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THÈSE

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Development and characterization of parenteral *in situ* gelling chitosan/glucose-1-phosphate depot systems for controlled drug release

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A ma famille

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« Jamais savant n'est tombé du ciel. »

« Es ist noch kein Meister vom Himmel gefallen. »

Proverbe alsacien / allemand

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Résumé en français

Développement et caractérisation d'hydrogels formés *in situ* de chitosane / glucose-1-phosphate pour la libération contrôlée de principes actifs

Contexte et objectifs de la thèse

Au cours des dernières décennies, le développement de formulations injectables formant des dépôts *in situ*, « *in situ* forming depot » (ISFD) en anglais, a suscité un intérêt considérable pour diverses applications pharmaceutiques et biomédicales. Ce sont des formulations liquides qui, après injection intramusculaire, sous-cutanée ou intra-tumorale forment un implant solide ou semi-solide *in situ* dans les conditions physiologiques [1,2].

Les ISFDs représentent une alternative intéressante qui permet de remédier aux limites des formes parentérales à libération immédiate (telles que les solutions, suspensions ou émulsions) de par leur capacité à prolonger la libération d'un principe actif sur plusieurs jours voire plusieurs mois tout en maintenant des taux plasmatiques suffisants pour produire l'effet thérapeutique souhaité. Ainsi, ces systèmes permettent de réduire la fréquence d'administration du médicament, assurant une meilleure observance thérapeutique. Comparé aux autres formes dépôts telles que les microsphères ou les implants, les ISFDs offrent l'avantage supplémentaire d'améliorer le confort des patients grâce à une administration plus aisée et peu invasive à travers une aiguille de petit diamètre. Le procédé de fabrication de ces formes galénique est d'une grande simplicité, ce qui permet de réduire les coûts de production. De plus, l'incorporation du principe actif au sein d'une matrice polymère permet de le stabiliser, notamment dans le cas des peptides et des protéines qui sont plus particulièrement sujets à dénaturation dans un milieu physiologique [3].

Un ISFD idéal devrait répondre aux critères suivants : *(i)* être composé d'excipients biocompatibles, biodégradables et approuvés pour un usage pharmaceutique, *(ii)* être suffisamment stable pour assurer une durée de vie acceptable et pouvoir être présenté dans des seringues pré-remplies prêtes à l'emploi, *(iii)* avoir une faible viscosité permettant une préparation aisée, une incorporation homogène du principe actif et une injection aisée à travers de fines aiguilles, *(iv)* présenter un mécanisme de solidification *in situ* suffisamment rapide afin d'éviter un temps de latence entre l'injection de la solution et la formation de

l'implant qui entrainerait une libération brutale du principe actif et une propagation de la solution dans les tissus environnants.

Pour ce travail de thèse, les systèmes à base de chitosane (CS) formant des hydrogels *in situ* à température corporelle ont été choisis parmi les différentes catégories d'ISFDs, pour la biocompatibilité et biodégradabilité reconnue de ce polymère ainsi que pour l'absence, dans leur composition, de solvants organiques et d'initiateurs ou d'agents réticulants toxiques. Après une description de l'état de l'art concernant les différents systèmes thermo-gélifiants à base de CS existants et de leurs utilisations (*Chapitre I*), nous nous sommes intéressés en détail aux mécanismes sur lesquels reposaient la formation des hydrogels de CS / agent gélifiant et plus particulièrement au rôle de la partie polyol de l'agent gélifiant dans ce mécanisme (*Chapitre II*). Dans une troisième partie (*Chapitre III*), nous nous sommes concentrés sur le développement d'un nouveau système associant le CS au glucose-1-phosphate (G1-P), sélectionné en tant qu'agent gélifiant alternatif au glycérophosphate (GP) conventionnellement utilisé dans ce type de formulation. Dans cette même partie, des essais de tolérance locale sous-cutanée des hydrogels de CS / G1-P ont été réalisés sur un modèle murin. Enfin, l'objectif de cette thèse étant de développer ces ISFDs pour la libération contrôlée de principes actifs, la dernière partie (*Chapitre IV*) est consacrée à l'étude de la libération *in vitro* de différents composés modèles incorporés dans le réseau polymère de CS / G1-P.

Chapitre I : Les hydrogels thermosensibles à base de chitosane et leurs applications pharmaceutiques et biomédicales.

Développées il y a une dizaine d'années, les solutions thermo-gélifiantes à base de CS ont fait l'objet de nombreuses études depuis lors, tant pour comprendre leur mécanisme de gélification que pour évaluer leurs potentielles applications dans le domaine pharmaceutique et biomédical. Le CS est un polysaccharide d'origine naturelle, obtenu par désacétylation partielle de la chitine. C'est un hétéro-polymère linéaire composés d'unités de N-acétyl-β-D-glucosamine et de β-D-glucosamine (Figure 1). De par la présence de fonctions amines primaire libres, le CS est un poly-électrolyte cationique (pK_{ap} de l'ordre de 6,2) soluble uniquement en milieu acide. Lorsque le pH devient supérieur au pK_{ap}, le polymère précipite [4,5].

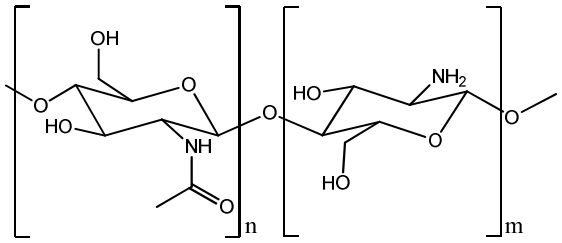


Figure 1: Structure chimique du chitosane

Cependant, Chenite *et al* [6] ont démontré qu'une solution de CS devient thermo-gélifiante à pH physiologique en présence de GP. Le mécanisme de gélification de la solution de CS / GP repose sur la formation d'un réseau tridimensionnel lors de l'augmentation de la température à 37°C, ceci dû aux effets synergiques de *(i*) la réduction progressive des forces de répulsion électrostatique entre les chaines de CS, *(ii*) la destruction de la couche d'eau protectrice structurée autour des molécules de CS par la partie glycérol du GP et *(iii*) la mise en place de forces attractives (interactions hydrophobes et liaisons hydrogènes) inter-polymères.Il apparait que les caractéristiques du CS tels que son degré de désacétylation (DD) et sa masse molaire (Mw), ainsi que le pH du système, le rapport CS : GP et le type d'acide utilisé pour la dissolution du polymère influent sur le processus de gélification et/ou sur la structure de l'hydrogel. Ainsi, le temps et la température de gélification peuvent être ajustés en faisant varier le rapport de CS et GP, tandis que la turbidité du gel est modulable en faisant varier le DD et la distribution des monomères du CS et que la morphologie du gel dépend fortement de la concentration et du Mw du CS ainsi que du type d'acide utilisé. Les études *in vitro* et *in vivo* confirment la biocompatibilité et la biodégradabilité de l'hydrogel dans les conditions physiologiques. Par conséquent, les hydrogels de CS / GP se formant *in situ* paraissent prometteurs tant pour la libération contrôlée de principes actifs que pour l'ingénierie tissulaire. En effet, la libération de principes actifs de faible masse molaire est significativement réduite par leur incorporation dans le système thermo-gélifiant par comparaison avec une solution de CS pure, permettant une libération prolongée par diffusion sur une période de plusieurs jours *in vitro*. Les thermogels de CS ont également été étudiés pour l'administration de peptides ou de protéines, tels que l'insuline ou des anticorps anti-TNFα, de par leur aptitude à prolonger la libération sur plusieurs semaines tout en protégeant les macromolécules des dégradations chimiques ou enzymatiques. Enfin, ces systèmes ont également fait leurs preuves dans le domaine de la thérapie cellulaire et de l'ingénierie tissulaire. Pour ces applications, des cellules souches associées à des facteurs de différenciation et de croissance sont mélangés à la solution thermo-gélifiante qui, une fois

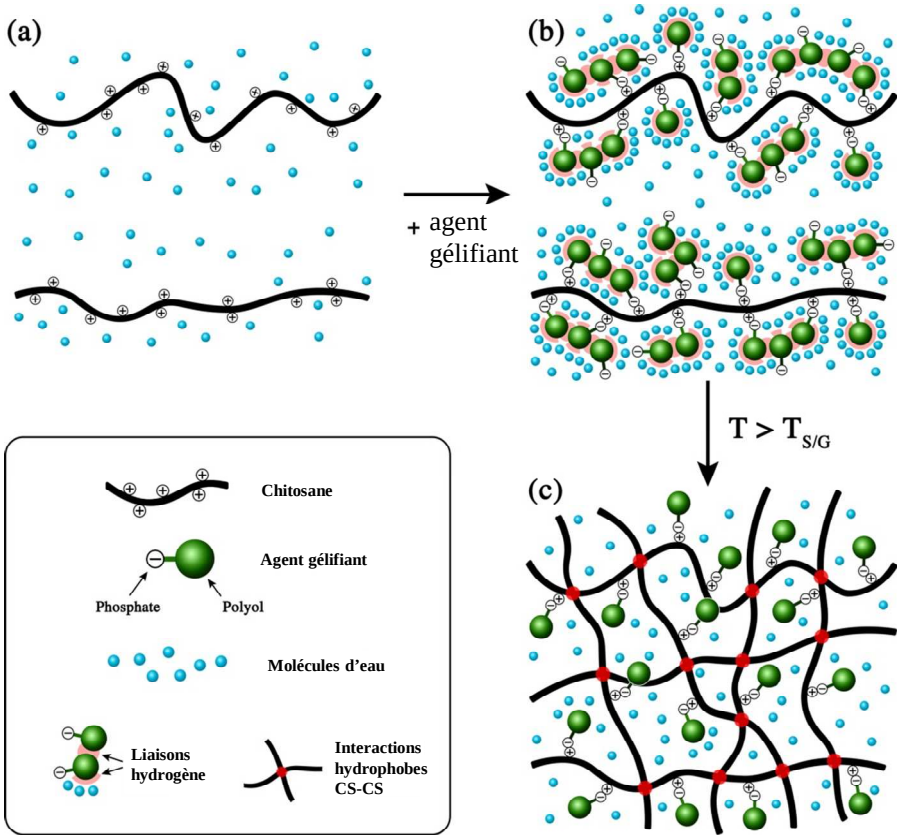
injectée au niveau des tissus endommagés, forme un hydrogel dans lequel les cellules peuvent se différencier et proliférer afin de favoriser la régénération des tissus. Afin de parer aux inconvénients du système conventionnel CS / GP, tels que la libération rapide des composés hydrophiles de faible masse molaire ou le manque de rigidité du gel, différents stratégies ont été mises en œuvre. Ainsi, les cinétiques de libération peuvent être modulées en combinant le système avec des formes vectorisées, telles que des liposomes ou des microsphères, tandis que l'adjonction d'autres polymères (de type gélatine, collagène ou dérivés de cellulose) au système permet d'améliorer les propriétés mécaniques du gel. Enfin, une dernière stratégie mise en œuvre consiste à modifier chimiquement le CS, dans le but d'augmenter sa solubilité ou sa sensibilité au pH ou encore d'améliorer la rigidité des hydrogels. Malgré les efforts déployés pour améliorer les thermogels de CS, il reste à remédier à certaines limites de ces systèmes, notamment le manque de stabilité de la solution à basse température.

<u>Chapitre II</u> : Etude rhéologique des systèmes chitosane / polyol-phosphate : influence de la partie polyol dans le mécanisme de gélification thermo-induite.

Afin de mieux comprendre le mécanisme de formation des hydrogels à base de CS ainsi que le rôle de la partie polyol de l'agent gélifiant, différentes combinaisons de CS / agent gélifiant ont été soumises à une étude détaillée de leur comportement rhéologique. Une étude préliminaire a été réalisée avec différents agents gélifiants potentiels tels que des sels de phosphate inorganiques (Na_2HPO_4, $(NH_4)_2HPO_4$), de l'α-méthoxy-ω-phosphate polyéthylène glycol et des polyols-phosphates (à savoir du β-glycérophosphate (β-GP), du glucose-1-phosphate (G1-P) et du glucose-6-phosphate (G6-P)). Cette étude a permis de démontrer que la présence d'une partie polyol est indispensable dans la structure de l'agent gélifiant afin d'empêcher la précipitation immédiate de la solution de CS à pH physiologique et de rendre le système thermosensible. Les polyols sont connus pour protéger les protéines contre la dégradation thermique en stucturant les molécules d'eau autour de ces dernières de manière à former une couche protectrice. Les résultats des différents tests rhéologiques (balayage en fréquence, en température et en temps) réalisés sur les systèmes CS / polyol-phosphates et CS / Na_2HPO_4, complétés par des mesures spectroscopiques ^{31}P-NMR et des mesures de pH, ont mis en évidence le rôle protecteur de la partie glucose ou glycérol vis-à-vis des chaines de CS. Il est légitime de présumer que, tout comme pour les protéines, cet effet protecteur est dû

à l'effet structurant du polyol sur les molécules d'eau environnantes (au travers de liaisons hydrogènes) à basse température, réduisant ainsi les interactions attractives entre les molécules de CS à $pH > pK_{ap}$ (Schéma 1 (b)). Sous l'effet de l'augmentation de la température, le pK_{ap} du CS diminue, entrainant une réduction progressive des forces de répulsion électrostatique entre les chaines de CS, tandis que l'agitation thermique des molécules du système augmente, réduisant ainsi la cohésion de la couche protectrice polyol / eau. Ainsi, lorsque la température du système est supérieure à la température de gélification ($T_{S/G}$), les forces attractives entre molécules de CS entrainent la formation d'un réseau tridimensionnel par l'intermédiaire d'interactions hydrophobes et de liaisons hydrogènes (Schéma 1 (c)).

Schéma 1: Représentation du mécanisme de gélification des solutions de CS / polyol-phosphate.



L'augmentation de la température de gélification des différents systèmes CS / polyol-phosphate est observée dans l'ordre suivant : $T_{S/G}$ (β-GP) < $T_{S/G}$ (G1-P) < $T_{S/G}$ (G6-P), ce qui indique une augmentation de la stabilité de la couche protectrice polyol / eau lorsque la taille de la partie polyol augmente.

Chapitre III : Le glucose-1-phosphate, un nouvel agent gélifiant pour les systèmes thermo-gélifiants à base de chitosane

Après avoir étudié l'influence de la partie polyol sur le mécanisme de gélification dans le Chapitre II, et au vu des résultats obtenus en utilisant le G1-P en tant qu'agent gélifiant, ce composé a été sélectionné pour poursuivre les recherches dans le but d'optimiser les thermogels à base de CS. Dans un premier temps, les propriétés viscoélastiques du système CS / G1-P ont été déterminées par rhéologie. Les résultats montrent que le temps et la température de gélification varient en fonction du type et de la concentration de CS ainsi que de la concentration en G1-P. Les formulations les plus adéquates pour une application parentérale, c'est-à-dire ayant un $T_{S/G} \sim 37°C$ et formant un gel relativement rigide en quelques minutes à température physiologique, ont ainsi pu être sélectionnées. Le caractère non réversible de la transition sol / gel, observé par rhéologie lors de cycles de chauffage-refroidissement successifs, a été confirmé par analyse DSC et peut être attribué à la persistance d'interactions attractives entre les chaines polymères à basse température. La détermination de la viscosité et de l'injectabilité des solutions de CS / G1-P montrent que ces solutions sont rhéofluidifiantes et que leur injection à travers de fines aiguilles (entre 23 et 30 G) est aisée. La stabilité à long terme de deux solutions de CS / G1-P (composées de 1,5 % de CS et de 0,27 et 0,40 mmol/g G1-P respectivement), d'une solution de CS / β-GP et d'une solution de CS pure, a été évaluée dans des conditions réfrigérées et à température ambiante. Les propriétés physiques évaluées lors de cette étude sont l'apparence, le pH, la viscosité et le temps de gélification à 37°C des solutions ainsi que la masse molaire du CS, déterminée par chromatographie par perméation de gel (GPC). La stabilité de la solution dépend fortement de l'agent gélifiant utilisé et de sa concentration. Ainsi, la solution de CS / β-GP a formé un gel en moins d'une journée à 20 - 25°C et en moins de 30 jours à 2 - 8°C, confirmant le manque de stabilité de cette formulation standard, tandis que la solution de CS / G1-P à 0,40 mmol/g de G1-P a conservé sa stabilité physique 2 mois à température ambiante et au-delà de 9 mois dans des conditions réfrigérées. L'utilisation de G1-P comme agent gélifiant permet donc d'améliorer significativement la stabilité de la solution thermo-gélifiante, de telle sorte qu'une présentation en seringues pré-remplies prêtes à l'emploi pourrait être envisagée. Enfin, la dernière partie de cette étude porte sur la tolérance locale sous-cutanée des hydrogels de CS / G1-P dans un modèle murin. Pour cela, la solution polymère a été stérilisée au préalable par filtration stérilisante et un dosage des endotoxines bactériennes selon la méthode LAL (Lysat d'Amaebocytes de Limule) a été réalisé, montrant que la teneur en endotoxines

bactériennes de la solution est au moins 100 fois inférieure à la limite. L'examen des tissus sous-cutanés entourant l'hydrogel a mis en évidence une réaction inflammatoire à un corps étranger, semblable à celle rapportée dans la littérature pour des systèmes dépôts conventionnels tels que les microsphères de PLGA ou les hydrogels de CS / glycérophosphate. L'injection de la formulation entraine une inflammation aiguë puis chronique, qui s'estompe au cours des trois semaines de l'étude, accompagnée de la formation d'une capsule fibreuse autour du dépôt.

Chapitre IV : Evaluation *in vitro* des hydrogels de CS / G1-P pour la libération contrôlée de composés modèles

Dans ce dernier chapitre, l'utilisation des thermogels de CS / G1-P pour la libération prolongée de principes actifs est évaluée *in vitro*. Le bleu de méthylène (composé de faible Mw, mono-cationique à pH physiologique), le principe actif A (composé mono-anionique de faible Mw), l'éosine Y (composé di-anionique de faible Mw), et un peptide faiblement soluble dans l'eau ont été utilisés en tant que substances modèles. Ces composés ont été incorporés dans la solution afin d'étudier l'influence de leurs propriétés physico-chimiques (Mw, solubilité dans l'eau, charge électrique) sur leur cinétique de libération dans un milieu de dissolution à pH 7,4 et à 37°C. La comparaison des profils de libération des composés de faible Mw a mis en évidence l'aptitude de l'hydrogel à réduire significativement la libération initiale rapide (ou « burst effect » en anglais) de ces substances, en comparaison avec une solution de CS pure. Ainsi, l'ajout du G1-P, en rendant la solution thermo-gélifiante, permet de réduire la libération du bleu de méthylène et du principe actif A de plus de 45 % au bout de 2 h. De plus, la libération se prolonge ainsi sur 1 à 2 jours, comparé à 6 h sans G1-P. Au vu des cinétiques de libération similaires obtenues avec le bleu de méthylène et le principe actif A, il semble que les interactions électrostatiques potentielles entre les fonctions aminées protonées du CS et la charge négative portée par le principe actif A n'affectent pas le mécanisme de libération. Une réduction significative du « burst effect » a également été observée pour les hydrogels contenant de l'éosine Y (17,5 % en 6 h versus 50,1 % sans G1-P) pour lesquels la libération est prolongée sur une durée de plus de 11 jours, versus 4 jours sans agent gélifiant. Cette libération prolongée est attribuée aux interactions ioniques entre l'éosine Y et le CS. En effet, en raison du caractère polycationique du CS, les anions multivalents peuvent réticuler le polymère au travers d'interactions électrostatiques. Ce principe est

couramment utilisé pour préparer des hydrogels de CS, notamment en utilisant du tripolyphosphate ou du citrate en tant qu'agent réticulant. Ainsi, comme le démontre le comportement rhéologique du système, l'éosine Y joue le rôle d'agent réticulant entre les chaines de CS, transformant la solution de CS / G1-P en un gel réversible à basse température. Des modèles mathématiques ont été appliqués au profil de libération *in vitro* de l'éosine afin d'élucider le mécanisme de libération. Les résultats démontrent que la cinétique de libération est contrôlée par la diffusion du composé à travers la matrice polymère.

En raison de son caractère hydrophobe, le peptide modèle a été incorporé dans la solution de CS / G1-P par dispersion. Grâce à la formation du réseau polymère, le « burst effect » a été significativement diminué (2,5 % de libération en 6 h *versus* 60,6 % sans G1-P) et la libération du peptide a été prolongée sur plus de 3 mois, comparé à une libération complète en 3 jours à partir d'une solution de CS pure. Le profil de libération peut être divisé en 2 phases distinctes : une première phase de libération rapide due à la dissolution et à la diffusion du peptide durant la gélification du système ainsi que du peptide incorporé à la surface de l'hydrogel en contact avec le milieu de dissolution, suivi par une seconde phase de libération progressive et contrôlée du peptide encapsulé dans le réseau macromoléculaire. La cinétique de libération obéit dans ce cas au modèle d'Higuchi. L'addition de lysozyme dans le milieu de dissolution permet d'augmenter sensiblement le taux de libération du peptide. En effet, si quasiment aucune dégradation du CS n'a été observée par GPC dans le milieu de dissolution, l'ajout de 10 µg/mL de lysozyme entraine une dégradation progressive de la matrice polymérique (réduction de 20 % du Mw du CS en 24 jours) en hydrolysant les liaisons glycosidiques β-(1–4) entre les unités glucosamine et N-acétylglucosamine du polymère. Ainsi, l'évaluation de la libération *in vitro* des différents composés modèles a démontré que le profil et la durée de libération varient significativement en fonction des caractéristiques des substances encapsulés, notamment leur Mw, leur pKa et leur caractère mono- ou multivalent ainsi que leur solubilité dans l'eau. De manière générale, des résultats prometteurs ont été obtenus avec le système thermo-gélifiant CS / G1-P quant à la libération contrôlée et prolongée de principe actifs pour des durées de quelques jours à plusieurs mois.

Conclusion et perspectives

Ce travail de thèse a permis le développement d'un nouveau système thermo-gélifiant à base de CS et de glucose-1-phosphate, destiné à la délivrance parentérale prolongée de

principes actifs. La solution de CS / G1-P de faible viscosité est aisément injectable à travers de fines aiguilles au niveau du site désiré, formant alors un hydrogel *in situ* en quelques minutes dans les conditions de pH et de température physiologiques. Cette solution présente notamment l'avantage d'être stable sur plus de 9 mois dans des conditions réfrigérées, permettant ainsi d'envisager une présentation en seringues pré-remplies prêtes à l'emploi. De plus, les essais *in vivo* sur le rat ont permis de démontrer que l'hydrogel est raisonnablement bien toléré après administration en sous-cutanée. Enfin, les profils de libération obtenus *in vitro* pour différents composés modèles ont mis en évidence l'aptitude des hydrogels de CS / G1-P à prolonger significativement la libération des substances incorporées. Ainsi, la libération d'un peptide modèle a pu être prolongée sur plus de 3 mois. Ces résultats encouragent la mise en œuvre d'études de pharmacocinétique *in vivo* qui permettraient de confirmer la capacité du système à contrôler la cinétique de libération du peptide sur des périodes de plusieurs semaines à plusieurs mois. Il serait également intéressant de compléter la characterisation physico-chimique des systèmes CS / G1-P contenant des principes actifs. Ainsi, les interactions moléculaires entre le CS et les composés anioniques pourraient par exemple être analysées par titration calorimétrique isotherme (ITC). Il serait aussi souhaitable d'étudier la stabilité à long terme des solutions thermo-gélifiantes contenant différents principes actifs pour déterminer l'influence de leurs caractéristiques physico-chimiques (telles que leur Mw, leur charge ou leur solubilité). Du point de vue méthodologique, les essais de libérations *in vitro* pourraient être optimisés en utilisant par exemple un système à flux continu (USP IV) et/ou en modifiant le milieu de dissolution. Enfin, des études approfondies seraient nécessaires afin de comprendre l'influence des caractéristiques des matières premières (telles que la masse molaire et le DD du CS) et des paramètres de formulation (tels que le rapport CS:G1-P et le pH) sur les profiles de libération *in vitro* des composés incorporés dans l'hydrogel.

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Abbreviations and acronyms

A

AHP	Ammonium hydrogen phosphate

B

β-GP	β-glycerophosphate
BSA	Bovine serum albumin

C

CD8+	Cytotoxic T cell
CF	Carboxyfluorescein
CHC	Carboxymethyl-hexanoyl-chitosan
C_{max}	Maximum plasma drug concentration
CS	Chitosan
CS-TGA	Thiolated chitosan

D

DBM	Demineralized bone matrix
DD	Deacetylation degree
DFO	Desferrioxamine
DGP	Disodium glucose-phosphate
DHO	Dipotassium orthophosphate
DSA	Drug substance A
DSC	Differential scanning calorimetry

E

EL	Endotoxin limit
ESEM	Environmental scanning electron microscopy
EY	Eosin Y

F

FBRM	Focused beam reflectance measurements
FDA	Food and drug administration

G

G'	Storage modulus
G"	Loss modulus
G1-P	α-D-glucose 1-phosphate disodium salt
G6-P	D-glucose 6-phosphate disodium salt
GP	Glycerophosphate
GPC	Gel permeation chromatography

H

H&E	Hematoxylin and eosin
HCl	Hydrochloric acid
HAD	Oxidized hyaluronic acid
hMSC	Human mesenchymal stem cells
HPLC	High-performance liquid chromatography
HTCC	N-[(2-hydroxy-3-trimethylammonium) propyl] chitosan chloride

I

I.D.	Inner diameter
ISFD	*In situ* forming depot
ITC	Isothermal titration calorimetry

L

LAL	Limulus amoebocyte lysate
LSCM	Laser scanning confocal microscopy

M

MB	Methylene blue
MRT	Mean residence time
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Mw	Molecular weight

N

NMR	Nuclear magnetic resonance
NP	Nucleus pulposus

O

O.D.	Outer diameter
OVA	Ovalbumine

P

PBS	Phosphate buffered saline
PEG	Poly(ethylene glycol)
PHBHHx	Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate))
PVA	Poly(vinyl alcohol)

R

RBC	Red blood cells

S

s.c.	Subcutaneous
SANS	Small angle neutron scattering
SEM	Scanning electron microscopy
SHP	Sodium hydrogen phosphate
SNP	Silica nanoparticles

T

T_{gel}	Gelation temperature
t_{gel}	Gelation time
TNFα	Tumor necrosis factor alpha

U

UV	Ultraviolet

V

VEGF	Vascular endothelial growth factor
VH	Venlafaxine hydrochloride

W

WBC	White blood cells

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Introduction

In the last few decades, the development of parenteral *in situ* forming depots (ISFD) has attracted considerable attention as an innovative type of depot systems for various pharmaceutical and biomedical applications. ISFDs can be defined as liquid formulations, that can be injected into the desired site of the body (e.g. intramuscularly, subcutaneously or intratumorally) via a syringe and turn into a semi-solid depot *in situ*, under physiological conditions [1]. ISFD drug delivery systems were first reported in the late 1980s by Dunn *et al.* [2] at Southern Research Institute. They developed an innovative injectable and degradable sustained-release drug delivery platform, based on a solution of poly(lactic acid) (PLA) or poly(*D,L*-lactide-co-glycolide) (PLGA) in a water miscible solvent that generated a solid implant *in situ* after parenteral injection via a solvent-exchange precipitation mechanism.

Injectable depot systems overcome the limitations of conventional immediate release parenteral formulations (i.e. solutions, suspensions and emulsions) due to their ability to prolong the release of the incorporated drug over a period of weeks or months, while maintaining the drug concentration within a desired range. They allow a reduction of the dosing frequency and offer thereby a significant convenience and compliance benefit to patients. Furthermore, drug entrapment within a polymeric matrix often stabilizes the therapeutic agent, particularly in the case of peptides and proteins, and help preventing their denaturation in physiological environment. As compared to other depot technologies, like microparticles or implants, ISFD systems offer several benefits such as simple manufacturing processes allowing lower production costs, administration in a minimally invasive way through a small gauge needle and potential for self-administration via the use of auto-injectors. Ideal ISFD intended as drug delivery systems should fulfill several conditions: *i*) they need to be composed of pharmaceutically acceptable, biocompatible and biodegradable components, to obviate the need for a surgical procedure to remove the empty depot after drug release completion, *in vivo* degradation products should also be biocompatible; *ii*) the ISFD solution should be stable enough to ensure an acceptable drug product shelf life and enable a ready-to-use presentation, to avoid the need for extemporaneous reconstitution; *iii*) the viscosity of the polymeric solution should be low enough to allow easy formulation, homogeneous loading with therapeutic agents and easy injection through thin needles; *iv*) excipients and *in situ* solidification or gelation mechanism should be safe (e.g. no toxic organic solvents or crosslinking agents, no heat released at the injection site); *v*) they should solidify rapidly under physiological conditions, to avoid any lag time between the injection of

the system and the formation of the implant that could lead to high initial burst release or spreading in the surrounding tissues.

According to their mechanism of solidification under physiological conditions, injectable depot systems can be classified into *i*) thermoplastic pastes, having a solidification point near body temperature, *ii*) *in situ* cross-linked polymer systems, induced either by chemical-, physical- or photo-initiation, *iii*) *in situ* polymer precipitation systems, based on phase separation, and *iv*) thermally induced gelling systems that solidify at physiological temperature [3]. All these systems have been the subjects of a number of reviews [1,3-5]. Recently, Li *et al* [6] have summarized the progresses on injectable biodegradable hydrogels in view of their gelation, biodegradation and biomedical applications, Agarwal *et al* [7] have reviewed the use of injectable implants for the sustained release of protein and peptides while Fitzpatrick *et al* [8] and Gong *et al* [9] have focused on temperature-sensitive polymers and polymeric hydrogel as drug delivery systems.

This thesis is dealing with thermally induced gelling systems and more precisely is focusing on chitosan-based thermosensitive hydrogels which have been recognized as an attractive depot drug delivery technology. *Chapter I* reviews the recent progresses on injectable chitosan-based hydrogels as ISFD in pharmaceutical and biomedical applications. It introduces the chitosan-based thermogels, showing their specific advantages over other ISFD technologies in the field of sustained drug delivery. The standard chitosan / glycerophosphate system gelation mechanism has been described as well as the characterization of the hydrogel properties, depending on the formulation parameters applied. The application of the thermogel has been detailed for the delivery of various types of drug substances but also as a scaffold for cell encapsulation and tissue engineering. Improvement of the conventional system by addition of carrier particles, use of other gelling agents, incorporation of additional biodegradable polymers or via chemical modifications of chitosan has been subsequently discussed, highlighting the benefits and drawbacks of each approach.

The first objective of this thesis was to get a complete understanding of the sol / gel transition mechanism of the chitosan / glycerophosphate thermosensitive hydrogels and to investigate different types of gelling agents as alternative to glycerophosphate. Therefore, in the subsequent *Chapter II*, the mechanisms inducing the *in situ* gelation of the chitosan-based system in response to temperature changes, from ambient to body temperature, were

investigated. For this purpose, several polyol-phosphate and polyol-free phosphate salts were considered, according to their ability to turn the polymeric solution into a thermogelling system. Comprehensive rheological studies, completed by ^{31}P-NMR and pH measurements, were performed to establish a relationship between the chemical structure of the gelling agent and the macroscopic gelling behavior of the solutions, that is, transition temperature, gelation time, and gel strength. These investigations highlighted the key role of the gelling agent polyol part in preventing the interactions between the chitosan chains at ambient temperature or below.

Based on the results of Chapter II, Glucose-1-Phosphate (G1-P) was selected as an alternative gelling agent for the optimization of the chitosan-based ISFD system, described in *Chapter III*. Physico-chemical characterization of the chitosan / G1-P system was performed, i.e. viscosity and viscoelastic behavior upon heating and cooling was assessed using rheology. Long term storage stability of the solution was evaluated through the appearance, pH, viscosity and gelation time of the solution and through the changes in chitosan molecular weight, determined by gel permeation chromatography. The results emphasized an enhanced stability of the solution containing G1-P, at room temperature as well as at refrigerated conditions, compared to the classical chitosan / β-glycerophosphate solution. Furthermore, the injectability of the system in the liquid state was evaluated under variable injection parameters. Following a final sterilization step and a bacterial endotoxins test, the *in vivo* tolerability of the optimized placebo formulation was evaluated on a murine model.

Finally, *Chapter IV* has been dedicated to the investigation of the *in vitro* drug release kinetics from chitosan / G1-P hydrogel. Release profiles of different types of low molecular weight hydrophilic model compounds (cationic and anionic) are presented and show that the hydrogel network is effectively able to retard the release of the model compounds for several hours to days by physical entrapment. Comparison with the results on a poorly soluble peptide shows that the solubility and molecular weight of the drug significantly influenced the drug release.

