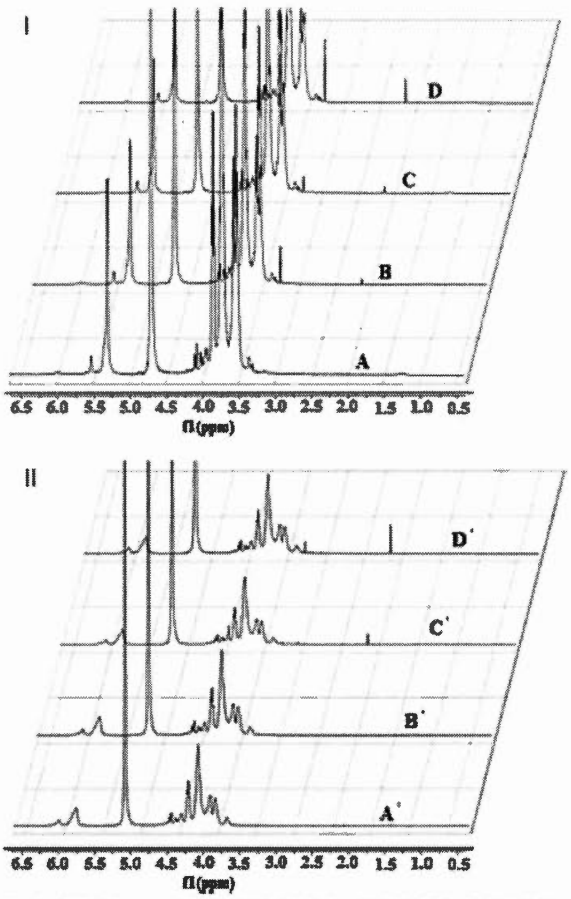

Fig. 6. ^{1}H NMR spectra (*I*): (*A*) CM-St (DS 0.02), (*B*) CM-AE-St (DS 0.02-0.024), (*C*) CM-AE-St (DS 0.02-0.049), and (*D*) CM-AE-St (DS 0.02-0.064) and (*II*) of (*A'*) CM- St (DS 0.05), (*B'*) CM-AE-St (DS 0.06-0.019), (*C'*) CM-AE-St (DS 0.06-0.045), and (*D'*) CM-AE-St (DS 0.06-0.059)

groups in SGF (Fig. 7). The same CM-St (DS 0.02) after grafting with different amounts of AE groups was able to control the release of metformin (Fig. 7I). By increasing the amount of AE groups, the release of metformin was prolonged in both SGF and in SIF. For instance, CM-AE-St (0.02–0.024) in 2 h released 95% of metformin in acidic medium while CM-AE-St (0.02–0.064) released 55% of metformin. Similar results were observed with CM-St (DS 0.06) after grafting with increasing amounts of AE groups (Fig. 7II).

The release kinetic of metformin in SGF and in SIF was evaluated based on the equations presented in Table I, where Q_t in equations A, B, C, and E is the amount of the drug released at time t, whereas Q_t in equation D is the amount of drug remaining in pharmaceutical dosage form at time t. Q_0 is the initial amount of drug in the pharmaceutical dosage form and K_0, K_1, K_H, K_S, and K_P are respectively the zero-order, first-order, Higuchi's, Hixson–Crowell's, and Korsmeyer–Peppas release rate constants and n is the release exponent. If the n value is 0.5 or less, the release mechanism follows Fickian diffusion, whereas at higher values for mass transfer in the range $0.5 < n < 1$, the system will follow a non-Fickian model (anomalous transport). The system follows a zero-order drug release and a case-II transport if the n value is equal to 1. Here the relaxation process of the macromolecules occurring upon water uptake by the system is the rate-controlling step. Water acts as a plasticizer and decreases the glass transition temperature of the polymer (55). For the values of n higher than 1, the mechanism of drug release is regarded as super case-II transport (39). The kinetic release

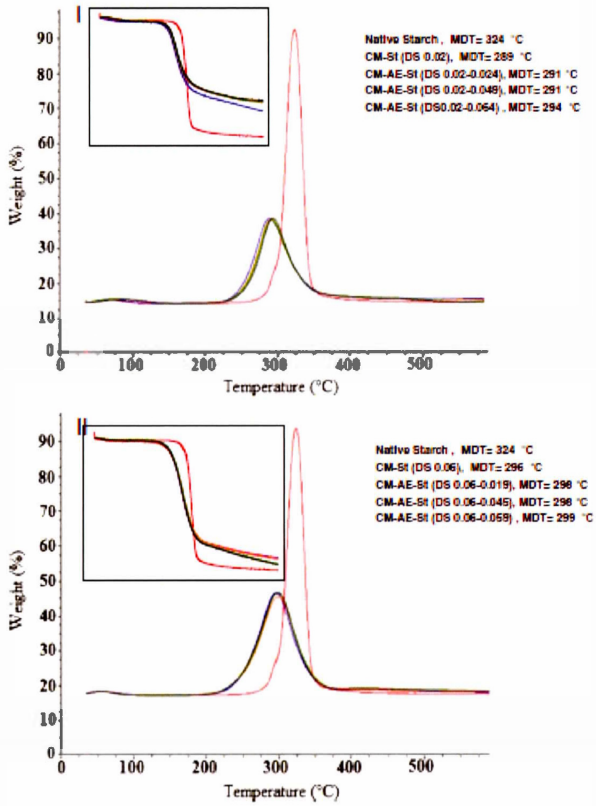

Fig. 5. Thermogravimetric (TG) and its derivative (DTG) diagram of native starch (*red*) and of (*I*) (*blue*) CM-St (DS 0.02), (*black*) CM-AE-St (DS 0.02-0.024), (*green*) CM-AE-St (DS 0.02-0.049), and (*brown*) CM-AE-St (DS 0.02-0.064) and of (II) (*blue*) CM-St (DS 0.06), (*black*) CM-AE-St (DS 0.06-0.019), (*green*) CM-AE-St (DS 0.06-0.045), and (*brown*) CM-AE-St (DS 0.06-0.059)

profiles of metformin in SGF and SIF from tablets based on CM-AE-St with different degrees of substitution, respectively, DS 0.02–0.064 and DS 0.06–0.059 (Table I) appear to fit well ($r^2 > 0.99$) the first-order model with the Korsmeyer's release exponent $n < 0.5$, which can be related to a diffusion controlled release of the drug associated with a small contribution of polymer relaxation.

Based on these results, the CM-AE-St (DS 0.06–0.059) derivative was selected to undergo further investigations and was compared to the commercial product Glumetza®. To better understand the effect of gastric acidity of the ampholytic starch matrix, tablets have been incubated 2 h in SGF and then transferred in SIF to follow the metformin release for a period of 12 h. This study was conducted with ampholytic starch loaded with 50% metformin (similarly to 1000 mg Glumetza tablets with 500 mg metformin loading) for a better comparison of the two dosage forms.

An oblong punch was used to prepare CM-AE-St tablets of 1 g (50% metformin loading), similar to Glumetza® in shape and weight (Fig. 8I, insert).

Figure 8I shows the dissolution profiles of metformin released from the commercial tablet Glumetza® and from tablets based on ampholytic starch CM-AE-St (DS 0.06–0.059), both with 50% metformin loading. The insert presents photographs of Glumetza® (G) and of tablet based on CM-AE-St (DS 0.06–0.059) at time 0 h and after 2 h incubated in SGF at pH 1.2. Table I illustrates drug release kinetic data with similarity factor (f_2) and difference factor (f_1), where "n" in Eqs. (F) and (G) in Table I is the number of sampling points, R_j and T_j are percent of the dissolved reference (R) and tested (T) tablets at each time point (j) respectively. For curves to be considered similar, f_1 values should be close to 0, and f_2 values should be close to 100. Generally, f_1 values up to 15 (0–15) and f_2 values greater than 50 (50–100) ensure sameness or equivalence of the two curves and,

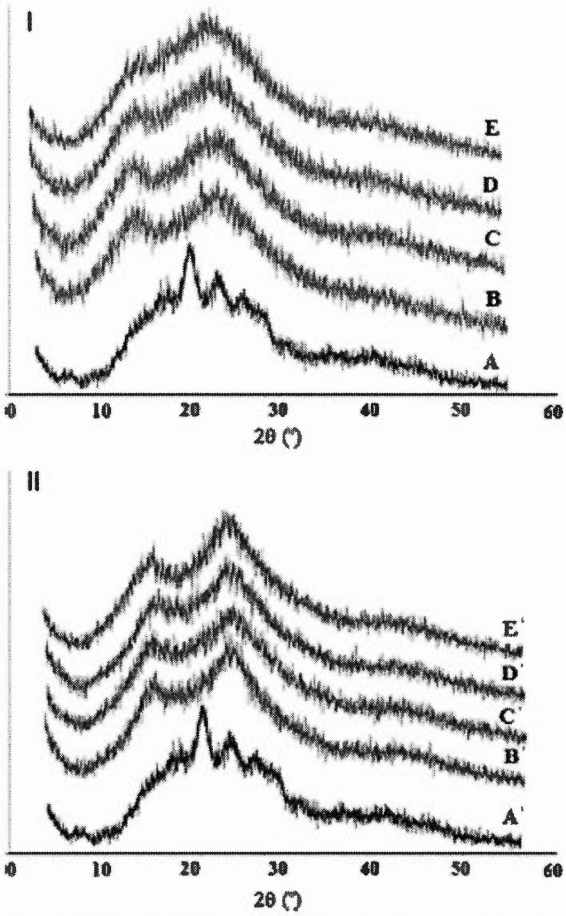

Fig. 6. X-ray diffraction patterns of (*A*) native starch and of (*I*) (*B*) CM-St (DS 0.02), (*C*) CM-AE-St (DS 0.02-0.024), (*D*) CM-AE-St (DS 0.02-0.049), and (*E*) CM-AE-St (DS 0.02-0.064) and (*II*) (*B'*) CM-St (DS 0.06), (*C'*) CM-AE-St (DS 0.06-0.019), (*D'*) CM-AE-St (DS 0.06-0.045), and (*E'*) CM-AE-St (DS 0.06-0.059)

thus, of the performance of the test (*T*) and reference (*R*) products (56,57). Up to 6 h, the dissolution profile of CM-AE-St tablet was similar to that of Glumetza®, with about 85% release of metformin. The kinetic analysis of the release profiles of metformin from all tested tablets (Table I) shows a good fit to the Higuchi's model ($r^2 > 0.99$), with the Korsmeyer's diffusional exponent $n \approx 0.5$ which can be related to diffusion with a small amount of relaxational controlled release of metformin.

Determination of the Fluid Uptake and Erosion

Fluid uptake and erosion tests are shown in Fig. 8II. It is noticed that Glumetza® tablets have more ability to absorb fluid, and therefore generate a higher swelling compared to oblong tablets based on CM-AE-St (DS 0.06–0.059). Furthermore, Glumetza® tablets showed a similar degree of erosion in comparison with the CM-AE-St oblong ones after 2 h in SGF, while after 12 h the tablets based on CM-AE-St (DS 0.06–0.059) showed higher erosion due to the greater polymer solubility in SIF in comparison to the limited solubility of the carrier used for Glumetza®. Axial and radial dimension changes (mm) of tablets were also observed at 2 and 12 h (Fig. 8III). It appeared that the axial and radial sizes of both CM-AE-St (DS 0.06–0.059) tablet and Glumetza® were almost similar after 2 h. After 12 h of incubation in acidic SGF, Glumetza® tablets showed a slightly greater radial increase from the initial tablet width of 10.13 mm up to 16.20 mm, when compared CM-AE-St (DS 0.06–0.059) with an initial width of 10.31 and 15.20 mm after 12 h of incubation in SIF.

DISCUSSIONS

Many attempts have been made to sustain the release of metformin, either by changing the metformin salts from hydrochloride to succinate (30) or by selecting longer and heat involving

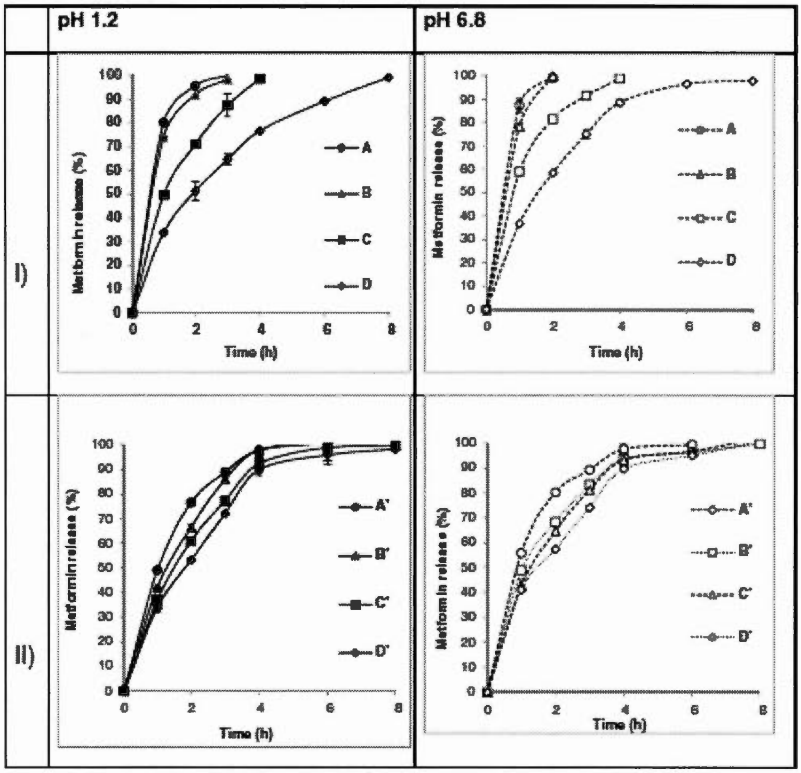

Fig. 7. Release profiles of metformin from 1000 mg tablet (60% loading) of (*I*): (*A*) CM-St (DS 0.02), (*B*) CM-AE-St (DS 0.02-0.024), (*C*) CM-AE-St (DS 0.02-0.049), and (*D*) CM-AE-St (DS 0.02-0.064) and (*II*) of (*A'*) CM- St (DS 0.06), (*B'*) CM-AE-St (DS 0.06-0.019), (*C'*) CM-AE-St (DS 0.06-0.045), and (*D'*) CM-AE-St (DS 0.06-0.059) at pH 1.2 (*left*) and pH 6.8 (*right*) side

methods of formulation like twin screw melt granulation/compression, hot melt extrusion, and injection molding (58,59). Also floating (17) or gastro-retentive tablets (60), and coated pellets (61,62) have been used. All these forms are using either complicated method of preparation or lower concentration of metformin, and none of them can be compared in terms of loading and dissolution profile with the commercial available product Glumetza®. Between pharmaceutical excipients currently used for controlled drug delivery systems, modified starches emerged as multitasking materials able to fulfill multiple roles: binders, fillers, matrix-formers, etc. (63). This multifunctionality associated with easiness of chemical modification was valorized for a wide range of applications. It was previously shown that the carboxylic groups on starch chains have a major role in the stabilization of the CM-St matrix in gastric fluid (2,3), having also a marked impact on matrix swelling, erosion, and dissolution contributing thus to the mechanisms of drug release. Previous studies (8) reported the release of metformin from monolithic dry-coated and uncoated tablets of CM-St, CM-St, and chitosan physical mixture or polyelectrolyte complex (PEC) of CM-St and chitosan, with metformin 20% loading. All these dry-coated or uncoated formulations released more than 80% of metformin within 3 h, except dry-coated formulations containing physical mixture of CM-St and chitosan of high molecular weight which prolonged the release of 80% of metformin to about 4 h. Based on the previous study (8), the ampholytic starch was proposed with both amino and carboxyl groups on the same starch backbone chain (Fig. 1a). Our concept is that self-stabilization of ampholytic starch may occur in both acidic (gastric) and intestinal (neutral) media (Fig. 1b). This self-stabilization seems to be proven by structural analysis. The fusion of CM-AE-St granules after amination observed in SEM could be attributed to the introduction of aminoethyl groups on the CM-St backbone, which could increase the hydrogen bonding and also inducing an ionic stabilization between the carboxymethyl and amine groups. It was also interesting to note that in a low degree of substitution, the carboxymethylation has no impact on polymer stability (64). By comparing the MDT values of CM-AE-St derivatives to those of CM-St for both DS (0.020 and 0.06), it appears that after amination the MDT was raised to 294°C for CM-AE-St (DS 0.02) and to 299°C for CM-AE-St (DS 0.06), suggesting an enhanced stability of CM-AE-St compared to CM-St. This may be related to additional hydrogen association and ionic stabilization in the case of the ampholytic CM-AE-St. When the hydroxyl groups were modified, the hydrogen bonds were mostly broken and consequently starch lost its crystallinity. The most ordered ampholytic starch excipient was that obtained with the highest DS (0.06–0.059) due to an additional ionic stabilization involving the