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Chapter I :

Thermosensitive chitosan-based hydrogels in pharmaceutical and biomedical applications

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Abstract

Introduction: In the last decade, thermogelling chitosan (CS) / glycerophosphate (GP) solutions have been reported as a new type of parenteral *in situ* forming depot (ISFD) system. These free-flowing solutions at ambient temperature or below turn rapidly into semi-solid hydrogels after parenteral administration, due to the increasing temperature at the injection site, without requiring the presence of any organic solvent or toxic initiator or cross-linker.

Areas covered: The mechanisms responsible of the thermo-induced gelation, identified as a reduction of electrostatic repulsion between the polymeric chains combined with synergistic chitosan - chitosan attractive forces, are discussed. Formulation parameters such as CS physico-chemical characteristics, CS / gelling agent ratio or pH of the system, were acknowledged as key parameters affecting the solution stability, the sol / gel transition behavior and/or the final hydrogel structure and biocompatibility. In this review, the use of the standard chitosan / glycerophosphate thermogels for various biomedical applications, including drug delivery, cell encapsulation and tissue engineering, is also discussed. Furthermore, this manuscript reviews the different strategies implemented to improve or modify the hydrogel characteristics, such as (*i*) combination with carrier particles; (*ii*) replacement of GP by another gelling agent; (*iii*) addition of a second biodegradable polymer and (*iv*) chemical modification of CS.

Expert opinion: The recent advances in the formulation of CS-based thermogelling systems already overcame several crucial challenges faced by the standard CS / GP system. Dispersion of drug-loaded carrier particles (e.g. liposomes or microspheres) into the thermogels allowed achieving prolonged release profiles for low molecular weight drugs; incorporation of an additional polymer enabled to strengthen the network while the use of chemically modified CS led to enhanced pH sensitivity or biodegradability of the matrix. Despite these major advances compared to the conventional CS / GP formulation, further investigations are needed to address the remaining issues, such as the lack of stability upon storage of the ISFD system.

1. Introduction

The development of *In Situ* Forming Depot (ISFD) has increasingly gained attention over the last decades as an attractive new class of controlled release systems for various pharmaceutical and biomedical applications (Figure I-1) [1,2]. ISFDs are defined as liquid formulations which form a solid or semi-solid depot *in situ* after injection into the body via a syringe [3]. As parenteral depot systems, ISFDs allow a controlled and prolonged release of the drug substance(s). This grants a reduction of the dosing frequency while maintaining the drug concentration within the therapeutic window and avoiding undesired peak-and-valleys delivery profiles [4]. ISFDs offer several advantages over other parenteral depot systems (e.g. microparticles or implants), such as more simple manufacturing processes allowing lower production costs, more patient convenient administration through smaller gauge needles and potential for self-administration via the use of auto-injectors. Beside those advantages, these delivery systems may also show some potential drawbacks like stability issues (requiring an extemporaneous preparation step), high viscosity causing an increased pain during injection, high initial drug burst due to depot gelation lag time, drug substance dependent release rate and local tolerability/toxicity [5-8]. ISFD technologies can be divided in four main categories, based on their mechanism of solidification or gelation under physiological conditions: 1) thermoplastic pastes, 2) *in situ* cross-linked polymer systems, 3) *in situ* polymer precipitation, and 4) thermally induced gelling systems [4].

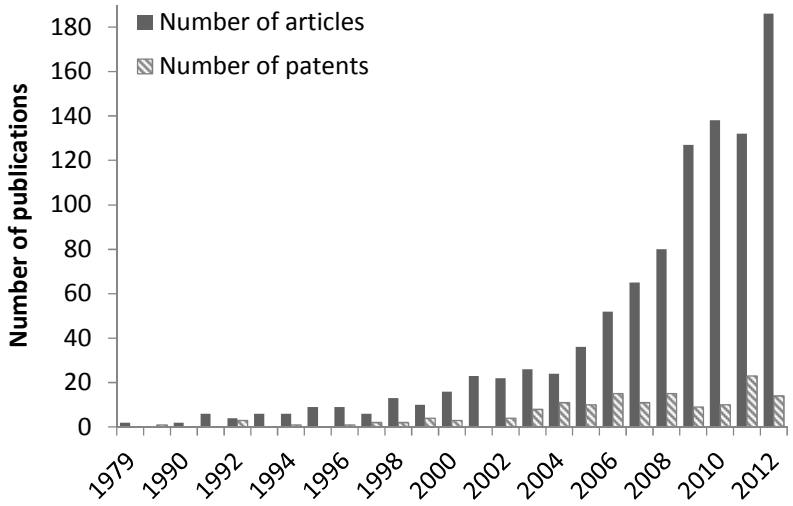

Figure I-1: Number of publications on *in situ* forming depot systems over the years. (Sources: Web of Knowledge and PatBase; End date: Dec 2012)

Among the thermally induced gelling systems, chitosan-based thermogelling solutions, that undergo sol / gel transition upon temperature increase, have been extensively investigated in the last decade. Chitosan (CS) is a natural linear polysaccharide, obtained from the partial deacetylation of chitin, which is extracted from the exoskeleton of crustaceans or insects and from the cell walls of fungi [9]. It is a heteropolymer of glucosamine units (β (1–4)-linked 2-amino-2-deoxy-D-glucopyranose) and N-acetylglucosamine units (2-acetamido-2-deoxy-D-glucopyranose) (Figure I-2). Due to the presence of the free amino groups, CS is a cationic polyelectrolyte (pK_{ap} of about 6.2), which can be dissolved in aqueous acidic solutions in the ionized state. At pH higher than the pK_{ap}, the neutralization of the CS chains induces the formation of gel-like precipitates [10]. The physico-chemical properties of CS, like molecular weight, deacetylation degree (DD) (i.e. the proportion of glucosamine unit) and distribution of glucosamine and acetylglucosamine units mainly depends on the source and preparation process of CS. CS is of great interest for the production of hydrogels for pharmaceutical and biomedical applications due to its intrinsic properties such as its non-toxicity, biocompatibility, biodegradability, bioadhesivity, bacteriostatic effects and penetration enhancer properties [11]. CS is not a thermosensitive polymer on its own, but as demonstrated by Chenite *et al* a CS solution becomes thermoresponsive at physiological pH upon addition of glycerophosphate (GP) [12]. CS-based thermosensitive hydrogels present specific advantages over others ISFD systems, including enhanced safety thanks to the absence of organic solvents or toxic initiators or cross linkers and easy entrapment of cells and/or of various types of drugs (small molecules, peptides, proteins, etc.).

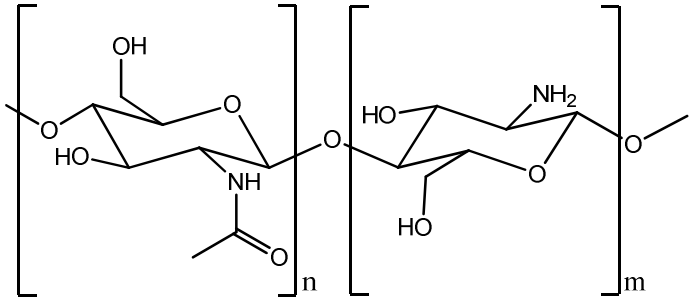

Figure I-2: Chemical structure of chitosan

This review article focuses on injectable thermosensitive CS-based ISFD solutions, which form semi-solid hydrogels after administration in response to temperature increase from ambient to physiological temperature. In the first chapter, the synergistic mechanisms involved in the thermo-induced sol / gel transition of the CS / GP system are discussed. The

main impacts of the formulation parameters on the gelation behavior and final hydrogel characteristics are highlighted. The use of the thermogelling system for pharmaceutical and biomedical applications, as an injectable drug delivery system or a cell carrier in tissue engineering, is also described. Finally, the strategies implemented to improve or tailor the hydrogel properties (e.g. through the combination with carrier particles, the use of other gelling agents, the addition of another biodegradable polymer or the chemical modification of CS) to meet the needs of the different applications are discussed.

2. Thermosensitive chitosan / glycerophosphate solution

CS-based thermogelling systems were first reported in 2000, when Chenite *et al* developed an innovative injectable, neutral and thermally sensitive gel-forming aqueous solution, made of CS and glycerophosphate [12]. CS was dissolved in a diluted acidic solution and was chilled in an ice bath together with a GP solution. The cold GP solution was then added dropwise to the CS solution under stirring. The resulting clear and homogeneous solution remained liquid at physiological pH as long as it was kept at low temperatures, but turned quickly into a gel upon heating at around body temperature. This technology was registered under the name BST-Gel^{TM} by BioSyntech (Canada) [13]. Three types of possible interactions were presumed to be involved in the heat-induced gelation process, namely (1) electrostatic attraction between the amino groups of CS and the phosphate groups of GP, (2) hydrogen bonding between the CS chains thanks to the decrease of electrostatic repulsion induced by the neutralization of the polymer by the addition of GP and (3) hydrophobic interactions between the CS molecules. It was also postulated that the polyol part of GP slowed down the gelation process at low temperature by promoting the protective hydration of the CS chains [14]. Nevertheless, further studies were necessary to elucidate the exact gelation mechanism.

2.1. Synergistic interactions promoting CS / GP heat-induced sol / gel transition

Several literature reports focused on understanding the thermo-induced gelling mechanism of CS-based ISFD solutions. The effect of temperature on CS / GP sol / gel transition was first investigated by Cho and coworkers [15]. Based on pH measurements of the CS / GP system and evaluation of CS and GP pKa values as a function of temperature, CS and GP degree of ionization and CS / GP solution ionic strength were calculated. The authors reported a significant decrease of the degree of ionization of CS upon heating, which suggests

a reduction of CS solubility and possible ionic interactions between the CS positively charged glucosamine units and the negatively charged GP. Energy dispersive X-ray analysis of phosphorous, performed on washed hydrogel, confirmed that GP was not bound to CS and diffused freely out of the gel [16,17]. Therefore, ionic bridging between CS and GP is not the main driving force for the sol / gel transition. Instead, synergistic effects between CS chains hydrophobic interactions and hydrogen bonding are assumed to play a key role in the gelation mechanisms. Furthermore, Cho *et al* also expected an increase of the CS / GP system ionic strength with temperature, due to an increased ionization of divalent GP anions upon heating [15]. The presence of a higher number of GP anions enables a reduction of the electrostatic repulsion force between the positively charged amine functions of CS. Moreover, increasing the ionic strength and temperature create favorable conditions for hydrophobic interactions between CS chains, presumed to be the main driving force of the gelation mechanism [18].

However, in an additional attempt to understand the unique CS / GP thermogelling process, Filion *et al* also characterized the ionization and solubility behavior of CS upon temperature increase [19]. Their results questioned the impact of the ionic strength on the gelation mechanism. The study revealed (*i*) a temperature-dependent reduction of the proton dissociation constant of CS (pK_{ap}) of 0.023 units per degree Celsius and (*ii*) that the pKa of GP was temperature-independent. That finding is opposed to the hypothesis made by Cho *et al* in the previous study, where a significant decrease of the pKa value of GP upon heating was assumed for the calculation of the ionization degree of the gelling agent [15]. Therefore, an alternative hypothesis was proposed for the mechanism of sol / gel transition, where the temperature increase induces a transfer of protons from CS amine groups to GP, thereby leading to a progressive neutralization of CS. The reduction of the CS ionization degree would result in a lower electrostatic repulsion between CS chains and would allow the attractive hydrophobic interactions and hydrogen bonding to occur, inducing gel formation. To confirm this hypothesis, and based on experimental results, a theoretical model was developed to calculate the CS degree of ionization as a function of temperature. This model allowed predicting a significant proton transfer from CS to GP upon heating. These results were corroborated by ^{31}P-NMR measurements, which showed that the proportion of monovalent versus divalent GP anions in the thermogelling solution increased upon heating due to proton uptake [20]. Therefore, as opposed to Cho *et al* conclusions, the CS / GP system ionic strength does not increase, but rather slightly decreases, upon heating [15]. As a consequence, based on this theoretical and experimental approach, electrostatic repulsion,

rather than ionic strength, was identified as being most impacted by the CS / GP system temperature. The thermosensitive behavior of the system was explained by CS - CS interchain attractive forces (i.e. hydrogen bonding and hydrophobic interactions) kicking off the gelation mechanism.

Additional studies including laser scanning confocal microscopy (LSCM) analysis of the CS / GP hydrogel, aimed to gain a better understanding of the gelation mechanism through the observation of the hydrogel morphology in its native hydrated state [21]. The images displayed a heterogeneous microstructure of the hydrogel. The network was found to result from the agglomeration of polymeric aggregates, which suggested that the mechanism of gelation may be nucleation and growth. After examination of the molecular conformation of CS chains by small angle neutron scattering (SANS) and ultra SANS, Crompton *et al* reported that the polymeric chains were effectively arranged into solvent-rich domains and polymer-rich aggregates of about 1-2 µm diameter [22]. On the nanoscale, a second level of two-phasic structure was also disclosed within these polymer-rich aggregates, composed of hydrophobic domains (having a characteristic length of approximately 350 Å) and hydrophilic domains (with a correlation length of about 120 Å). Atomic force microscopy observations and dynamic light scattering measurements of diluted CS / GP solutions at 34°C evidenced as well the formation of submicrometric chain aggregates, driven by hydrophobic interactions and hydrogen bonds. These analysis confirmed the granular nanostructure of the system at the initial stage of the sol / gel transition, due to the assembling of CS molecules into polymeric aggregates, which then form larger CS agglomerates [23].

More recent studies from Qiu *et al* allowed concluding on the CS/GP system gelation mechanism [23]. Urea (a hydrogen bonding disrupting agent) or isobutanol (a non-solvent of CS) were added to the CS / GP solution. The gelation process was slowed down with urea addition whereas it was accelerated with isobutanol, thereby confirming a synergistic effect of hydrogen bonding and hydrophobic interactions in the gelation process. Investigation on the influence of the GP polyol part on the gelation mechanism revealed that the glycerol-moiety plays a protective role at low temperature by creating a hydration layer around the CS molecules [24]. This water shield inhibits the CS-CS attractive interactions at ambient temperature or below, thereby preventing the gelation mechanism. The removal of the water shield upon heating allows the sol / gel transition to occur via the synergistic mechanisms disclosed above.

2.2. Impact of formulation parameters on CS/GP hydrogel characteristics

The effects of the formulation parameters on the characteristics and structure of the CS / GP hydrogels have been widely evaluated. Due to the different interactions taking place during the sol / gel transition, the gelation process and/or the final hydrogel network was significantly affected by the following parameters: pH of the CS / GP system, concentration of GP, deacetylation degree, molecular weight and concentration of CS and acidic solution used for CS dissolution.

2.2.1. Gelation time and temperature

The gelation time and temperature are crucial parameters of CS-based ISFDs since gelation should occur rapidly at physiological temperature in order to limit the diffusion of the solution from the injection site and to avoid a potential high initial drug burst [25]. These two parameters can be measured through a simple and rapid test tube inverting method, or, in a more accurate way, via rheological analysis of the viscoelastic properties of the sample. The gelation time and temperature could be adjusted by varying the GP and CS ratio [26-29]. Raising the GP concentration leads to an increase of the pH value of the system which in turn induces a reduction of both the CS protonation and intermolecular electrostatic repulsion, thereby promoting the sol / gel transition. Furthermore, increasing the polymer concentration from 1.0 to 2.0 % w/v allows faster gelation, due to an increased number of CS-CS entanglements [30]. However, a further increase of CS content leads to a highly viscous solution, hindering the diffusion of CS and GP and slowing down the gelation process [31]. Equally, a reduction of the gelation temperature is obtained by lowering the viscosity of the solution using lower molecular weight CS. At high GP concentrations and pH values, the effect of CS molecular weight becomes negligible [32,33]. The thermosensitive characteristics of the CS / GP solution is also significantly influenced by the CS deacetylation degree (DD), i.e. lower gelation time and temperature are obtained with an increased DD [14,28]. Chen *et al* demonstrated that CS chains with higher DD (i.e. more glucosamine and less acetylglucosamine monomers) showed more flexibility, facilitating therefore the molecular rearrangement during the gelation process [34]. The gelation temperature was also affected by varying the acid used as solvent for the solubilization of CS. Monovalent acid solutions (e.g. formic, acetic, propionic, hydrochloric or lactic acid) were appropriate for the preparation of thermogelling CS / GP solutions, whereas multivalent acids failed in turning the system into a gel upon heating at physiological temperature [35]. Furthermore, at the same

solution concentration, the highest and lowest gelation temperatures were reported when using acetic acid and lactic acid respectively [36]. Studies on the influence of drug loading on the gelation kinetic showed different behavior depending on the physico-chemical characteristics as well as on the concentration of the incorporated compounds. Thus, no significant change of the gelation properties were reported upon addition of dextran, betamethasone sodium phosphate or silver nanoparticles to the CS / GP solution [28,32], while the incorporation of meglumine antimoniate or of increasing concentrations of iodine contrast agents substantially slowed down the gelation process [37,38]. Finally, the structure of the GP used as gelling agent, either α-GP or β-GP, did not significantly affect the gelation behavior, corroborating the findings that ionic crosslinking between CS and GP are negligible in the sol / gel transition process [31]. The possibility to fine tune the time scale and temperature of gelation of the CS / GP hydrogel by varying the above mentioned formulation parameters makes it particularly attractive as ISFD system for various applications.

2.2.2. Gel strength

The mechanical strength of the CS / GP hydrogels can be evaluated by rheological frequency sweep tests, through the value of the storage modulus (G') (which represents the elastic behavior of the gel) and by the difference between G' and the loss modulus G" (reflecting the viscous behavior of the gel) [39]. Overall, CS / GP systems display strong gel behavior with G' values comprised between 1 and 10 kPa and with G' >> G" over the whole frequency range [26,29,40]. As long as the GP concentration was sufficient to induce gel formation at a given temperature, the strength of the three-dimensional network was found to be independent of the gelling agent concentration [24]. As the gel network is formed through intermolecular interactions of CS chains, the GP content was not expected to influence the final gel state. However, Kempe *et al* reported a slight increase of the gel strength upon doubling the GP content from 8 to 16 % [29]. This may be attributed to an incomplete heat-induced sol / gel transition process, since the rheological characterization was performed at 25°C whereas the gelation temperatures were 50°C and 37°C respectively.

2.2.3. Thermoreversibility

The thermoreversibility of the CS-based hydrogels could be investigated through rheological thermoanalysis, via heating and cooling cycles. The evolution of the storage modulus G' and the loss modulus G" upon heating of the CS / GP system showed a clear

transition from the solution state to the gel state. However, even if a gradual decrease of the gels strengths was observed upon cooling, no inverse gel / sol transition could be observed down to 5°C. The hydrogels obtained after heat-induced gelation are therefore not thermoreversible [12,15,29]. The incomplete thermoreversibility of the gel could be attributed to the two types of attractive interactions involved in the gelation process, since CS-CS hydrophobic interactions may be weakened during the cooling cycle whereas inter-chain hydrogen bonding are not affected by the temperature changes.

2.2.4. Turbidity

The gelation process turns the homogeneous and clear CS / GP solution into a turbid hydrogel. The reduction of CS solubility and the formation of CS-CS interactions lead to an increase of the optical density of the system, through the formation of hydrophilic and hydrophobic domains with different refractive indexes. Therefore, the change in turbidity of the system was sometimes used as an indicator of the sol / gel transition [27,30,36]. Berger *et al* reported that the gel turbidity could be modulated by varying the DD and the distribution of the CS monomers [41]. The authors demonstrated that transparent hydrogels could be achieved by homogeneously re-acetylating the polymer to a DD of 50 %.

2.2.5. Morphology

The morphology of the CS / GP hydrogels has been examined using various microscopy techniques, scanning electron microscopy (SEM) being the most widely used. SEM micrographs of freeze-dried CS / GP hydrogels revealed a porous structure [14,42-44] that became more compact and regular upon increasing CS concentration and Mw whereas an increase of the DD induced a more compact but irregular structure [30]. The gel matrix structure was also shown to be strongly dependent on the type of acid used in the preparation of the CS solution. For instance, hydrogels prepared using acetic or hydrochloric acid displayed an open, interconnected highly porous structure on SEM micrographs. In contrast the hydrogels obtained with propionic acid and lactic acid exhibited a flake porous structure and much bigger pores diameters [36]. Furthermore, the pore aperture size was also related to the ionic strength of the acidic solvent [35]. Further characterization was carried out by environmental SEM (ESEM), which allows investigation of the hydrogel structure details in hydrated conditions. Thus, potential artifacts induced by freeze-drying of the sample are avoided. ESEM imaging confirmed the uniform porous continuous network showing pore

diameters between several micrometers and 20 µm [16]. LSCM investigations revealed a polymeric aggregates based network structure with a fractal-like morphology [21].

2.2.6. Storage stability

The stability of the CS-based solutions is a critical parameter that needs to be assessed during pharmaceutical drug product development to ensure an acceptable shelf life. Despite its importance, there are limited reports in the literature on the CS / GP solutions stability. Viscosity changes were monitored over 3 months storage at room temperature and refrigerated conditions and revealed that the sol / gel transition occurred at higher temperature and also with time [14]. The stability was mainly affected by the DD of the polymer. When using 84 % deacetylated CS, the viscosity remained constant over 3 months, whereas the solution based on a mixture of 84 % and 95 % deacetylated CSs evolved to the gel state in less than 7 days at a temperature as low as 4°C. In an attempt to define storage approaches which would prevent the sol / gel transition, Schuetz *et al* compared refrigeration, freezing or lyophilization of CS-based thermogelling solutions prepared with different polyols as gelling agents [45]. The formulations' injectability and viscoelastic behavior upon raising temperature from 4 to 37°C were evaluated. As previously observed for the CS / GP systems, gelation occurred in less than 1 month storage at 4°C, confirming the lack of stability of the solutions at refrigerated conditions. As for the freezing approach, a significant reduction of the gelation time and a viscosity increase were observed for a thawed versus a freshly prepared solution. Thus, this storage method is also not considered as an appropriate alternative. Finally, depending on the polyol used as gelling agent, the reconstituted formulations obtained after freeze-drying were either in the gel state immediately after reconstitution or in the solution state but showing higher viscosities, shorted gelation times and increased gel strengths compared to the freshly prepared solutions. In summary, lyophilization was also not able to maintain the viscoelastic properties of the CS-based thermogelling solution. Thus, further investigations are needed to improve the stability of the CS / GP formulation, e.g. by the use of alternative gelling agents or additional stabilizing compounds.

2.2.7. Sterilization

Sterility of the CS-based ISFD solutions is a prerequisite for parenteral administration. Terminal sterilization processes requiring high temperatures, such as steam or heat

sterilization, cannot be applied due to the irreversible thermogelling properties of the CS / GP solution upon heating [46]. Gamma irradiation, another potential final sterilization technique, results in an important decrease of the CS solutions' viscosity due to polymer chain degradation, even when performed at -80°C [47,48]. Furthermore, terminal sterilization by sterile filtration through 0.2 µm porosity membranes may be difficult, depending on the viscosity of the polymeric solution, or not feasible if drug substances or cells are dispersed in the solution. Therefore, the CS and GP solutions are generally sterilized separately and mixed under aseptic conditions. Steam sterilization of the CS solution, prior to the addition of GP, is the most commonly used technique [38,42,49-51]. However, this method induces significant CS depolymerization, directly impairing the hydrogel characteristics (e.g. decrease of viscosity, gelation rate and gel strength) [52]. Addition of polyols, such as glycerol, glucose or poly(ethylene glycol), to the CS solution in order to protect the polymer from hydrolysis upon autoclaving showed only a modest inhibition of the heat-induced depolymerization [48]. Sterile CS powders or solutions were also obtained through ultraviolet, gamma or beta irradiation, but all these methods resulted in important degradation of the polymer. Thus, they were not deemed appropriate for CS sterilization [48,53,54]. The most convenient technique appears to be steam sterilization of the CS powder dispersed in water. Therewith, the polymer Mw is preserved. Subsequently, CS is dissolved by addition of acidic solvent under sterile conditions.

2.2.8. Biocompatibility

CS is generally regarded as a non-toxic, biocompatible polymer for parenteral applications [55,56]. Equally, GP is a biocompatible component naturally present in the body, approved by the U.S. Food and Drug Administration (FDA) as a phosphate source in emulsions for parenteral nutrition [57,58]. However, the safe use of the CS / GP hydrogels as parenteral ISFD systems required a thorough assessment through comprehensive *in vitro* and *in vivo* biocompatibility studies.

Cytotoxicity may be investigated via several *in vitro* techniques, however results comparison and interpretation is challenging due to the variety of testing conditions (i.e. hydrogel prepared using different Mw and DD CS, different acidic solvent, variable GP concentrations, and use of different cell type for the *in vitro* assays). The most commonly used cytotoxicity assays are the MTT (3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide), MTS (3-(4,5-Dimethyl-thiazol-2-Yl)-5-(3-Carboxymethoxyphenyl)-2-(4-Sulfo-

phenyl)-2h-Tetrazolium) or Alamar Blue assays, which uses the mitochondrial activity as an indicator of cell viability. Ahmadi *et al* reported, based on an Amalar Blue assay, that extract from 0.5 to 2 % CS with 5 % GP is biocompatible and enhances mesenchymal stem cells proliferation, whereas higher GP content reduces the cell growth compared to cell culture in control medium, presumably due to the high osmolalities of the extracts [51]. These results were confirmed by an MTS test on human fibroblasts which showed that hydrogels prepared with 0.088 M of GP allowed the growth and proliferation of the cells, whereas a cytotoxic effect was observed when increasing the GP content up to 1.155 M [42]. Using the MTT assay, relative growth rates between 80 and 100 % were observed for mouse embryo fibroblasts in contact with CS / GP hydrogel extracts prepared with acetic acid, lactic acid or hydrochloric acid and therefore a good biocompatibility was claimed. On the other hand, extracts of hydrogels prepared with formic acid and propionic acid were reported to induce significant cytotoxicity [36]. The compatibility was also tested via an hemolysis test that showed no hemolytic activity of the thermogel [59]. The overall trend indicates that CS / GP hydrogels show a certain level of cytotoxicity which is dependent on the GP and acid content. It has been generally reported that the selection of the appropriate formulation conditions led to good *in vitro* biocompatibility [33,35,53].

In spite of the good results observed through *in vitro* assays, *in vivo* biocompatibility studies remain essential to establish the safety of the CS-based hydrogels. *In vivo* toxicity of the depot system was mainly assessed through histopathological evaluations of the tissues in contact with the hydrogel. Chenite *et al* reported that the CS / GP solution turned rapidly into a gel after subcutaneous injection in rats and that the hydrogel biocompatibility depended on the deacetylation degree of CS, with higher DDs inducing less inflammatory response [12]. The increased degradation rate of CS with lower DD was presumed to lead to the generation of polymer fragments promoting the inflammatory reaction [49]. It was demonstrated as well that the importance of the inflammatory effect is linked to the injection site, e.g. a more pronounced reaction was observed after injection in the hind paw of rats compared to subcutaneous injection in the dorsal region. In general, in *vivo* administration of the thermogels generated no or only minimal toxic effects [27,60]. Even though an inflammation of the depot surrounding tissues was reported in some cases, no significant difference was observed when compared to the body response induced by surgery thread as negative control [59]. Furthermore, the inflammatory response decreased after the first week post-injection,

indicating therefore a good biocompatibility of the hydrogels [36,43]. Drug-loaded depots' safety remains to be further evaluated on a case-by-case basis.

2.2.9. Biodegradability

Biodegradation of the depot system presents a large convenience advantage compared to not biodegradable systems which need to be surgically removed after release duration completion. CS is known to be degraded *in vivo* by specific hydrolytic enzymes, mainly lysozyme [55,61]. Lysozymes can be found in various human body fluids and tissues, with concentration between 4 mg/L and 13 mg/L reported in serum [62]. Several investigations were published on the *in vitro* degradation behavior of CS-based hydrogel in contact with lysozyme. Monitoring of the hydrogel degradation by gravimetric analysis and gel permeation chromatography demonstrated the dependency between the deacetylation degree of CS and the enzymatic degradation, since lower degradation rates were observed for higher DDs [28]. Similarly, slower degradation occurred when increasing the CS concentration from 1 to 2 %. Analysis of the remaining depots after 21 days incubation with lysozyme revealed that degradation occurred via the hydrogel surfaces. In addition, the use of a ninhydrin assay to determine the amount of free glucosamine units in the incubation medium demonstrated a continuous degradation of the hydrogel through hydrolysis of acetylated residues [42]. A further evaluation of the degradation rate of the CS / GP hydrogel showed about 5 % weight loss after 10 days in PBS compared to about 50 % with addition of 500 mg/L lysozyme, indicating that a complete degradation of the depot could be achieved with time [63]. However, in view of the lower lysozyme concentration in serum, complete *in vivo* degradation of the hydrogel could take several months.

Biodegradation of CS thermogels after *in vivo* administration has been little investigated. *In vivo* data following subcutaneous injection in mice have shown a 75 % decrease of the volume of the depot after 50 days [27]. Sun *et al* corroborated these findings by comparing the hydrogels' morphology after *in vitro* incubation in PBS or *in vivo* subcutaneous implantation [43]. The porous structure of the hydrogel network was preserved during 7 days *in vitro* while it was significantly modified *in vivo*, displaying a more compact structure over time with a reduction of the pore size. Positive results have also been reported recently by Zhou and coworkers, who observed a loosening of the structure on the hydrogel surface after 8 weeks intra-muscular implantation and a start of disintegration after 12 weeks, thereby confirming the gradual biodegradation of the hydrogel over time [59]. The variations

of the network structure observed *in vivo* should be taken into consideration when studying the pharmacokinetic profiles obtained from drug-loaded hydrogels, as they may impact the long-term drug release.

2.3. Pharmaceutical and biomedical applications of CS / GP thermogelling systems

From the above mentioned characteristics, CS / GP hydrogel appears as a promising ISFD system. The gelation kinetic as well as the hydrogel morphology can be controlled by selecting the most suitable polymer (Mw and DD) and by varying the formulation parameters (e.g. ratio of CS and GP, pH, acidic solution). Moreover, by selecting the appropriate formulation parameters, the hydrogel exhibits good biocompatibility and biodegradability results. Consequently, the use of this thermogel for the controlled release of various types of drugs (e.g. hydrophilic and hydrophobic small molecules, proteins and peptides as well as vaccine delivery) and also cell encapsulation or tissue engineering has been extensively studied *in vitro* as well as *in vivo* in the past few years.

2.3.1. CS / GP systems for the delivery of low molecular weight compounds

Ruel-Gariepy *et al* first described the *in vitro* release of model compounds loaded into the CS / GP system and demonstrated its ability to significantly reduce the initial burst of chlorpheniramine maleate compared to a CS solution without gelling agent [14]. The release of this positively charged low Mw compound was reduced from 53 % to 15 % after 4 h by addition of GP to the CS solution. Furthermore, the release of the compound was sustained by the CS / GP hydrogel over 2 days, whereas a complete release was achieved in less than 1 day from the pure CS solution. Comparison of positively charged methylene blue and negatively charged calcein revealed that the release was not affected by the drug electrostatic charge. The two compounds displayed similar profiles, characterized by a small burst of less than 20 % within 4 h, followed by a sustained release over 1 to 2 days, thereby showing that there was no ionic bonds between the compounds and the CS matrix. By increasing the compound Mw, the release duration could be extended to several days, as observed for albumin- or dextran-loaded gels, whereas addition of lysozyme increased the release rate due to degradation of the network. Interestingly, addition of low Mw model compounds in the CS solution (before GP addition) rather than in the final CS / GP solution led to slower release rates, likely due to a different distribution of the compounds into the gel network [30]. It was also surprising to

notice that the hydrophilic adriamycin showed slower *in vitro* release kinetics than the hydrophobic 6-mercaptopurine [30]. However, almost complete release was achieved in less than 1 day for both compounds. Additionally, slower releases were observed when increasing the CS Mw from 1130 to 1360 kDa, due to the compaction of the hydrogel structure induced by higher Mw.

Comprehensive investigations of the release mechanism from CS-based hydrogel were performed using venlafaxine hydrochloride (VH), a highly water soluble compound [64]. By applying several mathematical models to the *in vitro* data, it was found that the release kinetic was best described with the first-order model and that the release was diffusion controlled. Evaluation of the effect of several formulation variables through a Box-Benken experimental design showed that an increase in drug load and GP content led to higher initial release and rate constants, whereas CS concentration did not substantially affect the release. Additionally, *in vivo* pharmacokinetic studies demonstrated a reduction of the maximum plasma drug concentration (C_{max}) and a prolongation of the mean residence time (MRT) of the drug after subcutaneous administration of the optimized VH-loaded hydrogel compared to a VH solution. Thus, the delivery of low molecular weight drugs may be prolonged for few hours to few days when the compounds are entrapped in CS / GP hydrogel.

The use of CS / GP hydrogel for the local delivery of anti-cancer drugs after intra-tumoral injection has been actively investigated. CS-based thermogels sustained the release of paclitaxel or camptothecin over more than 1 month *in vitro* [50,65]. The paclitaxel release rate was inversely correlated to the initial drug loading, since hydrogels loaded with 6.4 and 64 mg/ml paclitaxel resulted in release rates of 4.2 % and 2 % per day respectively. Paclitaxel is a hydrophobic molecule, which is therefore dispersed in the hydrogel. Thus, the concentration-dependent release rate is attributed to the necessity of a first dissolution step prior to diffusion from the matrix. *In vivo* efficacy of the CS / GP / paclitaxel formulation was assessed on an EMT-6 murine mammary carcinoma model, where one injection of the hydrogel showed similar inhibition of tumor growth than four intravenous injections of Taxol® while inducing less toxicity. Evaluation of the *in vitro* proliferation of two cancer cell lines (human U87 glioblastoma and rat C6 glioma) in contact with ellagic acid-incorporated CS / GP hydrogel demonstrated a significant and concentration-dependent reduction of the cell viability after 3 days incubation [42]. Another study tested the intratumoral injection of a doxorubicin-loaded CS / GP hydrogel in combination with a vaccinia virus-based vaccine as chemoimmunotherapy and showed improved antitumoral effect compared to monotherapy in

mice [66]. It may be noted that CS / GP hydrogels also demonstrated sustained *in vitro* release and *in vivo* activity of low Mw drugs in other applications than parenteral depots systems, in particular for wound dressing and inner ear drug administration [32,33,67,68].

2.3.2. CS / GP systems for the delivery of peptides and proteins

CS-based thermosensitive systems have also been studied for parenteral delivery of therapeutic peptides or proteins, due to their prolonged release properties but also due to their ability to protect the macromolecules against enzymatic degradation [12,69]. It was found that insulin loaded in the gel was released in its native form over about 2 weeks in phosphate buffer by diffusion [29]. A second *in vitro* release study of insulin-loaded CS / GP hydrogel confirmed this result, since a controlled release of the peptide over more than 10 days could be achieved [70]. The burst release of about 10 % observed in the first hours of the *in vitro* test was assigned to the lag time before complete gelation and to the immediate release of the insulin at the surface of the gel. Bovine serum albumin (BSA), used as a model protein encapsulated in CS-based hydrogel, showed a sustained *in vitro* release over about 2 weeks [71,72]. Shamji *et al* demonstrated that CS / GP thermogels can prolong the release of several anti-tumor necrosis factor alpha (TNFα) proteins, like soluble TNF receptor Type II (18.9 kDa) and a monoclonal anti-TNF antibody (150 kDa), up to 10 days while preserving their therapeutic activity in murine fibrosarcoma cells [72].

2.3.3. CS / GP systems for the delivery of vaccines

Gordon *et al* compared the potential of CS nanoparticles and CS / GP thermosensitive hydrogels for vaccine delivery, using ovalbumin (OVA) as a model protein antigen [73]. *In vitro* OVA release from CS hydrogel was slower than from CS nanoparticles (10 % versus 50 % release respectively in 10 days), presumably due to electrostatic interactions between the protein and the matrix. Raman spectroscopy indicated the existence of physico-chemical interactions between OVA and CS, in contrary to the observation of Ruel-Gariepy *et al* for the low Mw negatively charged calcein [14]. The electrostatic interactions between OVA and CS may be favored by the increased size and the high number of negatives charges of the protein compared to calcein at pH 7.4, which could behave as a cross-linker forming ionic bonds with the positively charged CS chains. Subcutaneous administration of OVA-loaded CS depot system induced both $CD4^+$ and $CD8^+$ T cells proliferation and OVA-specific antibody production in mice. Therefore, as opposed to OVA-loaded CS nanoparticles which showed no

substantial immune stimulation, CS / GP system containing the antigen showed promising results in the field of vaccines delivery.

2.3.4. CS / GP systems applications in cell encapsulation and tissue engineering

CS / GP hydrogels may be applied as injectable scaffolds mimicking the extracellular matrix. Cell encapsulation and tissue engineering through the use of CS-based thermogels has been investigated due to the proven biocompatibility, biodegradability, high water content and porosity of the system [74]. Dang *et al* reported the use of the hydrogel for the three dimensional culture of lymphoid cells, which showed good activity after 20 days [53]. The CS-based system was examined for the co-delivery of human mesenchymal stem cells (hMSC) and desferrioxamine (DFO) as a pro-angiogenic therapy for the treatment of critical limb ischaemia [75]. The formulation allowed the *in vitro* proliferation of the encapsulated hMSCs together with a sustained release of DFO over 7 days, resulting in an increased VEGF expression in human cells. Furthermore, significant improvement of stem cell engraftment, survival and homing by the use of CS / GP hydrogels as carriers were also reported [76]. This confirmed the feasibility of using CS-based gels as cell delivery vehicles. Therefore, CS-based thermogels have been extensively studied for bone and cartilage tissue engineering [77-80]. Hoemann *et al* showed that injecting a mixture of CS / GP and autologous whole blood in microfracture defects in sheeps significantly improved cartilage repair compared to control defects [81]. These results were confirmed in rabbits, where the injection of CS / GP / whole blood into marrow-stimulated chondral defects resulted in an increased synthesis of chondral repair tissue [82]. This innovative bio-orthopaedic product for cartilage repair, named BST-CarGel®, has received the European market approval in 2012. In the bone regeneration context, CS-based thermosensitive gels loaded with bioactive glass nanoparticles also proved their ability to induce the formation of bone-like apatite after immersion in simulated body fluid [83]. In a different approach, Kim and coworkers reported the use of the hydrogel for the local and prolonged delivery of osteoinductive growth factors [84]. Furthermore, CS / GP was recently reported for the delivery of demineralized bone matrix (DBM) powder [63]. *In vivo* studies in rats demonstrated a correlation between DBM content and inductive bone formation. Thus, biocompatible CS / GP hydrogels were identified as a promising material having the suitable properties for bone and cartilage regeneration applications, and can also be used as an appropriate depot that can stimulate cell proliferation and delivery.

3. Improvement of chitosan-based thermogelling systems

Since the discovery of the CS / GP system and the proof of its suitability in various pharmaceutical and biomedical applications, different strategies have been implemented to improve or modify the characteristics of the standard system. Depending on the intended use (e.g. drug delivery system, biomimetic scaffold, etc.), the thermogelling system properties could be varied by (*i*) the combination with carrier particles, (*ii*) the replacement of GP by other gelling agents (Figure I-3), (*iii*) the addition of another polymer to the system or (*iv*) the chemical modification of CS.

3.1. CS / GP systems combined with carrier particles

CS hydrogels may be coupled with drug-loaded carrier particles, i.e. microspheres, nanoparticles or liposomes, as a smart approach to further prolong the release period of the entrapped compound while maintaining the drug carrier at the desired site [85]. This approach is of particular interest for low Mw hydrophilic compounds, for which the delivery period from the standard CS / GP hydrogel is typically in the order of few hours to few days [14]. Ruel-Gariepy *et al* demonstrated good compatibility between carboxyfluorescein (CF)-loaded liposomes and the CS / GP hydrogel [86]. Addition of liposomes slightly reduced the sol / gel transition time and increased the hydrogel strength. *In vitro* studies showed a sustained release of CF from the liposome / hydrogel system over more than 2 weeks, compared to 1 day for CF-loaded hydrogel. The release profile was mainly dependent on the liposomes characteristics (e.g. size and composition). *In vivo* pharmacokinetics studies in rats showed a prolonged cytarabine delivery from liposome / hydrogel mixtures over more than 60 h, compared to shorter mean residence times (MRTs) of 47 and 10 h from conventional liposome suspensions or from CS / GP hydrogels respectively [87].

In a similar approach, the incorporation of meglumine antimoniate (MGA)-loaded CS-microspheres into the CS / GP system also significantly slowed down the *in vitro* release in PBS, with 22 % lower MGA released in the first 24 h compared to CS / GP hydrogel alone [37]. In another recent study, the *in vitro* release profiles of free insulin, insulin-loaded CS / GP and CS / GP containing insulin phospholipid complex-loaded PHBHHx (poly(3-hydroxybutyrate-co-3-hydroxyhexanoate)) nanoparticles (NP-CS/GP) were compared [44]. Complete release of the free insulin was achieved within 8 h, whereas the incorporation of the peptide into the CS / GP hydrogel or within nanoparticles entrapped in the hydrogel

significantly reduced the release rate, with respectively 30.45 % and 5.64 % of the total insulin released within 8 h. The same trend was observed after subcutaneous injection of the three formulations in diabetic rats. While the relative blood glucose level decreased to 70 % for only several hours with free insulin, the therapeutic effect was prolonged over 17 h for insulin-loaded CS / GP and over more than 5.5 days by using the NP-CS/GP system [44]. As part of the investigation of OVA-loaded CS / GP thermosensitive hydrogels as vaccine delivery system, the formulation was optimized by incorporating silica nanoparticles (SNP) containing OVA into the CS / GP solution [73]. This system demonstrated increased cell-mediated and humoral immune responses *in vivo* compared to OVA-loaded SNP administered in PBS, confirming therefore the ability of the combination of the CS / GP system with SNP to act as an effective vaccine delivery system [88].

Intratumoral injection of radioactive agents has been widely employed for cancer treatment. One of the major drawbacks associated with this approach is the leakage of the radionuclides from the tumor site to non-target organs or tissues. In order to overcome this problem, the combination of Rhenium-188 colloid and *in situ* gelling CS / GP solution for use in local radiotherapy was investigated [89,90]. Rhenium-188 colloid entrapped into the hydrogel was maintained at the injection site without remarkable diffusion after injection in rats, thereby reducing systemic exposure and associated toxicity. Furthermore, the intra-tumoral delivery of the isotope significantly reduced the hepatic tumor size from 1.5 cm^3 to around 0 cm^3 within 4 weeks.

3.2. Combination of CS and other gelling agents

3.2.1. Inorganic salts

The first attempt to replace GP by an inorganic phosphate salt was made with ammonium hydrogen phosphate (AHP) [91]. Viscosity measurements at 37°C confirmed the occurrence of the sol / gel transition in few minutes, depending on the AHP content. Furthermore, at equivalent molar concentration, gelation at body temperature was faster when using AHP compared to GP. CS / AHP was not a clear solution like the CS / GP, but rather translucent [92]. The delivery of a local anesthetic, ropivacaine, using a CS / AHP thermogel showed effective nerve blockade for up to 48 h in rats [60]. In a similar approach, dipotassium orthophosphate (DPO) also induced the thermo-gelation of CS solutions, while no sol / gel transition could be observed with monobasic phosphate salt and immediate precipitation

occurred with tribasic phosphate, due to the inappropriate pH ranges of these solutions [93]. CS / DPO solution loaded with doxorubicin successfully showed anti-tumor activity in a murine model [94]. Interestingly, the combination of CS and sodium hydrogen phosphate (SHP) underwent two phase transitions upon heating [95]. The system displayed a gel behavior below 30°C and above 43°C, and turned into a solution in between. Despite this unfavorable thermogelling behavior, camptothecin nanocolloids loaded-CS / SHP hydrogel could be formed 2 h after subcutaneous injection in mice [96]. Further investigations questioned the potential of CS / inorganic phosphate salts as ISFDs. Microscopic analyses of these systems recently showed inhomogeneous dispersions rather than clear solutions at low temperatures, with the formation of gel-like precipitates instead of homogeneous gel upon heating [97]. Indeed, addition of dibasic phosphate salts to CS solutions resulted in a salting-out effect (i.e. CS solubility was decreased, resulting in its precipitation). This effect was observed for different CS : phosphate salt ratios even at low temperatures [98]. These results highlight the essential role of the polyol-moiety of GP in preventing immediate CS precipitation at physiological pH. Liu *et al* adopted a different approach by adding sodium bicarbonate ($NaHCO_3$) to the CS solution. Bicarbonate ions increase the pH of the CS solution by reacting with H^+ ions and by releasing CO_2 [99]. Therefore, sol / gel transition occurred due to the slow CS neutralization induced by CO_2 generation at physiological temperature. Thus, the CS / $NaHCO_3$ solution remained stable in sealed and CO_2 concentrated vials and turned into a gel once CO_2 was released. Despite the long gelation time observed *in vitro* for this system, subcutaneous injection *in vivo* in rats confirmed its *in situ* gelling behavior.

3.2.2. Polyols and polyoses

Several phosphate-free polyols have also been evaluated as gelling agents [45,100]. Addition of sodium hydroxide was necessary to increase the pH in the vicinity of 6.8, since a pH value above the CS pKa is essential to the gelation of the polymeric solution. This way, the addition of 1,3-propanediol, 1,2-propanediol, glycerol, mannitol or trehalose to the CS solution led to thermally-induced gelation. However, the gelation times at 37°C observed for these systems varied between 60 min and 120 min, making them inappropriate as ISFD drug delivery systems. It can be noted that CS / mannitol and CS / trehalose solutions allowed to improve the shelf-life of the formulations by lyophilization, but with significant changes in

the thermogelling behavior (e.g. gelation time, gel strength) compared to freshly prepared solutions.

3.2.3. Polyol-phosphates

Beside the widely used GP salt, only few other polyol-phosphate salts have been tested with regards to their capacity to induce thermosensitive gelation of CS-based solutions for parenteral applications. Recently, CS / glucose 1-phosphate and CS / glucose 6-phosphate disodium salt solutions were reported as thermogelling systems. However, these systems displayed longer gelation times at 37°C compared to CS / GP solution of equivalent molar concentration, due to the stabilizing effect of the glucose-moiety on the polymer [24]. Thermogelling CS solutions have also been prepared by adding glucosamine carbonate or glucosamine phosphate solutions as gelling agents [101]. However, these solutions, prepared by co-dissolving glucosamine hydrochloride with sodium carbonate or tribasic potassium phosphate, were unstable and potentially underwent Maillard reaction at room temperature, as indicated by the brownish coloration of the solutions.

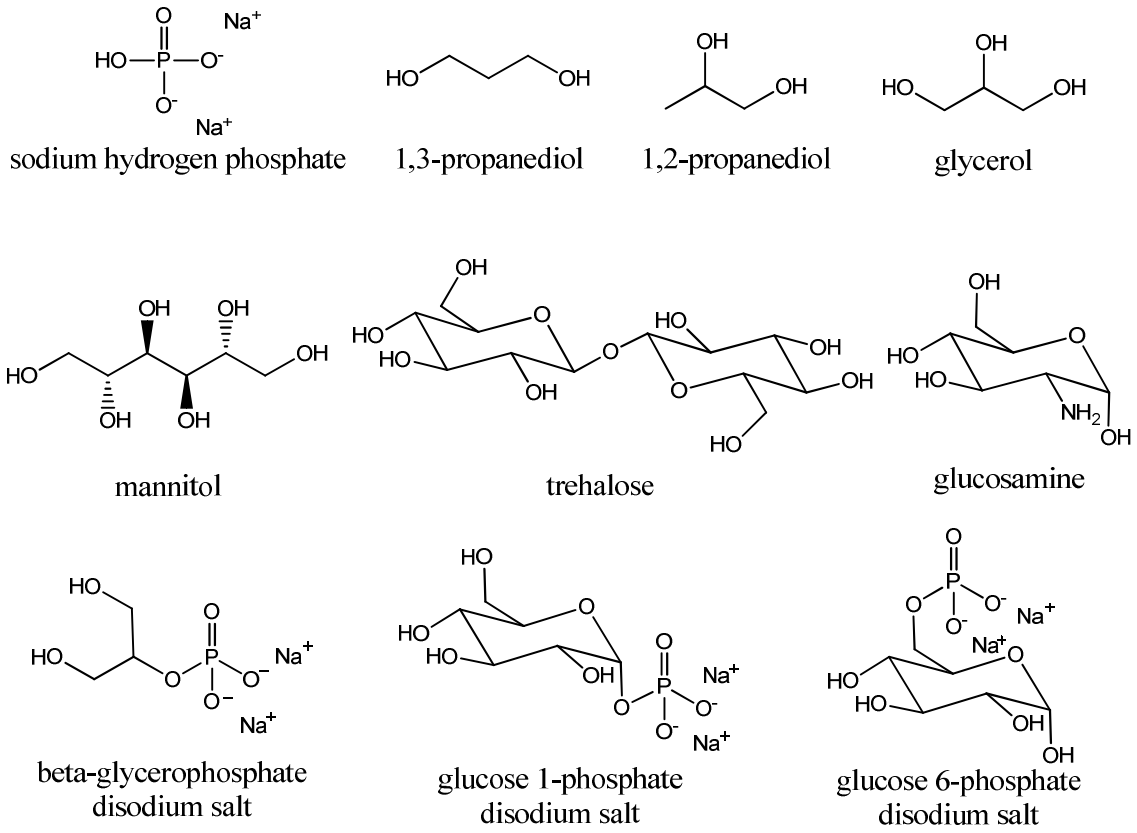

Figure I-3: Molecular structures of various gelling agents used for chitosan-based thermogelling solutions.

3.3. Combination of CS / GP and other polymers

3.3.1. Natural polymers

Several groups evaluated the benefits of the addition of a second biocompatible natural polymer to the CS-based solution. Indeed, CS / GP may lack appropriate mechanical properties for given applications, especially for cell carrier systems. To circumvent this limitation, blending the CS / GP system with another polymer has been proposed. Due to their well-known biocompatibility, biodegradability and ability to support cellular growth, collagen and its derivative, gelatin, were among the first polymers tested. Thermosensitive hydrogels based on CS / gelatin / GP blends have been successfully used for nucleus pulposus (NP) regeneration, either as a cell carrier for NP cells, or as a controlled release system for ferulic acid delivery [102,103]. Addition of 2 % gelatin in the CS / GP solution reduced the gelation temperature from 36 to 31°C and shortened the gelation time from 10 min to 2.3 s at 37°C. Mechanical strength of the hydrogel also increased proportionally to the gelatin concentration.

For instance, the gel strength increased 600-fold with the addition of 2 % gelatin in the CS / GP system, due to the enhanced molecular interactions between gelatin and CS. Furthermore, the CS / gelatin / GP hydrogel kept the same good biocompatibility properties as the CS / GP hydrogel, as demonstrated via cytotoxicity studies on NP cells. Nevertheless, the addition of gelatin reduced the stability of the solution at ambient temperature. No gelation occurred within 15 min for the CS / GP solution while addition of 2 % gelatin led to sol / gel transition within 8 min at 25°C, thereby impeding the handling of the solution before injection. Yang and coworkers added mouse insulinoma, encapsulated as microspheres, in the CS / gelatin / GP solution [104,105]. Subcutaneous injection in diabetic rats led to insulin secretion and allowed maintaining the non-fasting blood glucose concentration below 200 mg/dL for 25 days. Therefore, CS / gelatin / GP hydrogel has the potential to be used as cell carrier for an injectable bioartificial pancreas.

Similarly, CS / collagen / GP solutions also turned more rapidly into gels at physiological conditions than CS / GP. For instance, thermogelling solutions composed of a 65 : 35 CS : collagen ratio showed a gelation time of about 5 min, versus 18 min for CS / GP. Additionally, the stiffness of the hydrogel containing collagen was 3-fold higher than CS / GP gels, due to the collagen fibrillogenesis induced by the addition of GP as a weak base and the temperature [106,107]. SEM images showed the collagen fibers inserted in the CS network, resulting in narrower pore size than pure CS-based gels. *In vitro* studies demonstrated that the addition of collagen enhanced the proliferation of several types of human stem cells within the matrix [108]. The feasibility of using CS / collagen / GP system as injectable *in situ* forming scaffold for cell delivery was assessed in rats, where the encapsulated cells survived over at least 28 days in the matrix [109,110].

Due to its natural origin and presence in many tissues, hyaluronic acid is also well suited for biomedical applications. Biodegradation studies showed a slightly slower degradation rate for CS / oxidized hyaluronic acid (HDA) / GP hydrogels compared to CS / GP. However, the addition of HDA into thermosensitive CS / GP solutions induced an almost instantaneous gelation, due to the formation of Schiff's base links between CS amino groups and HDA aldehyde groups, as confirmed by infrared spectroscopy [111]. The lack of thermosensitivity of the HAD-containing hydrogels made them unsuitable for *in situ* forming depot applications. Nevertheless, the covalent crosslinking between CS and HAD increased the stability of the hydrogel, which can support cell encapsulation and growth for up to 1 month.

CS / starch / GP combinations with a CS : starch ratio of 4 : 1 exhibited a reduced gelation temperature of 37°C compared to the 40 - 44°C observed for CS / GP solutions [112]. However, addition of higher starch content led to an immediate precipitation of the polymeric solution upon GP addition, thereby suppressing the thermosensitivity of the system. Pre-gelatinized starch addition significantly affected the structure of the hydrogel by enlarging the average hydrogel pore size (26.4 µm versus 19.8 µm without starch). Consequently, the water absorption capacity increased from 13 to 20 % when starch is added to the hydrogel. *In vitro* enzymatic degradation test in PBS containing 0.02 mg/mL lysozyme showed a decrease of the wet weight of the CS / starch / GP hydrogel from 100 % to 67 % within 56 days, compared to 79 % observed for the standard CS / GP. These results suggest a higher degradation rate for the system combined with starch, due to the looser polymeric network and, as consequence, enhanced accessibility of lysozyme. *In vitro* cell culture indicated that CS / starch / GP hydrogel could promote the proliferation of chondrocytes while maintaining their phenotype. Furthermore, subcutaneous injection in rats confirmed the ability of the solution to form a depot *in situ*. In view of the good cell viability results, faster gelation kinetic and higher degradation rates observed for the CS / GP system containing starch as an additional polymer, this hydrogel present desirable properties for use as an injectable scaffold for tissue engineering. Nevertheless, due to the looser network and larger pore size compared to the standard CS / GP hydrogel, this system may not be appropriate for sustained drug delivery, especially for low Mw compounds.

3.3.2. Synthetic polymers

The incorporation of a synthetic polymer into the CS / GP system also demonstrated slightly modified physical properties of the hydrogels. Concentrated methylcellulose solutions underwent gelation on their own upon heating, due to intra- and inter-molecular hydrophobic interactions. However, due to sol / gel transition temperatures in the range of 50 – 70°C, methylcellulose solutions are not appropriate as ISFD systems. Addition of methylcellulose solution to the CS / GP solution did not change significantly the gelling behavior of the system, since only a slight sol / gel transition temperature increase from 32°C to 33°C was observed [113]. High mechanical strength and uniform microporous network structure of the CS / methylcellulose / GP hydrogel were showed by rheological and SEM microscopic analysis. Cell viability assay indicated that thermogelling CS / methylcellulose / Na_3PO_4 blend is promising as a suitable injectable scaffold for chondrocytes for tissue engineering. In

a similar approach, CS / hydroxyethyl cellulose (HEC) / GP thermosensitive solution have been developed and tested as a cell carrier for cartilage repair by Hoemann *et al* [114]. The study compared the thermogelling behavior of CS / GP and CS / HEC / GP solutions prepared with similar CS and GP concentrations and with different grades of HEC. The results showed that no sol / gel transition occurred for the CS / GP solution at 37°C, due to the low level of GP which did not allow sufficient neutralization of the solution (pH 6.8). Interestingly, gelation of the system could be induced by commercial-grade HEC in 10 to 30 min, whereas medical-grade HEC also failed to induce gelation of the system. Further investigations revealed that glyoxal, an additive commonly used to delay hydration and prevent lumping of HEC during industrial processing, was responsible of the CS / HEC / GP system gelation by covalently cross-linking CS. Therefore, in contrast to the standard CS / GP solution, gelation did no longer occur only through physical interactions (i.e. hydrogen bonding and hydrophobic interactions) but via chemical cross-linking. Moreover, this system might not be suitable as parenteral ISFD depot system due to the reported cytotoxicity of glyoxal on fibroblast cell.

CS / poly(vinyl alcohol) (PVA) blends, mixed with sodium bicarbonate as thermogelling agent, were also reported as forming hydrogels under physiological conditions [115]. PVA is a hydroxy polymer that can stabilize CS at low temperature by the formation of a water shield around the polymer chains, similarly to the glycerol part of GP. Upon heating, the water molecules protecting CS are removed and CS chains associate via hydrophobic interactions, leading to gel formation. Due to the protective effect of PVA, the gelation temperature and time increased with the PVA content. The addition of 1 to 5 wt.% PVA into a 1 wt.% CS solution raised the gelation temperature from 21 to 34°C, extended the gelation time from few seconds to about 8 min and decreased the gel strength from around 2 to 0.3 Pa at 40°C, indicating the formation of relatively weak gels. SEM imaging showed that increasing PVA concentrations from 0.2 to 5 wt.% also significantly reduced the pore size of the hydrogel, due to physical entanglements between CS and PVA. Owing to the increased lag time for gelation when PVA is added to the CS solution, this system revealed an initial burst of the entrapped compounds. For instance, BSA-loaded CS / PVA / $NaHCO_3$ hydrogel showed a release of around 40 % of the entrapped protein in 5 h [115], whereas less than 5 % release was observed from a BSA-loaded standard CS / GP hydrogel within the same time frame by Kim *et al* [71]. Nevertheless, the protective effect of PVA might be able to prolong the stability of the system at ambient temperature or refrigerated conditions. Further

investigations would be needed to determine the improved stability properties of CS / PVA compared to the pure CS-based system. As previously observed for the standard CS / GP system, the addition of a drug carrier, like CS nanoparticles or hydroxyapatite, to the CS / PVA system reduced the burst and prolonged the release of several model compounds [116,117]. For example, the release of diclofenac sodium decreased from 19 % to 10 % in 5 h when the drug was loaded into positively charged CS nanoparticles before addition to the hydrogel, and CS / PVA hydrogels containing hydroxyapatite prolonged the release of BSA over more than 48 h, versus 24 h for the pure hydrogel [117].

3.4. Modified-CS / GP thermogelling systems

Chemical modification of CS has been reported as an additional way to improve the physico-chemical properties of CS-based thermosensitive hydrogels (Figure I-4). As mentioned previously, quaternized CS, also named N-[(2-hydroxy-3-trimethylammonium) propyl] chitosan chloride (HTCC), was synthesized by the reaction of CS and glycidyltrimethylammonium chloride [118]. HTCC showed enhanced properties compared to CS, e.g. water solubility, permeation enhancing effects and bioadhesive properties. Furthermore, in contrast to the turbid CS / GP hydrogels obtained when using highly deacetylated CS, HTCC / GP hydrogel prepared with 89 % deacetylated CS were transparent. HTCC / GP hydrogels also exhibited improved pH sensitivity due to the increased amount of amino groups on CS [119]. pH-sensitive swelling studies showed that standard CS / GP hydrogel shrank when placed either in acidic or basic solutions, whereas HTCC / GP hydrogel dissolved quickly in acidic solutions and swelled slightly in basic solutions. The solution could therefore be used as an intelligent carrier system which turned into a gel at physiological temperature and released the incorporated drug substance in a pH dependent manner. This was confirmed by release studies performed with doxorubicin (DOX) as model drug. While almost 80 % DOX was released in 6 h in buffer solution at pH 5, only 9 % was release within the same period of time at pH 8, with a prolonged release over more than 4 days. However, the viscosity of the hydrogel obtained after 30 min at 37°C decreased 5-folds when using HTCC instead of CS. This may render the HTCC / GP hydrogel unsuitable for specific applications, like the use as scaffold for tissue engineering, where high gel strength is required. Therefore, mixtures of CS and HTCC with GP have been developed and the obtained thermosensitive hydrogels demonstrated excellent *in vitro* and *in vivo* biocompatibility. *In vitro* release profiles obtained for ornidazole-loaded hydrogels in buffer

pH 6.8 showed about 60 % and 90 % of drug released from the CS / HTCC / GP and the CS / GP hydrogels respectively within 3 h, demonstrating that the addition of HTCC slowed down the release, presumably due to enhanced hydrophobic interactions in the system [120]. Furthermore, basic fibroblast growth factor loaded in the gel showed enhanced periodontal regeneration of the tissues in dog compared to the CS / HTCC / GP hydrogel alone, thereby confirming the potential of this system as a tissue engineering scaffold for periodontal treatment [121].

Thiolated CS (CS-TGA) / GP thermosensitive hydrogel was also investigated as a double crosslinking hydrogel with improved mechanical properties [122]. CS-TGA was obtained by the covalent binding of TGA to the CS amine functions. The system combined physical crosslinking via CS / CS hydrophobic interactions and hydrogen bonding, governed by the gelling agent GP and temperature, and chemical crosslinking via the disulfide bonds. The cumulative effect of this double crosslinking confers an increased mechanical strength to the hydrogel compared to the reference CS / GP gel. CS-TGA / GP hydrogel exhibited a sol / gel transition within 2 min at physiological temperature and sustained the release of the model protein BSA over about 2 weeks, similarly to what has been observed for standard CS / GP hydrogel by Shamji *et al* [72]. *In vitro* cytotoxicity and *in vivo* biocompatibility studies revealed a durable and biocompatible gel with good potential as an injectable ISFD drug delivery system.

Finally, CS modification by partial substitution with hydrophilic carboxymethyl groups and hydrophobic hexanoyl groups allowed obtaining amphiphilic carboxymethyl-hexanoyl-CS (CHC). CHC / GP mixture was investigated for the formation of a thermogelling network [123]. Due to its amphiphilic nature, CHC self-assembled into positively charged nanocapsules in aqueous solutions. In the presence of GP and upon increasing the temperature to 37°C, the CHC-nanocapsules aggregated to form a gel in few minutes. Microscopy analysis confirmed the presence of nanocapsules in the formed hydrogel. Thus, the CHC / GP hydrogel structure is considerably different compared to the conventional CS / GP network. Unlike the standard CS / GP hydrogel which remains a stable depot, the CHC / GP nanogels disintegrated into small fragments upon addition in aqueous media, thereby questioning the ability of the CHC / GP system to be used as parenteral ISFD drug delivery system. However, CHC / GP nanocapsule-based hydrogel loaded with ethosuximide, a highly water-soluble antiepileptic drug, was subcutaneously administered in rats and a therapeutic effect was evidenced up to 4 days. Furthermore, a simultaneous clearance of the gel from the injection

site was revealed by noninvasive magnetic resonance imaging. This result, together with the fast disintegration observed *in vitro* in aqueous media, suggest that CHC / GP system degrade much faster than the reference CS / GP hydrogels due to its nanocapsule-gel structure.

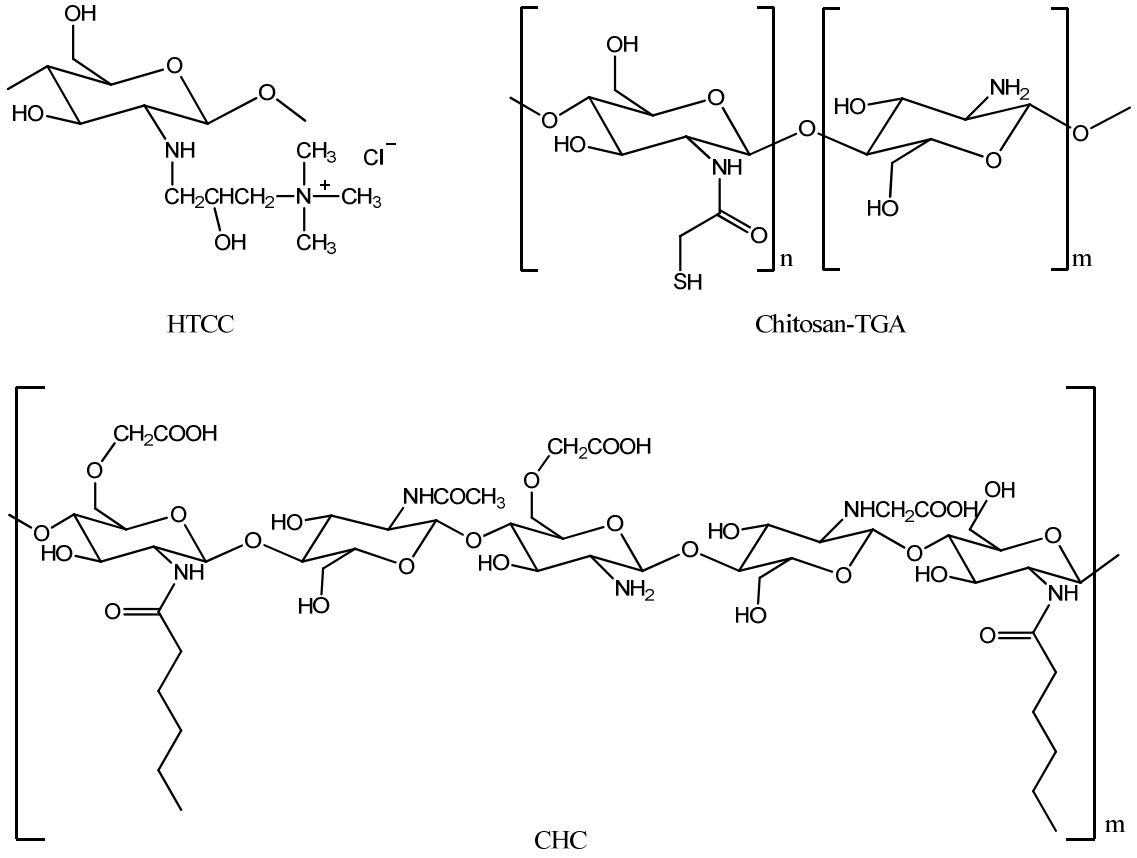

Figure I-4: Molecular structure of modified-chitosans used in CS-based thermogelling solution (N-[(2-hydroxy-3-trimethylammonium) propyl] chitosan chloride (HTCC), thiolated CS (CS-TGA) and carboxymethyl-hexanoyl-CS (CHC)).