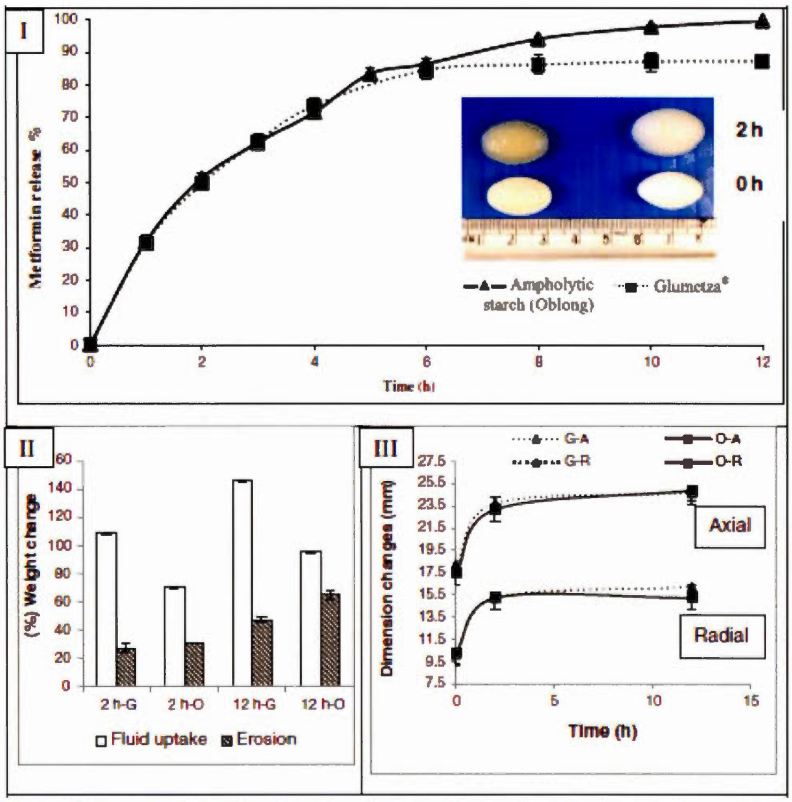

Fig. 8. Release profiles (*I*), fluid uptake and erosion (*II*), axial and radial tablet dimension changes (*III*) of Glumetza® (*G*) and tablet based on CM-AE-St (DS 0.06-0.059) oblong (*O*) of 1 g (50% loading). Oblong tablet was incubated: 2 h in SGF at pH 1.2 and up to 12 h in SIF at pH 6.8, whereas Glumetza® 500 mg incubated only in SGF at pH 1.2 up to 12 h. *Insert* in (*I*), photographs of Glumetza® (*G*) and of tablet based on CM-AE-St (DS 0.06-0.059) at time 0 h and after 2 h incubated in SGF at pH 1.2

CM and AE groups (Fig. 6II). The presence of CM groups on the polysaccharidic chains may offer gastro-resistance, whereas AE groups will reduce the polymer solubility in SIF. Moreover, a polyelectrolyte complexation between the anionic CM and cationic AE groups located on neighboring chains may also contribute to CM-AE-St stabilization. In gastric fluid, the ampholytic starch undergoes protonation of both carboxylic (generating a compaction of the outer layer around the tablet) and aminoethyl groups which produce an outer hydrogel able to release metformin in the early stage of gastric residence. Due to the ionic stabilization involving carboxylic and ethylamine groups in case of CM-AE-St-based tablets, the fluid uptake was moderate (69% after 2 h and 95% after 12 h), whereas in the case of Glumetza® it was higher (108% after 2 h and 146% after 12 h). The pill might be able to continue the intestinal transit through the pylorus, with the gastric emptying waves differing thus from gastro-retentive dosage forms (i.e., Glumetza®). In the intestinal fluid, the protonated carboxyl groups of the ampholytic CM-AE-St, located at the outer gel layer of the monolithic tablets, will gradually change the protons for Na^+ cations mostly arriving with the $NaHCO_3$ secretion of the pancreas. This will facilitate hydration, fluid uptake, erosion, and polymeric material dissolution in the simulated intestinal medium. In the case of CM-AE-St-based tablets, the erosion increased from 30% after 2 h in SGF to 64% after 12 h in SIF. In comparison with CM-St alone, CM-AE-St polymer solubility was lowered due to lesser solubility of amine groups in neutral media. The metformin release from Glumetza® is controlled by hypromellose (HPMC) (insoluble) as matrix forming agent. About 85–88% of metformin was released in the first 6 h, without further liberation in SGF, which may be due to a tight gel formed around the tablets. It is considered that when HPMC is hydrated in aqueous media, more than one state of water exists in the surface gel layer of a HPMC matrix (65,66). It was postulated that water may exist as (a) tightly bound water that interacts with polymer chains and is nonfreezable, (b) free water which is freezable, and (c) water that exists in bound states between these two extremes (65,66). The high fluid uptake of Glumetza® (108% after 2 h and 146% after 12 h) is required to get a tablet ensuring gastro-retentive releasing medication. Taking into consideration the tight bond between HPMC and water, other factors relating to the drug itself can alter the mechanism of drug liberation. Highly soluble drugs are thought to be released from tablet formulated with HPMC, principally but not exclusively, by diffusion (the case of

Glumetza® having $n = 0.60$ in Korsmeyer–Peppas equation) while poorly soluble drugs are released primarily by erosion. In addition, highly soluble drugs may act as pore formers that may make the pathways within gel structures less tortuous (67). With regard to HPMC, another factor to be taken into account is that ionically charged drugs are less mobile due to their potential interaction with the gel. This may increase the time taken for such drugs to diffuse through the gel structure (68,69). Both tablet types, CM-AE-St and Glumetza®, are in compliance with USP requirements (70) for the dissolution of metformin in the controlled release dosage form. The $f_1 = 2$ and $f_2 = 89$ values confirm also that *in vitro* drug release of CM-AE-St tablets is similar to that of commercial product Glumetza®. It is noteworthy that the remaining unabsorbed metformin accumulates in the gut mucosa prior to be ultimately eliminated with feces. Buse, Fineman et al (71) provided clinical evidence suggesting the primary hypoglycemic effect of metformin resides in the human gut and they described a novel delayed-release metformin formulation. It was hypothesized that gut exposure of metformin, but not circulation, accounts for most of its antihyperglycemic effect (71). This is why our ampholytic starch may be an excipient of interest for gut delivery.

CONCLUSION

The ampholytic CM-AE-St excipient was able to control the release of metformin in SGF and SIF for 8 h. The new proposed polymeric excipient can be self-stabilized and compacted in acidic medium due to protonation of CM groups and generating an outer gel by hydration of AE groups. Furthermore, the AE groups become insoluble in neutral intestinal medium. Thus, the presence of the two groups on the same macromolecular backbone will generate a beneficial effect for the usage of such polymer as carrier for the formulation of highly soluble drugs helping to control the release of drug during the transit along the gastrointestinal tract. In comparison with commercial product, the tablets of CM-AE-St (DS 0.06–0.059) with 50% drug loading have a similar dissolution profile to Glumetza® but releasing the drug over the whole intestinal tract and follow USP requirements for sustained release tablets of metformin. This opens a new perspective of ampholytic starch derivatives as novel pharmaceutical excipients for the challenging formulation of highly soluble drugs with required high loading. Further studies are going to investigate the pharmacokinetic performance of CM-AE-St (DS 0.06–0.059) in comparison with commercial products.

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Research Paper

Ampholytic starch excipients for high loaded drug formulations: Mechanistic insights

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ABSTRACT

Ampholytic starch derivatives are proposed as a new class of excipients carrying simultaneously anionic carboxymethyl (CM) and cationic aminoethyl (AE) groups on starch (St) polymeric chains. Three different types of derivatives were obtained by using the same reagents and varying only the order of their addition in the reaction medium: in one step method (OS) the two reactants were added simultaneously, whereas in two steps method (TS) either CMSt or AESt were prepared separately in the first step, followed by subsequent addition of the second reactant. It was found that all ampholytic derivatives were able to generate monolithic tablets by direct compression and allowed 60% loading of acidic (Acetylsalicylic acid), basic (Metformin), zwitterion (Mesalamine) or neutral (Acetaminophen) as drug models. The in vitro dissolution tests followed for 2 h in SGF and then in SIF, showed that the mentioned starch derivatives were stabilized by self assembling and generated matrices able to control the release of drugs for about 24 h. The addition order of reagents has an impact on ampholytic starch properties offering thus a high versatility of this new class of starch excipients that can be tailored for challenging formulations with high dosages of several drugs.

1. Introduction

Last decades were marked by innovative concepts of new drug delivery systems focused on improved effectiveness of medication and patient compliance. Molecular self-assembly based on noncovalent, weak interactions established between macromolecules represents a simple way to generate various supramolecular structures with a wide range of properties (Mateescu et al., 2006). This versatility is related to the possibility to tailor the polymeric structure in order to obtain properties suiting a specific application (Rattanakit et al., 2012; Hayashi et al., 2017). Hydrogels are largely used in controlled delivery of therapeutic agents and, despite the variety of their structures, there are two main features that can be modulated: hydrophilicity/hydrophobicity ratio and the ionic charges. Many research studies were dedicated to natural sourced such as cellulose (Hiremath and Saha, 2008; Vanhoorne et al., 2016), starch (Ispas Szabo et al., 1999, 2017), chitosan derivatives (Polk et al., 1994; Souza et al., 2014; Santos Menegucci et al., 2015), or synthetic polymers i.e. methacrylates (Okor and Obi, 1990), polyethylene glycol (Okor and Obi, 1990) and derivatives, in order to better control the drugs release. Most of self-assembled molecules present hydrophobic and /or hydrophilic domains where the hydrophilic zones can carry charges (anionic, cationic or zwitterionic) or may be uncharged but polar (Okor and Obi, 1990; Aji Alex et al., 2017; Yan et al., 2017). Intramolecular interactions may be responsible for the conformation of the molecules and conduct to specific secondary structures (helix, coils, sheet) whereas the intermolecular non-covalent interactions may be involved in self-assembled macromolecular structures like nanogels (Lamprecht et al., 2014), polyelectrolyte capsules (Szczepanowicz et al., 2014, 2016), cubosomes (Yang et al., 2014a), etc. Due to its biocompatibility and abundant source, starch is widely used in pharmaceutical and biomedical applications (Calinescu et al., 2005, 2007; Sakeer et al., 2017a). The availability of three hydroxyl groups per glucose unit offers the possibility for chemical modification enriching thus the panel of starch properties and applications. Starch modification was achieved through various reactions such as cross-linking (Lenaerts et al., 1998), esterification, etherification (Mulhbacher et al., 2001; Calinescu et al., 2007; Sakeer et al., 2010a), and grafting (Kaur et al., 2007) of functional groups onto the carbohydrate backbone. The esterification (i.e. by derivatization with acetic anhydride) can reduce starch hydrophilicity generating matrices for drug release (Mulhbacher et al., 2001) or provide interesting film-forming properties (Sakeer et al., 2017a). Differently, starch etherification with sodium monochloroacetate produced carboxymethyl starch (CMSt): an ionic matrix forming excipient for drug delivery with multiple applications such as protection of the active pharmaceutical ingredient (API) from gastric acidity (Calinescu et al.,

2005, 2007), drug controlled release (Lemieux et al., 2009) or chronodelivery medication (Ispas-Szabo et al., 2017). Very limited studies investigated aminoethyl starch (AESt) as biomedical material (Mell et al., 1968; Sakeer et al., 2017a). AESt can be obtained by aminoethylation of starch by etherification with chloroethylamine hydrochloride. Some previous studies illustrated amphoteric starch carrying quaternary ammonium (QA) and phosphate groups (Lin et al., 2012; Peng et al., 2016) or QA and CM groups (Shimei et al., 2006; Yang et al., 2014c) or QA and succinate groups (Lekniute et al., 2013) but the majority of these ampholytic derivatives were used as flocculants in environmental processes (Xu et al., 2005; Shimei et al., 2006; Lekniute et al., 2013; Yang et al., 2014b, c; Peng et al., 2016). Another approach consisted in a physical dry mixture of CMSt with polymers containing amine groups (i.e. chitosan) or the preparation of polyelectrolyte complex (PEC) obtained by co-processing which are able to sustain the release of neutral and acidic APIs in SIF at 20% drug loading (Assaad et al., 2011).

In the present study, we are investigating a new class of ampholytic starch derivatives carrying both anionic CM and cationic AE groups on the starch backbone chains. In a previous study we investigated only one type of starch derivative as matrix for metformin delivery (Sakeer et al., 2017b) while now we are exploring three types of ampholytic starch obtained by three different methods using the same reagents. Considering the simultaneous presence of CM and AE functional groups and their eventual pH-dependent ionization on different dissolution media, it was of interest to understand the capacity of these excipients to control the liberation of various drugs. Thus neutral (acetaminophen), acidic (acetyl salicylic acid – ASA), basic (metformin) and zwitterion (mesalamine) drug active molecules were used as tracers in monolithic tablets with the aim to evaluate the usefulness of the new class of excipients for drug formulation mainly for controlled delivery of high loaded dosage forms.