4. Conclusion

In this review, the recent advances in the development of injectable thermosensitive CS-based hydrogels were summarized. The aqueous polymeric solutions obtained at room temperature or below undergo sol / gel transition upon heating at physiological temperature. This thermogelling property led to extensive investigations as suitable *in situ* forming depots systems for various pharmaceutical and biomedical applications. The polymeric solution could be easily mixed with drug substances and/or cells at low temperature, and formed a semi-solid depot after injection. The standard CS / GP system showed prolonged release from several hours to days for hydrophilic and low molecular weight compounds, and up to weeks

for poorly water soluble drugs, peptides and proteins, with the additional benefit to protect macromolecules against physiological degradation. CS / GP hydrogels may also serve as scaffold for tissue engineering, due to their biomimetic properties, porous structure and biodegradability. Implementation of several strategies to improve the conventional system (e.g. use of different gelling agents or chemically modified CSs, addition of carrier particles or a second biodegradable polymer in the system) showed great potential, notably in the field of drug delivery.

5. Expert opinion

Parenteral injectable formulations which turn into depots *in situ* by temperature modulation are interesting technologies for various pharmaceutical and biomedical applications. Among these systems, CS / GP thermogelling solutions, that undergo sol / gel transition at physiological temperature, have been extensively investigated for their interesting characteristics, including the absence of organic solvents or of toxic crosslinking agents, the biocompatibility and biodegradability of the system and its use for various applications such as drug delivery, cell encapsulation and tissue engineering. As reported in the current review, the solutions properties (e.g. viscosity, stability, injectability), thermogelling properties (i.e. sol / gel transition time and temperature) and the final hydrogels characteristics (e.g. gel strength, morphology, turbidity, biodegradability, drug release profile) can be fine-tuned by varying the Mw and DD of the polymer as well as by varying the formulation parameters (e.g. ratio of CS and gelling agent, pH, acidic solution). However, further adaptations of the conventional thermogel release properties or mechanical characteristics might be necessary for specific applications. For instance, combination with PHBHHx-nanoparticles, CS-microspheres or liposomes allowed prolonging the release of low Mw compounds from several hours or days to weeks, while reducing significantly the initial drug burst. Thus, continuous delivery over a period of several months may be achieved by entrapping drug-loaded long-term sustained release carriers, such as poly(*D,L*-lactide-co-glycolide)-microspheres, in the CS / GP depot system. Likewise, addition of a second biodegradable polymer, natural or synthetic, could improve the mechanical strength of the hydrogel, making it more suitable as cell delivery or tissue engineering scaffold.

In spite of the considerable efforts made recently to improve the CS-based thermosensitive depot system, several limitations remain to be overcome. CS, as a natural

polymer, is subjected to batch to batch variability that should be controlled, in view of the major role of the polymer characteristics (e.g. DD, Mw, viscosity) in the gelation mechanism and final hydrogel attributes. Furthermore, CS is not yet approved by the FDA for parenteral applications although it has shown great potential for medical/pharmaceutical applications, *in vitro* as well as *in vivo*. As for all ISFD systems, the control of the shape and size of the final depot formed at the injection site remains a challenge, and may lead to heterogeneous *in vivo* drug delivery kinetics and degradation rates. In our opinion, the combination of several strategies, such as the use of additional polymers combined with carrier particles and / or chemically modified CS, could be a smart approach to further optimize the stability, gelling behavior and drug release properties of these promising systems.

6. Article highlights

- Thermosensitive CS-based hydrogels are of great interest as drug delivery systems or tissue engineering scaffolds since they are water-based, non-toxicity, biocompatible and biodegradable.

- Synergistic mechanisms (electrostatic repulsion, hydrophobic interactions, hydrogen bonding) are involved in the thermo-induced sol / gel transition of the CS / gelling agent systems.

- Thermogelling behavior and hydrogel characteristics can be fine-tuned by varying the formulations parameters such as CS Mw and DD, ratio of CS and gelling agent, pH, acidic solution.

- The major impediments of these systems are the lack of stability at or below room temperature, the high release rate observed for low molecular weight hydrophilic drug substances and the weak mechanical strength of the hydrogel.

- Use of other gelling agents than the conventional GP, combination of the system with additional biodegradable polymers or use of chemically-modified CS are smart approaches to overcome the limitations of the CS-based thermogels.

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Chapter II :

Rheological study of chitosan/polyol-phosphate systems: influence of the polyol part on the thermo-induced gelation mechanism

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Abstract

Thermo-sensitive gelling systems, like chitosan / polyol-phosphate, are candidates with a high potential for the design of biodegradable drug delivery systems, notably for *in situ* forming depots. They consist of stable and low viscosity aqueous solutions, liquid at room temperature, which turn into a gel state upon an increase of temperature (e.g. after subcutaneous administration). This technology enables a sustained release of potentially encapsulated active substances. Despite these thermo-gelling solutions being widely studied for the development of parenteral drug delivery systems, most commonly using β-glycerophosphate (β-GP) as gelling agent, the mechanism inducing the gelation and the role of the polyol part in this mechanism has not been clearly elucidated. In order to investigate the mechanism of the gelation process, comprehensive rheological studies were performed, comparing different chitosan / polyol-phosphate systems varying in the chemical structure of the polyol parts of the gelling agents. As reference, β-GP was compared to glucose-1-phosphate (G1-P) and glucose-6-phosphate (G6-P), and to a polyol-free phosphate salt, Na_2HPO_4. Frequency sweep experiments at different temperatures or different gelling agent concentrations, temperature and time sweep tests, completed by ^{31}P-NMR and pH measurements, were performed as complementary experimental approaches. The results disclosed significant trends with widespread implications, establishing a relationship between the chemical structure of the polyol part and the macroscopic gelling behavior of the solutions, i.e. transition temperature, gelation time and gel strength. The new results presented in this study show that increasing the size of the polyol part prevents the interactions between the chitosan chains, strongly influencing the gelling process.

1. Introduction

The development of thermo-sensitive gelling systems has received considerable attention in recent years. Typically, these formulations are polymeric aqueous solutions of low viscosity at room temperature, and undergo a sol/gel transition upon a temperature rise, intrinsically induced by the *in vivo* parenteral injection. These systems offer several advantages: *(i)* ready-to-use formulations, *(ii)* easy administration, *(iii)* easy and cost-effective manufacturing, *(iv)* absence of any organic solvents, and mostly *(v)* these are simple systems to control the sustained release of drugs or to be mixed with cells for tissue engineering applications [1-3].

Chitosan (CS) is a biopolymer obtained by partial deacetylation of chitin from crustacean shells. It is a linear polysaccharide composed of glucosamine units (β (1–4)-linked 2-amino-2-deoxy-D-glucose) and N-acetyl glucosamine units (2-acetamido-2-deoxy-D-glucose). CS possesses well-known beneficial biological properties like low toxicity, biocompatibility and biodegradability and has therefore attracted a lot of attention in the pharmaceutical field, *e.g.* for the design of drug delivery systems [4-6]. CS is typically only soluble in acidic aqueous media, at a pH lower than the CS pK_{ap} around 6.2. At low pH, the free amine functions are protonated, giving rise to electrostatic repulsion between the polymer chains, and thus allowing their solvation. In contrast, at pH higher than 6.2, the neutralization of the CS chains induces the formation of gel-like precipitates [7].

In 2000, Chenite *et al.* [8] developed a new thermo-gelling system based on the combination of CS and β-glycerophosphate (β-GP). The addition of β-GP increased the pH, but without phase separation or gelation. The ternary system CS / β-GP / water remained in a liquid state at low temperatures but underwent a sol / gel transition when heated at 37°C. This new gelling system opened new doors for the design of *in situ* forming drug delivery systems, and has been extensively studied for that purpose [9-12]. As well, in recent years, many research efforts were dedicated to understand the gelation mechanism at the molecular level. Overall, the sol / gel transition can be summarized as representing a competition between several intermolecular interactions, including: *(i)* the electrostatic repulsion between positively charged CS molecules, *(ii)* a screening effect on this repulsion, induced by the negatively charged β-GP and *(iii)* attractive interactions due to hydrophobicity and hydrogen bonding between the CS molecules [13-16]. It is noteworthy that these three points do not

explain the effect of temperature on the gelation, which is likely related to the glycerol moiety of β-GP, and to the thermo dependence of the pK_{ap} of CS that allows the proton transfer between CS and β-GP [14,17].

Even though a large number of studies were focused on this CS / β-GP system, the role of the polyol moiety of β-GP in the thermo-gelling mechanism has not been clearly elucidated. Several studies [18-20] assumed that the glycerol part creates a protective hydration layer around the CS chains, thus preventing their self-interactions and aggregation, at low temperature and even at neutral pH. On the other hand, other studies [14,21-23] showed that inorganic phosphate salts alone (*i.e.* without polyol moieties, like ammonium hydrogen phosphate, dibasic sodium phosphate or dipotassium hydrogen orthophosphate) could also confer to the CS a thermo-sensitive gelling behavior. Finally, different authors [24,25] have shown the role of polyols in the thermo-gelling properties of the CS solutions. They demonstrated that solutions of CS and phosphate-free polyols (gelling agents), with a control of pH around 6.5 - 6.8 achieved by addition of sodium hydroxide, have a sol / gel transition sensitive to temperature as well.

To summarize, even if the physicochemical and rheological properties of given CS / gelling agent systems are well known and characterized, the impact of the chemical structure of the gelling agents, and notably the polyol group, on the overall gelation behavior, is still not well understood and elucidated. The present study is precisely focused on this open question, in order to disclose the role of the polyol-moiety in the gelation mechanism at the molecular level. After a preliminary screening of potential gelling agents able to maintain the thermosensitive behavior of CS-based systems, we propose a comparative rheological study of different thermo-gelling CS / polyol-phosphate systems, only varying in the chemical structure of the polyol groups. The chosen representative gelling agent candidates were: β-GP (as reference), α-D-glucose 1-phosphate (G1-P), D-glucose 6-phosphate (G6-P), and inorganic phosphate salt (without polyol group) [26,27]. The different thermo-gelling systems were compared and studied through sweep tests, as a function of several physicochemical parameters impacting on the gelation mechanism, such as concentration, temperature and time.

2. Materials and Methods

2.1. Materials

A CS of technical grade: Chitoscience® 90/50 (deacetylation degree (DD) 89.7 %, Mw: 122 kDa) and a CS of pharmaceutical grade: Chitoceutical® 90/50 (DD 90.7 %, Mw: 135 kDa) were used in this study. Both CSs are derived from shrimp shell and were purchased from Heppe Medical Chitosan (Halle, Germany). *Gelling agents* (see formulas in Figure II-1): ammonium hydrogen phosphate ($(NH_4)_2HPO_4$, Mw: 132.1 g/mol), disodium hydrogen phosphate (Na_2HPO_4, Mw: 141.96 g/mol), β-glycerophosphate disodium salt hydrate (β-GP, Mw: 288.10 g/mol), α-D-glucose 1-phosphate disodium salt hydrate (G1-P, Mw: 376.16 g/mol) and D-glucose 6-phosphate disodium salt hydrate (G6-P, Mw: 358.15 g/mol) were purchased from Sigma-Aldrich. The variation of pKa with temperature for the polyol-phosphate compounds have been already reported in literature [28-30]. The thermo-dependence of the pK_{ap} of CS (-0.023 pK units/°C) is significantly higher than the ones of the studied polyol phosphates (< +0.002 pK units/°C) [14]. α-methoxy-ω-phosphate polyethylene glycol (MP-PEG, Mw: 500 g/mol) was kindly provided by Chemicell (Berlin, Germany). Hydrochloric acid was purchased from Merck. All other reagents were of analytical grade and were used without further purification. Ultrapure® water was obtained using a MilliQ® Millipore filtration system (Millipore, Molsheim, France).

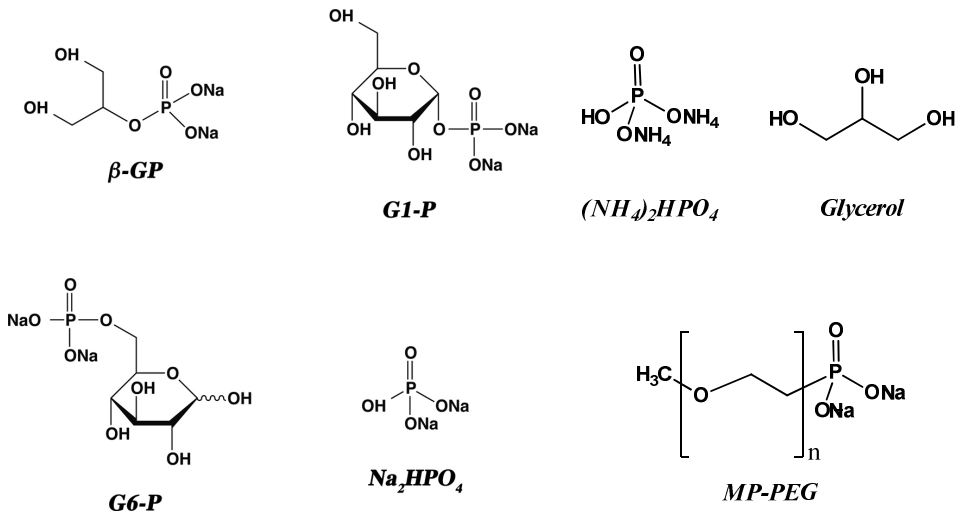


Figure II-1: Molecular structures of the gelling agents studied: β-glycerophosphate (β-GP), α-D-glucose 1-phosphate disodium salt (G1-P), $(NH_4)_2HPO_4$, Glycerol, D-glucose 6-phosphate disodium salt (G6-P), Na_2HPO_4 and α-methoxy-ω-phosphate polyethylene glycol (MP-PEG).

2.2. Preparation of chitosan / gelling agent solutions

Effect of gelling agent type on thermogelling behavior: a 2.2 wt.% CS solution was prepared by dissolving Chitoscience® 90/50 in hydrochloric acid (HCl) 0.1 M overnight at room temperature under constant magnetic stirring. The solution was subsequently filtered through 11 µm pore membranes and precooled to 4°C for 15 minutes. Gelling agent solutions were prepared in MilliQ water and chilled along with the CS solution. An appropriate amount of gelling agent solution was then added drop-by-drop to the CS solution under magnetic stirring and cooling in an ice bath. After mixing, the CS / gelling agent solutions were stirred for another 15 minutes. The resulting formulations, composed of 1.7 wt.% CS and between 0.2 and 0.4 mmol/g gelling agent, were stored at 4°C. The appearance, pH value and thermo-induced gelation ability were evaluated for each CS / gelling agent system.

CS / gelling agent systems used for rheological study: CS solutions were obtained by dissolving 3.75 wt.% Chitoceutical® 90/50 in HCl to a molar ratio of 0.9:1 with CS amine groups. Gelling agent solutions were prepared in MilliQ water. CS and gelling agent solutions were precooled to 4°C for 15 minutes. Then, solution of gelling agent (6 g) at fixed concentrations (between 50 and 350 mg/ml) was added dropwise to CS solution (4 g), under gentle magnetic stirring and cooling in an ice-bath. After mixing, the CS / gelling agent solutions were stirred for another 15 minutes. The resulting formulations were stored at 4°C. The CS / β-GP, CS / G1-P and CS / G6-P systems were transparent, clear, and homogeneous solutions with pH (at 4°C) ranging from 7.2 to 7.5, for concentrations of gelling agents ranging from 0.17 to 0.43 mmol/g. In contrast, the CS / Na_2HPO_4 systems appeared as inhomogeneous white dispersions, with pH (at 4°C) ranging from 7.6 to 7.9 for concentrations of disodium hydrogen phosphate ranging from 0.17 to 0.43 mmol/g.

2.3. Determination of gelation ability

The gelation ability of CS / gelling agents systems was determined in glass vials at 37°C. 1 mL CS / gelling agent system was injected in a 10 mL vial (inner diameter: 22 mm) at 37°C, in a temperature-controlled bath. In order to control whether the solution underwent gelation, the vial was inverted horizontally every 30 s (without removing it from the bath) over 30 min, and gelation was defined as the state when the solution no longer flowed.

2.4. Rheological measurements

The rheological measurements were performed with a Haake Rheostress1 rheometer (Thermo Fisher Scientific, Karlsruhe) with a circulating environmental system for the control of temperature. The viscoelastic properties of the CS / gelling agent samples were assessed by measuring the storage modulus (G') representing the elastic behavior of the material, and loss modulus (G") reflecting the viscous behavior. 8 mL of freshly prepared degassed sample were introduced between the concentric cylinders and then covered with a solvent trap to prevent evaporation during processing. The value of the strain amplitude was set at 10 % and was checked to ensure that all measurements made with CS / polyol-phosphate systems were carried out within the linear viscoelastic range, where G' and G" are independent of the strain amplitude.

Rheological characterizations of the CS / gelling agent aqueous solutions consisted of different experiments as follows. Dynamic frequency sweep tests were performed at 10, 20, 25, 30, 35 and 40°C at fixed gelling agent concentration (0.26 mmol/g) and *vice versa* at fixed temperature for different gelling agent concentrations, 0.17, 0.26, 0.35 and 0.43 mmol/g. G' and G" were measured over angular frequencies (ω) increasing through logarithmic steps, from 0.1 to 100 rad/s. Likewise, gel strength was evaluated in terms of the value of G' at low frequencies. Temperature sweep tests were carried out at a constant frequency (of 1 rad/s), according to a temperature increase from 7 to 50°C, with a rate of 0.5°C/min. Gelation time of the thermo-gelling systems was assessed by time sweep tests, at constant angular frequency of 1 rad/s and constant temperature of 37°C. Finally, thermo-reversibility of the hydrogels was investigated by increasing (from 7 to 50°C) and decreasing (from 50 to 7°C) the temperature, with a rate of ± 0.5°C/min. For all experiments, the sol / gel transition temperature (T_{gel}) or time point (t_{gel}) correspond to the intersection of the curves of G' and G".

A remark on the rheological behavior of pure chitosan solutions

It is noteworthy that the rheological behavior of unlinked polymer solutions, like CS in an acidic aqueous solution, showed the formation of a temporary network in the high frequency ranges. This phenomenon is not related to the mechanisms described here involving the formation of a gel. Nevertheless, it is important to consider it in the discussion. This phenomenon is due to the fact that, at high frequencies, the CS molecules could no longer glide along each other, gradually inducing entanglements, giving rise to a temporary gel-like

rheological behavior. As an illustration, Figure II-2 reports frequency sweep tests of a CS solution (1.5 wt.%, at pH 5.8). In the low frequencies range, the curves show the dominance of the viscous portion (G" > G'), the polymer exhibits the behavior of a viscoelastic liquid. At higher frequencies (in gray in the figure), the results indicate a slight dominance of the elastic portion (G' > G"). In addition, since similar curves were obtained for 20, 30 and 40°C, it is clear that this phenomenon does not influence the thermo-gelling process studied herein. However, it was decided to systematically show this part of the results in the present study underlayed in grey like shown in **Figure II-2**.

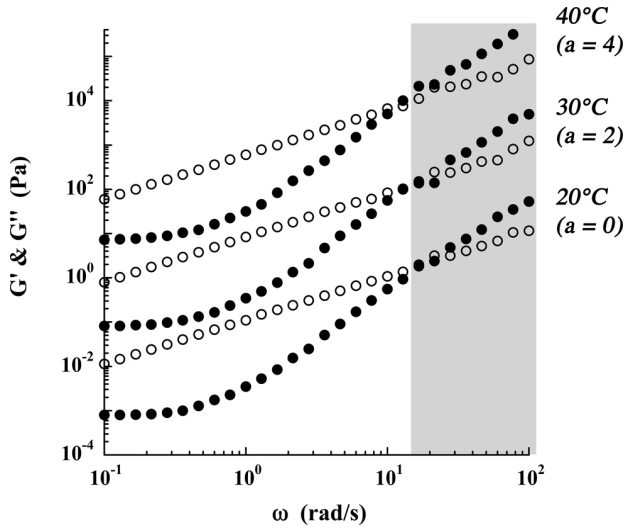


Figure II-2: Storage modulus (G', ●) and loss modulus (G", ○) of 1.5 wt.% CS solution, as a function of angular frequency (ω) at 20, 30 and 40°C. The data are shifted along the vertical axis by a factor of 10^a to avoid overlapping.

2.5. pH value measurements over the gelation process

The pH values of the CS / β-GP, CS / G1-P and CS / G6-P samples with 0.43 mmol/g gelling agent were measured as a function of time at 37°C using a 780 pH Meter (Metrohm, Herisau, Switzerland). The cold (4°C) CS / gelling agent samples were placed in a water-bath at a constant temperature of 37°C and pH and temperature values were recorded in a continuous manner during the gelation process.

2.6. ^{31}P-NMR measurements

^{31}P-NMR spectra were obtained on a Bruker AV-III-500 MHz spectrometer, equipped with a 5 mm BBO probe. Pulse program: zg30 1D ^{31}P experiment with 30 degrees pulse with no ^{1}H-decoupling. The ^{31}P shifts were referenced internally to the phosphoric acid signal at 0.0 ppm. The NMR tube containing the CS / polyol phosphate sample was stored at 4°C. Then, it was introduced into the spectrometer stabilized at 17°C. The program ran a temperature gradient from 17°C to 45°C by steps of 2°C. After each step, a stabilization time of 60 s was applied before acquisition. The time-lapse before the first measurement was about 5-10 min.

3. Results

3.1. Effect of gelling agent type on thermogelling behavior

Beside the commonly used β-GP, five other potential gelling agents bearing a phosphate group were evaluated for their ability to induce a thermosensitive sol / gel transition of CS-based solutions with pH values close to physiological pH (Table II-1). The standard CS / GP system was a clear solution at 4°C and turned quickly into a turbid hydrogel at 37°C (Figure II-3 (a)). Consistently with literature [22,31], it was found that CS solutions containing inorganic phosphate salts, i.e. $(NH_4)_2HPO_4$ or Na_2HPO_4 at a molar concentration of 0.2 mmol/g, were inhomogeneous white dispersions at low temperatures and formed gel-like precipitates upon heating (Figure II-3 (b)). Furthermore, the addition of glycerol prior to $(NH_4)_2HPO_4$ did neither change the appearance nor the gelling behavior of the system (Figure II-3 (c)). A new low molecular weight polyethylene glycol (PEG) derivate, α-methoxy-ω-phosphate PEG (MP-PEG), was also investigated as gelling agent, due to its structural similarities with β-GP (i.e. presence of a mono-phosphate head and oxygen in the polymer backbone, as shown in Figure II-1). However, the formation of a white precipitate was observed instantaneously following MP-PEG addition to the CS solution (Figure II-3 (d)). Finally, two glucose-phosphate salts, G1-P and G6-P, were tested and showed their ability to replace β-GP for the preparation of CS-based thermosensitive solutions. Addition of G1-P or G6-P increased the CS solution pH to the physiological range while keeping it in a clear solution state (Figure II-3 (e) and (f)). Furthermore, sol / gel transition occurred within few minutes upon heating to body temperature, leading to the formation of homogeneous turbid gels.

Table II-1: CS/gelling agents systems pH, appearance and ability to gel at 37°C

Formulation	Gelling agent concentration (mmol/g)	Terminal pH value at 4°C	Appearance at 4°C	Homogeneous gelation at 37°C
CS / β-GP	0.2	7.2	Clear solution	Yes
CS / Na_2HPO_4	0.2	7.0	Inhomogeneous white dispersions	No
CS / $(NH_4)_2HPO_4$	0.2	7.0	Inhomogeneous white dispersions	No
CS / Glycerol / $(NH_4)_2HPO_4$	0.4 / 0.4	7.8	Inhomogeneous white dispersions	No
CS / MP-PEG	0.2	n.a.*	White precipitate	No
CS / G1-P	0.2	7.2	Clear solution	Yes
CS / G6-P	0.2	7.3	Clear solution	Yes

*: terminal pH value of the CS / MP-PEG formulation could not be measured due to the formation of a pasty white precipitate, as can be observed in Figure II-3.

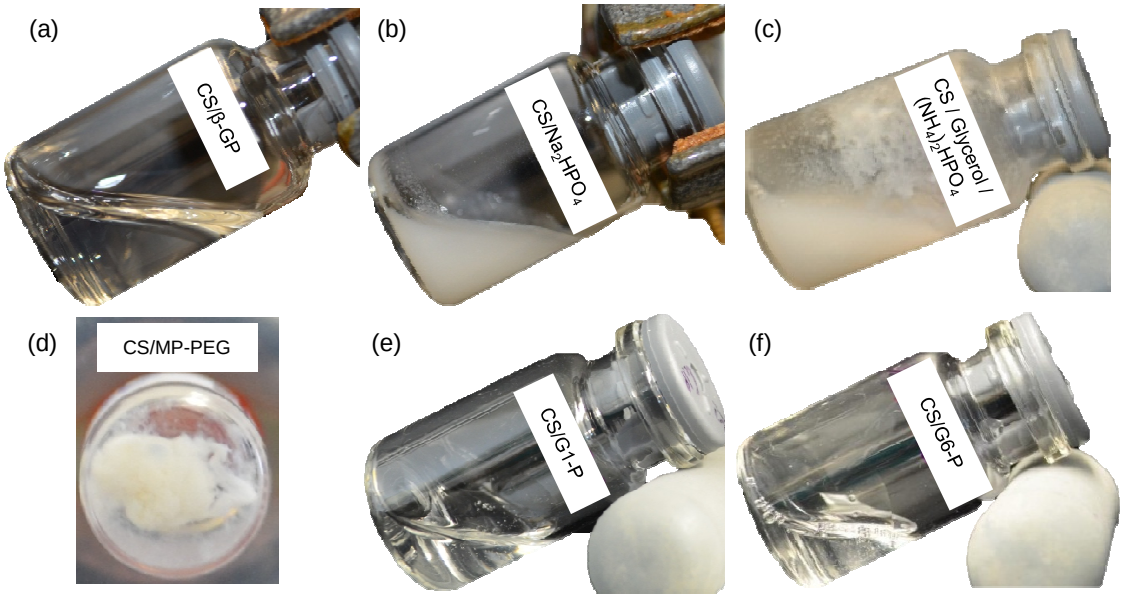


Figure II-3: CS / gelling agents systems visual appearance at 4°C.

3.2. Frequency sweep experiments – Effect of the temperature

The first rheological results, reported in Figure II-4, concern the frequency sweep tests of CS / gelling agent solutions at different temperatures, and for a constant and representative gelling agent concentration. The four gelling agents selected, β-GP, G1-P, G6-P and Na_2HPO_4, were compared at the same molar concentration (0.26 mmol/g), from 0.1 to 100 rad/s, and from 10 to 40°C. Overall, the three gelling agents containing polyol functions present a similar behavior, but with slightly different T_{gel}. The β-GP induces a T_{gel} around 30°C, for G1-P it is shifted to around 35°C, and even higher for G6-P, between 35 and 40°C. Below these sol/gel transitions temperatures, the systems displayed viscoelastic liquid behavior, similar to the one of the pure CS 1.5 wt.% solution shown in Figure II-2. Then, at $T > T_{gel}$, the storage moduli G' were higher than the loss moduli G". The results show values of G' and G" in the form of almost parallel straight lines throughout the entire frequency range and the value of G' was more than one order of magnitude greater than that of G". Thus, it can be concluded that the systems formed strong gels under this conditions [32-34]. This transition is even visible for G1-P at 35°C, where G' gradually increased to exceed G", indicating that a weak gel has been formed. However, for higher temperature, *i.e.* 40°C, G' and G" exhibited straight parallel lines (as for β-GP), indicating that a strong gel is definitively formed.

In contrast, the CS / Na_2HPO_4 system showed G' > G" in the whole frequency range at all tested temperatures (Figure II-4 (Na_2HPO_4)). This indicates that this gelling agent induces a gelling of the CS solutions independent of the experimental and thermodynamic conditions. A slight break of the moduli at high frequency could also be observed, likely due to a structural change of the gel induced by high shear.

These results confirm that all three polyol-phosphate salts are able to neutralize the CS solution up to physiological pH, while preventing its gelation at, or below, room temperature, thus allowing control of the sol / gel transition with a temperature rise. When compared to the behavior of polyol-free phosphate salt (like Na_2HPO_4), it is clear that this thermo-sensitive CS gelation phenomenon is induced by the presence of the polyol moiety in the gelling agent structure.

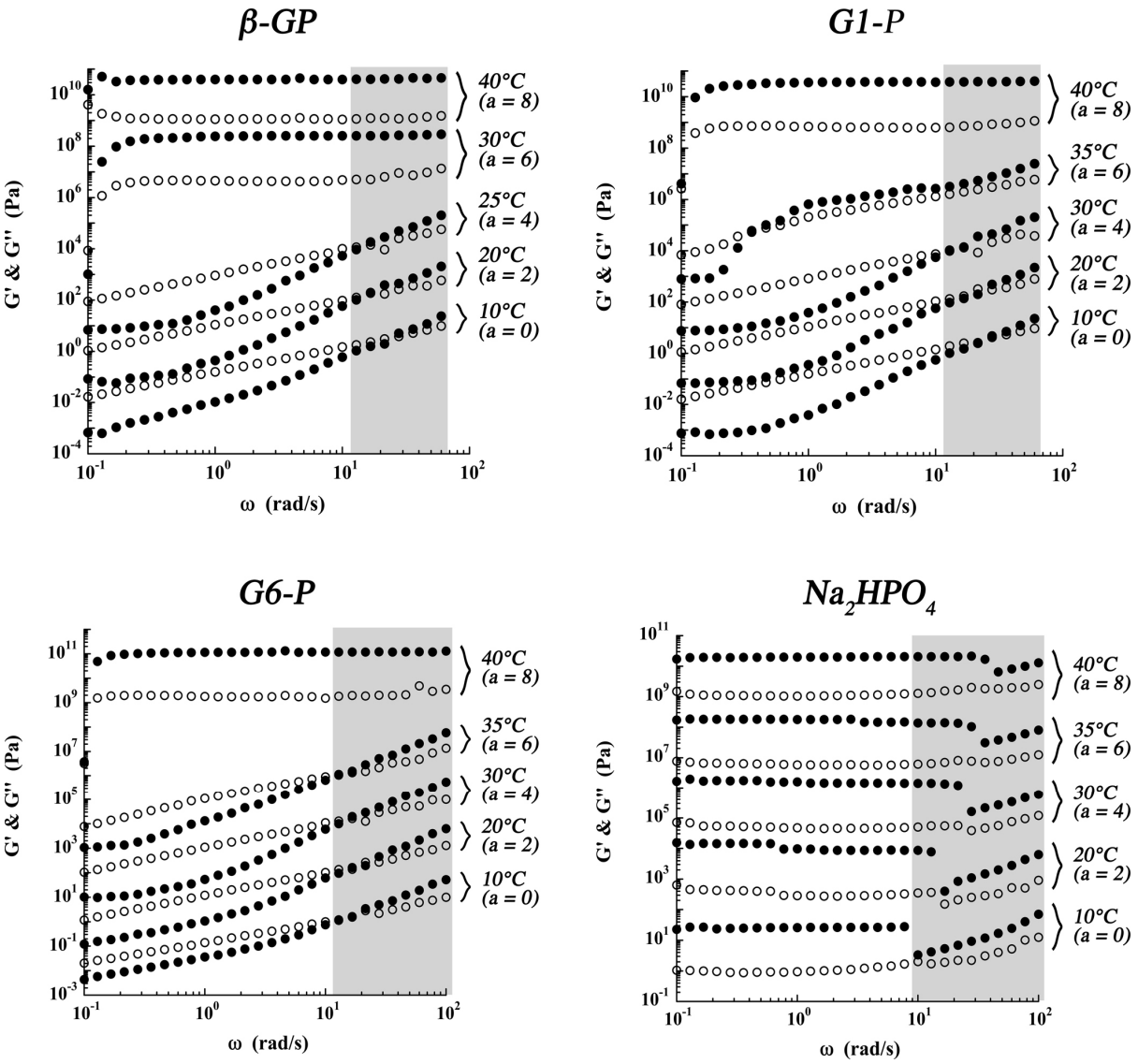


Figure II-4: Frequency dependence of storage modulus (G', ●) and loss modulus (G", ○) of CS / β-GP, CS / G1-P, CS / G6-P and CS / Na_2HPO_4 solutions (CS concentration: 1.5 wt.% and gelling agent concentration: 0.26 mmol/g) at different temperatures. The data are shifted along the vertical axis by a factor of 10^a to avoid overlapping.