2. Materials and methods

2.1. Materials

High amylose starch (Hylon VII) was supplied by National Starch/Ingredion (Bridgewater, NJ, USA). Mesalamine (pharmaceutical grade) was a product of PharmaZell (Raubling, Germany). Metformin (1,1-dimethylbiguanide hydrochloride) was from MP Biomedicals (Solon, OH, USA) and acetylsalicylic acid (ASA) was from Fisher Scientific (Hampton, NH, USA). Acetaminophen and sodium monochloroacetate (SMCA) were from Sigma-Aldrich (Germany) and 2-chloroethylamine hydrochloride (CEAHC) was from Fluka (Switzerland). The other reagents were chemical grade and used without further purification.

2.2. Preparation of starch derivatives

To investigate the effect of ionic charges on the control of drug delivery various starch derivatives were synthesized by two different procedures (Fig. 1):

A) One step (OS) approach: the two reagents sodium monochloroacetate (SMCA) and 2-chloroethylamine hydrochloride (CEAHC) were rapidly dissolved in water and then added simultaneously to gelatinized starch.

B) Two steps (TS) approach: the polymers were prepared in two ways by changing the order to introduce the functional groups: (1) the anionic (CM) groups were grafted first and then followed by cationic (AE) ones. (2) the cationic groups were first introduced on polysaccharidic chains followed by anionic ones. Thus AESt was prepared first by dissolving CEAHC in water and added to gelatinized starch. The obtained polymer was precipitated and then re-dissolved and reacted with SMCA in order to obtain AECMSt.

Practically, in OS method an amount of 12.50 g of starch was dispersed in 50 mL of distilled water at 60–70 °C and then gelatinized with 75 mL of 5 M NaOH under continuous stirring for 1 h at 60–70 °C. An amount of 9.37 g of SMCA was rapidly solubilized in a minimal water volume. Separately, an amount of 9.37 g CEAHC was dissolved in a minimal water volume and rapidly added to gelatinized starch simultaneously with the SMCA continuing the stirring for 1 h maintaining the pH 9–10. Then, the preparation was cooled down and neutralized with glacial acetic acid. The carboxymethyl aminoethyl starch CMAESt (OS) powder was separated by precipitation from the slurry with an equivalent volume of methanol:water (70:30 v/v). The derivative was washed with methanol:water (70:30 v/v) until a final conductivity (Fisher Scientific Accumet Research AR20, San Diego, CA, USA) of filtrate decreased to less than 75 μS/cm. Pure methanol and acetone were used for final drying; the collected powder was left overnight at room temperature for complete air drying and sieved to obtain particles of less than 300 μm.

For the two steps (TS) method, the starch was first gelatinized as previously described for the OS procedure. To produce Carboxymethyl starch (CMSt), the proper amount of SMCA was quickly solubilized in a minimal water volume and added to the gelatinized starch maintaining the stirring for 1h at 60–70 °C at pH 9–10. Separately, the Aminoethyl starch (AEST) was obtained by rapidly solubilising the proper mass of CEAHC in water and then added to the gelatinized starch continuing the stirring for 1 h at 60–70 °C and pH 9–10. Then, the solution was cooled down and neutralized with glacial acetic acid. The polymer powders were obtained by precipitation with methanol and dried as described previously. The CMSt or AESt were each re-dispersed in 50 mL of distilled water followed by addition of 75 mL of 5 M NaOH and heated at 60–70 °C for 1 h under continuous stirring for gelatinization. Amounts of 9.37 g CEAHC (in case of CMSt), to obtain CMAESt (TS) or 9.37 g SMCA (in case of AESt), to obtain AECMSt (TS) were solubilized in a minimal water volume and added to gelatinized starch keeping the stirring for 1 h at 60–70 °C and pH 9–10. Then, the solutions were cooled down and neutralized with glacial acetic acid. The CMAESt (TS) and AECMSt (TS) powders were each obtained by precipitation with methanol and drying as described above.

2.3. Evaluation of the degree of substitution

A) The degree of substitution (DS) with CM groups was determined by back-titration as previously described by Stojanović et al. (2005) with small modification. Briefly, 100 mg of polymer (n = 3) were solubilized in 10 mL of 0.05 M NaOH, and phenolphthalein was added as indicator. The excess of NaOH was titrated with 0.05 M HCl. The blank (10 mL of 0.05 M NaOH) was also titrated by the same method. The amount of –COOH groups and the DS were calculated (Stojanović et al., 2005) using the Eqs. (1), (2):

$$n = (V_b - V) \times C_{HCl} \quad (1)$$

$$DS = \frac{162 \times n}{m - W \times n} \quad (2)$$

where Vb (mL) is the volume of HCl used for the titration of the blank; V (mL) is the volume of HCl used for the titration of the sample; C_{HCl} (mol/L) is the concentration of HCl; 162 (g/mol) is the molar mass of glucose unit; W = (58) (g/mol) is the increase in the mass of glucose unit by substitution with a CM- group, and m (g) is the mass of dry sample.

B) The degree of substitution (DS) expressed in terms of amino groups on the final derivative was determined with the ninhydrin reagent (2 g ninhydrin dissolved in 75 mL dimethylsulfoxide under nitrogen flushing and completed with 25 mL of 4 M lithium acetate buffer, pH 5.2). A volume of 0.5 mL of the ninhydrin reagent was added to a volume of 0.25 mL of ampholytic starch solution (15 mg/mL) in deionized water (in triplicate). The vials were immediately capped, vortexed, and heated in a covered boiling water bath for 30 min. Vials were then cooled below 30 °C in a cold water bath. The absorbance at

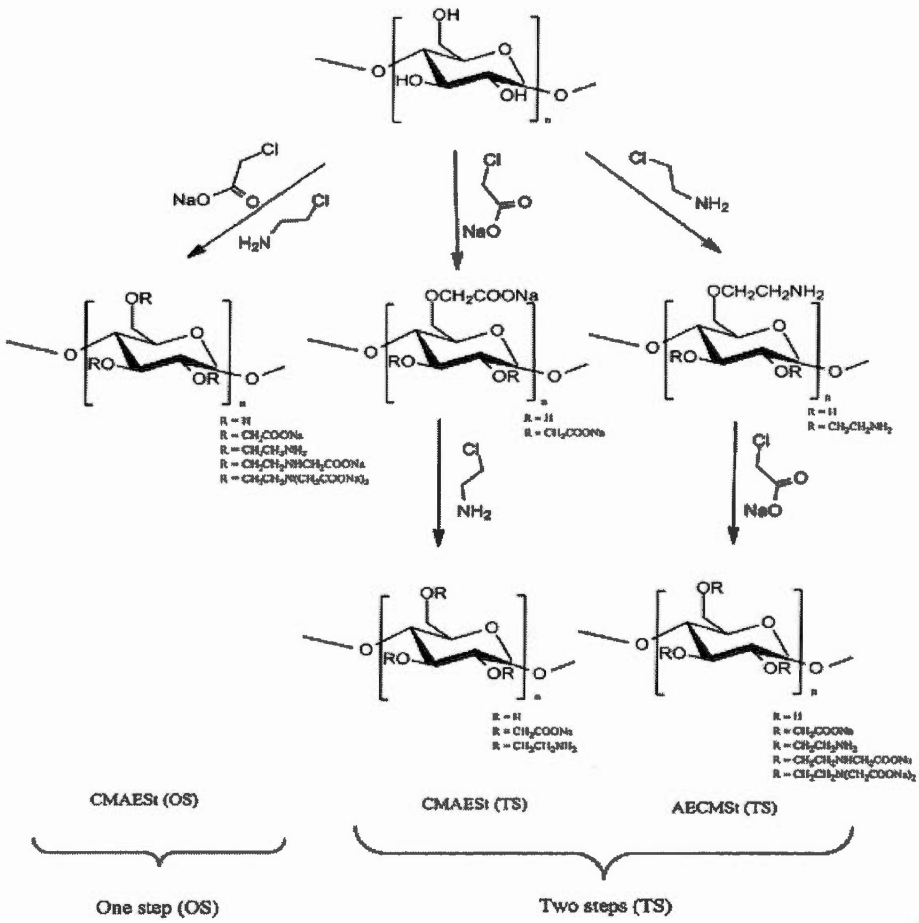


Fig. 1. Schematical presentation of starch derivatization with sodium monochloroacetate and with chloroethylamine hydrochloride to generate amphotytic starch carrying carboxymethyl (CM) and aminoethyl (AE) functions obtained by one step (OS) or two steps (TS) preparation methods.

570 nm was measured on a UV/VIS spectrophotometer with zero set against a similarly treated blank of water. Glycine (Sigma) solutions (0.12–0.51 mg/mL) were used to generate a standard curve (Le Tien et al., 2003; Le Dévédec et al., 2008).

2.4. Zeta potential (ζ) and pH determination

Measurements of surface charge of various polymer solutions were performed using ZetaPlus/Bl-PALS (Brookhaven Instrument Corp, Holtsville, NY, USA). The measurements were carried-out at 25 °C. Analysis of the particle surface charge was done in triplicates. The pH of polymer solution 1% (w/v) in water at room temperature was measured by a Fisher Scientific Accumet Research AR20 pH-meter.

2.5. Viscosity determination

The viscosity of starch derivatives in concentration of 1% (w/v) in water and in phosphate buffer pH 6.8 at 25 °C was measured on a Brookfield viscometer (DV-II pro viscometer, Middleboro, MA, USA). The experiment was done with a spindal CPE 40 at 10 rpm after 4 h and after 48 h.

2.6. Fourier transform infrared (FT-IR) analysis

The FT-IR spectra of native starch (Hylon VII) and of its derivatives as powders were recorded (64 scans at a resolution of 4 cm^{-1}) using a Thermo-Nicolet 6700 FT-IR spectrometer (Madison, WI, USA) equipped with a deuterated triglycine sulfate-KBr (DTGS-KBr) detector and a diamond smart attenuated total reflection (ATR) platform.

2.7. ^1H NMR measurements

To determine the starch functionalization pattern, ^1H NMR spectra were collected using a high-field 600 MHz, Bruker Avance III HD spectrometer running TopSpin 3.2 software and equipped with a 5 mm TCI cryoprobe. The temperature of samples was regulated at 27 °C. The samples were dissolved either in deuterium oxide (D_2O) or in deuterated dimethyl sulfoxide-d6 (DMSO-d6) with both methyl groups deuterated, then heated at 65 °C for 30 min, and kept at 4 °C for 2 h.

2.8. Thermogravimetric analysis

Thermogravimetric analysis (TG) of the samples was carried out in platinum crucible at a heating rate of 10 °C/min between 25 and 900 °C under nitrogen atmosphere (flow rate 100 mL/min) using a TA® Instruments incorporated high-resolution thermo gravimetric analyzer Seiko TG/DTA 6200 (Japan).

2.9. Scanning electron microscopy

The microstructure of the sample particles was examined by a Hitachi (S-4300SE/N) scanning electron microscopy with variable pressure (Hitachi High Technologies America, Pleasanton, CA, USA) at voltage of 3–15 kV and magnifications of 100× and 1000×. Samples were mounted on metal stubs and sputter-coated with gold.

2.10. X-ray diffraction

X-ray diffractometry of polymers was performed using a Siemens D-5000, (Munich, Germany) device. The samples were exposed to X-ray radiation (Cu Kα with wavelength of 1.789 Å) and a scanning rate of 0.05 °/min. Samples (ground into powders with an agate mortar and pestle) were loaded on a low background quartz plate in an aluminium holder and analyzed through a 2θ range of 5–50°. The XRD spectra were treated using DiffracPlus software.

2.11. Micromeritics properties of starch derivatives powders

Micromeritics study a number of characteristics, including particle size, size distribution, particle shape, angle of repose, porosity, true volume, apparent density and bulkiness. The USP compendial methods were applied to investigate the flow properties by measuring the angle of repose according to "1174" USP powder flow procedure, whereas the bulk and tapped densities of the polymer powders were determined by calculating Carr's index and Hausner's ratio according to the "616" USP method, using a Vankel tapped density tester (Varian, NC, USA). The angles of repose of ampholytic starch powders were measured by using the fixed funnel method. Briefly, the ampholytic starch powder was poured into a funnel which was fixed at a position so that the outlet orifice of the funnel was 2 cm above a black surface. The powder was flowed down from the outlet orifice to form a cone on the surface and the angle of repose was then calculated by measuring the height of the cone (h) and the radius of its base (r) with the help of calibrated scale (Patil-Gadhe and Pokharkar, 2014). The angle of repose (Θ) was calculated in triplicate according to the Eq. (3)

$$\theta = tan^{-1}\left(\frac{h}{r}\right) \tag{3}$$

To measure tapped density of the obtained powder a known mass of ampholytic starch powder was poured into a calibrated measuring cylinder and the volume occupied by this powder was recorded (n = 3). The tapped density ρ_{tap} was determined by volume measurement of the tapped mass until no further changes in the powder volume were observed (Shah et al., 2008). The Carr's index (Carr, 1965) and Hausner's ratio (Hausner, 1967) were calculated according to Eqs. (4) and (5):

$$CI = \frac{\rho_{tab} - \rho_{bulk}}{\rho_{tab}} \times 100 \tag{4}$$

$$HR = \frac{\rho_{tap}}{\rho_{bulk}} \tag{5}$$

Where CI is Carr's compressibility index; HR is Hausner's ratio; ρ_{tap} is the tap density; and ρ_{bulk} is the bulk density.

2.12. Preparation of tablets

Monolithic tablets (with 60% drug loading) were obtained by direct compression (2.5 T/cm²) of a homogenous mixture of excipient and drug powder with a total tablet weight of 833 mg for acetaminophen, metformin or aspirin and 667 mg in case of mesalamine using flat-faced punches with 12.95 mm diameter and a Carver hydraulic press Model C 3912 Hydraulic Cylinder (Wabash, IN, USA). The above loadings were chosen in line with commercial products for a better comparison.

2.13. Determination of the fluid uptake and erosion

Fluid uptake and erosion properties have been investigated with placebo tablets obtained by direct compression (2.5 T/cm²) using flat-faced punches with 9.50 mm diameter. The choice of diameter of 12.9 mm for tablets with medication was to better fit with the commercial formulations. The diameter 9.50 mm was chosen for placebo tablets in order to better understand the role of polymeric excipients only. The studies were carried out by immersing the weighed dry tablet (W1) in 40 mL of either simulated gastric fluid (SGF, pH 1.2) or simulated intestinal fluid (SIF, pH 6.8 phosphate buffer). The immersed tablets were incubated in 40 mL fluid and submitted to rotation at 50 rpm (Glas-Col rotator, Terre Haute, IN, USA). After 2, 4, 6, 8 h, tablets were withdrawn and carefully weighed (recorded as W_2). The recovered tablets were then placed into an oven at 35–40 °C for two days until a constant weight was obtained. The final weight of the tablets was measured (W_3) after complete drying at constant mass (Sakeer et al., 2010a,b). Fluid uptake was determined according to Eq. (6):

$$\%\,Weight\ change = \frac{W_2 - W_1}{W_1} X100 \tag{6}$$

The degree of erosion was determined according to Eq. (7):

$$\%\,Erosion = \frac{W_1 - W_3}{W_1} X100 \tag{7}$$

2.14. In vitro dissolution tests

The dissolution tests were carried out for all tablets either separately in (SGF) and in (SIF) dissolution media, or after incubation of tablets for 2 h in SGF followed by SIF. An USP paddle apparatus 2 was used. The dissolution volume (mL), the paddle speed (rpm) and wavelength (λ nm) were selected for each drug as per pharmacopeial requirements (USP, 2015): for mesalamine in SGF (500 mL, 100 rpm, λ 300 nm) and in SIF (900 mL, 50 rpm, λ 330 nm), for acetaminophen in SGF and SIF (900 mL, 50 rpm, λ 243 nm), for metformin (1000 mL, 100 rpm, for SGF λ 218 nm and for SIF λ 232 nm), and finally for ASA in SGF (900 mL, 100 rpm, λ 280 nm) and in SIF (λ 227 nm). The concentrations of the released active molecules at different times were calculated based on a standard curve of each drug in SGF and in SIF.