3.3. Frequency sweep experiments – Effect of the gelling agent concentration

Frequency sweep tests were performed as in the above section, but with a constant temperature, and with increasing gelling agent concentrations. Temperatures were fixed, in function of the gelling agent and based on the results shown in Figure II-4, to allow observation of the sol / gel transition in the studied concentration range. Results are reported in Figure II-5, and, as above, show similar behaviors for the three polyol-phosphate salts.

With an increase of the gelling agent concentration from 0.17 to 0.43 mmol/g, the CS / gelling agent systems show a "liquid / weak gel / strong gel" transition. The slight differences observed confirm those previously presented. They are due to differences in the values of T_{gel}, which varies in function of the gelling agents. As mentioned before, the CS / Na_2HPO_4 system presents a gel-like behavior whatever the gelling agent concentration, with a structural break at high frequencies.

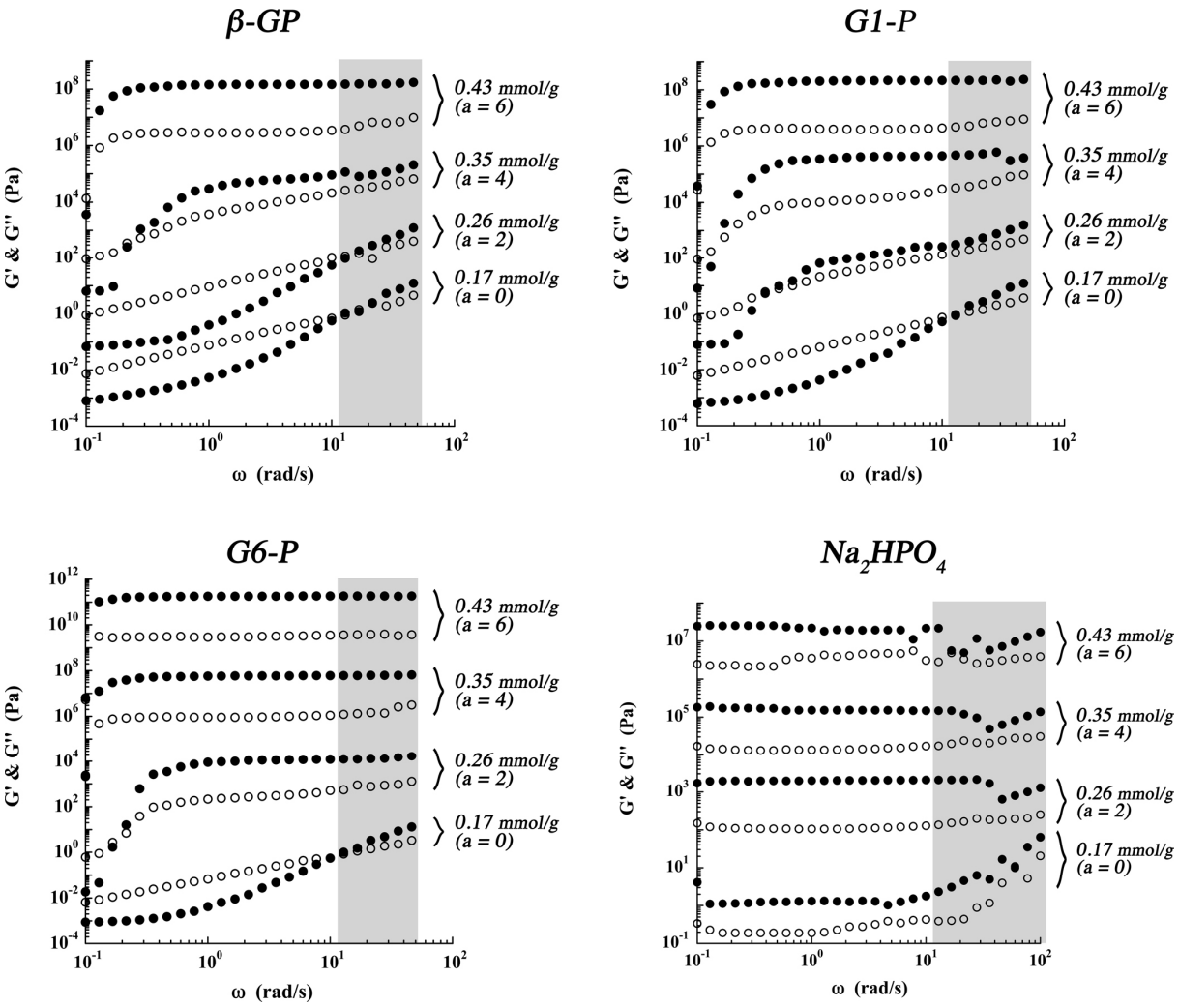


Figure II-5: Frequency dependence of storage modulus (G', ●) and loss modulus (G", ○) of CS / β-GP at 25°C, CS / G1-P at 35 °C, CS / G6-P at 37 °C and CS / Na_2HPO_4 at 40°C (CS concentration: 1.5 wt.%) at different gelling agents concentrations. The data are shifted along the vertical axis by a factor of 10^a to avoid overlapping.

Moreover, once strong gels are formed (*e.g.* at higher temperatures than those of Figure II-5), frequency sweep tests show that the strength of the gels remains constant

independently of a further increase of gelling agent concentration. A representative example is presented in Figure II-6, with CS / β-GP at 30°C, giving a G' value of 220 ± 15, 230 ± 10 and 220 ± 15 Pa for 0.26, 0.35 and 0.43 mmol/g respectively. The same behavior was observed for the two other CS / glucose-phosphate systems studied.

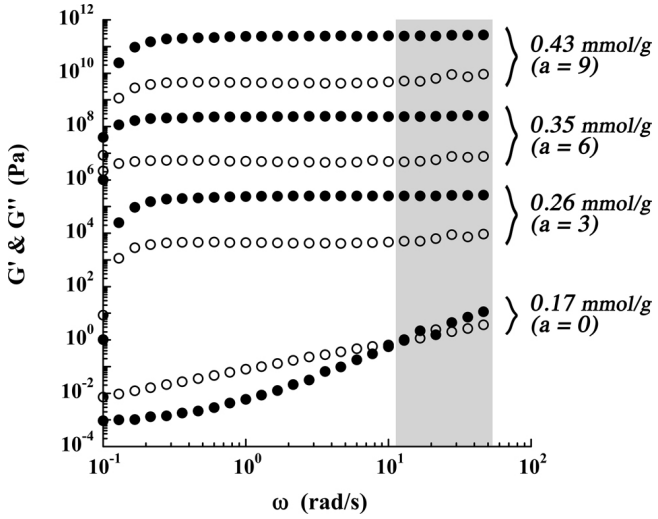


Figure II-6: Frequency dependence of storage modulus (G', ●) and loss modulus (G", ○) of CS / β-GP at 30°C (CS concentration: 1.5 wt.%) at different gelling agents concentrations. Data are shifted along the vertical axis by a factor of 10^a to avoid overlapping.

3.4. Temperature and time sweep experiments

Temperature sweep tests were conducted at a given rate and constant gelling agent concentration. Representative results, obtained with 0.43 mmol/g, are reported in Figure II-7 (a). As expected, the progression of G' and G" show a clear sol / gel transition for each CS / gelling agent system, when G' becomes higher than G". The gelation temperatures increase from T_{gel} = 26.8°C with β-GP, to T_{gel} = 35.8°C with G1-P, to finally reach the higher value of T_{gel} = 38.8°C with G6-P. These results disclose the clear influence of the chemical structure of the gelling agents on the gelation temperature, confirming the trend shown in Figure II-4 with a complementary approach.

Time sweep tests were performed at 37°C and at a constant concentration of gelling agents. As for the temperature sweep tests, the gelling agent concentration of 0.43 mmol/g provides representative results, reported in Figure II-7 (b). Between the different gelling agents, the difference in the chemical structure of the polyol parts results in strong differences

in the gelation times, with t_{gel} (β-GP) = 2 min < t_{gel} (G1-P) = 12 min < t_{gel} (G6-P) = 24 min. For given experimental conditions, these time sweep experiments confirm the impact of the nature of the gelling agents on the gelation times.

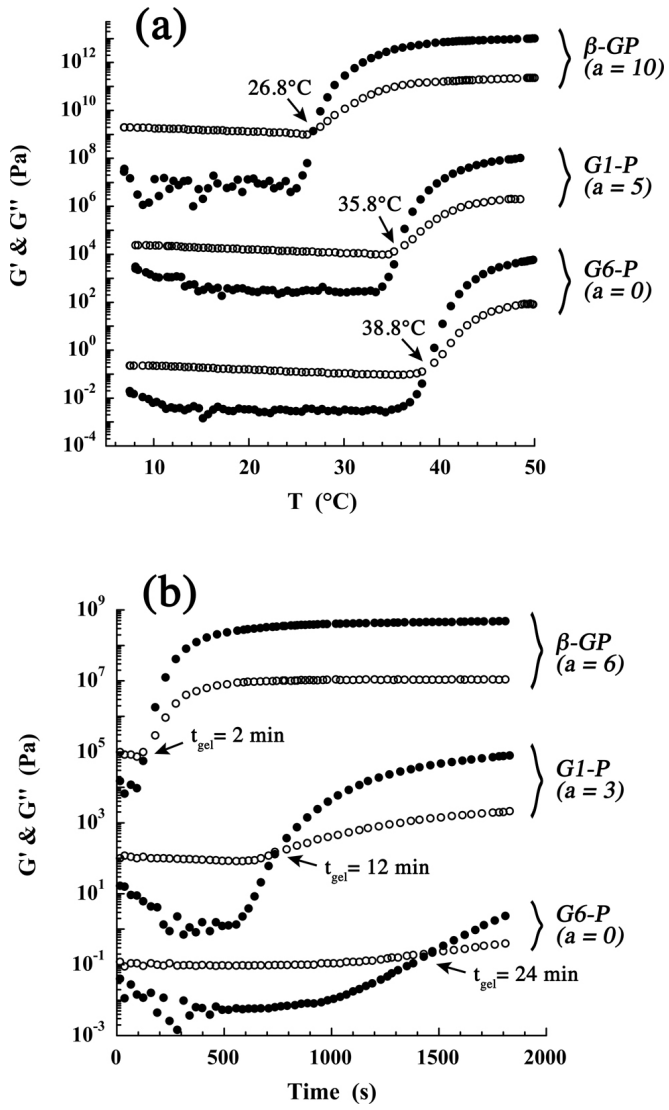


Figure II-7: (a) Temperature dependence of storage modulus (G', ●) and loss modulus (G", ○) of CS / β-GP, CS / G1-P and CS / G6-P solutions for a gelling agent concentration of 0.43 mmol/g. (b) Time dependence of storage modulus (G', ●) and loss modulus (G", ○) of CS / β-GP, CS / G1-P and CS / G6-P solutions for a gelling agent concentration of 0.43 mmol/g, at 37°C. The data are shifted along the vertical axis by a factor of 10^a to avoid overlapping.

3.5. Thermo-reversibility

The thermo-reversibility of gelation of CS / gelling agent solutions was investigated by performing a heating and cooling cycle between 7 and 50°C. As presented in Figure II-8, a similar rheological behavior was observed, whatever the gelling agent, β-GP, G1-P or G6-P. As presented above, the heating process results in the formation of strong gels. However, upon cooling, these gels remain stable and do not revert to the liquid state. Thus, once formed, the gels are not thermo-reversible. It is to be noted that during cooling from 50 to 7°C, G' moduli decreased gradually from about one order of magnitude (*i.e.* from 1470 to 160 Pa for CS / β-GP, from 1750 to 90 Pa for CS / G1-P and from 3930 to 360 Pa for CS / G6-P), lowering the gels strengths.

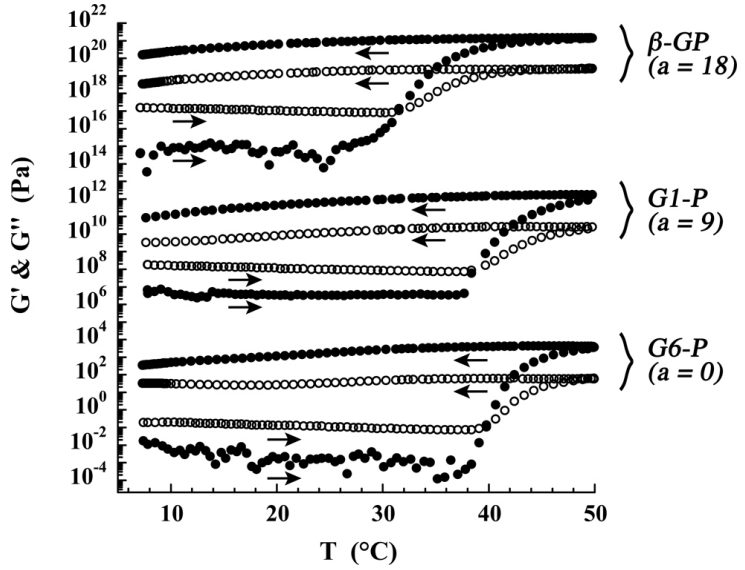


Figure II-8: Temperature dependence of storage modulus (G', ●) and loss modulus (G", ○) of CS / β-GP, CS / G1-P and CS / G6-P solutions (gelling agent concentration: 0.26 mmol/g) in a heating and cooling process. The data are shifted along the vertical axis by a factor of 10^a to avoid overlapping.

3.6. pH value measurements along the gelation process

pH and actual temperature of the CS / polyol phosphate solutions were monitored in function of time after placing the cold samples at 37°C. Results are presented in Figure II-9 (a). First, a significant pH decrease of the thermogelling solutions along the sol / gel transition from 7.38 ± 0.03 to 6.82 ± 0.02 with increasing the temperature from 4 to 37°C is observed. Once gelation is achieved at 37°C, the pH values of the CS / gelling agent

hydrogels are stable over time. In addition, the pH curves can be divided in two regions, their limits are indicated in the figure by the arrows and correspond to the gelation points. This is confirmed by presenting the pH in function of the temperature (Figure II-9 (b)). The temperature corresponding to these transitions (*i.e.* 26°C, 34.5°C and 37°C, respectively for CS / β-GP, CS / G1-P and CS / G6-P) appears very close to the gelation temperatures obtained by rheology (see Figure II-7 (a) 26.8°C, 35.8°C and 38.8°C, respectively for CS / β-GP, CS / G1-P and CS / G6-P).

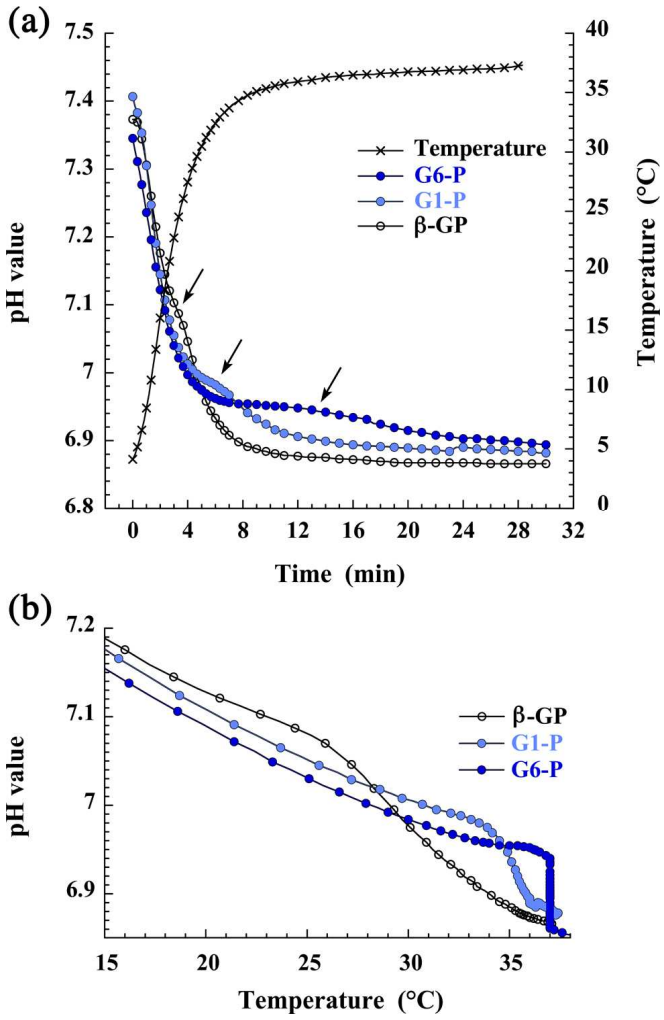


Figure II-9: (a) Time and (b) temperature dependence of pH values of CS / β-GP, CS / G1-P and CS / G6-P cold solutions for a gelling agent concentration of 0.43 mmol/g set up at 37°C.

3.7. ^{31}P-NMR measurements

To complete the characterization of the gelation process, ^{31}P-NMR was applied using the gelling agents as powerful probes. It is commonly known that the chemical shift is related to the temperature, pH, and environment of the phosphorus nucleus [13,35,36]. The shift of the ^{31}P along the gelation process (*i.e.* in function of temperature) was followed, and compared the gelling agents alone in solution with the CS / gelling agent systems. The results are presented in Figure II-10. In the case of gelling agents alone, and independent of the gelling agent, the results show a linear deshielding of the ^{31}P shift with temperature raise. This reflects the effect of the temperature on the environment of the phosphorus. In the presence of CS the first linear part of the curve (if any) can be attributed to the same phenomenon, *i.e.* the effect of temperature on the ^{31}P shift. Therefore, the change of the curve profiles (stabilization and decrease of the shift) in the second part of the curve indicates an additional effect due to a modification of the environment of the phosphorus, linked to the decrease of the interactions between CS and gelling agents. This transition reflects the gelation of the CS / gelling agent systems, showing a similar trend compared to the one disclosed by rheology and pH measurements (T_{gel} (CS / β-GP) < T_{gel} (CS / G1-P) < T_{gel} (CS / G6-P)). It is to be noted that due to the very fast gelation of the CS / β-GP system, the first linear part in the ^{31}P-NMR study was not observed for this system.

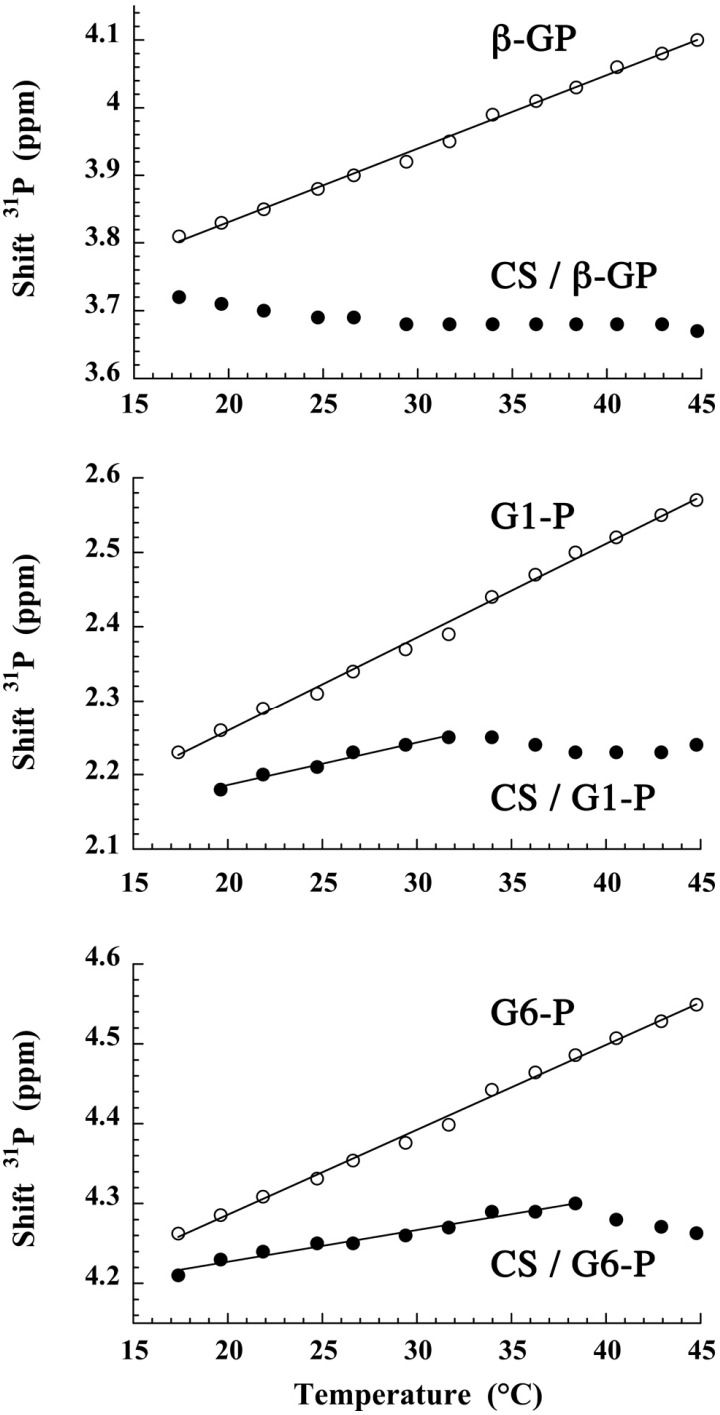


Figure II-10: Temperature dependence of the chemical shift of ^{31}P nuclei from 17 to 45°C of gelling agent solutions and CS / gelling agent systems (gelling agent concentrations: 0.43 mmol/g).

4. Discussion

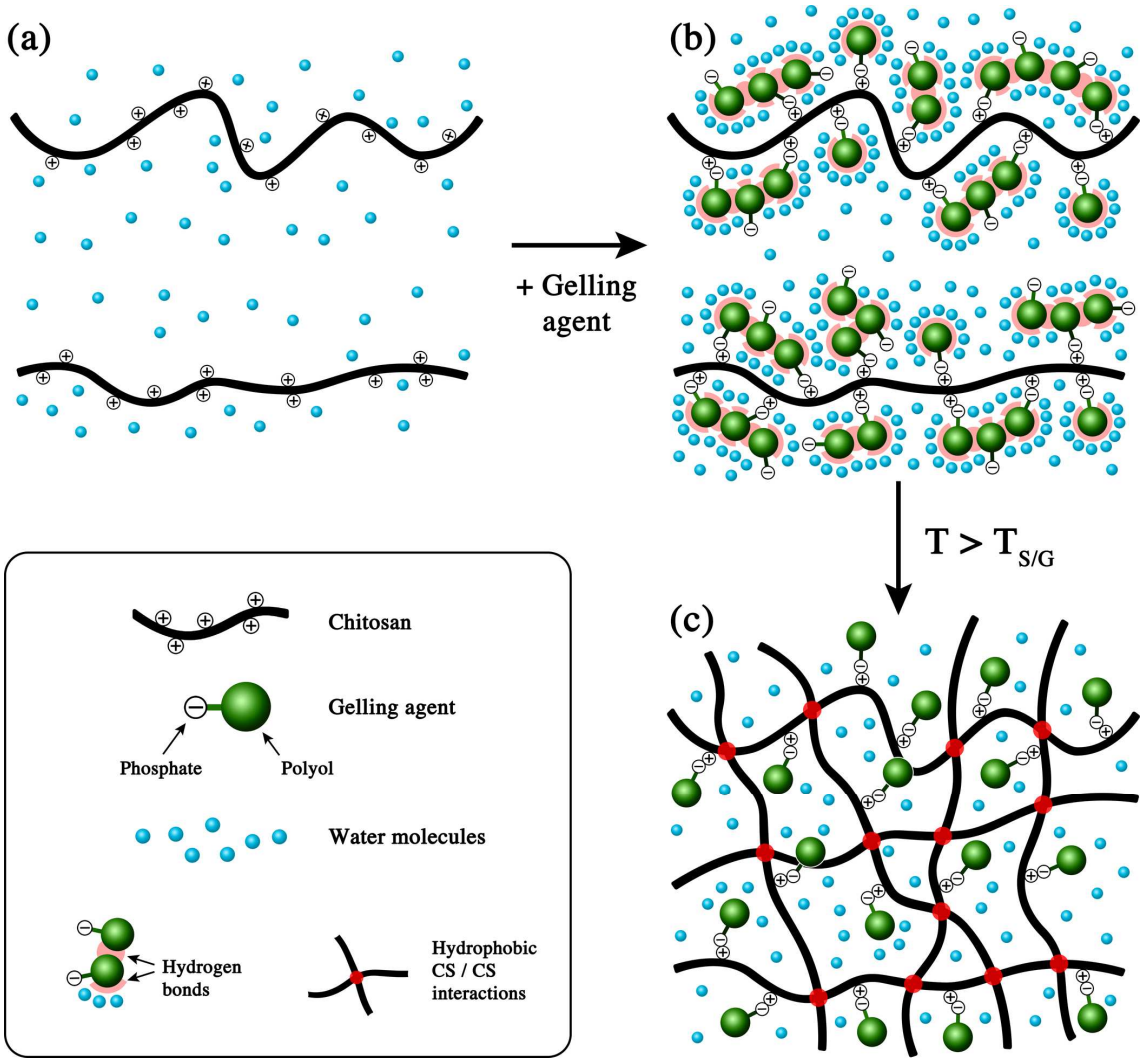
Through a comprehensive rheological characterization corroborated by pH measurements and ^{31}P-NMR analysis, it could be shown that the chemical nature of the polyol part of the gelling agent plays an important role in the gelation mechanism. In this discussion section, an understanding of this role at a molecular level is proposed. Overall, the thermogelling process of CS / polyol-phosphate solutions is divided in two steps, namely the maintaining of the CS molecules in solution at neutral pH at low temperature and the sol / gel transition upon heating.

Solubility of CS macromolecules in acidic conditions is ensured by the charge repulsion of amino groups. Upon addition of gelling agents, the phosphate parts neutralize the majority of the positive charges on CS. As a result, the charge repulsion between the polymer molecules are reduced, potentially inducing the formation of a gel-like precipitate. This was observed when using $(NH_4)_2HPO_4$ or Na_2HPO_4 (*i.e.* polyol-free phosphate gelling agents) as illustrated in Figure II-3 and 4 (Na_2HPO_4). However, the presence of polyol in the gelling agent structure prevents or delays the polymer precipitation and the gel formation, the CS remains soluble even at physiological pH, in function of temperature (see Figure II-3 and 4 (β-GP), (G1-P) and (G6-P)). Addition of glycerol to the CS solution prior to the inorganic phosphate salt did not suffice to prevent the formation of precipitates (Table II-1). MP-PEG did not prevent CS precipitation despite the oxygen atoms present in the polymer backbone (Figure I-3 (d)). The hydroxyl groups of the polyol-moiety are assumed to be key in the protection of CS chains against molecular interactions leading to precipitation.

In literature, polyols (like glycerol) are recognized to protect proteins against thermal degradation through adopting a particular conformation, creating a shell around the macromolecules [37-39]. In view of the rheological results revealed in the present study, it is legitimate to assume that the polyol part of the gelling agents plays this protective role on the CS molecules. Thanks to hydrogen bonds (H-bonds) formed with the surrounding water molecules, and also between each other, glycerol and glucose create a protective hydration layer around the polymer chains, as illustrated in Scheme 1 (b). This layer remains stable at neutral pH and low temperature, resulting in the reduction of the self-interactions between the CS chains, and thus preventing the formation of a macromolecular gel. Owing to the strong electrostatic attractions of the gelling agents to the polymer *via* the phosphate part, one can

easily imagine that the polymers are surrounded by the polyol groups (oriented towards the water phase), and largely hydrated thanks to H-bonds. The weak interactions between polymer-linked gelling agents and free gelling agents, as well, could contribute to the formation of a hydrated shell protecting the polymer molecules (see Scheme 1 (b)). Nevertheless, H-bond based interactions remain weak, and therefore cannot form a gel network, even if two polymer chains are linked through interactions of polyols groups.

Scheme 1: Representation of the gelation mechanism of CS / polyol-phosphate solutions.



The apparent proton dissociation constant of CS, pK_{ap}, is known to be strongly temperature-depend, with reduced pK_{ap} upon heating [17]. Therefore, increasing the temperature of the CS / gelling agent system can induce proton transfer from CS to the gelling agent, thereby inducing a further reduction of the degree of ionization of CS and leading to

less electrostatic interactions between the polymer and the gelling agents [14]. The proton transfer from CS to the gelling agent is confirmed by the pH decrease measured along the gelation process (Figure II-9 (a)), which indicates that the ratio of gelling agent in their acid form (β-GP^-, G1-P^- or G6-P^-) to gelling agent in their base form (β-GP^{2-}, G1-P^{2-} or G6-P^{2-}) in the systems increased upon heating. Furthermore, upon increasing temperature of the samples, *i.e.* the thermal agitation of the water molecules, the number of H-bonds is decreased along with the cohesion of the polyol protective shells around the CS molecules. As a result, see Scheme 1 (c), at $T \geq T_{gel}$, the hydration of the CS chains is not strong enough for maintaining their solubilization in the aqueous phase and preventing their interactions. In addition to the screening of the remaining repulsive charges of CS, the attractive interactions between the polymer chains can be achieved via hydrogen bonding and hydrophobic interactions, leading to gelation [40]. In addition, Figure II-6 reveals that, at a given temperature, once the concentration of gelling agent is sufficient to induce the sol / gel transition, a further increase of the gelling agent concentration does not influence the strength of the gels. It follows that the gel strength does not directly appear to be linked to the concentration of gelling agent.

An increase of T_{gel} between different CS / gelling agent systems means that the protective hydration layer is more stable (for systems having higher T_{gel}), and thus its disruption requires more thermal energy. In Figure II-4, the stability of this polyol protective layer increases in the following order: β-GP < G1-P < G6-P. This hypothesis is also corroborated by the literature which acknowledges that the ordering of the water layer increases with the polyol size [41]. Likewise, temperature sweep tests (Figure II-7 (a)), pH following (Figure II-9 (b)) and ^{31}P-NMR analysis (Figure II-10) corroborate these findings. In each case, it was observed that the sol / gel transition temperature is lower for CS / β-GP than for CS / G1-P, than for CS / G6-P.

Besides, whatever the gelling agent, an increase of concentration results in lowering of the transition temperature T_{gel}. Indeed, increasing the gelling agent content raises the pH of the thermogelling solution, reducing thereby the initial degree of ionization of CS and the possibility of electrostatic interactions between the polymer and the gelling agent. The protective hydration layer is thus also diminished so that the sol / gel transition occurs at lower temperatures.

Finally, the thermo-irreversibility of the process (Figure II-8) clearly emphasizes the two different sorts of intermolecular interactions governing the gelling process. The first concerns weak hydrogen bonds. They allow the formation of the protective hydration layer around CS chains and their number is sensitive to temperature. Then, upon an increase of the temperature, these weak interactions are broken in favor of stronger hydrophobic interactions between the CS molecules. Cooling of the formed gels results in a decrease of their strength, likely due to a weakening of the hydrophobic interactions, but being still strong enough to prevent their disruption.

To summarize, the physicochemical characterization of CS / gelling agent, through a study of the influence of the chemical nature of the polyol group on the gelation process, disclosed new aspects of the gelation mechanisms at the molecular level. The three polyol-phosphates tested were selected for their increasing size (between β-GP and glucose-phosphates), or for the different attachment between the polyol and phosphate moiety (G1-P and G6-P). It results in different efficiency of the CS protection against a temperature rise (impacting the transition temperature T_{gel}) and on the gelation kinetics.

It is noteworthy to remark that despite the close chemical structures of G1-P and G6-P, they induce significantly different gelation behaviors in all the rheological experiments. In G1-P, the phosphate group is linked to the hemiacetal hydroxyl group, whereas, for G6-P, it is attached to the primary alcohol group in C^6 position. This difference in structure implies that the distance between the phosphate and the glucose ring is slightly increased in the structure of G6-P. In view of the rheological results, this appears to be a decisive factor that improves the protection of the CS molecules against their self-interaction and gel formation.

5. Conclusion

In this work, rheological properties of CS aqueous solutions in the presence of four gelling agents were investigated as a function of angular frequency, temperature and time. The comparison of gelation behavior of CS solution combined with inorganic phosphate salts, β-GP, G1-P or G6-P, indicated that the glucose-phosphate salts had the same ability as the commonly used glycerophosphate to turn CS solutions into thermosensitive systems. G1-P and G6-P could neutralize CS solutions up to physiological pH while keeping them in the solution state at room temperature, and gelation could take place upon heating. Our study suggests that the polyol moiety of the gelling agents is responsible for the thermal sensitivity of the CS solution and that the chemical structure and size of the polyol plays a significant role in controlling the gelation process. It appears that polyols create a hydration protective layer around the CS chains, largely built through weak intermolecular interactions, like hydrogen bounds. A temperature increase disrupts this polyol layer and allows the polymers to interact with each other through stronger hydrophobic bonding, thus inducing the gel formation. This study disclosed that the size of the polyol part has an impact on the stability of this hydration layer, and thus on the transition temperature T_{gel} and kinetics of gel formation.

Acknowledgments

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Chapter III :

Injectable chitosan-based *in situ* forming depot - Investigating glucose-1-phosphate as a new gelling agent

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Abstract

Chitosan (CS)-based thermosensitive solutions that turn into semi-solid hydrogels upon injection at body temperature have increasingly drawn attention over the last decades as an attractive new type of *in situ* forming depot (ISFD) drug delivery system. Despite the great potential of the standard CS / β-glycerophosphate thermogelling solutions, their lack of stability over time at room temperature as well as at refrigerated conditions renders them unsuitable as ready-to-use drug product. In the present study, Glucose-1-Phosphate (G1-P) is investigated as an alternative gelling agent for improving the stability of CS-based ISFD solutions. Furthermore, the local tolerance of subcutaneously implanted CS / G1-P hydrogels was investigated by histological examination over three weeks. The thermogelling potential of CS / G1-P solutions, determined by rheology, is dependent on the polymer molecular weight and concentration as well as on the G1-P concentration. Differential scanning calorimetry measurements confirmed that sol / gel transition takes place at around body temperature and is not fully thermo-reversible. The long term storage stability was evaluated through the appearance, pH, viscosity and gelation time at 37°C of the solution and through the changes in CS molecular weight, determined by gel permeation chromatography. The results emphasized an enhanced stability of the CS / G1-P system, at room temperature and at refrigerated conditions, compared to the standard CS / β-glycerophosphate. CS solution with 0.40 mmol/g G1-P is stable for at least 9 months at 2 - 8°C, versus less than 1 month when using β-glycerophosphate as gelling agent. Furthermore, the solution is easy to inject, as evidenced from its low viscosity, its shear-thinning behavior and injectability evaluation using 23 G to 30 G needles. The inflammatory reaction observed in the tissue surrounding the hydrogel in rats was a typical foreign body reaction, similar to the one observed for common PLGA depot systems or standard CS / β-glycerophosphate hydrogels. These features confirm the potential of CS / G1-P solutions as an injectable ready-to-use *in situ* forming hydrogel.

1. Introduction

Among ISFD systems, thermogelling solutions which form semi-solid hydrogels after administration in response to temperature increase from ambient to physiological temperature are of particular interest. Such injectable *in situ* forming hydrogel, based on CS as biocompatible and biodegradable polymer, was first described by Chenite *et al* in 2000 [1], who reported an aqueous solution of CS and β-glycerophosphate (β-GP) at physiological pH which had the property of turning into an semisolid gel at temperature around 37°C and concluded that this polyol-phosphate salt is able to turn the polymeric solution into a temperature-controlled pH-dependent gelling system. The sol / gel transition of the CS / β-GP solution, the gelation mechanisms, and their control for potential biomedical applications were extensively investigated in the last decade [2-7]. These studies revealed that gelation of the CS / β-GP solution is a consequence of several intermolecular interactions. In brief, the effect of β-GP on CS solutions (solubilized in acidic aqueous medium) is to partially neutralize the polymer chains along with preventing precipitation by the formation of a protective hydration layer around the CS molecules. Then, increasing the temperature has two effects: *(i)* it induces a proton transfer from CS to β-GP, thereby promoting CS neutralization, and *(ii)* it results in breaking hydrogen bonds at the origin of the protective shell cohesion around the CS molecules. As a result, polymer chains become hydrophobic and create physical crosslinking between each other, forming a hydrogel.

CS / glycerophosphate thermogels have been studied as drug delivery systems for the controlled release of various types of drugs (e.g. low molecular weight hydrophilic and hydrophobic compounds, proteins and peptides) *in vitro* as well as *in vivo* and showed potential to achieve prolonged release over days to weeks [7-11]. Furthermore, the hydrogels were also reported as promising injectable scaffolds for cell encapsulation and tissue engineering, especially for bone and cartilage repair, due to the biocompatibility, biodegradability, high water content and porosity of the three dimensional matrix [12-16].

The feasibility of developing analogous *in situ* thermogelling systems using other gelling agent than β-GP, like inorganic phosphate salts, polyols or polyoses, was evaluated in several studies, with more or less success [17-20]. However, storage stability of CS-based thermogelling solutions has received only little attention and the few results reported, based on viscosity changes over the storage period, revealed a lack of long term stability at room

temperature as well as at refrigerated conditions. The fact that gelation occurs with time even at refrigerated conditions is a concern with CS / β-GP systems since other storage or formulation approaches, like freezing or freeze-drying respectively, are less suitable and would not allow the thermogelling system to be presented as a convenient ready-to-use product in pre-filled syringes [21]. Storage stability is an essential condition in view of a pharmaceutical application as *in situ* forming hydrogels, and this point still remains very challenging for such pharmaceutical purpose [19,22].

In the previous chapter, the impact of the chemical nature of the gelling agent on the rheological properties and gelation mechanisms was studied, and Glucose-1-Phosphate salt (G1-P) showed very similar ability as the commonly used β-GP, to turn CS solutions into thermosensitive systems [23]. It was emphasized that G1-P allowed increasing the ordering of the protective water layer around CS at low temperatures, which could be likely related to the stability of the thermogelling solution. Thus, it appears that G1-P could be an alternative gelling agent to β-GP in order to prepare *in situ* forming hydrogels with optimized physicochemical properties with real potential for drug delivery applications. Therefore, the objectives of the present study are to evaluate *(i)* the feasibility of using G1-P as alternative gelling agent for CS-based ISFD system for subcutaneous (s.c.) applications, through the determination of the sol / gel transition temperature, gelation time at physiological temperature, gel strength, viscosity and injectability of CS / G1-P solutions ; *(ii)* the storage stability of CS / G1-P solutions at room temperature and refrigerated conditions, in comparison with the standard CS / β-GP solution, and *(iii)* the histological biocompatibility of the CS / G1-P hydrogel.

2. Materials and Methods

2.1. Materials

Two different CS of technical grade: Chitoscience® 90/50 (DD 89.7 %, Mw: 122 kDa) and Chitoscience® 90/200 (DD 89.7 %, Mw: 267 kDa), and two CS of pharmaceutical grade: Chitoceutical® 90/50(1) (DD 90.7 %, Mw: 135 kDa) and Chitoceutical® 90/50(2) (DD 88.7 %, Mw 149 kDa) were used in this study. All CS are derived from shrimp shell and were purchased from Heppe Medical Chitosan (Halle, Germany). β-glycerophosphate disodium salt hydrate (β-GP, Mw: 288.10 g/mol), α-D-glucose 1-phosphate disodium salt hydrate (G1-P, Mw: 376.16 g/mol) and phosphate buffered saline (PBS) were purchased from Sigma. Hydrochloric acid (HCl) was purchased from Merck. All other reagents were of analytical grade and were used without further purification. Ultrapure® water was obtained using a MilliQ® Millipore filtration system (Millipore, Molsheim, France).

2.2. Physical characterization of chitosans using triple detection GPC

All CS were characterized by triple detection gel permeation chromatography (GPC) on a Viscotek Triple Detector Array max system (Viscotek, USA) using two ViscoGel A6000M (mixed Bed) columns (Malvern Instruments GmbH, Germany). The set up consisted of a size exclusion chromatograph connected to a light scattering cell; a refractive index detector and a viscometer, allowing for simultaneous determination of the absolute polymer molecular weight (Mw), hydrodynamic radius and intrinsic viscosity (η). The analysis were performed at 30°C with a 0.3 M acetic acid, 0.3 M sodium acetate, 1 % ethylene glycol mobile phase and using a flow rate of 0.7 mL/min and a dn/dc of 0.163 mL/g. CS samples were prepared at concentrations of 1 mg/mL, dissolved 24 h under agitation in the same buffer and then filtered using a 0.2 µm filter prior to analysis. The injection volume was 100 µL. Calibration of the system was made using a Pullulan standard (Mw = 133 kDa, [η] = 0.515 dL/g, polydispersity index Mw/Mn = 1.08) obtained from Viscotek. For the evaluation of the physical data OmniSEC software from Viscotek was used. Each characterization of CS samples was performed in duplicate.

2.3. Preparation of CS / G1-P solutions

CS solutions were obtained by dissolving 3.75 wt.% CS in HCl according to a molar ratio for CS amine groups / HCl equal to 0.9/1. G1-P solutions were prepared at various concentrations in MilliQ water. CS and G1-P solutions were separately cooled to 4°C for 15 minutes. Then, G1-P solution was added drop-by-drop into the CS solution placed in an ice-bath under magnetic stirring. The obtained CS / G1-P solution was further stirred for 15 minutes. The resulting formulations were stored at 2 - 8°C. CS / β-GP solution was prepared according to the same procedure.

2.4. Determination of gelation time

Gelation times of CS / G1-P thermogelling solutions were determined in PBS at 37°C. After 7 min equilibration at room temperature, 1 mL CS / G1-P solution was injected in a 10 mL vial (inner diameter: 22 mm) containing 5 ml PBS pH 7.4 at 37°C (in a temperature-controlled bath). In order to control whether the solution underwent gelation, the vial was inverted horizontally every 30 s (without removing it from the bath) over 20 min, and the gelation time was defined as the time at which the solution no longer flowed.

2.5. Rheological measurements

Rheological measurements were performed using a Haake Rheostress1 rheometer (Thermo Fisher Scientific, Karlsruhe) with a circulating environmental system for temperature control. The sample volume was about 0.5 mL, introduced between a cone-plate geometry (2° cone angle, 35 mm diameter). A solvent trap was used to minimize water evaporation during all tests. To determine their viscosity at 20.00 ± 0.20°C, freshly prepared degassed samples, prepared with 1.5 wt.% Chitoceutical® 90/50(1) and G1-P concentration ranging from 0.00 to 0.53 mmol/g, were subjected to rotational tests at controlled shear rates, during which the shear rate was kept constant for 60 s and then increased stepwise from 5 to 10^3 s^{-1}. The apparent shear viscosity values were calculated as the mean of the apparent shear viscosities determined after 30 s equilibration at each shear rate. The viscoelastic properties of the thermogelling solutions were assessed by measuring the storage modulus (G') and loss modulus (G"), representing the elastic behavior and the viscous behavior of the material, respectively. The value of the strain amplitude was set at 1 % to ensure that all measurements were carried out within the linear viscoelastic range. Three kinds of tests were performed:

(i) temperature sweep tests were carried out at a constant frequency of 1 Hz, with a temperature increase from 7 to 50°C and a heating rate of 1°C/min to determine gelation temperatures; *(ii)* time sweep tests, to determine gelation times, were carried out at constant angular frequency of 1 Hz and constant temperature of 37.00 ± 0.20°C. The sol / gel transition temperature (T_{gel}) or time (t_{gel}) correspond to the intersection of the curves of G' and G"; *(iii)* the strength of the hydrogels was evaluated by performing frequency sweep tests on hydrogels formed either in contact with air or in PBS. Gels were made as follows, 1 mL CS / G1-P solution were poured in round shaped molds and were incubated 6 h, 24 h and 48 h at 37°C to allow formation of hydrogels, either in an atmosphere saturated in moisture to avoid water evaporation or in 1 mL PBS pre-heated at 37°C. Then, dynamic frequency sweeps tests were performed on the hydrogels at 37.00 ± 0.20°C and G' and G" were measured over frequencies decreasing through logarithmic steps from 30 to 0.1 Hz. The gel strength was evaluated in terms of the value of G' at low frequencies. Measurements were done in triplicate. Finally, the thermoreversibility of the CS / G1-P system was investigated in duplicate through two successive heating and cooling cycles carried out at a constant frequency of 1 Hz, during which the temperature was increased from 5 to 40°C at a heating rate of 1.0°C/min, maintained at 40°C for 30 min, then decreased from 40 to 5°C at a cooling rate of 0.5°C/min and maintained at 5°C for 30 min.

2.6. Differential Scanning Calorimetry (DSC) analysis

The sol / gel transition and thermoreversibility of a CS / G1-P solution containing 1.5 wt. % Chitoceutical® 90/50(2) and 0.40 mmol/g G1-P was further analyzed using a differential scanning calorimeter Q2000 from TA Instruments (Alzenau, Germany). The sample was hermetically sealed into a DSC sample pan and an empty pan was used as reference. The scanning range of temperature was 0 to 50°C at a heating rate of 1.0°C/min, followed by a cooling cycle from 50 to 0°C at a rate of 10.0°C/min and a second heating cycle from 0 to 50°C at a rate of 1.0 °C/min. All measurements were performed in duplicate.

2.7. Osmolality measurements

Measurement of the osmolality of CS / G1-P solutions containing 1.5 wt.% Chitoceutical® 90/50(1) and concentration of G1-P ranging from 0.27 to 0.53 mmol/g was performed using the freezing-point depression method. Calibration of the osmometer (Model

3320, Advanced Instruments, Inc., USA) was done with reference standards and osmolalities were recorded using 0.25 mL sample. Measurements were performed in triplicate.

2.8. Stability of CS / gelling agent solutions

Stability tests were carried out with different CS solutions: *(i)* a pure CS solution, 1.5 wt.% Chitoceutical® 90/50(2); two CS / G1-P solutions *(ii)* 1.5 wt.% Chitoceutical® 90/50(1) / 0.27 mmol/g G1-P and *(iii)* 1.5 wt.% Chitoceutical® 90/50(1) / 0.40 mmol/g G1-P, and *(iv)* a CS / β-GP solution, 1.5 wt.% Chitoceutical® 90/50(1) / 0.40 mmol/g β-GP. These solutions were filtered through 0.22 µm filters, filled in 2R glass vials and purged with nitrogen prior to hermetic sealing. Mean headspace oxygen content determined by frequency modulated spectroscopy using the Lighthouse FMS-760 Instrument (Lighthouse Instruments, LLC) was 0.70 ± 0.07 %. Samples were stored at refrigerated conditions (i.e. 2 - 8°C) and at room temperature (i.e. 20 - 25°C), and analyzed after t = 1, 7, 14, 30, 60, 90, 135, 180 and 270 days for the samples stored at 2 - 8°C and at t = 1, 7, 14, 30, 60 and 180 days for the samples stored at 20 - 25°C respectively. At each time point, a panel of different characteristics were followed for each samples: *(i)* the formulation was visually inspected for any change in the physical appearance, i.e., color, turbidity, consistency (liquid or gel state); *(ii)* the pH value was determined using a 691 pH meter (Metrohm, Herisau, Switzerland) ; *(iii)* the viscosity at 20.00 ± 0.20°C was measured by performing a step test as described in "2.5. Rheological measurements" ; *(iv)* the gelation time was determined by a time sweep test as defined in "2.5. Rheological measurements" ; *(v)* the relative molecular weight of the polymer was determined by GPC. Samples were diluted to approximately 1 mg/mL CS in mobile phase. GPC analysis was carried out in a Water 2695 series liquid chromatograph coupled with a 2414 refractive index detector. 100 µl were injected into a series of two columns (Viscotek A6000M General Mixed Aqueous Columns, length: 300 mm, diameter: 7.8 mm, particle size: 13 µm) at an oven temperature of 30°C. The mobile phase consisted of 0.3 M acetic acid, 0.3 M sodium acetate and 1 % ethylene glycol. The system was calibrated using Varian polysaccharide standards (708000, 200000, 21100, 5900 and 667 Da). Sample injection was performed in duplicate and weight average molecular weight (Mw) was calculated with a Chromeleon 6.8 Datasystem (Dionex Corporation, Sunnyvale, CA, USA). The molecular weights of the samples were calculated relative to the Mw at t = 0. All measurements were performed in triplicate.

2.9. Evaluation of injectability

The injection force for a CS / G1-P solution containing 1.5 wt.% Chitoceutical® 90/50(1) and 0.40 mmol/g G1-P was measured using an electronic tensile tester (Zwick Z 2.5, Ulm, Germany) and analyzed with the testXpert II software (version 3.0, Zwick, Ulm, Germany). 1 mL thermogelling solution was injected into an empty glass vessel, using a 1 mL syringe (1 mL Luer-Lok Tip syringe, BD, Franklin Lakes, NJ, USA). Several needles were tested: *(i)* 23 G (HSW FINE-JECT® 23 G x 1", Henke-Sass Wolf GmbH, Tuttlingen, Germany), *(ii)* 25 G (Terumo® Hypodermic Needles, 25 G x 1" Thin Wall Needle, Terumo Medical Corporation, Somerset, NJ, USA) and *(iii)* 30 G (BD Microlance™ 3, 30 G x ½", BD, Franklin Lakes, NJ, USA). Before measurement, all syringes were equilibrated 10 min at room temperature and primed to expel the air bubbles until a small amount of the solution came out. Injection force was measured at two different injection speeds: 80 and 100 mm/min, for each needle size. All measurements were replicated 10 times.

2.10. Sterile filtration

CS / G1-P solution containing 1.5 wt.% Chitoceutical® 90/50(1) and 0.40 mmol/g G1-P were filtered through 0.22 µm syringe filters (hydrophilic, 33 mm diameter, 4.5 cm^2 filtration area, Millex-GV Filters, Millipore). The influence of sterile filtration on the solution characteristics was evaluated on three batches by studying the viscosity and gelation time of the solution at 37°C, determined by rheology as described in "2.5. Rheological measurements", as well as the molecular weight of CS, determined by GPC, measured before and after sterile filtration. The molecular weights of the CS in the filtered solutions were calculated relative to the Mw of non-filtered solutions. All measurements were performed in **triplicate.**

2.11. Bacterial endotoxin test

Endotoxin levels in the two pharmaceutical grade CS (i.e. Chitoceutical® 90/50(1) and Chitoceutical® 90/50(2)) as well as in two batches of CS / G1-P solution composed of 1.5 wt.% CS (either Chitoceutical® 90/50(1) or Chitoceutical® 90/50(2)) and 0.40 mmol/g G1-P were determined using the semi-quantitative gel clot technique (method B) with limulus amoebocyte lysate (LAL), according to the European Pharmacopoeia monograph 2.6.14 [24]. The endotoxins limit (EL), which represents the maximum concentration of endotoxin that is

allowed in a dose of a specific drug product, was calculated for CS / G1-P solution using equation 1:

$$EL = K / M \quad (1)$$

were K is 5 EU/kg, which represents the maximum tolerated amount of endotoxin for humans for injectables, and M is equal to the maximum daily dose of product per kg body mass. A standard body weight of 70 kg is taken into account for the calculation.

2.12. Local tolerability of CS / G1-P hydrogel

2.12.1. Animal study

The local tolerability of CS / G1-P hydrogels over three weeks was examined in healthy 10 week old Hans Wistar male rats. 0.5 mL sterile CS / G1-P thermogelling solution (1.5 wt.% Chitoceutical® 90/50(2), 0.40 mmol/g G1-P), equilibrated at room temperature, was subcutaneously injected using a 23 G needle in the interscapular region of nine rats under general anesthesia. The same volume of sterile saline solution was subcutaneously injected in their caudal dorsal area as a negative control. The health status and body weight of the animals were monitored. Blood samples were collected under isoflurane by sublingual puncture at day -3 (predose) for hematology analysis and by vena cava puncture on the day of necropsy for hematology and biochemistry analysis. After 1, 7 and 21 days, three rats were sacrificed via exsanguination from vena cava puncture after exposure to isoflurane and the tissues at the injection sites were excised for histological examination.

2.12.2. Hematology

Blood samples for hematology were collected into EDTA anticoagulant (BD Microtainer® tube, K2EDTA, reference 365975). The parameters listed in Table III-1 were determined, using an ADVIA 120® analyzer.

Table III-1: Hematology parameters

Red Blood Cell Count	White Blood Cell Count
Hemoglobin	Neutrophil Count
Hematocrit	Lymphocyte Count
Mean Corpuscular Volume	Monocyte Count
Mean Corpuscular Hemoglobin	Eosinophil Count
Mean Corpuscular Hemoglobin Concentration	Basophil Count
Red Cell Distribution Width	Large Unstained Cells Count
Reticulocyte Count	Platelet Count

2.12.3. Clinical Chemistry

Blood samples for clinical chemistry were collected into plain tubes (SARSTEDT PC Micro Tube, reference 41.1500.005). The parameters listed in Table III-2 were determined, using a Synchron CX5® analyzer.

Table III-2: Clinical chemistry parameters

Sodium	Magnesium
Potassium	Glucose
Chloride	Cholesterol
Calcium	Triglycerides
Aspartate Transaminase	Total Bilirubin
Alanine Transaminase	Total Protein
Alkaline Phosphatase	Albumin
Urea	Globulin
Creatinine	Creatine Kinase
Phosphorus	

2.12.4. Histological examination

Tissue samples used for histological examination were fixed in 10 % v/v neutral buffered formaldehyde, embedded in paraffin and sectioned at 3 µm thickness. The sections were then stained with hematoxylin and eosin (H&E) or with 0.01 % Fast Green in conjunction with Safranin-O [25]. For histopathological assessment, the stained sections were examined using light microscopy. Each animal served as their own control. Microscopic findings were entered in Pristima software and graded using a semiquantitative scale (minimal to severe); minimal (+) change contained one or a few small foci/cells; mild (++) change was

composed of small to medium size foci/cells; moderate (+++) change contained frequent and moderately sized foci/cells, marked (++++) change had extensive, confluent foci affecting most of the tissue and severe (+++++) change was effacing completely the normal structure of the tissue.

2.13. Statistical analysis

Data were recorded as mean ± standard deviation. The significance of differences between groups was assessed using a t test when two groups were compared. ANOVA followed by Tukey's tests were applied for comparisons involving 3 or more groups. All significance tests were performed in Minitab 16 (Minitab, State College, PA), and a p value less than 0.05 indicated a statistically significant difference.

3. Results and discussion

3.1. Physical characterization of CS by triple detection GPC

A preliminary physicochemical characterization of the polymer concerned the determination of the molecular weight (Mw), intrinsic viscosity and hydrodynamic radius. This was achieved by triple detection GPC on the four CS batches used in this study. Results are reported in Table III-3. Chitoscience® 90/50 and the two batches of Chitoceutical® 90/50 were supposed to have approximately the same Mw. Indeed, the viscosity of a polymeric solution depend on the Mw of the dissolved polymer [26] and, according to the supplier, the viscosity of all CS 90/50 batches (1 % solution in 1 % acetic acid at 20°C) is about 50 mPas. As expected, they showed Mws in the same range of 122, 135 and 149 kDa respectively, whereas Chitoscience® 90/200, for which the viscosity is stated about 200 mPas, revealed a higher Mw of 267 kDa. The molecular weight distribution, characterized by the polydispersity index Mw/Mn, is rather broad, ranging from 1.57 to 1.90 for all samples. Furthermore, the polydispersity index as well as the intrinsic viscosity and the hydrodynamic radius increased slightly with increasing polymer size.

Table III-3: Physical parameters obtained for the different chitosan batches.

Name	Absolute Mw (kDa)	Mw/Mn	Intrinsic viscosity (dL/g)	Hydrodynamic radius (nm)
Chitoscience® 90/50	122	1.57	3.62	18.33
Chitoceutical® 90/50(1)	135	1.61	3.86	19.28
Chitoceutical® 90/50(2)	149	1.62	4.11	20.30
Chitoscience® 90/200	267	1.90	6.01	27.74

3.2. Sol / gel transition behavior

CS / G1-P thermogelling solutions should undergo sol / gel transition *in situ* due to body temperature; therefore their gelation time was determined in PBS at 37°C. The sol / gel transition should not be too fast to avoid needle clogging during injection, but should be rapid enough to avoid drug burst from the liquid formulation [27]. The influence of the CS molecular weight and of the CS and G1-P concentrations on the gelation time (Table III-4) was studied. First, consistently with literature, decreasing the polymer Mw, from 267 to

122 kDa, resulted in a significant decrease of the gelation time from 15 to 3.5 min [28]. The same effect was observed with the CS concentration, *i.e.* concentration variation from 2 to 1.5 wt.%, gave rise to a strong drop of the gelation time from 20 to 2.5 min. However, it is to be noted that a further decrease of the CS content to 1.25 wt.% did not further accelerate the gelation. Finally, increasing the G1-P concentration from 0.20 to 0.40 mmol/g resulted in reducing the gelation time from 7 to 2.5 min, in agreement with previous reports on CS / β-GP systems [6,29]. Actually, the effect of the CS concentration and molecular weight on sol / gel transition time was likely linked to viscosity effects: the higher the concentration or Mw, the higher the solution viscosity, and the lower the mobility of the gelling agent and polymer chains, slowing down the gelation kinetics [30]. When increasing the G1-P concentration, consequently higher pH induced a reduction of the degree of ionization of CS. Electrostatic interactions opportunities between the polymer and the gelling agent were lowered, thereby reducing the protective hydration layer around CS. Furthermore, this leads also to a reduction of the electrostatic repulsion between CS chains, therefore promoting gelation at 37°C.

Table III-4: Gelation time at 37°C and pH value of CS / G1-P solution as a function of CS Mw, CS concentration and G1-P concentration.

CS		**G1-P**	**CS / G1-P**	
Mw (kDa)	**Concentration (wt. %)**	**Concentration (mmol/g)**	**t_{gel} (min)**	**pH**
267	1.5	0.33	15	7.1
122 : 267 (1:1)	1.5	0.33	7	7.2
122	1.5	0.33	3.5	7.4
122	2	0.40	> 20	7.3
122	1.75	0.40	11	7.2
122	1.5	0.40	2.5	7.4
122	1.25	0.40	3	7.4
122	1.5	0.27	4.5	7.3
122	1.5	0.20	7	7.2

3.3. Rheological characterization

3.3.1. Viscosity of CS / G1-P solutions

Viscosity of injectable ISFD solutions is a key parameter that needs to be assessed to ensure on the one hand an easy filling of the syringe and on the other hand an easy injection through thin needles. Apparent shear viscosity of 1.5 wt.% CS solutions containing increasing G1-P concentrations as a function of shear rate at 20°C is shown in Figure III-1 (a). In agreement with previous studies on CS solutions, all samples exhibited a shear-thinning behavior, characterized by a drop of the viscosity from around 150 to 80 mPas upon shear rate raise from 5 to 1000 s^{-1} [31-33]. Due to the high Mw and the concentration of the polymer, CS macromolecules in solution entangle loosely with each other at rest and disentangle upon applying the shear rate, lowering thereby the viscosity of the solution [34]. This shear-thinning flow behavior is advantageous for an easy administration of the thermogelling solution. Addition of G1-P concentrations between 0.27 and 0.53 mmol/g results in a linear increase in viscosity of the CS solution, without changing the flow behavior (Figure III-1 (b)). Two opposite effects arise from G1-P addition. Due to the phosphate part of the gelling agent, addition of G1-P increased the pH of the CS solution from 5.6 to around physiological values (i.e. 7.35 to 7.49 for G1-P concentration from 0.27 to 0.53 mmol/g respectively). At low pH conditions ($pH < pK_{ap} \approx 6.2$), CS behaves as a polycationic electrolyte due to the protonation of the free amine groups. The electrostatic repulsion between the macromolecules results in an extended conformation of the polymer. Increasing the pH reduces the charge density of CS molecules, resulting in a more contracted conformation of the polymer. Thus, the viscosity of the CS / G1-P solution, which is related to the macromolecular volume in solution, should be reduced compared to the pure CS one [33,35]. However, due to the glucose moiety of G1-P and its water-structuring effect (via hydrogen bond interactions), the gelling agent increased the apparent viscosity of the solution in a concentration-dependent manner [36,37]. For G1-P concentration as high as 0.53 mmol/g, viscosity was shown to be below 300 mPas at a shear rate of 5 s^{-1}. Since it was demonstrated that ISFD formulations with viscosities below 600 mPas were suitable for injection using needles sizes $\leq$ 23 G [38], CS / G1-P solutions seem to be appropriate injectable formulations. This characteristic will be further assessed through a comprehensive evaluation of the lead formulation's injectability in a next part (Part 3.7. Determination of injectability).

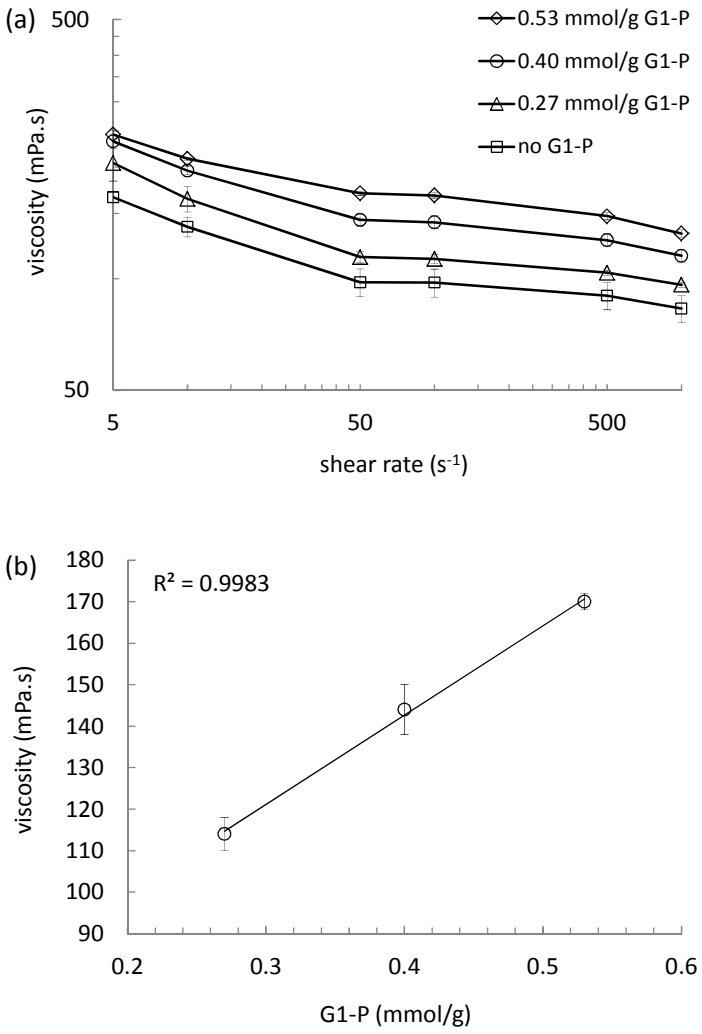

Figure III-1: Influence of G1-P concentration on apparent shear viscosities of 1.5 wt.% CS solution over a step test (a) and at a shear rate of 50 s^{-1} (b) for G1-P concentrations ranging from 0.27 to 0.53 mmol/g (n = 3).

3.3.2. Viscoelastic properties of CS / G1-P solutions

The viscoelastic behavior of CS solutions with different G1-P concentration is summarized in Figure III-2. The sol / gel transition temperature (T_{gel}) is defined as the temperature at which the storage modulus (G') is equal to the loss modulus (G"). As shown in Figure III-2 (a), at temperatures below T_{gel}, CS / G1-P systems exhibited viscoelastic liquid behavior, with the curves showing the dominance of the loss modulus (G" > G'). Upon temperature increase, at $T > T_{gel}$, the elastic portion of the system became dominant (G' > G"), clearly indicating that the solutions turned into gels. Following a similar trend, and corroborating the results of Table III-4, the time sweep tests at 37°C (Figure III-2 (b)) show a

decrease of the gelation time along with the increase of G1-P concentration. Furthermore, once the gelation process was initiated, the two moduli increase gradually up to the end of the test, indicating that the gel network formation is progressive and was not completed during the time of the measurements. Figure III-3 shows the appearance of a 1.5 wt.% CS / 0.40 mmol/g G1-P formulation in the solution state at room temperature and in the gel state at 37°C. The decrease of the gelation temperature of CS / G1-P systems goes along with a reduction in gelation time at physiological temperature and therefore can be linked to the increase in the pH value of the thermogelling solution when adding more gelling agent. These results confirm the trend observed above in vials. The differences in the gelation times observed by rheology and those determined in vials can be attributed to the different experimental approaches and methodologies.