2.15. Release kinetic patterns

The drug release kinetic parameters were evaluated according to: zero-order kinetics (Costa and Sousa Lobo, 2001), first-order kinetics (Costa and Sousa Lobo, 2001), Higuchi's model (Higuchi, 1961, 1963), Hixson–Crowell's model (Hixson and Crowell, 1931) and Korsmeyer–Peppas model (Korsmeyer et al., 1983).

2.16. Statistical analysis

All tests were done in triplicate and data are reported as means ± SD. For statistical analysis, the one way ANOVA was followed by Fisher's post hoc tests with a minimum confidence level ($P < 0.05$) for statistical significance.

3. Results and discussions

3.1. Structural insights

The hydroxyl groups on glucopyranose ring are susceptible to react with the proposed reagents SMCA and/or CEAHC in the order C_2, C_6 and C_3. In ampholytic starch, the hydroxyl group can be involved in substitution with CM (CH_2COONa) groups after reaction with SMCA or substitution with AE ($CH_2CH_2NH_2$) groups by reaction with CEAHC, whereas secondary interactions between CEAHC and SMCA may produce ethylamino carboxymethyl (EACM) ($CH_2CH_2NHCH_2COONa$) or ethylamino dicarboxymethyl (EADCM) $CH_2CH_2N(CH_2COONa)_2$ groups. In the case of one step polymer preparation (Fig. 1) at least three different types of structures can theoretically occur: 1) CM and AE starch derivatives; 2) CM and secondary and/or tertiary amines, but exhibiting terminal aminoethyl (AE) groups; 3) ethylamine groups substituted with CM groups exhibiting AECM or AE$(CM)_2$ (EADCM). In two steps method, when CM groups are grafted firstly, followed by introduction of AE groups, one type of starch derivative is expected presenting CM and AE groups. Differently, for AECMSt, when AE groups are grafted first and then followed by CM groups, there is a good probability of amine groups to be substituted with CM groups and to generate also EACM and EADCM groups (Fig. 1). The degrees of substitution (DS) with CM determined by back-titration of the ampholytic samples were 0.024, 0.041 and 0.028 for CMAESt (OS), CMAESt (TS) and AECMSt (TS), respectively. These DS values represented the average number of carboxymethyl groups per glucose unit. The DS in terms of primary amine (AE) groups were 0.012, 0.012 and 0.015 mmol/g for CMAESt (OS), CMAESt (TS) and AECMSt (TS), respectively. The ionic charge of the aqueous solutions of ampholytic starches calculated as Zeta potential (ζ) was −25.9 mV for CMAESt (OS), whereas ζ values of −42.98 mV were found for CMAESt TS and −52.5 mV for AECMSt (TS). These values are consistent with the chemical modification of starch by CM, AE, EACM or EADCM groups. The highest value for AECMSt (TS) could be ascribed due to the higher amount of EACM and/or EADCM providing a stronger negative charge (Wongsagonsup et al., 2005a, b). For the 1% (w/v) polymer solution in water the pH values were 6.42, 6.2 and 6.9 for CMAESt (OS), CMAESt (TS), and AECMSt (TS) respectively, indicating that almost all ampholytic starch were neutral. The viscosity of CMAESt (TS) after 4 h in water was 8.34 cP, higher than that of AECMSt (TS) and of CMAESt (OS), whereas after 48 h all polymers showed almost similar viscosity. These values indicate that all polymers are hydrated almost at the same extent in water but with different rates.

On the other hand, CMAESt (OS) showed higher viscosity after 4 h and after 48 h in phosphate buffer pH 6.8 in comparison with CMAESt (TS) and AECMSt (TS) probably due to the higher solubility of both CMAESt TS and AECMSt in phosphate buffer, whereas CMAESt (OS) undergo swelling rather than solubilization in SIF (Table 1).

3.2. Fourier transform infrared (FT-IR) analysis

For starch and its derivatives (Fig. 2) a broad band (3200–3400 cm^{-1}) with a maximum at 3296 cm^{-1} is due to the stretching vibrations of —OH, and a small band at 2927 cm^{-1} is attributed to the —CH stretching vibration. The band at 1079 cm^{-1} was ascribed to CH_2 —O —CH_2 stretching vibrations. The bands at 1589 cm^{-1} and at 1417 cm^{-1} were ascribed to —COO^- group (Stojanović et al., 2005; Ispas-Szabo et al., 2017). The higher intensity in case of CMAESt (TS) and AECMSt (TS) could be due to higher amount of carboxylate groups. The high intensity of the band at 999 cm^{-1} could be ascribed to C —N stretching vibrations, whereas the weak shoulder between 1630–1735 cm^{-1} could be assigned to —NH bend (Deng et al., 2006; Assaad et al., 2011). The presence of these additional bands confirms the grafting of CM, AE, EACM or EADCM onto the starch backbone.

Table 1
Viscosity (cP) of starch derivatives at 1% w/v in distilled water (W) and in SIF measured after 4 h and respectively after 48 h, at 25 °C.

	CMAESt (OS)		CMAESt (TS)		AECMSt (TS)	
Time/Media	W	SIF	W	SIF	W	SIF
4 h	7.94	7.57	8.34	2.82	7.75	2.88
48 h	8.95	5.82	8.77	3.00	8.41	3.00

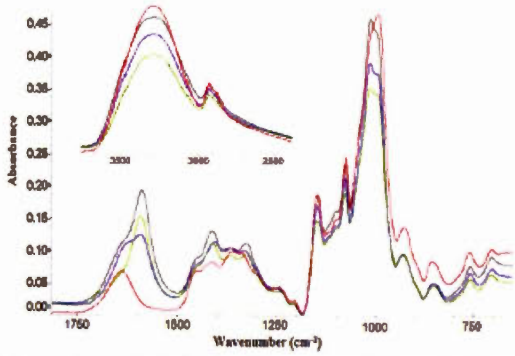


Fig. 2. FT-IR spectra of Native Starch (red), CMAESt (OS) (blue), CMAESt (TS) (black) and AECMSt (TS) (green).

3.3. ^1H NMR measurements

Fig. 3I, II, presents the ^1H NMR spectra of the native starch (Hylon VII) and its ampholytic derivatives in D_2O and in DMSO-d6 respectively. When D_2O was used as a solvent, the proton signals at 5.3 ppm were ascribed to H1 and those at 3.3–3.9 ppm were assigned to H2-6 (Yang et al., 2014b). The most significant peaks at δ = 2.17–2.20, δ = 3.16–3.18 were found for ampholytic starch derivatives and not for native starch. They belong to the hydrogens of aminoethyl groups (Noga et al., 2012; Amar-Lewis et al., 2014). The lower intensity of starch pattern is due to the limited solubility of starch in D_2O. With DMSO-d6 as solvent the peaks were sharp. The peak at 3.31 ppm was ascribed to H2, and those at 3.36 ppm to H4, at 3.57 ppm to H5 and at 3.64 ppm to H3. The chemical shifts of —OH(2), —OH(3), and —OH(6) were possibly at origin of peaks between 4.58 and 5.50 ppm, whereas the proton signals at 5.3 ppm were assigned to H1(Namazi et al., 2011; Yang et al., 2014b). The most significant peaks are at δ = 3.16–3.18, which belong to the hydrogens of aminoethyl group. The peak at δ = 1.6 is related to the methylene group of CM (Namazi et al., 2011).

3.4. Thermogravimetric analysis

The thermogravimetric pattern (TGA and DTG curves) of native starch and of its derivatives investigated in a temperature range of 25 °C–600 °C are presented in Fig. 4. All samples showed a two-steps weight loss below 600 °C. The first one was minor and seems to correspond to the loss of intramolecular and intermolecular water around 50–110 °C. The second one may be related to material decomposition (Li et al., 2010; Zhang et al., 2014). Water is considered one of the main products of decomposition at temperatures below 270 °C. Further heating up to 600 °C resulted in carbonization and ash formation. The maximal decomposition temperature (MDT) was 291 °C for AECMSt (TS), whereas MDT for CMAESt (OS) was 294 °C and MDT for CMAESt (TS) was 293 °C. All values are lower than the MDT of native Starch (324 °C). The reason seems to be the substitution of hydroxyl groups of

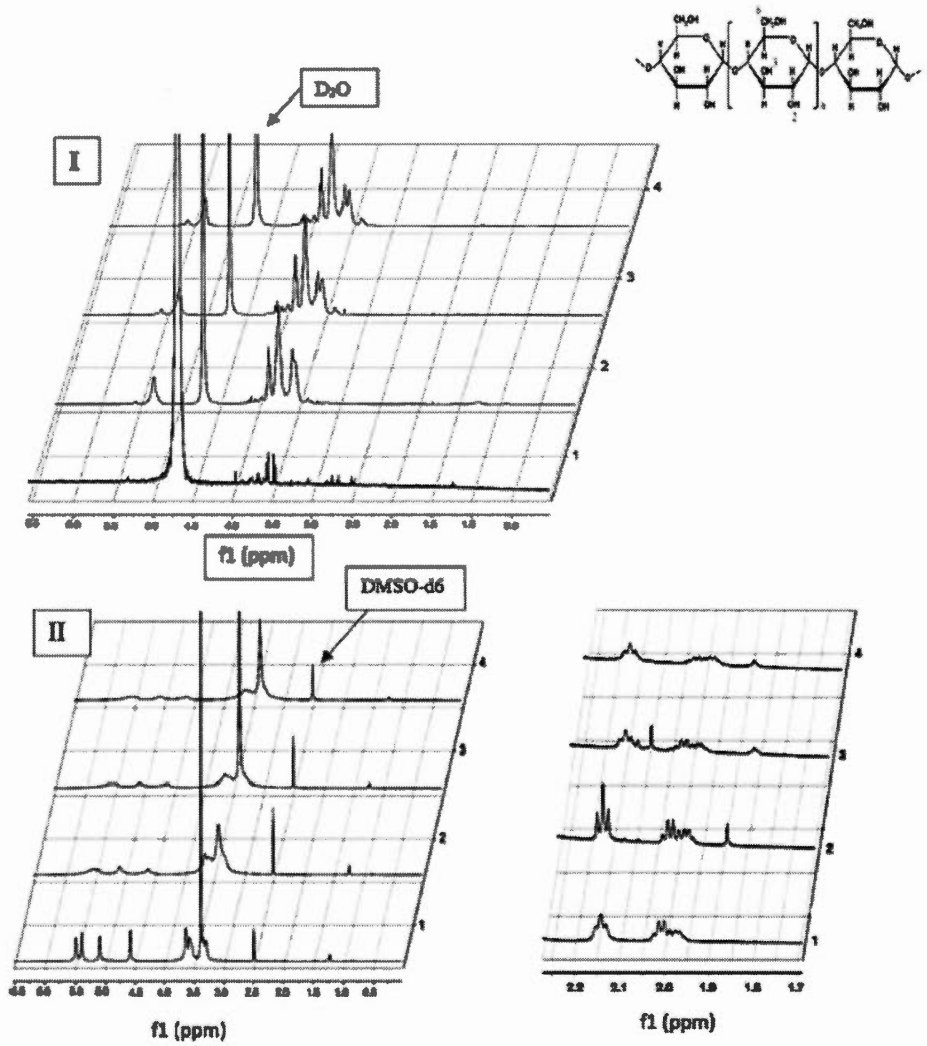


Fig. 2. ^{1}H NMR spectra in D_2O (I) and in DMSO-d6 (II) for Native Starch (1), CMAESt (OS) (2), CMAESt (TS) (3) and AECMSt (TS) (4).

starches with CM and/or AE and/or EACM or EADCM groups. By calculating the area under the curve (AUC) for the main stage of polymer degradation, the decomposed component could be qualitatively determined. The AUC of the main decomposition stage of native starch was 80.6 and it is higher than AUC of other derivatives, which means that native starch is decomposed as a function of temperature at a different pattern than other ampholytic starch derivatives, due to their functionalization starch with CM, AE, EACM or EADCM groups. The AUC for CMAESt (TS) and AECMSt (TS) are very close each other, suggesting that similar amounts of resembling components are decomposed as a function of temperature. (Tiwari and Hihara, 2009, 2012).

3.5. *X-ray diffraction*

X-ray diffraction of native starch (Hylon VII) powder and of its derivatives (Fig. 5) showed the starch as the most ordered organization with diffraction peaks at $2\theta = 16.78°$, $17.48°$, $19.84°$, $25.88°$ corresponding to a type B structure and at $2\theta = 23.02°$, $13.38°$ corresponding to a V type structure. By derivatization, a pronounced reduction in order degree was observed with peaks decreased or disappeared (those at $2\theta = 19.84°$, $25.88°$), suggesting the loss of the B-type double helix, possibly due to the effect of derivatization of the hydroxyl groups with either CM groups (Wang et al., 2010; Gao et al., 2011) or AE groups (Kuo and Lai, 2007; Pi-Xin et al., 2009; Chang et al., 2014) or EACM or EADCM groups. However, hydrogen bonds contributed to maintain the starch order in a certain extent. The CMAESt (OS) pattern presented sharper peaks at $2\theta = 13°–16°$ and $2\theta = 21°–25°$ than CMAESt (TS) or

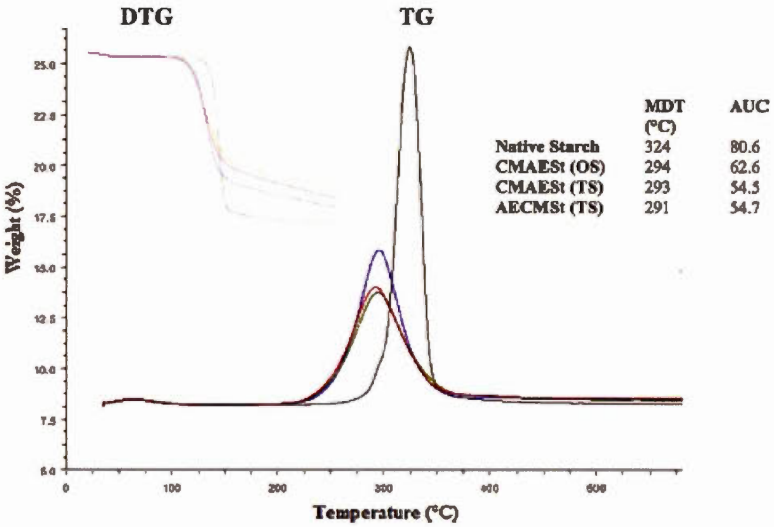


Fig. 4. Thermogravimetric (TG) and its derivative (DTG) diagrams of Native Starch (black), CMAESt (OS) (blue), CMAESt (TS) (green) and AECMSt (TS) (red).