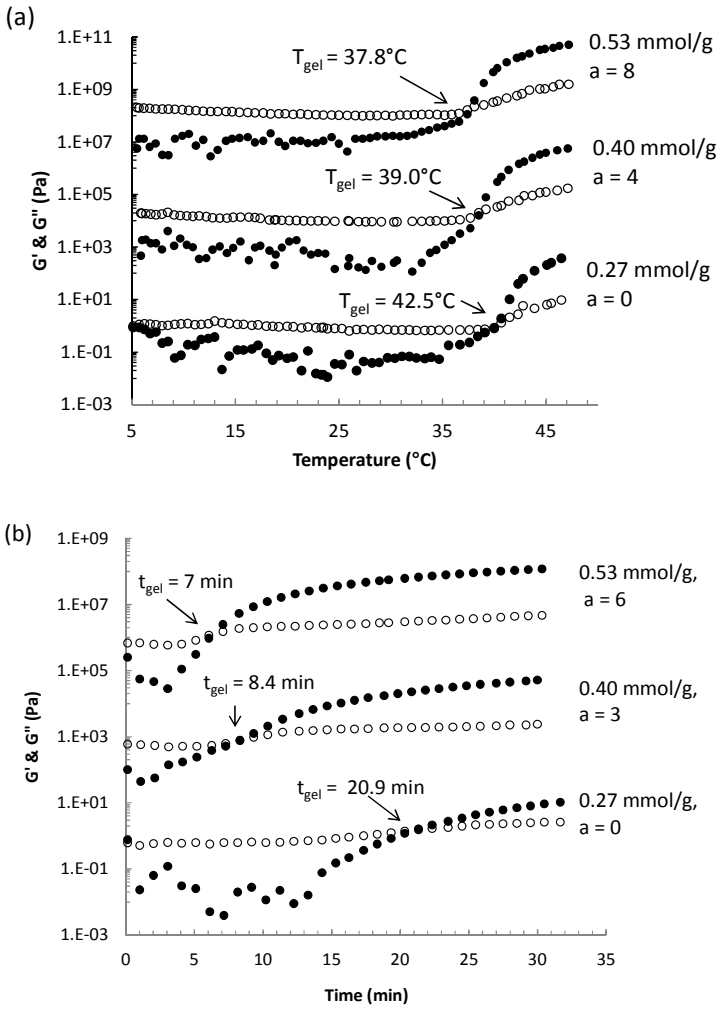

Figure III-2: (a) Temperature and (b) time dependence at 37°C of storage modulus (G', ●) and loss modulus (G", ○) of CS / G1-P solutions (1.5 wt.% CS) for G1-P concentrations ranging from 0.27 to 0.53 mmol/g. The data are shifted along the vertical axis by a factor of 10^a to avoid overlapping (n = 3).

Figure III-3: 1.5 wt.% CS / 0.40 mmol/g G1-P formulation in the solution state at room temperature and after gelation at 37°C.

Results obtained by frequency sweep experiments concern the value of the hydrogels strength once formed after different incubation times in a saturated water vapor atmosphere at 37°C. As shown in Figure III-4 (a), the system display G' > G" over the entire frequency range, with a value of G' independent of the frequency. Furthermore, G' and G" curves exhibit almost parallel lines in the low frequency range, and the G':G" ratio is herein higher than 20:1, which typically indicates a gel-like character [26]. In addition, the strengths of the gel formed after 6 h, 24 h and 48 h in a saturated water vapor atmosphere at 37°C, appeared to significantly increase over time (Figure III-4 (b)), in alignment with the observations made from the temperature and time sweep tests. This phenomenon is consistent with the molecular gelation mechanisms, induced by the temperature increase, and attributed to the gradual removing of the protective hydration layer formed by the gelling agent around the CS chains. Therefore, even if the first hydrophobic attractive interactions occur between the polymer chains, inducing the formation of a network, the number of CS - gelling agent interactions is still further reduced with time, leading to a gradual increase of the gel strength. Furthermore, once the gelation process was initiated, the increased viscosity of the system slows down the diffusion of G1-P away from the polymer and the self-association of the CS chains. Therefore, the number of junction points between CS molecules rises with time until complete gelation is achieved.

Additionally, the strengths of CS / G1-P hydrogels formed either in a saturated water vapor atmosphere or in PBS at 37°C were compared (Figure III-4 (b)). Gelation in PBS led to significantly stronger gels, with G' values in the range of 1000 Pa versus 200 to 400 Pa in a saturated water vapor atmosphere. Indeed, in the aqueous media at pH 7.4, G1-P can diffuse freely out of the gel during the gelation process while the pH is maintained higher than the pK_{ap} of CS [2,39]. Thus, CS - CS attractive interactions are no longer prevented by the protective hydration layer and the steric hindrance caused by G1-P, thereby creating more

physical junctions of the polymer network. CS / G1-P thermogelling solutions are intended to be administered subcutaneously, where the gelling agent is likely to diffuse out of the depot and into the surrounding tissue, since fluid flow in s.c. tissue usually ranges from 7 to 53 mL/100 g per min [38,40]. Thus, strong hydrogels are expected to form progressively at the site of injection.

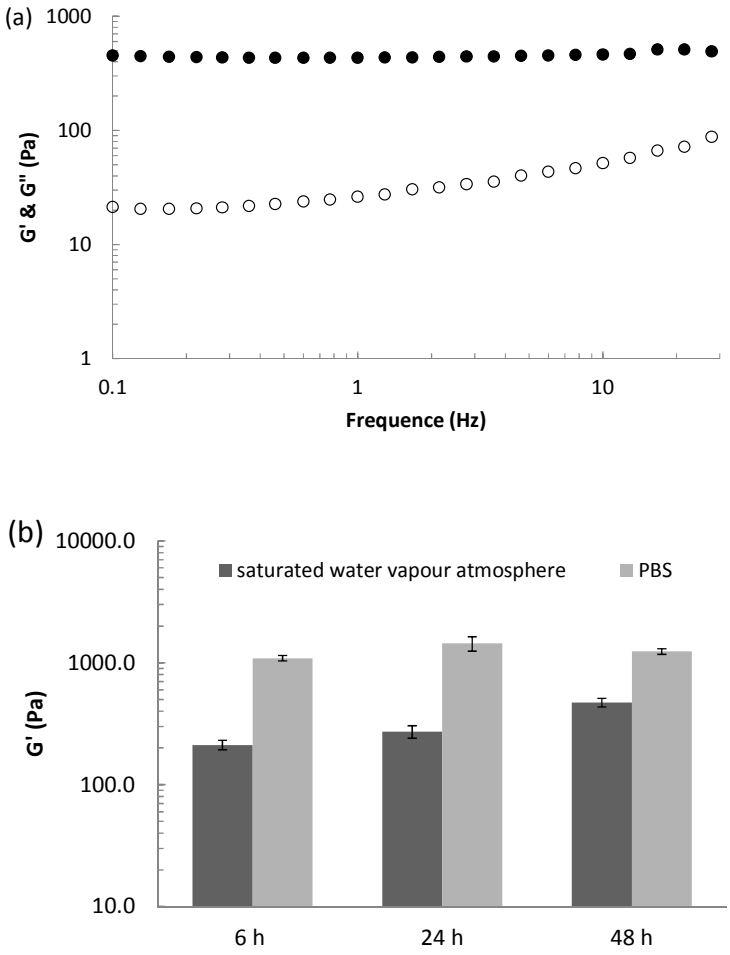

Figure III-4: (a) Frequency dependence of storage modulus (G', ●) and loss modulus (G", ○) of CS / G1-P hydrogel (1.5 wt.% CS and 0.40 mmol/g G1-P) after 6 h in a saturated water vapor atmosphere at 37°C. (b) Strength of CS / G1-P hydrogels (1.5 wt.% CS and 0.27 mmol/g G1-P) after 6 h, 24 h and 48 h incubation either in a saturated water vapor atmosphere or in PBS at 37°C (n = 3).

3.3.3. Thermo-reversibility of CS / G1-P solutions

To investigate the thermoreversibility of the hydrogel, CS / G1-P solution was submitted to two successive heating-cooling-cycles in the temperature range of 5 to 40°C (representative results are shown in Figure III-5). Upon the first increase in temperature, sol / gel transition occurred at around 39°C, as observed previously in Figure III-2 (a). The value of the storage modulus kept increasing while the temperature was maintained at 40°C, due to the growing formation of the three-dimensional network of the hydrogel. Cooling to 5°C caused a 10-fold drop of the value of G' while the value of G" remained constant, indicating a transition from strong to weak gel without reversion to the liquid state, in agreement with previous reports on CS / polyol-phosphate systems [1,41]. The same behavior was observed for the second heating-cooling cycle, for which a sharp increase and decrease of G' occurred during the heating and cooling phases respectively, but with G' remaining above G", confirming that CS / G1-P hydrogel is not fully thermoreversible.

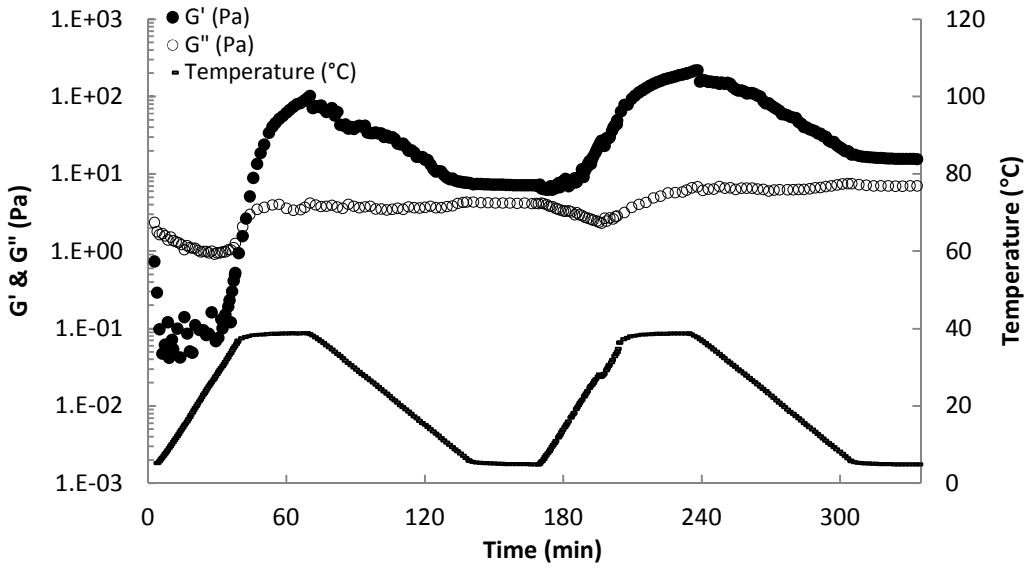

Figure III-5: Temperature dependence of storage modulus (G', ●) and loss modulus (G", ○) of CS / G1-P solution (1.5 wt.% CS, 0.40 mmol/g G1-P concentrations) over heating and cooling cycles (n = 2).

3.4. Differential scanning calorimetry

DSC has been used to further characterize the gelation process and thermoreversibility of CS / G1-P solutions. The first heating scan (Figure III-6, thermogram a) shows a broad

endothermic peak (ΔH = 0.30 J/g) between 33 and 48°C, with a maximum temperature of 37.1°C. This peak can be related to sol / gel transition of the CS / G1-P solution. This endothermic process could correspond to both the disruption of the hydrogen bonds forming the hydration shell surrounding the CS chains, and to the formation of intra- and inter-polymeric hydrophobic interactions [42,43]. This is confirmed by the fact that the sol / gel transition temperature disclosed here is consistent with the gelation temperature given by rheological experiments at the same heating rate (see Figure III-2 (a)).

Likewise, a second DSC scan (*i.e.* this time, after gel formation and cooling to 0°C) was performed on the CS / G1-P sample under identical conditions (Figure III-6, thermogram b). The transition appears thermo-irreversible since no endothermic peak appears for the second measurement, confirming thereby the results of the rheological thermo-analysis performed previously.

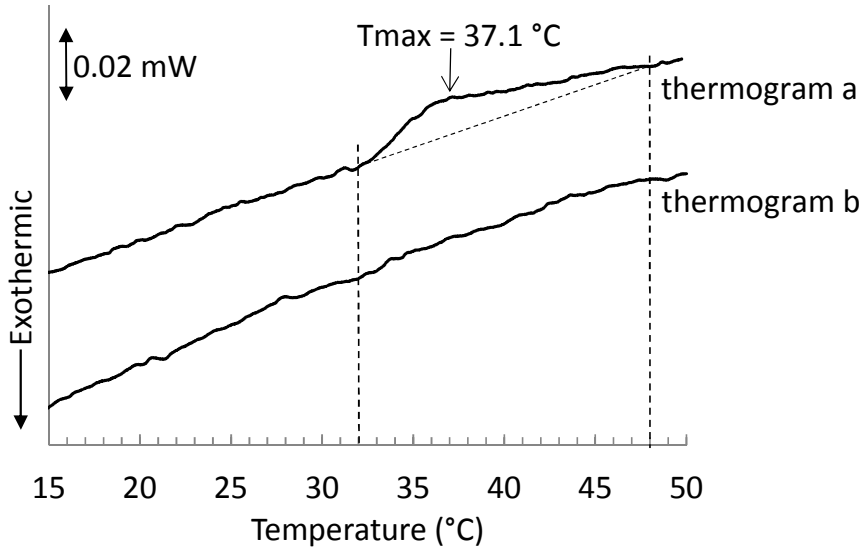

Figure III-6: DSC thermograms of a CS / G1-P solution (1.5 wt.% CS and 0.40 mmol/g G1-P) recorded at a heating rate of 1°C/min. Thermogram a and b correspond to the first and second heating scan respectively (n = 2).

3.5. Osmolality

Addition of the gelling agent in the CS solution strongly contributes to the tonicity of the formulation. As shown in Figure III-7, the osmolality of CS / G1-P solutions ranged from 760 to 1520 mOsm/kg, linearly related with the increase of gelling agent concentration from 0.27 to 0.53 mmol/g. The tonicity of parenteral formulations should be as close to

physiological conditions (e.g. 280 - 300 mOsm/kg) as possible, in order to minimize pain and/or tissue irritation. However, for subcutaneous (s.c.) injection, hypertonic solution are known to facilitate the drug absorption and could therefore be sometimes even desired [44]. Moreover, preparations with high osmolalities (up to 1100 mOsm/kg) have been reported in literature for intra-muscular or s.c. administration, without causing a significant increase of the burning or pain sensation [45-47]. Thus, injectable CS / G1-P solutions containing up to 0.40 mmol/g gelling agent were postulated to be acceptable.

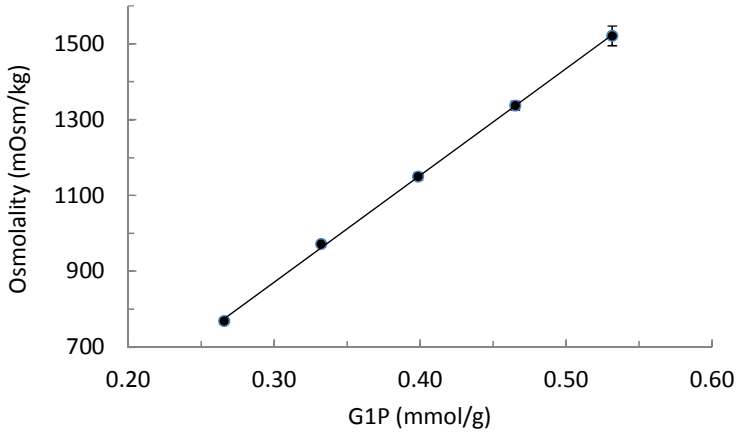

Figure III-7: Osmolality of CS / G1-P solutions (n = 3).

3.6. Storage stability of CS / gelling agent solutions

Storage stability is a crucial parameter that needs to be assessed during pharmaceutical development of an *in situ* forming hydrogel system. This type of formulations should have an acceptable shelf life, i.e. their physical thermogelling properties should be maintained throughout defined storage conditions up to administration. This section presents the stability study of two representative CS / G1-P thermogelling solutions (with 0.27 and 0.40 mmol/g G1-P) along with the comparison with the classical CS / β-GP thermogelling solution (with 0.40 mmol/g β-GP) and pure CS solution (at the same polymer concentration: 1.5 wt.%). The study included following-up over 6 to 9 months, for two different storage temperatures (2 - 8°C and 20 - 25°C), the appearance, pH, viscosity and gelation time of the solutions as well as CS molecular weight.

3.6.1. Appearance and pH

Overall, no change in the appearance (liquid state) nor in the pH values of the CS and CS / G1-P solutions were observed over the 6 to 9 months storage (for both temperatures). The pH of the pure CS solution remained 5.2 ± 0.1 while those of CS / G1-P solutions were 7.1 ± 0.1 and 7.2 ± 0.1, for 0.27 and 0.40 mmol/g G1-P, respectively. On the contrary, CS / β-GP solution turned into a turbid gel in less than 30 days at 2 - 8°C and after only 1 day at 20 - 25°C, with a drop of the pH value from 7.4 to 7.0.

3.6.2. Viscosity

Apparent shear viscosities of the CS solution and CS / gelling agent systems are reported in Figure III-8. Results show that for storage periods as long as 6 to 9 months and at refrigerated conditions, the apparent shear viscosity of the pure CS and CS / G1-P solutions remained unchanged. On the contrary, the viscosity of CS / β-GP increased significantly within 30 days, from about 180 mPas up to 7500 mPas at a shear rate of 5 s^{-1}. Likewise, the viscosity of CS / G1-P solutions was stable at room temperature over 2 months and only slightly increases at low shear rates after 6 months, whereas the viscosity of the pure CS solution very slightly reduced over time and the viscosity of the CS / β-GP solution raised drastically after only 7 days. These results are fully aligned with former observations, i.e. the pure CS solution as well as the CS / G1-P solutions remained in the liquid state over time for both storage conditions while the CS / β-GP solution turned into gel with time. Storage under refrigerated conditions allowed maintaining the CS / β-GP formulation in the liquid state for just over 14 days versus less than 7 days at room temperature. Even, as previously observed by monitoring the gel strengths (Figure III-4 (b)), once the sol / gel transition occurred, the viscosity of the CS / β-GP gels continue to increase up to stabilization (more appreciable at 2 - 8°C in Figure III-8 (g)).

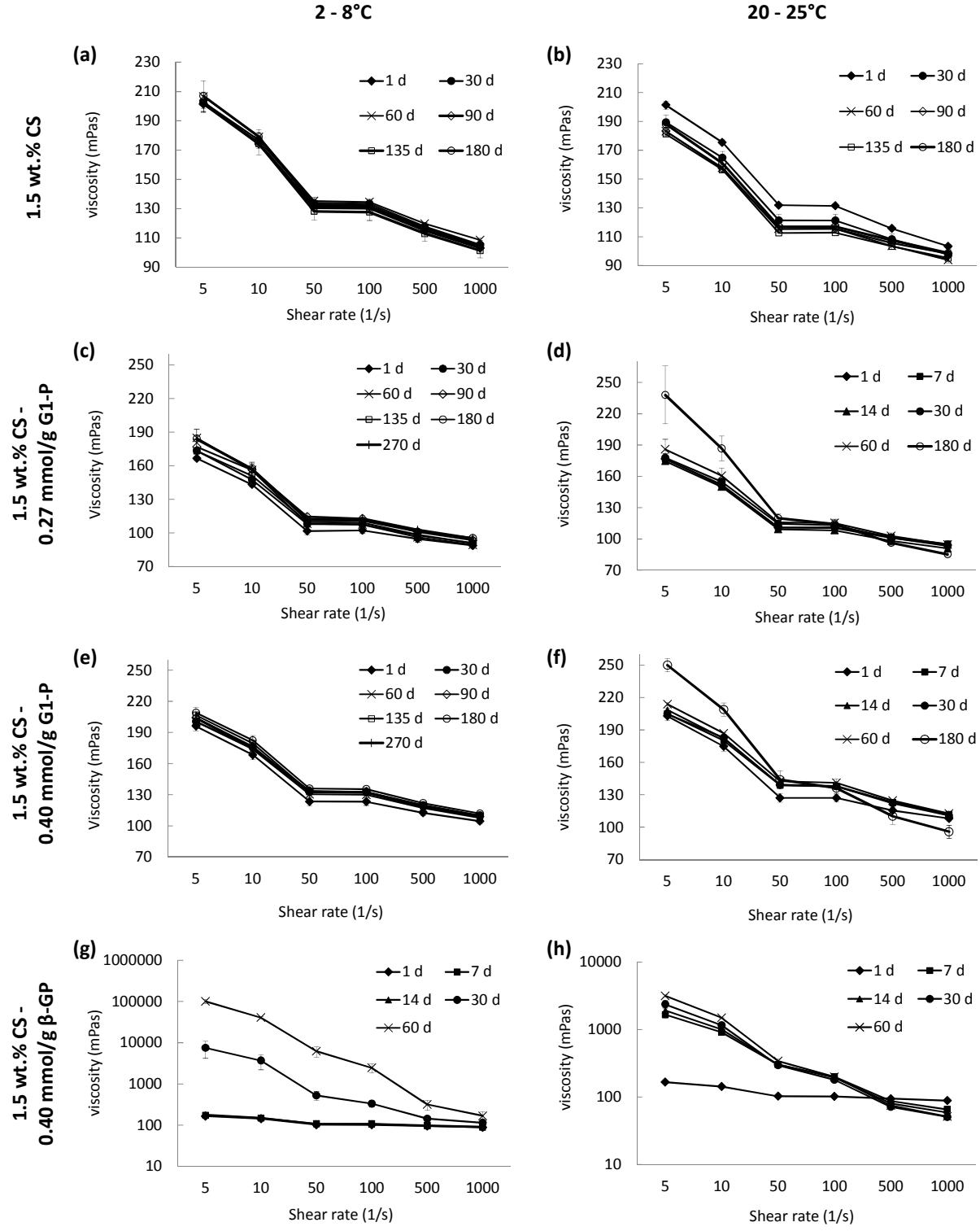

Figure III-8: Apparent shear viscosity at 20.0 ± 0.2°C for CS solution and CS / gelling agent systems stored at 2 - 8°C and at 20 - 25°C (n = 3).

3.6.3. Gelation time

The sol / gel transition times of the different CS / gelling agent formulations set up at 37°C were followed over the same storage period and reported in Figure III-9. It appears that the gelation time of the solution composed of 0.27 mmol/g G1-P is not stable over time, and decreases from about 40 min initially, to 35 min and 27 min after 6 months storage at 20 - 25°C and 2 - 8°C respectively, with significant variations observed over time under 20 - 25°C conditions. These variations vanish when the G1-P concentration is increased to 0.40 mmol/g. Indeed, the gelation time observed for the CS / G1-P solution containing 0.40 mmol/g G1-P stored at 20 - 25°C was stable over 2 months, but slightly decreased afterwards. However, when stored under refrigerated conditions, this solution displayed a constant gelation time of 12.2 ± 1.0 min over 9 months. With regards to the CS / β-GP solutions, the samples underwent a spontaneous gelation in less than 30 days at 2 - 8°C and 1 day at 20 - 25°C. These observations were confirmed by the rheological measurements of the gelation time, since the CS / β-GP system exhibited a gel-like behavior (G' > G") from the beginning of the time sweep test. However already before these time points, the gelation times were nevertheless very fast, in the range of 1 to 2 min. So the CS / β-GP solution was not stable when stored either at room temperature or under refrigerated conditions. Conversely, the addition of 0.40 mmol/g G1-P as alternative gelling agent to the CS solution significantly improved the stability of the system, in particular when stored at 2 - 8°C.

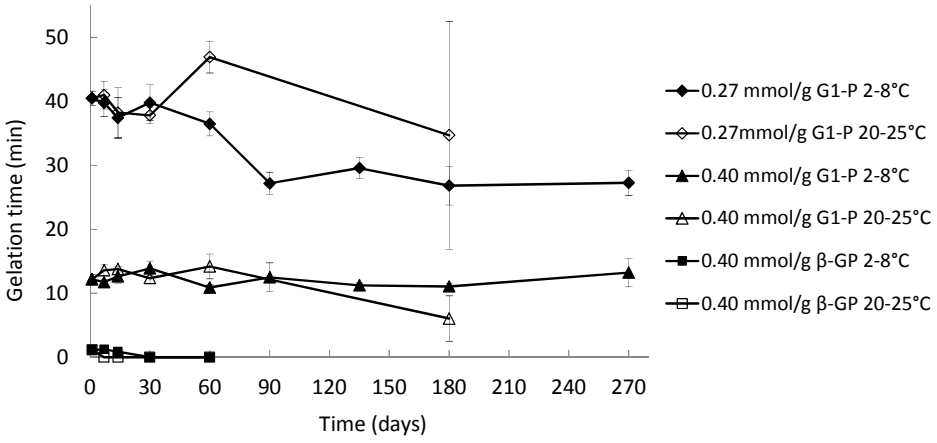

Figure III-9: Evolution of the gelation time, determined by rheology at 37°C, of CS / gelling agent solutions stored at 2 - 8°C and at 20 - 25°C (n = 3).

The lack of stability of CS / β-GP and other CS / polyol thermogelling solutions, even stored at refrigerated conditions, was already reported by Ruel-Gariepy *et al* [22] and Schuetz *et al* [19]. They concluded that the most appropriate storage method consisted in lyophilization of the formulations, to prevent any evolution to the gel state. However, this approach is expensive and would require a reconstitution step before injection. In addition, it also induced substantial changes in the gelation times and viscosities compared to freshly prepared solutions. In the present study, increasing the size of the polyol part of the gelling agent (*e.g.* using G1-P instead of the commonly used β-GP) was shown to improve the stability of the CS-based thermosensitive solutions. In view of our previous studies on the gelation mechanism (see Chapter II), increasing the size of the gelling agent polyol part likely enhances the stability of the protective hydration layer surrounding the CS chains, thus allowing extending the shelf life of the CS / gelling agent solution.

3.6.4. CS molecular weight

To complete the stability study, this last part deals with following the CS molecules integrity along the storage period as a function of storage temperature, through the determination of their molecular weight. The two CS / G1-P solutions were selected for this study, as well as pure CS solution for comparison. The molecular weight were analyzed by GPC and the results, reported in Figure III-10, revealed that no significant degradation of the polymer occurred in the presence of G1-P over 9 months of storage at 2 - 8°C. Conversely, around 20 % of the CS is degraded in pure CS solution without G1-P after 3 months under refrigerated conditions. When the same solutions were kept at room temperature, the Mw of all formulations, with or without gelling agent, decreased by 16 to 20 % within 6 months.

CS degradation is commonly known to occur with time in acidic aqueous solutions, due to acid-catalyzed hydrolytic chain scission of the glycosidic linkages of the polymer [48,49]. This chain scission results in a decrease of the CS molecular weight. In this study, the addition of G1-P to the CS solution was assumed to prevent the degradation mechanism through two complementary effects. First, addition of the gelling agent, which is a weak base, increased the pH from 5.2 to 7.2 - 7.3 and thus prevented the acid-catalyzed hydrolytic degradation of the polymer [50]. Secondly, as explained previously in the context of the comparison of the viscosity of CS versus CS / G1-P solutions, in a pure CS solution with pH < pKa, the polymer is in an expanded conformation, due to the electrostatic repulsion between the positively charged glucosamine monomers. Therefore, acid hydrolysis is

facilitated in these solutions since the glycosidic linkages are exposed. Increasing the pH via G1-P addition led to a partial neutralization of the polymer and, consequently, to a contraction of the CS chains, thereby hindering the hydrolysis [51]. Thus, addition of G1-P prevents the acidic depolymerisation mechanism of CS over at least 9 months at 2 - 8°C. Increased storage temperature (20 - 25°C) showed a significant impact on CS degradation. These results are consistent with those of Chen *et al* [51] and Varum *et al* [52], which showed that the hydrolysis rate increased with temperature.

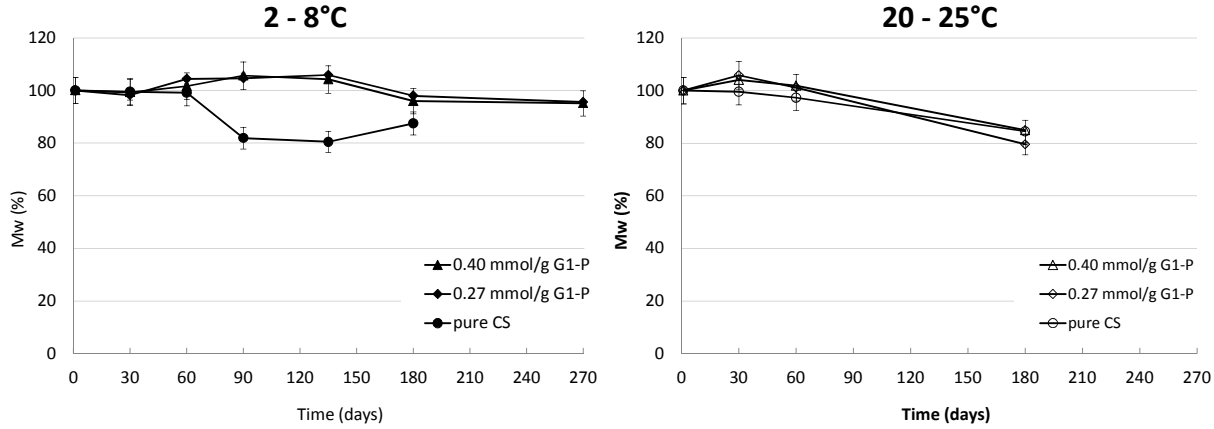

Figure III-10: Evolution of the relative Mw of CS in a pure CS solution, in a CS / 0.27 mmol/g G1-P solution and in a CS / 0.40 mmol/g G1-P solution during storage at 2 - 8°C and at 20 - 25°C (mean ± SD, SD = 5 %, *n* = 3).

To sum-up, investigating the stability of CS / gelling agents at different storage temperatures allowed disclosing the superior protective effect of G1-P compared to the classical β-GP. It allowed identifying the optimized formulation and storage conditions to provide an acceptable shelf life as well. Actually, CS / G1-P solution with a gelling agent concentration of 0.40 mmol/g was stable for at least 9 months when stored at refrigerated conditions. Thus, this thermogelling solution has the potential to be presented in the solution state, without requiring any reconstitution step before administration, and ready-to-use pre-filled syringes can therefore be considered.

3.7. Determination of injectability

In the development of ISFD systems, an important issue also lies in the ease of injection through the thin needles commonly used for s.c. administration [53,54]. Therefore, the FDA Guidance for Industry on container closure systems for packaging human drugs and

biologics requires the evaluation of the performance of a syringe by establishing the force necessary to inject the dosage form. Needle size recommended for s.c. injection range between 25 G (outer diameter (O.D.) = 0.51 mm, inner diameter (I.D) = 0.26 mm) and 30 G (O.D.= 0.31 mm, I.D. = 0.16 mm), and the volume of dosage form injected should be between 0.5 and 2.0 mL [44,55,56]. In this section, the force required to inject 1 mL thermogelling solution containing 1.5 wt.% CS and 0.40 mmol/g G1-P (chosen as the optimized formulation) through 23, 25 and 30 G needles was mesured. The pressure resistance of s.c. tissue at the injection site was not taken into consideration. Two injection speeds were studied, 80 and 100 mm/min, which correspond to 40 and 33 seconds for completing the injection, respectively. It is also to notice that the upper limit of 20 N was set as acceptance criteria for s.c. injections, according to Schoenhammer *et al* [38]. The results are presented in Figure III-11, and show that, in the conditions of our study, only the needle size significantly impacted the injection force. The force necessary to inject the CS / G1-P solution was below 5 N when using 23 G and 25 G needles, which may be related to very smooth injections. Using a thinner needle of 30 G required an increased injection force of about 17.5 N, which still did not exceed the upper limit of 20 N. In contrary to other ISFD solutions which were generally administered through large needles of 18 to 23 G due to their high viscosity [57-59], the CS-based formulation can be considered as easy to inject through needles as thin as 30 G, as assumed previously based on the low viscosity of the CS / G1-P solution (see Figure III-1 (a)). Administration via small needle size is known to cause less pain at the injection site, thereby improving patient comfort and compliance [60].

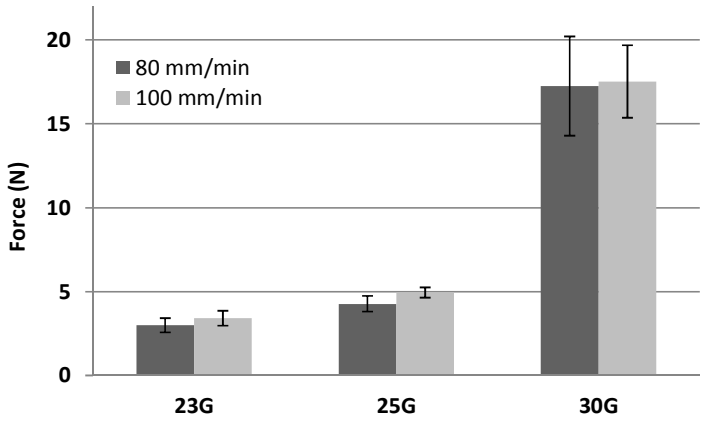

Figure III-11: Injection forces of 1 mL CS / G1-P solution (1.5 wt.% CS and 0.40 mmol/g G1-P) injected in air at two injection speeds with 23 G, 25 G and 30 G needles (n = 10).