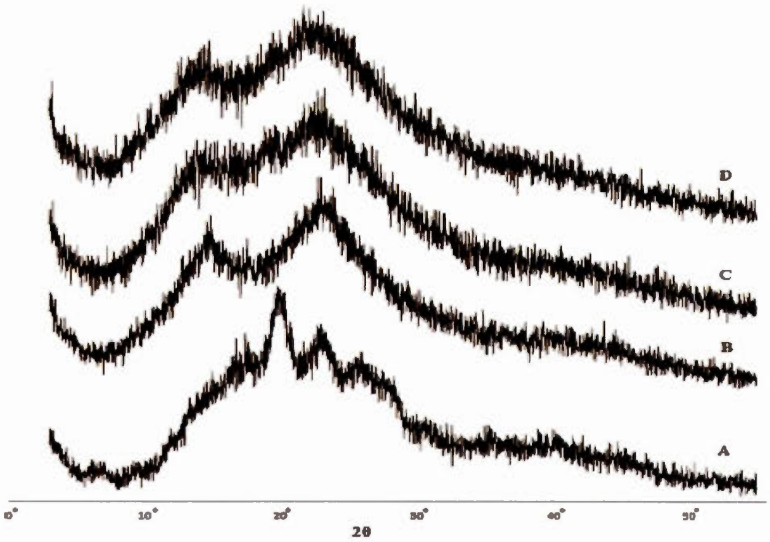


Fig. 5. X-ray diffraction patterns of Native Starch (A), CMAESt (OS) (B), CMAESt (TS) (C) and AECMSt (TS) (D) powders.

AECMSt (TS).

3.6. Scanning electron microscopy

The aspects of native starch (Hylon VII) and of CMAESt (OS) of CMAESt (TS) and of AECMSt (TS) derivatives are different (Fig. 6) depending on modifications operated on the starch structure. The native starch (Hylon VII) granules (Fig. 6A) were predominantly round or oval in shape, with a smooth surface. This seems due to the high content of amylose (more than 70%), which is more crystalline, known to exhibit double helix-B form and to be strongly stabilized by hydrogen associations between the hydroxyl groups of glucopyranose units (Friciu et al., 2013). The CMAESt (OS) is characterized by an irregular shape with an uneven surface likely due to the association of numerous small particles forming larger granules (Fig. 6B). The presence of carboxylic groups probably reduced the network self-assembling by hydrogen associations between hydroxyl groups and promoted repulsion effects leading to the reorganization of the polymeric matrix (Lemieux et al., 2010). Smoother surfaces were observed Fig. 6C, D. in case of CMAESt (TS) and of AECMSt (TS) which might be due to the higher DS of CM (Sakeer et al., 2017b).

3.7. Micromeritic properties of starch derivatives powders

The flow properties of the powder formulations are important parameters from industrial perspective because improper flowability may lead to problems in storage of excipients, handling and processing operations. The micromeritic properties were assessed by determining

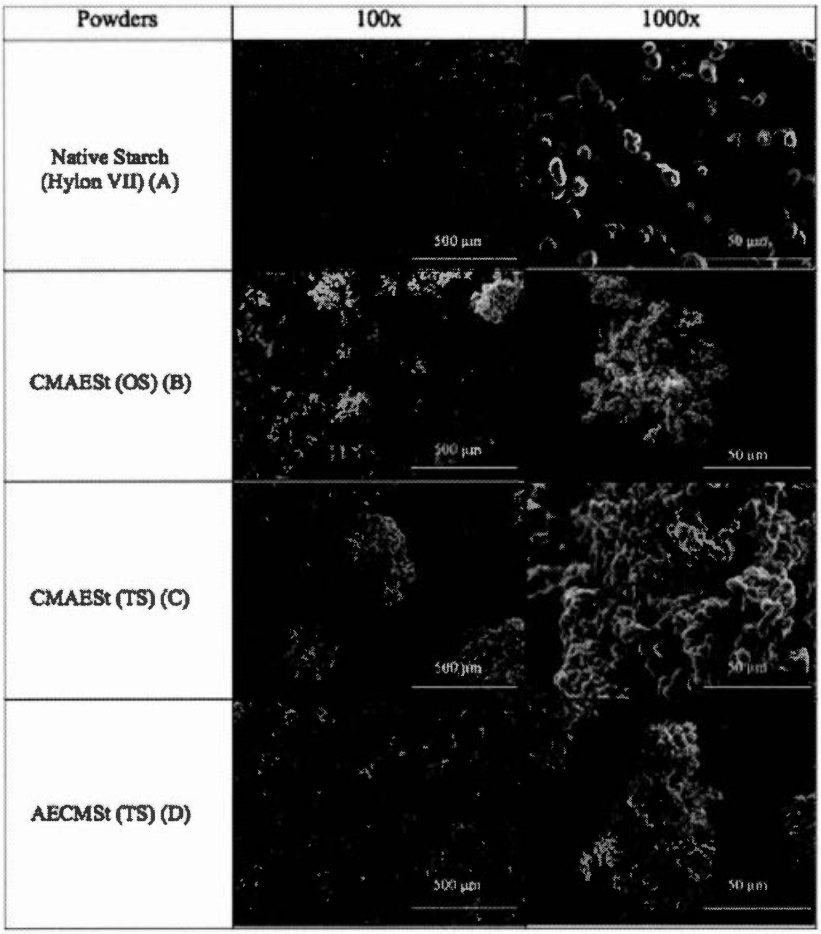


Fig. 6. Scanning electron microscopy micrographs of Native Starch (A), CMAESt (OS) (B), CMAESt (TS) (C) and AECMSt (TS) (D) powders at magnifications of 100x and 1000x.

Table 2
Micromeritic properties of starch derivatives powders.

	θ (°)	CI (%)	HR
CMAESt (OS)	28.30	28	1.6
CMAESt (TS)	33.02	26	1.35
AECMSt (TS)	27.92	17	1.2

angle of repose (Θ), Carr's index (CI) and Hausner's ratio (HR) and the results are summarized in Table 2.

The angles of repose for all the obtained starch derivatives powders were found to be in the range of 27–38 °, whereas CI and HR were in the range of 17–38% and 1.2–1.6, respectively. The angle of repose less than 30° indicates excellent flow properties, whereas a value of 56° indicates 'very poor' flow. Particles with high internal friction or cohesion present an increased value of angle of repose. Moreover, CI and HR should be less than 20% and 1.20, respectively, to ensure optimal flowability for powders (Singh et al., 2013). AECMSt (TS) showed the lowest angle of repose (27°), Carr's index (17%) and Hausner's ratio (1.2) providing the best micromeritics properties among all derivatives.

3.8. Determination of the fluid uptake and erosion

Fluid uptake and erosion tests in SGF and in SIF separately, are shown in Fig. 7I, II. It was noticed that AECMSt (TS) has a better ability to absorb SGF fluid and generates a higher swelling compared to CMAESt (OS) and CMAESt (TS). In SIF, CMAEST (OS) showed higher fluid uptake and hence maintain the integrity of the tablet. On contrary, AECMSt (TS) tablets were totally solubilized after 6 h. The erosion of tablets formulated with AECMSt (TS) in SGF was lower than that of CMAESt (OS) and of CMAESt (TS). On other hand, in SIF, the solubility of CMAESt (OS) was limited and thus its erosion was slower than that of CMAESt (TS) and of AECMSt (TS). The radial swelling (dimension changes, mm) of tablets was also observed in SGF and SIF (Fig. 7III, IV). It appeared that CMAESt (OS) and CMAESt (TS) have almost same swelling in SGF. On contrary, AECMSt (TS) showed the lower swelling in SIF, due to its simultaneous higher solubility in SIF. Fig. 8I–III presents the fluid uptake, erosion and diameter changes of tablets containing only the ampholytic starch derivatives (excipient-free) first incubated 2 h in SGF and then moved to SIF. The CMAESt (OS) showed the highest ability of fluid uptake (Fig. 8I) with lower erosion pattern (Fig. 8II) due to its limited solubility in SGF and in SIF. Differently, AECMSt (TS) showed the lower tendency to swell and higher erosion

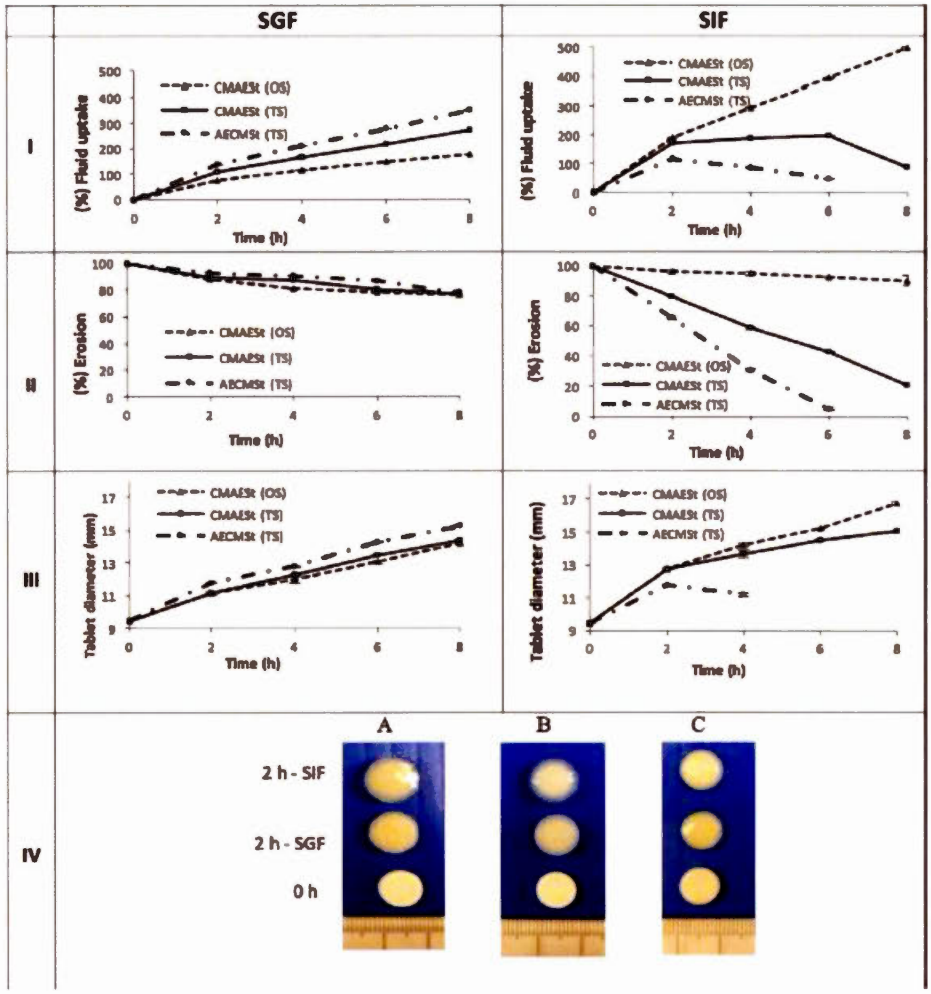


Fig. 7. Fluid uptake (I), erosion (II) and radial tablet swelling (diameter changes) (III) of drug-free tablets formulated with CMAESt (OS), CMAESt (TS) and AECMSt (TS) as matrix forming excipients in SGF or in SIF. (IV) Photographs of tablets based on CMAESt (OS) (A), CMAESt (TS) (B) and AECMSt (TS) (C) at time zero (0 h, dry phase), after 2 h incubation in SGF and after 2 h in SIF.

behaviour due to its solubility in SIF. The highest fluid uptake and limited erosion for CMAEST (OS) was in accordance with the highest dimension changes (Fig. 8III). CMAESt (OS) presented limited erosion in SGF but when moved to SIF a gel layer was formed on the outer surface of the tablet. Differently, the CMAESt (TS) tablets formed a compact gel in SGF which contributed to keep the integrity of the tablets and prevented further fluid penetration and dissolution of the hydrated gel layer formed on the outer surface of the tablet (Fig. 8, IV) when the tablet moved to SIF. The fluid uptake and erosion reflected well the supposed structures of the ampholytic matrices in function of the processing of derivatization. For instance, the better absorption of SGF by AECMST (TS) suggests a preferential carboxymethylation (step II) of the AE-groups already grafted in step I to give AECM or AE$(CM)_2$ groups, rather than of initial hydroxylic groups of starch. Similarly, for the higher substituted AECMSt (TS), the high SIF uptake can be explained by the external location of carboxylic groups in structures type ACEM and AE$(CM)_2$, in detriment of amine groups (which would generate immiscibility in SIF). Thus, for this ampholytic derivative, due to carboxymethylation (step II), there are less primary amine groups but more carboxylic groups, when compared with the CMAEST (OS) and with the CMAEST (TS). This fits the higher reactivity of primary amine groups than that of hydroxylic groups in nucleophilic substitution type SN1. Furthermore, it is also expected, during the first derivatization with chloroethyl amine, to have a continuation of substitution at level of primary amine groups to generate secondary and tertiary aminations, with a pattern of limited dendrimer which may be controlled by the ratio of chloroethylamine: starch. Moreover, irrespective of the type of amination, each graft will present primary amine groups that are susceptible to the first carboxymethylation step. Details of this process will be dealt with in another report.

3.9. *In vitro dissolution tests*

Ampholytic starch excipients were tested in terms of controlled drug

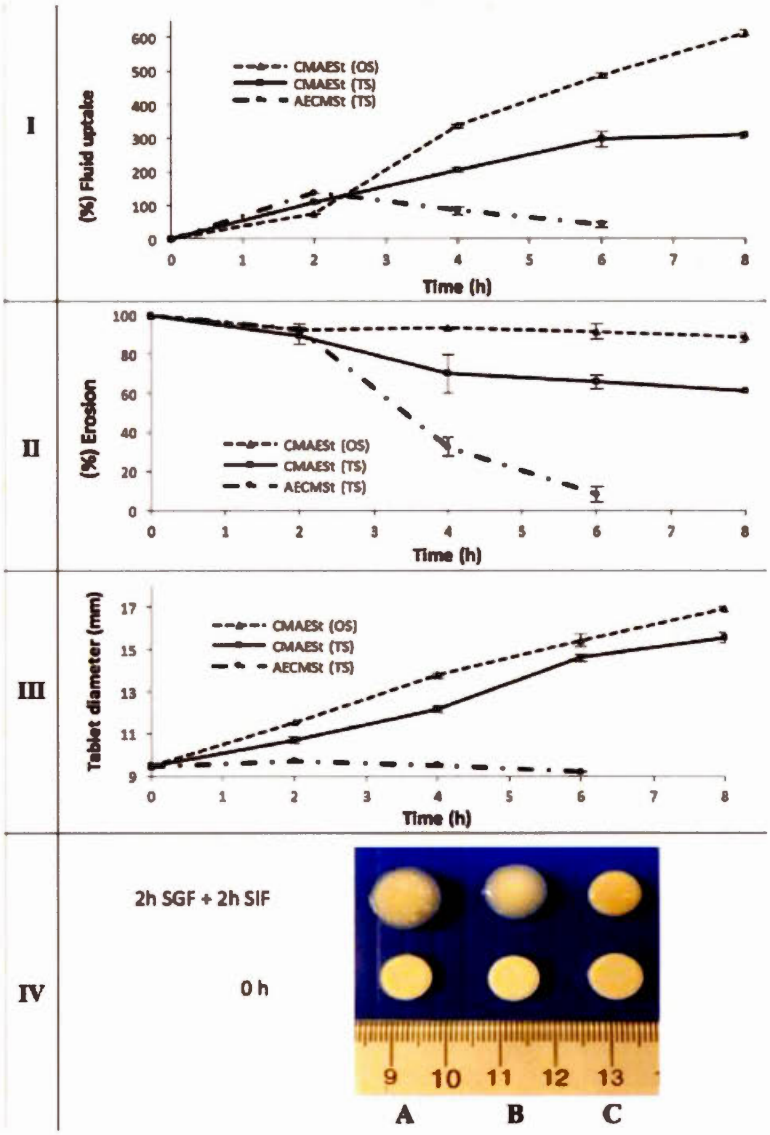


Fig. 8. Fluid uptake (I), erosion (II) and radial tablet swelling (III) of a drug-free tablet formulated with CMAESt (OS), CMAESt (TS) and AECMSt (TS) as a matrix forming excipients incubated 2 h in SGF and then in SIF for 6 h. (IV) Photographs of tablets formulated with CMAESt (OS) (A), CMAESt (TS) (B) and AECMSt (TS) (C) after incubation for 2 h in SGF and then for another 2 h in SIF.

release from high loaded formulations. Monolithic tablets with various drug loading (60%) were prepared and the dissolution patterns were first followed in SGF and SIF separately (Fig. 9). To find out the effect of drug properties on the behavior of ampholytic starch excipients, four tracers with different characteristics: ASA (acidic drug), metformin (basic drug), mesalamine (amphoteric drug), or acetaminophen (neutral drug) were selected to be formulated by direct compression of mixed powders directly with each ampholytic starch excipients and further evaluated for dissolution behaviour. Aspirin is an acetyl derivative of salicylic acid, with an acid dissociation constant pKa 3.5 at 25 °C (Dressman et al., 2012; Suwalsky et al., 2013). Metformin (Table 3) has acid dissociation constant values pKa of 2.8 and 11.5 and exists very largely as cationic species (hydrophilic chlorohydrate) at physiological pH values. The metformin pKa values make metformin a stronger base than most other basic drugs (Desai et al., 2014). Mesalamine (5-ASA) exhibits amphoteric properties (Table 3; its solubility increases at acidic pH values (pH < 3) in the stomach and at more alkaline values (pH > 5.5) in the lower part of the small intestine (French and Mauger, 1993). Acetaminophen, an extensively conjugated system, consists of a benzene ring substituted by one hydroxyl group and an amide group in the para position (1,4). The conjugation greatly reduces the basicity of the nitrogen, while making the hydroxyl acidic through delocalisation of charge developed on the phenoxide anion (Kääriäinen et al., 2017) (Table 3). The cumulative ASA release from

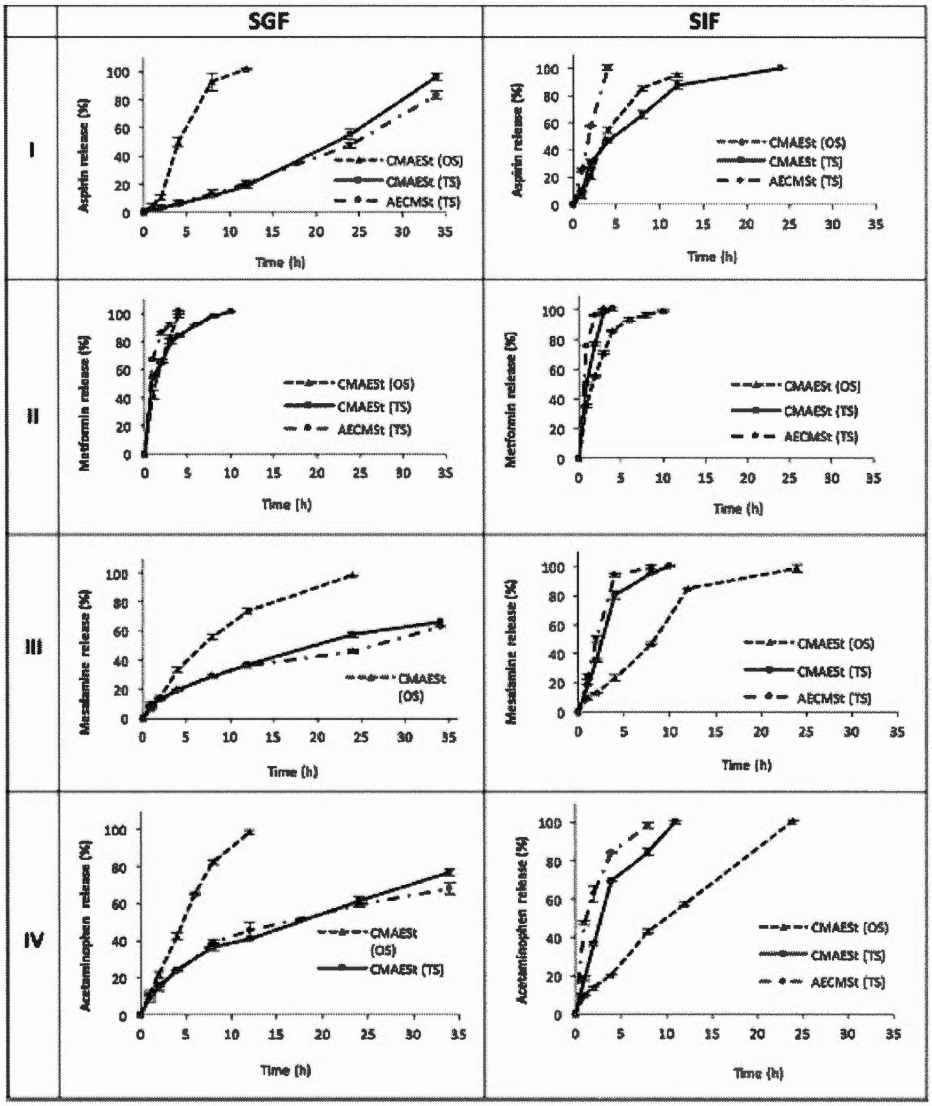
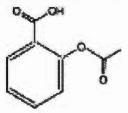
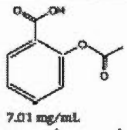
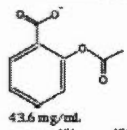
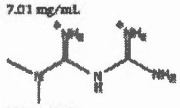
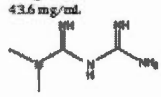
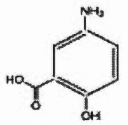
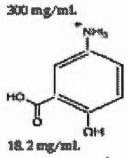
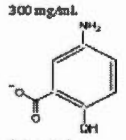
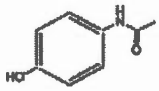
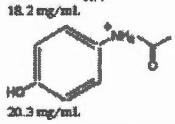
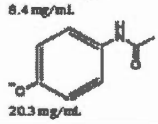


Fig. 9. Release profiles of Aspirin (I), Metformin (II), Mesalamine (III) and Acetaminophen (IV) in SGF or in SIF from tablets (60% loading) formulated with CMAESt (OS), CMAESt (TS) and AECMSt (TS) as matrix forming polymers.

tablets in SGF and in SIF are shown in Fig. 9, I. Both CMAESt (TS) and AECMSt (TS) derivatives were able to control the release of ASA in SGF, where 55%, of ASA was released within 24 h by tablets of CMAESt (TS) and 47% of ASA released by AECMSt (TS). In SIF only CMAESt (TS) was able to prolong the release of ASA for more than 12 h. The Fig. 9, II shows the release of metformin in SGF and in SIF.

Controlling the release of metformin is still a challenge for formulator due to its high solubility and aqueous solution pH 1.2–6.8 (Desai et al., 2014). Because of its short half-life (< 3 h), frequent administrations of high dosages (up to 2.5 g daily) are needed to maintain its required plasma concentration (Garber et al., 1997; Stepensky et al., 2001; Qin et al., 2014). It was found with our ampholytic starch that 92% of metformin was released within 6 h in SGF from tablets contained CMAESt (TS) as matrix forming excipients, whereas 93% was liberated within 6 h SIF from CMAESt (OS). The release of mesalamine in SGF and SIF is presented in Fig. 9, III. In SGF, all ampholytic starch polymers were able to prolong the release of mesalamine over 12 h. In SIF only CMAESt (OS) and CMAESt (TS) prolonged the release of mesalamine to 10 h. Fig. 9, IV shows the release of acetaminophen in both SGF and SIF. In SGF, CMAESt (TS) and AECMSt (TS) sustained the release of acetaminophen with 61.69% liberated from tablets of CMAESt (TS) and 59.3% acetaminophen liberated from tablets of AECMSt (TS) within 24 h. In SIF CMAESt (OS) released 57.75% of acetaminophen within 12 h, while CMAESt (TS) released 84.5% of acetaminophen

Table 3
Structures of bioactive molecules in neutral, SGF, and SIF media.

	Structure	SGF	SIF
Aspirin			
Solubility		7.01 mg/mL	43.6 mg/mL
Metformin			
Solubility		300 mg/mL	300 mg/mL
Mesalamine			
Solubility		18.2 mg/mL	8.4 mg/mL
Acetaminophen			
Solubility		20.3 mg/mL	20.3 mg/mL

within 8 h. In conclusion CMAESt (OS) was able to control the release of amphoteric or neutral drugs only in SIF. From the polymers prepared in two steps procedure either CMAESt (TS) or AECMSt (TS) was able to sustain the release of acidic, amphoteric, or neutral drugs in SGF. Differently in SIF, CMAESt (TS) were able to better control the release of medication in comparison with AECMSt (TS). Furthermore, to find out the impact of whole gastrointestinal tract (GIT) on the polymeric matrices, the tablets were first exposed to SGF for 2 h and then transferred to SIF at the same condition of dissolution test Fig. 10. The release profiles showed that all ampholytic starch excipients were able to control the release of acidic, basic and neutral drugs at high loading (60%). In case of ASA and acetaminophen, all ampholytic starch derivatives showed similar behaviour in controlling of the release of active principles (Fig. 10, Table 4). For metformin only the CMAESt (TS) was able to slower the release for a duration up to 6 h. The ability of CMAESt (TS) to prolong the release of metformin could be correlated with the higher DS of CM groups, 0.041 compared with DS 0.024 for CM groups in CMAESt (OS) and DS 0.028 of AECMSt (TS). Another explanation may be the formation of a kind of internal stabilization between carboxylic groups and the amine groups of ampholytic matrix. This is less probable in term of AECMST (TS), where carboxyl groups are probably lower in peripheral layer and less accessible to form complex. Evaluation of the release profile of metformin from tablets loaded on CMAESt (TS) prepared at different DS of CM or AE groups recently published (Sakeer et al., 2017b), showed that CMAESt (TS) at higher DS of both CM and AE can modulate the release of metformin up to 12 h (Sakeer et al., 2017b). In case of Mesalamine, CMAESt (OS) exhibits less variability with media changes compared with the other two derivatives synthesized via TS procedure. Knowing that Mesalamine is a zwitterion molecule carrying amino and carboxylic groups (Table 3) that will be ionized in SGF and SIF and potential drug-polymer ionic interactions can occur either with CM or AE ionized groups.

This could explain similar release profiles irrespective to the media changes (Fig. 10, Table 4). Same rationale seems not be applicable for CMAESt (TS) and AECMSt (TS) derivatives obtained by the two steps procedure. It is worth to note that both TS derivatives were more substituted (one for CM and the other for AE) compared with OS derivative where the DS for the two groups were close each other and this is reflected in higher Zeta potential values. Less control observed when tablets were exposed to SIF only (6 h and respectively 3 h) could be explained by repulsive forces between mesalamine and charged derivatives. The 90% drug release times were in line with the solubility data of the active principles. For instance the shortest release time was found for metformin which presents the highest solubility (Table 3), whereas long release times (28–30 h) were found for mesalamine and aspirin which both present relatively low solubility. For acetaminophen, intermediate release time 16–22 h fitted well its solubility in gastric and in intestinal fluids. Although relatively fast in comparison with the release of acetaminophen, aspirin and mesalamine, the release time of 5.5 h for metformin seems adequate for this medication to be liberated in the intestinal tract including the colon from monolithic tablets obtained with CMAESt (TS). The release kinetics of the four tracers in SGF and in SIF was evaluated based on the equations presented in Table 1 S, where Q_t in equations A–C and E is the amount of the drug released at time t, Q_t in equation D is the amount of drug remaining in pharmaceutical dosage form at time t and Q_0 is the initial amount of drug in the pharmaceutical dosage forms. The K_0, K_1, K_H, K_S, K_P are respectively the zero-order, first-order, Higuchi's, Hixson–Crowell's, Korsmeyer–Peppas release rate constants and n is the release exponent. If the n value is 0.5 or less, the release mechanism follows a Fickian diffusion, whereas at higher values for mass transfer in the range $0.5 < n < 1$, the system will follow a non-Fickian model (anomalous transport). The system follows a zero-order drug release and a case-II transport if the n value is equal to 1. Here the relaxation process of the macromolecules occurring upon water imbibition into the system is the rate- controlling step. For the values of n higher than 1, the mechanism of drug release is regarded as super case-II transport (Korsmeyer et al., 1983).

The kinetic release profiles of ASA in SGF and in SIF for all polymers was fitting zero order model and first order model respectively, with the Korsmeyer's release exponent $n > 0.5$ which can be related to case-II transport Table 1 S. Due to the high loading (60%) and solubility of metformin, the proposed polymeric excipients were not able to prolong the release of metformin for a duration longer than the commercial products (Sakeer et al., 2017b).

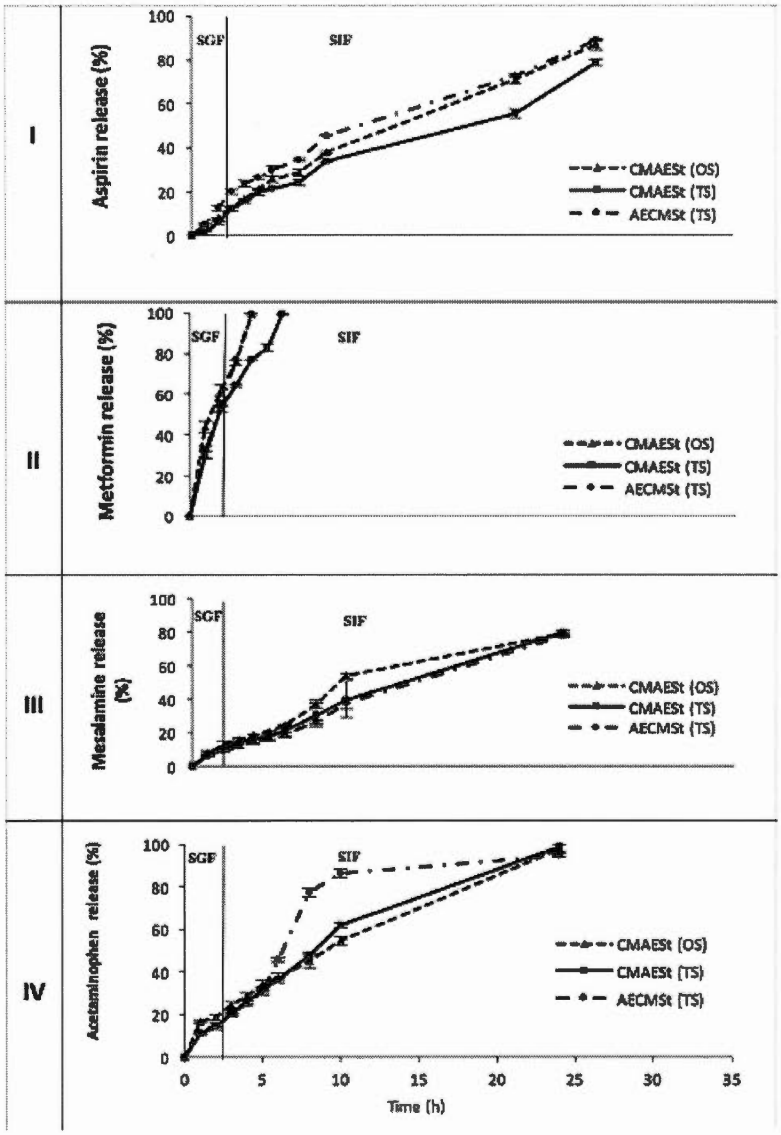


Fig. 10. Release profiles of Aspirin (I), Metformin (II), Mesalamine (III) and Acetaminophen (IV) from tablet (60% loading) formulated with CMAESt (OS), CMAESt (TS) and AECMSt (TS) as matrix forming polymers followed for 2 h in SGF and then transferred to SIF.

Table 4
Time (h) of 90% drug release from monolithic tablet contained amphiphilic starch derivatives as matrix forming agents in SGF, SIF and SGF followed by SIF.

Active molecule	CMAESt (OS)			CMAESt (TS)			AECMSt (TS)		
	SGF	SIF	SGF + SIF	SGF	SIF	SGF + SIF	SGF	SIF	SGF + SIF
Aspirin	8 h	12 h	30 h	33 h	17 h	33 h	> 35 h	3.8 h	30 h
Metformin	3.5 h	8 h	3.5 h	5.8 h	2 h	5.5 h	3 h	1.5 h	3.5 h
Mesalamine	19.5 h	20 h	28 h	> 35 h (60%)	6 h	28 h	> 35 h (60%)	3 h	28 h
Acetaminophen	10 h	21 h	22 h	> 35 h (70%)	10 h	22 h	> 35 h (70%)	7 h	16 h

Table 5
Kinetic parameters of Aspirin (i), Metformin (ii), Mesalamine (iii), and acetaminophen (iv) from tablets (60% loading) formulated with CMAESt (OS) (I), CMAESt (TS) (II) and AECMSt (TS) (III) as a matrix forming excipients in SGF then transferred to SIF.

Dosage forms		Kinetic analysis										
		A		B		C		D		E		
		K_0	r^2	K_1	r^2	K_H	r^2	Ks	r^2	n	KP	r^2
I	i	0.06	0.993	-0.008	0.990	1.69	0.96	0.004	0.992	0.95	0.14	0.97
	ii	-	-	-	-	-	-	-	-	-	-	-
	iii	0.05	0.93	0.006	0.992	0.018	0.993	0.002	0.96	0.85	0.60	0.98
	iv	0.05	0.990	-0.001	0.98	1.9	0.96	0.002	0.98	0.59	1.81	0.95
II	i	0.078	0.989	-0.009	0.993	1.9	0.995	0.006	0.997	1.15	0.5	0.96
	ii	0.21	0.95	-0.005	0.99	5.5	0.98	0.005	0.90	0.55	5.8	0.95
	iii	0.053	0.993	-0.006	0.979	1.28	0.98	0.002	0.93	0.79	0.65	0.98
	iv	0.06	0.95	-0.002	0.990	2.3	0.96	0.003	0.992	0.81	0.58	0.96
III	i	0.044	0.97	-0.009	0.95	2.2	0.98	0.005	0.89	0.79	0.78	0.97
	ii	-	-	-	-	-	-	-	-	-	-	-
	iii	0.052	0.994	-0.005	0.997	1.14	0.997	0.002	0.96	0.79	0.41	0.97
	iv	0.06	0.71	-0.001	0.885	2.7	0.86	0.004	0.97	0.98	1.13	0.98

The kinetic equation was applied only for CMAESt (OS) in SIF and for CMAESt (TS) in SGF. In both cases the drug release was fitting the first order model with the Korsmeyer's release exponent n close to 0.5 suggesting that the release is matching non-Fickian model (anomalous transport). The release of mesalamine was following different models in different dissolution media. The Korsmeyer's release exponent n was close to 0.5 in SGF indicating that the system fits well to Fickian diffusion. In SIF, n values were close to 1 (Table 1S) matching to non-Fickian model (anomalous transport). CMAESt (TS) was able to control the release of acetaminophen in SGF and in SIF and the release was fitting first order model in both media. CMAESt (OS) was able to prolong the release of acetaminophen only in SIF. Differently, AECMSt (TS) sustained the release of acetaminophen in SGF. The value of release exponent n was greater than 0.5 fitting non-Fickian models (anomalous transport). When the dissolution was conducted in simulating GIT transit (SGF for 2 h then followed by SIF), the ASA showed different release kinetics, with n between 0.5–1 (Table 5) indicating a non-Fickian model (anomalous transport). Metformin and mesalamine followed first order kinetics and zero order with n between 0.5–1 indicating anomalous transport which means that both swelling and diffusion are going together (Table 5). Acetaminophen followed a zero order for CMAEST (OS) and Hixson–Crowell's order for both CMAESt (TS) and AECMSt (TS) with between 0.5–1 indicating to anomalous transport of active ingredient. Therefore, interesting to note is that using the same reagents and changing only the order of their addition in the reaction, produced compounds with different properties. Thus, the ampholytic starch from one step CMAESt (OS) was able to control the release of acidic, amphoteric and neutral medication in SIF, whereas the polymers obtained from two steps method CMAESt (TS) and AECMSt (TS) were able to sustain the release of same tracers also in SGF. When the dissolution was done first in SGF then followed by SIF, all ampholytic starch showed an ability to prolong the release of high loading medications (60%) for up to 24 h. Self-stabilization of ampholytic starch CMAESt (OS) could be explained by the limited solubility of the polymer in SGF. The carboxylic groups are compacted following modulation protonation and amino groups may form stable macromolecular gels, lowering the solubility. In SIF the higher swelling ability and lower chain flexibility (as supported by viscosity measurement) and limited solubility of CMAEST (OS) are key factors of controlling the release of medications. Moreover, a polyelectrolyte complexation between the anionic CM- and cationic AE- groups located on neighboring chains, may also contribute to CMAESt (OS) gel formation and stabilization. On other hand, self-stabilization of ampholytic starch CMAESt (TS) could be explained by protonation of CM groups and generating an outer gel layer by hydration of AE groups in SGF. In SIF, the compacted gel layer generated in SGF alongside with limited solubility of AE, forming a barrier toward further penetration of intestinal fluid inside the gel layer, lowered the erosion and solubilization of outer gelled layer surface. Consequently, the tablet integrity is maintained prolonging the release of the active ingredients from the dosage form. Another aspect which could contribute to starch ampholytic derivatives behavior is the repartition of ionic charges on the starch backbone which would be more uniform in case of OS synthesis compared with TS procedure. It should be noticed that for TS an intermediate precipitation of the first derivative was done prior to introduction of the second functional group. This might influence the arrangements of chains which will be stabilized by self-assembling (polymer-polymer ionic interactions). This may impact the surface exposure for further interactions (introduction of the second ionic group, polymer-drug interactions, etc).

4. Conclusion

The presence of the two groups on the same macromolecular backbone will generate a beneficial effect for the usage of such ampholytic starch polymers as excipients for the challenging formulation of drugs with high loading affording the controlled release of APIs during the transit along the gastrointestinal tract. The proposed new starch derivatives offer a wide range of applications where the polymer-polymer or polymer-drug self-assembling via electrostatic interactions are able to control the drug release at high loading of actives molecules, even for particularly challenging APIs as mesalamine and as metformin. Further studies are going on CMAESt (OS), at different DS of CM or AE groups in order to determine the position of CM, AE, EACM or EADCM on starch backbone chain and for elucidation of contribution of starch morphological aspects.

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Appendix A. Supplementary data

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A.2. **Oral presentations:**

Self-stabilizing ampholytic starch excipients for sustained release of highly soluble drugs: the case of metformin. 19th Annual CBGRC, November 18th, 2016, Concordia University. Montreal, Quebec, Canada. 19th Annual CBGRC. November 18th, 2016. Concordia University. Canada.

Abstract: Self-stabilizing ampholytic starch excipients for sustained release of highly soluble drugs: The case of metformin

Khalil Sakeer*, Pompilia Ispas-Szabo, Mircea Alexandru Mateescu

The novel Ampholytic CarboxyMethyl-AminoEthyl-starch (CM-AE-St) polymer was synthesized first by starch treatment with sodium monochloroacetate to obtain CM-St and then by treatment with 2-chloroethylamine hydrochloride in order to introduce AminoEthyl (AE) groups. The CM-AE-St was characterized by physical methods, FT-IR, 1H NMR, SEM, TG, X-ray diffraction and by in vitro drug dissolution. Higher DS for both CM- and AE- groups favor the ability of ampholytic CM-AE-St to control the drugs release of high soluble drugs such as Metformin followed in simulated gastric fluid (SGF) and in simulated intestinal fluid (SIF). For instance, monolithic tablets with CM-AE-St (DS 0.06 for CM and 0.059 for AE groups) and with metformin loading 50% similar shape and weight to the commercial Glumetza®, were able to prolong liberation of active drugs up to 10h, exhibiting a similar dissolution profile to that of commercial product. The presence of both (cationic and anionic) functional groups generates a self-stabilized polymer with improved ability to control the drug delivery. The release profiles show a good fit to Higuchi's model ($r2 > 0.99$). The release pattern obtained with the ampholytic starch excipient seems to be in good agreement with the United States Pharmacopeia (USP) requirements for metformin controlled release tablets.

A.3. **Extended abstract:** New class of self-stabilizing ampholytic starch as excipients for drug sustained release. Control Release Society annual meeting, July 16-19, 2017. Boston, Massachusetts, USA

Deadline Reached

The deadlines for presentation submissions and modifications for this program have been reached.
View Submission (no changes allowed.)

Presentation #3669

New class of self-stabilizing ampholytic starch as excipients for drug sustained release

Khaleel Sakeer, Pompilia ispas-Szabo and M.Alexandru Mateescu. UQAM. montreal. QC. CANADA

Abstract Text:

New class of self-stabilizing ampholytic starch as excipients for drug sustained release

Khalil Sakeer[1], Pompilia Ispas-Szabo[1], Mircea Alexandru Mateescu[1]

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Introduction: There is a growing interest for sustained drug release of high loaded dosage forms. To respond to these challenging formulations, an adequate carrier should be able to keep the tablet size small enough to allow ease swallowing alongside with a simple method of tablets preparation. In this context we are proposing a new class of excipients with self-assembling properties which can generate stable structures and ensure a sustained drug delivery. Starch was functionalized with ionic (CarboxyMethyl CM) and cationic (AminoEthyl AE) groups and new ampholytic properties were obtained.

Methods: Two series of new ampholytic starch derivatives were synthesized in a) one step (OS) and b) two steps (TS) process. One step involves simultaneous grafting of anionic and cationic groups while in the two steps procedure the CM groups were grafted first on starch chains followed by functionalization of AE groups. The final product: CarboxyMethyl-AminoEthyl-Starch (CM-AE-St) could be obtained at different degrees of substitution by varying the number of CM and AE groups. Metformin, Mesalamine, Aspirin and Acetaminophen were selected as model drugs. Monolithic tablets with 50% and 60% loading were prepared by direct compression of the active molecule with various CM-AE-St derivatives.

Results: The *in vitro* drug dissolution tests showed that higher degrees of substitution for both CM- and AE- groups favor the ability of ampholytic CM-AE-St to control the drug release in simulated gastric fluid (SGF) and in simulated intestinal fluid (SIF). Metformin release was in compliance with USP requirements. Its dissolution was controlled for 12 h exhibiting a similar Higuchi's model release profile with the commercial product Glumetza®. For the other three active agents at 60% loading the CM-AE-St OS generated a matrix able to provide a sustained control in SIF due to predominance of amino groups having a decreased solubility. On other hand CM-AE-St TS was able to provide an increased protection in SGF. Structural studies (FT-IR, 1H NMR, SEM, TG, X-ray diffraction) confirm the starch derivatization and self-stabilization phenomena.

Conclusion: The presence of the two ionic groups on the same macromolecular backbone will generate a beneficial effect for the usage of such polymer as carrier for the challenging formulation i.e. highly soluble or charge drugs helping to control the drug release during the transit along the gastrointestinal tract.

Learning Objectives

1. Develop a new self-stabilized pharmaceutical excipients derived from starch
2. Formulating challenging active agents as monolithic tablet with CM-AE-St excipient
3. Explaining the phenomena standing behind the concept of ampholytic starch

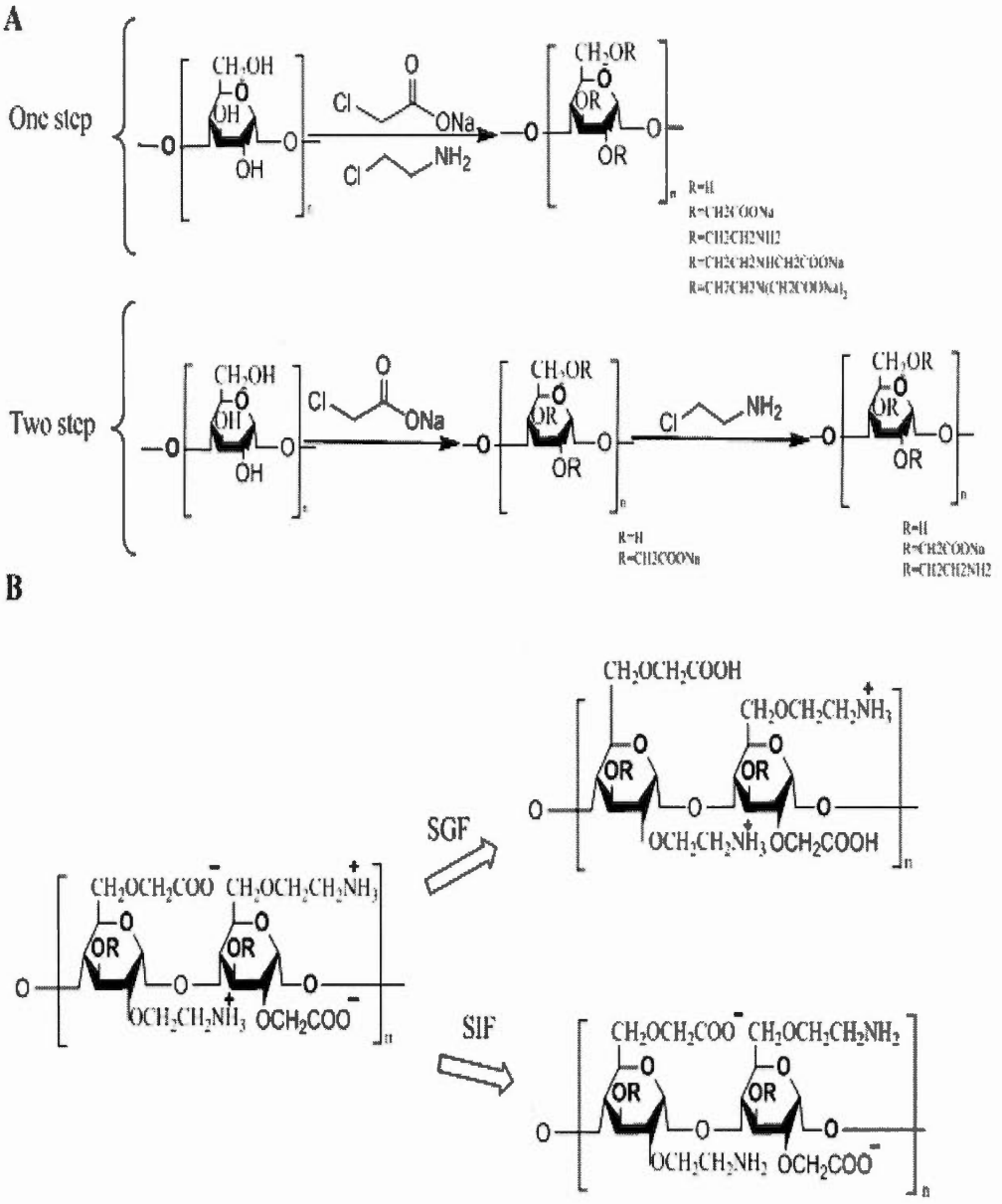


Fig. 1. Hypothetical presentation of starch derivatization (A) with sodium monochloroacetate and with chloroethylamine hydrochloride to generate ampholytic starch carrying carboxymethyl (CM) and aminoethyl (AE) functions by different methods of preparation. Hypothetical structure (B) of ampholytic starch in simulated gastric (SGF) and intestinal (SIF) fluid.

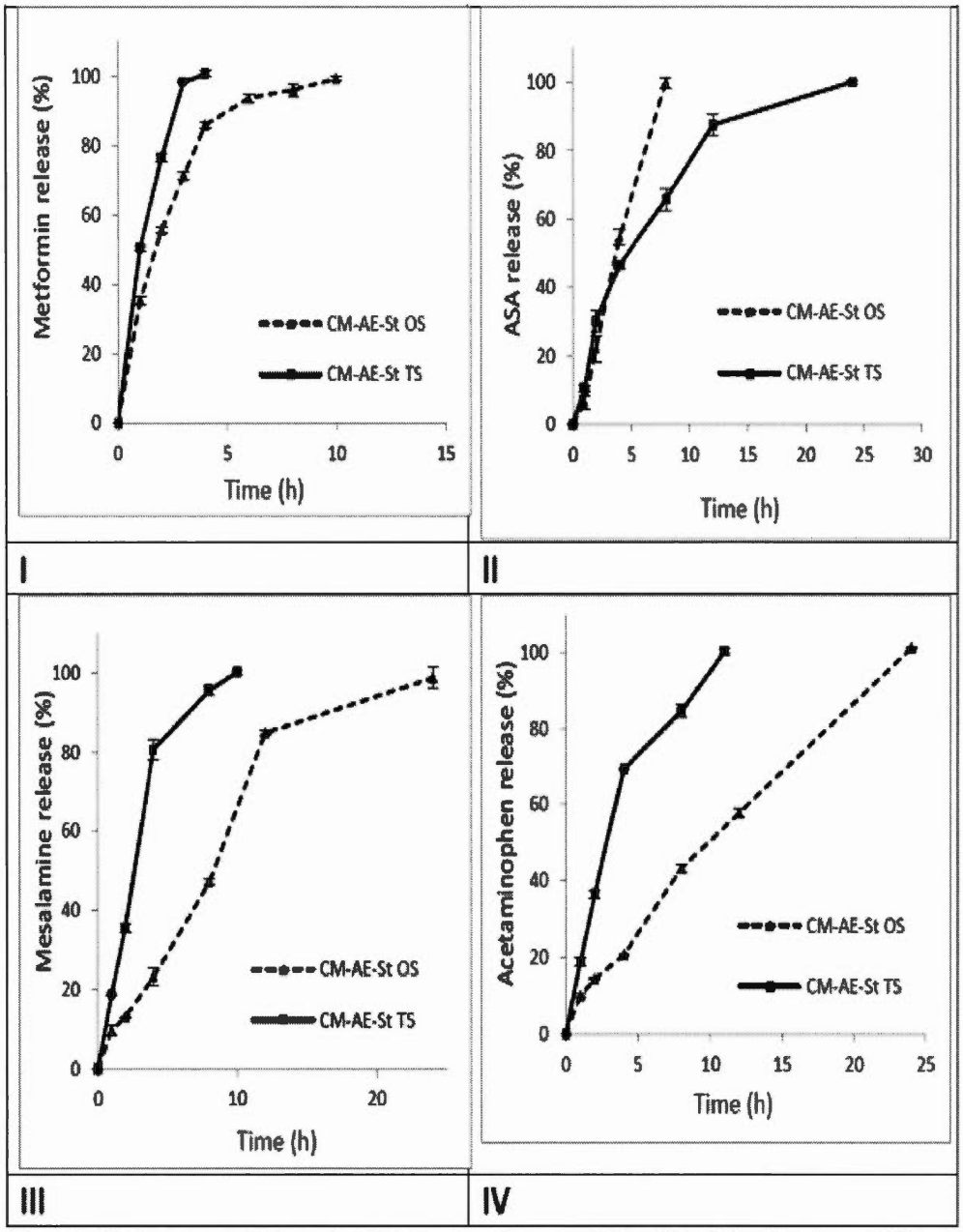
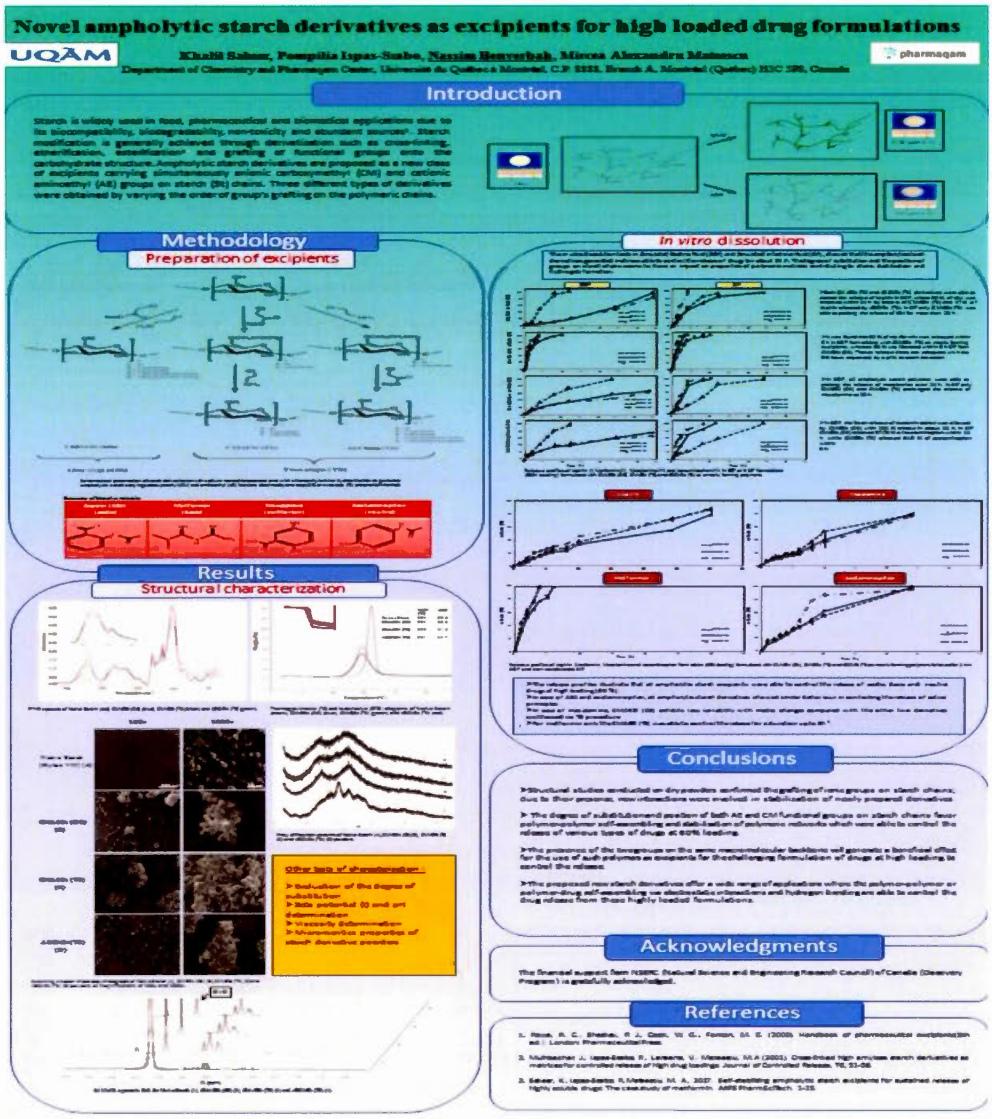


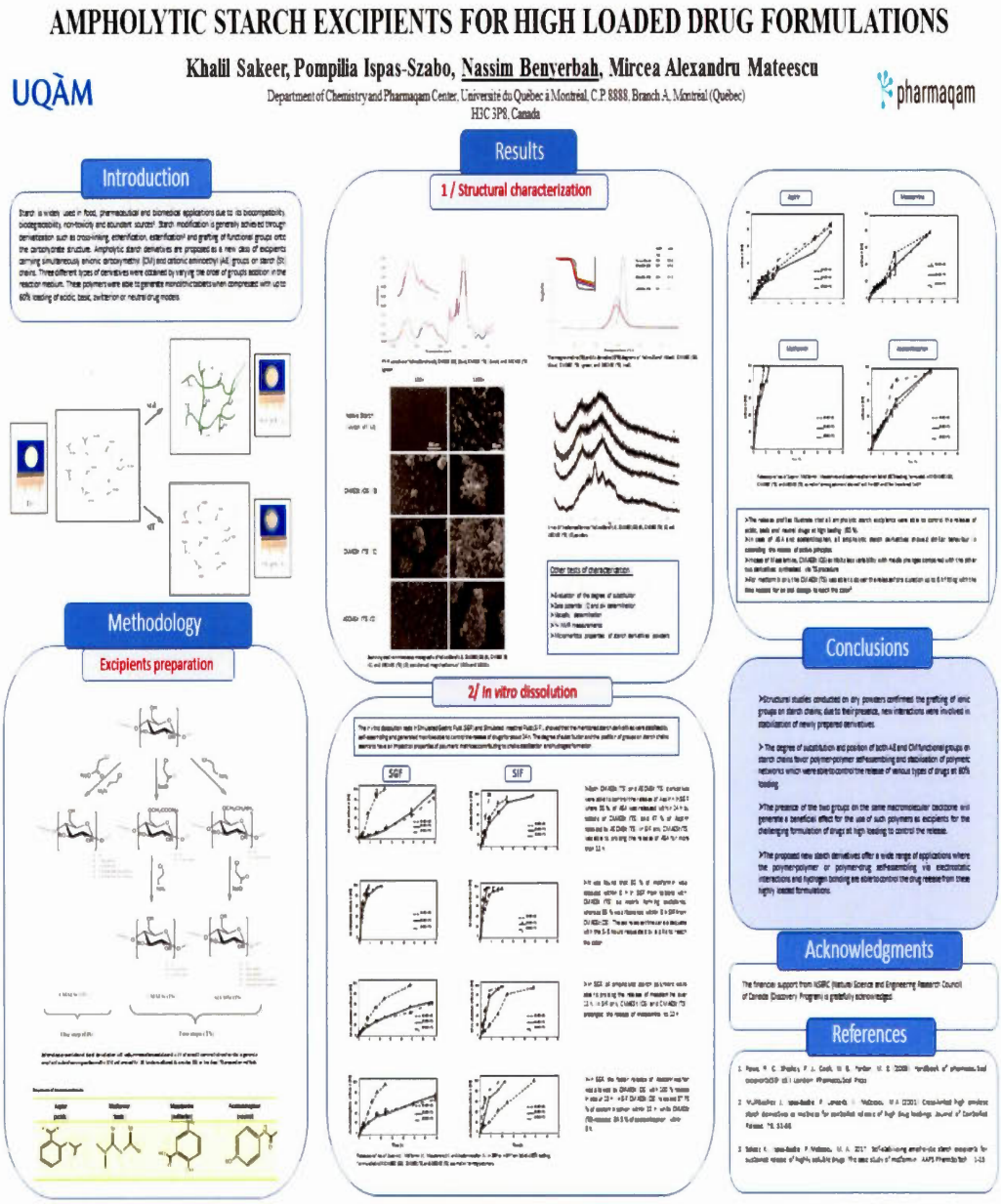
Fig. 2. Release profiles of Metformin (I), Aspirin (II), Mesalamine (III) and Acetaminophen (IV) from tablet (60% loading) formulated with CM-AE-St (OS) and CM-AE-St (TS) as a matrix forming agents in SIF.

A.4. Poster presentations

A.4.1. Ampholytic starch excipients for high loaded drug formulations. 7e Colloque Pharmaquam (Université du Québec à Montréal). 31 August, 2017. Montréal, Québec, Canada.



A.4.2. Novel ampholytic starch derivatives as excipients for high loaded drug formulations. 7e Colloque RQRM (Réseau québécois de recherche sur les médicaments). 30 Sept – 1 Oct, 2017. Québec, Canada



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