3.8. Sterile filtration of CS / G1-P solution

Since CS / G1-P solution is intended for parenteral injection, sterilization is essential for its successful clinical administration. Due to its thermogelling property, the solution cannot be subjected towards terminal heat or steam sterilization. Furthermore, as gamma irradiation is known to induce CS chain degradation, this terminal sterilization method is also not suitable for the CS / G1-P formulation [61,62]. Thus, another potential method is to sterilize the solution by filtration through a 0.2 µm membrane and to fill it under aseptic conditions into presterilized vials. Therefore, the influence of sterile filtration on the thermogelling solution was assessed through the determination of the viscosity and gelation time at 37°C of the solution and of CS molecular weight before and after filtration. Sterile filtration was easily performed on the CS / G1-P solution. As presented in Figure III-12 (a), the viscosity of the solution remained unchanged after filtration. GPC analysis (Figure III-12 (b)) confirmed this result, since only a slight decrease of about 1 % of the molecular weight of the polymer was observed between the non-filtered and the filtered formulation, this value being within the accuracy range of the method (± 5 %). Thus, no polymeric material seems to be lost on the filter during sterile filtration. On the other hand, as shown in Figure III-13, sterile filtration affected the time for sol / gel transition at 37°C, since it increased from 8.9 ± 0.8 min for the non-filtered samples to 10.3 ± 1.4 min after filtration. However, this slight increase of the gelation time is still acceptable for ISFD system. Thus, sterilization via filtration through 0.2 µm membrane can be applied to the CS / G1-P solution without inducing important variations of the formulation properties.

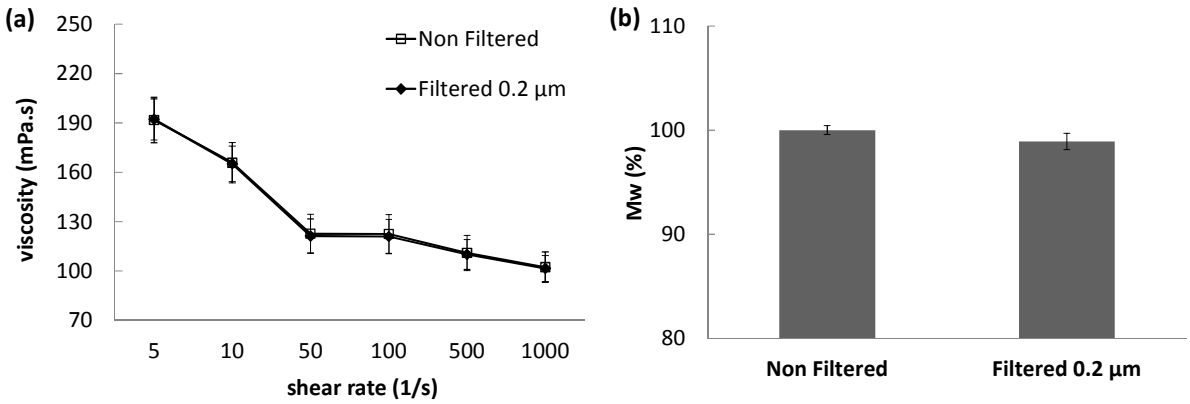

Figure III-12: (a) Apparent shear viscosity at 20.0 ± 0.2°C and (b) relative Mw of CS for three different batches of 1.5 wt.% CS / 0.40 mmol/g G1-P solution either non filtered or sterilized by filtration through a 0.2 µm membrane (n = 3).

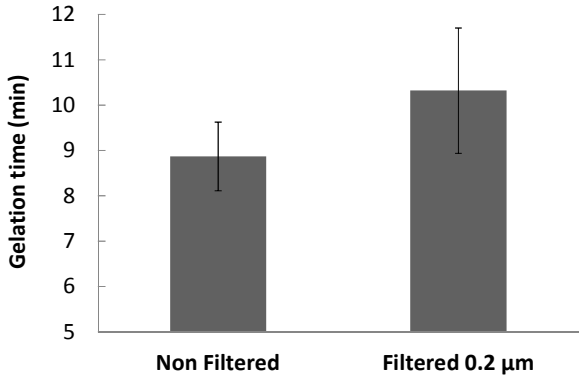

Figure III-13: Gelation time, determined by rheology at 37°C, of three different batches of 1.5 wt.% CS / 0.40 mmol/g G1-P solution either non filtered or sterilized by filtration through a 0.2 µm membrane (n = 3).

3.9. Bacterial endotoxin content of CS and CS / G1-P solutions

Microbiological acceptance criteria need also to be considered in view of the parenteral administration of ISFD systems. The endotoxin content of two pharmaceutical grade batches of CS and of the corresponding CS / G1-P solutions was determined with a semi-quantitative LAL assay; the results are presented in Table III-5. The tested CS varied in endotoxin level; Chitoceutical® 90/50(1) had an endotoxin level between 0.06 and 0.12 EU/mg whereas Chitoceutical® 90/50(2) showed a lower level between 0.03 and 0.06 EU/mg. The endotoxin level determined for the two thermogelling solutions were found to be between 0.0003 and 0.003 EU/mg. Considering that the maximum human dosage of the CS / G1-P solution is about 1000 mg per patient, the endotoxin limit is 0.35 EU/mg of thermogelling solution, according to equation 1. Thus, the maximum endotoxin exposure by CS / G1-P injections is at least 100-fold lower than the limit.

Table III-5: Bacterial endotoxin content of CS and CS / G1-P solutions.

Sample	Detected endotoxin content (EU/mg)
Chitoceutical® 90/50(1)	0.06 – 0.12
Chitoceutical® 90/50(2)	0.03 – 0.06
Chitoceutical® 90/50(1) / G1-P 1	0.0003 – 0.003
Chitoceutical® 90/50(2) / G1-P 2	0.0003 – 0.003

3.10. Histological examination

In order to investigate the local tolerance of implanted CS / G1-P hydrogels, the solution was injected through a thin needle into the s.c. tissue of nine rats. The injected solution turned into a gel *in situ* in few minutes under physiological temperature (Figure III-14). The histopathological effects resulting from the contact of the s.c. tissue with the hydrogel were monitored at 1, 7 and 21 days post-injection. As expected, the results show a typical foreign body reaction due to the implantation of the hydrogel.

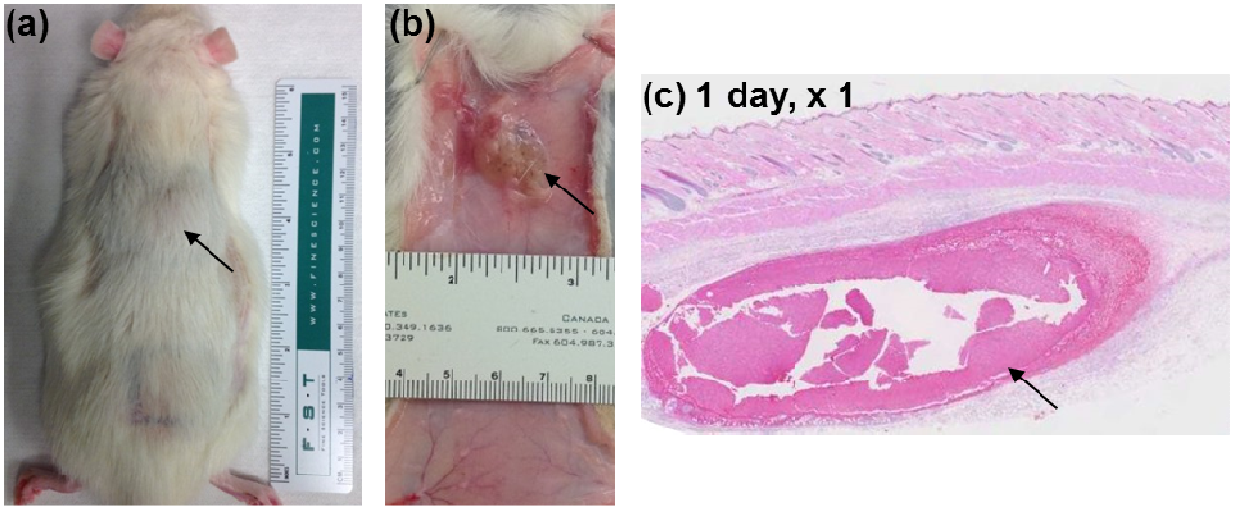

Figure III-14: (a) and (b) Local tolerance study in the subcutaneous tissue of rats at 1 day. (c) Histology of the CS / G1-P hydrogel and surrounding tissues at 1 day. The arrows indicate the location of the CS / G1-P hydrogel.

On the first day post injection, moderate to marked acute inflammatory response could be observed (see Figure III-15 (a) and (b)). Inflammatory cells, i.e. neutrophils, eosinophils and fewer macrophages, were present in the tissue surrounding the hydrogel. Furthermore, minimal to slight focal to multifocal hemorrhage accompanied the inflammation. Neutrophils and macrophages containing intracytoplasmic granular material were found at the interface with the hydrogel, showing that these cells phagocytized the CS-based depot.

After one week post injection, the depot was surrounded by necrotic neutrophils and cellular debris admixed with fibrin. Moderate to marked subacute inflammation was observed, characterized by the presence of macrophages and fewer neutrophils, eosinophils and lymphocytes in the s.c. tissues, as shown in Figure III-15 (c) and (d). Minimal to slight

fibrosis consisting in an immature granulation tissue, composed of sprouting capillaries and activated fibroblasts, was located in the surrounding area of the hydrogel. Minimal focal multifocal hemorrhage accompanied the inflammation. Phagocytosis of the depot was indicated by the presence of granular material in the cytoplasm of a few neutrophils and macrophages, as displayed in Figure III-16 (a).

Finally, after 21 days post implantation, the degree of inflammation of the s.c. tissue declined significantly compared to the earlier time points (Figure III-15 (e) and (f)). A slight chronic inflammation composed of lymphocytes and smaller amount of macrophages was present around the hydrogel. Minimal to slight fibrosis, consisting in a mature less vascularized granulation tissue, encapsulated the depot (Figure III-16 (b)). As previously, minimal focal to multifocal hemorrhage accompanied the inflammation and intracytoplasmic phagocytized material was present in few neutrophils and macrophages.

The histopathology results, summarized in Table III-6, showed that the inflammatory reaction reduced with time, while slight fibrosis progressively surrounded the implant site. These reactions are comparable to the tissue responses typically observed for other implantable drug delivery systems, like biodegradable PLA and PLGA microspheres, or standard CS / β-GP hydrogels, which also show acute inflammation followed by chronic inflammation, granulation tissue formation and fibrosis [63,64]. This suggests thereby reasonably good compatibility of the hydrogel in the s.c. tissues. For all animals, the hydrogel persisted over the 3 weeks of study. Furthermore, no detrimental effect of the hydrogel on the body weight or animal behavior was observed.

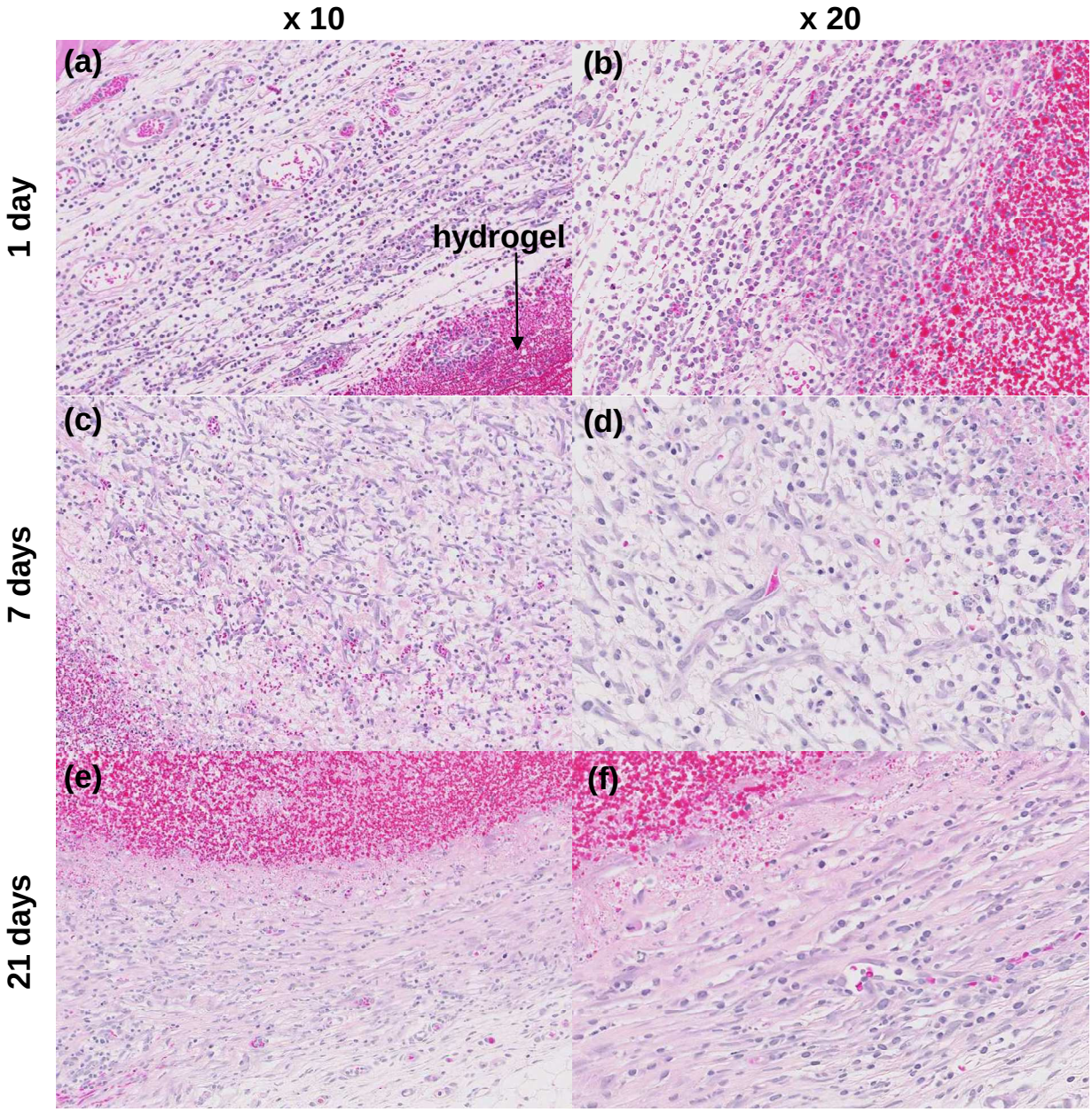

Figure III-15: Histopathological examination of inflammatory response (H&E staining) at the interface between the CS / G1-P hydrogel and the s.c. tissue: (a) and (b) 1 day, (c) and (d) 7 days, (e) and (f) 21 days post-injection.

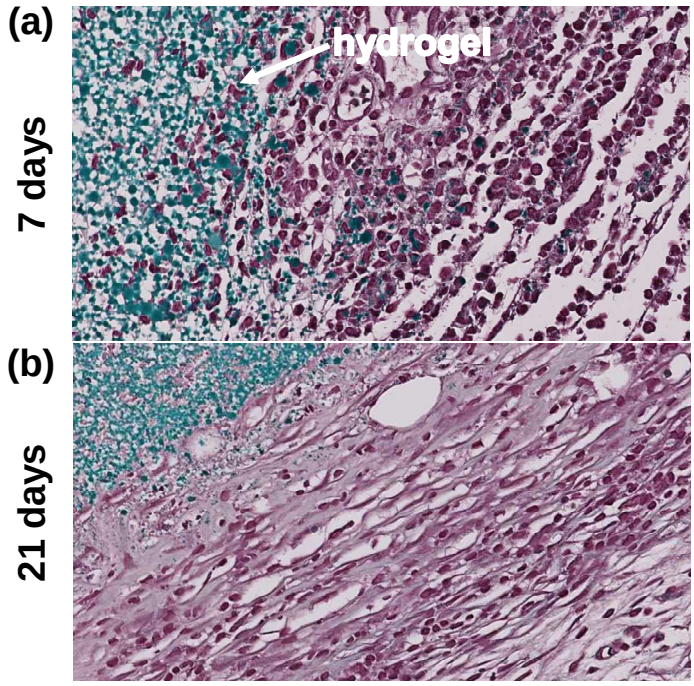

Figure III-16: Histopathological examination of inflammatory response (0.01 % Fast Green in conjunction with Safranin-O staining) at the interface between the CS / G1-P hydrogel and the s.c. tissue at 7 days post injection, original magnification x40 (a), and 21 days post injection, original magnification x20 (b).

Table III-6: Histological examination of s.c. tissue of rats implanted with CS / G1-P hydrogels.

Time point post injection	Inflammation	Fibrosis
1 day	+++ / ++++ acute	-
7 days	+++ / ++++ subacute	+ / ++ immature
21 days	++ chronic	+ / ++ mature

For inflammation, "++", "+++" and "++++" indicates mild, moderate and marked inflammatory response respectively.

For fibrosis, "-", "+" and "++" represents no, minimal and slight fibrosis respectively.

3.11. Hematological and biochemical examination

The complete blood count (white blood cells (WBC), red blood cells (RBC) and platelet count) of the rats was determined after 1, 7 and 21 days post injection and compared to the pre-test values obtained 3 days before injection. It could be observed that, 1 day post injection, all animals had an increased number of neutrophils, leading to increased WBC in 2/3 of the animals while the third one showed a decrease in WBC due to reduced lymphocytic counts. At 7 days post injection, animals had decreased RBC mass parameters with increased reticulocytes. Furthermore, a modest increase in neutrophils and platelet count was also observed. Finally, at 21 days, the rats had a modest decreased red blood cell mass parameter with increased reticulocytes. These slight hematology changes can be attributed to the presence of an inflammatory process. The results of biochemistry analysis were compared to reference ranges for Wistar Han rats. Increased in triglycerides was present in all rats at 7 days and one rat at 21 days post injection. Although this is likely due to the non fasting state of the animals, a direct effect of the CS / G1-P depot cannot be excluded. At all time points, all animals presented glucose values in the highest end range, likely due to the release of G1-P from the hydrogel. On the long term, this CS / G1-P-related hyperglycemia might affect the glucose metabolism of the rats and the effect on the insulin should be monitored in follow up studies.

4. Conclusion

To conclude, through a panel of different physico-chemical characterization methods, including viscosity and injectability evaluation, thermo-gelation and stability studies, the present work demonstrates the feasibility of CS / G1-P solution as interesting alternative to the standard CS / β-GP thermosensitive system forming a depot at physiological conditions. Rheological thermo-analysis and DSC scan confirmed the sol / gel transition temperature of the CS solution containing 0.40 mmol/g G1-P at around body temperature and demonstrated that the strong gel formed was not thermoreversible. Moreover, the storage stability problem of the formulation, actually one of the most important issue of standard CS-based thermogelling systems, could be solved by using G1-P as alternative gelling agent. The CS / G1-P *in situ* depot forming solution kept its thermogelling properties for minimum 2 months at room temperature and over 9 months at refrigerated conditions. Therefore, and in view of the easy injectability of the formulation though thin needles, a convenient presentation in ready-to-use prefilled syringes could be considered. The inflammatory reaction observed around the CS / G1-P formulation injected subcutaneously in rats was a typical foreign body reaction, similar to the tissue response reported for other depot systems like PLGA microparticles or standard CS / β-GP hydrogels. Following the injection of the formulation, acute to chronic inflammation occurred in a sequential order and inflammation tended to fade with time. Fibrosis accompanied the inflammatory response and surrounded the implant site; it started 7 days post injection with a well vascularized immature granulation tissue and towards 21 days, it formed a slightly vascularized thick capsule composed of fibroblasts and collagen fibers. Thus, the hydrogel exhibited acceptable tissue biocompatibility.

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Chapter IV :

In vitro evaluation of CS / G1-P for the controlled release of model compounds

Abstract

Chitosan / Glucose-1-phosphate (CS / G1-P) thermosensitive solutions showed suitable properties for the potential development of parenteral ISFD formulations. In this chapter, the feasibility to formulate compounds with different physico-chemical properties in CS / G1-P thermogelling solutions or dispersions as well as the *in vitro* release performance of these formulations were addressed. Methylene blue (MB), drug substance A (DSA) (an anionic low molecular weight compound), eosin Y (EY) and a hydrophobic peptide were used as model substances and were either dissolved (for MB, DSA and EY) or dispersed (for the peptide) in the thermogelling system. *In vitro* release experiments showed a sustained release over days to months, depending on the physico-chemical properties of the loaded substance (i.e. molecular weight, charge, solubility). Similar release profiles were observed for MB- and DSA-loaded hydrogels, thereby demonstrating that the reversible electrostatic interactions between the anionic DSA and the cationic CS polymer have no marked influence on the release behavior. Formulation loaded with EY showed a lower release rate than DSA-loaded depots. Further rheological characterization of the viscoelastic behavior of CS / G1-P / EY systems demonstrated that the multivalent EY anions acted as cross linkers, forming bridges between the polymeric chains. Hydrogels loaded with a poorly water soluble model peptide displayed a small burst and a sustained release for over 3 months. The release rate could be adjusted by varying the drug loading. Lysozyme induced degradation of the polymeric matrix translated in an increase of the peptide release rate and reduction of CS Mw over time.

1. Introduction

In the past few years, CS-based ISFD have been extensively studied as drug delivery systems for the sustained and controlled release of drugs [1-4]. These systems showed promising *in vitro* and/or *in vivo* results for various clinical applications, including chemotherapy, diabetes, antibiotherapy, anxiety disorder and vaccination. In literature, drug delivery from CS hydrogels is reported to be tunable by varying the raw material attributes such as the deacetylation degree (DD) and molecular weight (Mw) of CS, the formulation parameters like the ratio of CS and gelling agent, the drug physico-chemical properties as well as the drug loading [3-8]. In the previous chapter of this thesis, thermosensitive solutions obtained from a CS solution neutralized with G1-P showed suitable physicochemical characteristics for use as injectable ready-to-us ISFDs. This last chapter will focus on the feasibility to formulate compounds in CS / G1-P solutions yielding in thermogelling solutions or dispersions as well as the assessment of their *in vitro* release performance as controlled release drug delivery systems. In order to better understand the release mechanism(s) and the interactions between the CS-based hydrogel and the loaded substances, different types of model compounds were studied. Due to its polycationic nature, CS can interact with negatively charged entities (ions, ionic molecules or polymers) [9]. This characteristic was frequently used for the formation of CS nano- and microparticles by ionic gelation with tripolyphosphate as cross linker or for the preparation of CS / siRNA polyplexes [10-12]. Thus, the first purpose of this work was to investigate the release of CS / G1-P hydrogel loaded with three hydrophilic low molecular weight substances, i.e. methylene blue (MB), drug substance A (DSA) and eosin Y (EY), and to determine the influence of the charge of the ionic molecule on the release behavior.

Due to their short half-life, many authors investigated the use of injectable depot systems for the delivery of peptides [13-16]. Thus, as a second part of this study, the potential of the CS-based *in situ* forming hydrogel to sustain the release of a poorly water soluble peptide for a targeted period of 3 months was evaluated *in vitro* and the influence of drug loading on the release behavior was assessed. Furthermore, the influence of the hydrogel enzymatic degradation on peptide release and CS molecular weight by addition of lysozyme, recognized to be the main degradation mechanism of CS in human serum and tissues [17-19], was examined.

2. Materials and Methods

2.1. Materials

CS of technical grade: Chitoscience® 90/50 (DD 89.7 %, Mw: 122 kDa), and two CS of pharmaceutical grade: Chitoceutical® 90/50(1) (DD 90.7 %, Mw: 135 kDa) and Chitoceutical® 90/50(2) (DD 88.7 %, Mw 149 kDa) were used in this study. All CS are derived from shrimp shell and were purchased from Heppe Medical Chitosan (Halle, Germany). α-D-glucose 1-phosphate disodium salt hydrate (G1-P, Mw: 376.16 g/mol), methylene blue hydrate (Mw: 373.90 g/mol), eosin Y disodium salt (Mw: 691.85 g/mol), phosphate buffered saline (PBS), polysorbate 80, benzalkonium chloride, sodium azide and lysozyme (from chicken egg white, enzymatic activity: 81989 U/mg) were purchased from Sigma-Aldrich. DSA (Mw: ~ 330 g/mol) and a model peptide (Mw: ~ 1400 Da) was provided from Novartis Pharma AG (Basel, Switzerland). Hydrochloric acid (HCl) was purchased from Merck. All other reagents were of analytical grade and were used without further purification. Ultrapure® water was obtained using a MilliQ® Millipore filtration system (Millipore, Molsheim, France).

2.2. Preparation of the formulations

CS solution: CS solutions were obtained by dissolving 3.75 wt.% CS in HCl according to a molar ratio for CS amine groups : HCl equal to 0.9 : 1.

CS / G1-P solution: G1-P solution was prepared at 350 mg/mL in MilliQ water. CS and G1-P solutions were separately cooled to 4°C for 15 minutes. Then, 2.571 mL G1-P solution was poured drop-by-drop into 2.40 g CS solution placed in an ice-bath, under magnetic stirring. The obtained CS / G1-P solution was adjusted to 6.00 g with MilliQ water and further stirred for 15 minutes. The resulting formulation, containing 1.5 wt.% CS and 0.40 mmol/g G1-P, was stored at 2 - 8°C.

MB-, DSA- and EY-loaded CS / G1-P solutions: the model compounds were solubilized at different concentrations in the G1-P solution. The G1-P / model compound solution was cooled down to 4°C for 15 min prior to adding it drop-by-drop into the cold CS solution under magnetic stirring in an ice bath. The obtained CS / G1-P / model compound solutions were adjusted to final weight with MilliQ water and further stirred for 15 minutes.

The final formulations contained 1.5 wt.% CS, 0.40 mmol/g G1-P and different concentrations of each model compounds, as described in Table IV-1. All formulations were stored at 2 – 8°C until use.

Table IV-1: Overview of the CS / G1-P / low Mw model compounds formulations.

Formulation	Model compound	Model compound content (wt.%)	Model compound concentration (µmol/g)
CS / G1-P / MB 0.06	MB	0.06	1.60
CS / G1-P / DSA 0.06	DSA	0.06	1.80
CS / G1-P / EY 0.03	EY	0.03	0.43
CS / G1-P / EY 0.06	EY	0.06	0.87
CS / G1-P / EY 0.12	EY	0.12	1.73
CS / G1-P / EY 0.3	EY	0.30	4.33
CS / G1-P / EY 0.6	EY	0.60	8.67
CS / G1-P / EY 1.2	EY	1.20	17.34
CS / G1-P (placebo)	/	/	/

MB-, DSA- and EY-loaded CS solutions: Positive controls were prepared with the same protocol but without including G1-P as gelling agent *i.e.* MB, DSA or EY were solubilized in MilliQ water before addition to the CS solution.

Peptide-loaded CS / G1-P suspensions and particle size determination: the peptide was added into the G1-P solution, dispersed by magnetic stirring for 2 h and sonicated 2 min using an ultrasound probe (amplitude 80 %, cycle 1, Hielscher Ultrasound technology UP400S Ultrasonic processor, Stuttgart, Germany) under magnetic stirring in an ice-bath. 3.60 g G1-P / peptide suspension was then added dropwise into 2.40 g of cold CS solution under magnetic stirring in an ice bath. The obtained CS / G1-P / peptide formulation was further stirred until a homogeneous suspension was obtained, as verified by optical microscopy and focused beam reflectance measurements (FBRM) (Table IV-2). The final formulations, containing 1.5 wt.% CS, 0.40 mmol/g G1-P and 2.5 or 5.0 wt.% peptide, were stored at 2 – 8°C until use.

FBRM data were obtained using a Lasentec FBRM PI-14/206 probe (Mettler-Toledo, USA.) and analyzed with Lasentec FBRM software (Version 6.7.0 09/2005 Mettler Toledo, Columbia, USA). This light scattering technique measures the backscattered light from a rotating laser beam focused outside a sapphire window, in contact with the suspension. When

the laser light scans particles near the probe window, it generates backscattered light signals whose duration is translated into chord lengths (straight-line distance from one edge of the particle to another). Therefore, even if the chord length is related to the particle size, it does not measure directly the particle size distribution, but rather the chord length distribution [20].

Table IV-2: Chord length distribution in the CS / G1-P / peptide suspensions.

Formulation	Peptide content (wt.%)	Median square-weighted chord length (µm)	Mean square-weighted chord length (µm)	< 30 µm (%)
CS / G1-P / Peptide2.5	2.50	10.27	12.66	94.56
CS / G1-P / Peptide5	5.00	10.87	12.57	96.61

Peptide-loaded CS suspensions (positive controls): CS / Peptide suspensions as positive controls were prepared following the same protocol but dispersing the peptide in water instead of G1-P solution.

2.3. Peptide quantification by HPLC

The peptide was quantified using an Agilent 1100 HPLC system (Agilent, Santa Clara, CA, USA), with a Waters symmetry shield, RP18 3.5 µm, 50 x 4.6 mm column (Waters Corporation, Milford, USA). The mobile phase A consisted of water, acetonitrile, phosphoric acid (900 : 100 : 1 V/V/V) and mobile phase B of (100 : 900 : 1 V/V/V) at a flow rate of 1.0 mL/min using a 6 steps gradient. 10 µL samples were injected at an oven temperature of 40°C. The analysis was performed by UV detection at 230 nm. All samples were injected in duplicate. The resulting chromatogram was analyzed with Chromeleon 6.8 Datasystem (Dionex Corporation, Sunnyvale, CA, USA). The reference stock solution of peptide was prepared in methanol and the standard solutions of different concentrations were prepared with release medium of pH 7.4 (see Table IV-3). The resulting calibration curves ranged from 3 to 250 µg/mL with a correlation coefficient above 0.999.

2.4. *In vitro* release tests

In vitro release tests were performed in triplicate using two different methods. In method I, an amount of approximately 0.5 g (accurately weighed to 0.001 g) formulation was injected into a 50 mL tube and incubated 1 h in a reciprocating water bath (GFL, Burgwedel, Germany) maintained at 37°C to allow gel formation. 25 mL release medium pH 7.4 pre-

warmed to 37°C was then added slowly to the depot (t = 0) and mild shaking (10 %) was started. In method II, the same amount of formulation was injected directly into a 50 mL tube containing 25 mL (50 mL for the peptide-loaded formulations) release medium pH 7.4 pre-warmed to 37°C, in a reciprocating water bath (GFL, Burgwedel, Germany) maintained at 37°C (t = 0). After 1 h at rest, to allow gel formation, mild shaking (10 %) was started. For method I and II, at defined time points, the tubes were gently homogenized, centrifuged and 10 mL samples (25 mL for the peptide-loaded formulations) were removed from the media and replaced with fresh release medium to maintain the volume constant and to maintain the sink conditions. The amount of compound released was monitored by spectrophotometry using an Agilent 8453 UV-visible spectrophotometer, at a wavelength of 665 nm for MB, 517 nm for EY and 276 nm for DSA. All measurements were performed in duplicate. A CS / G1-P hydrogel (without MB, EY or DSA) was used as a blank control for absorbance correction. The amount of peptide released was determined by HPLC, as described previously. The composition of the release medium pH 7.4 used for each release test is listed in Table IV-3. The mechanism of drug release from CS / G1-P hydrogel was investigated by applying the mathematical models presented in Table IV-4 to the *in vitro* release data. By comparing the regression coefficient (R^2), the most appropriate equation can be found.

Table IV-3: Composition of the release media pH 7.4 used for the *in vitro* release tests.

Composition	**CS / G1-P / MB**	**CS / G1-P / DSA**	**CS / G1-P / EY**	**CS / G1-P / peptide**
Na_2HPO_4	1.09 g	1.09 g	1.09 g	1.09 g
KH_2PO_4	0.32 g	0.32 g	0.32 g	0.32 g
NaCl	8.06 g	8.06 g	8.06 g	8.06 g
Benzalkonium chloride	0.01 g	/	/	0.01 g
Sodium azide*	/	0.25 g	0.25 g	/
Polysorbate 80**	/	/	/	4.00 g
Lysozyme	/	+/- 0.01 g	/	+/- 0.01 g
Water	qs 1000.0 mL	qs 1000.0 mL	qs 1000.0 mL	qs 1000.0 mL

*: to avoid potential interactions between benzalkonium chloride and the anionic DSA and EY, sodium azide was used as an alternative preservative.

**: polysorbate 80 was added as surfactant to improve the peptide solubility in the release medium

Table IV-4 : Mathematical models.

Model name	Equation	Parameters definitions
Zero order	$Q_t=Q_0+K_0t$	Q_t : amount of drug released in time t Q_0 : initial amount of drug in the release medium K_0 : zero order release constant t : time
First order	$\log (100 - Q_t) = - K_1t$	Q_t : amount of drug released at time t K_1 : first order release constant t : time
Higuchi	$Qt = K_Ht^{1/2}$	Q_t : amount of drug released at time t K_H : release rate constant for Higuchi model t : time
Hixson-Crowell	$Q_0^{1/3}-Q_t^{1/3} = K_{HC}t$	Q_0 : initial amount of drug in depot Q_t : amount of drug remaining in the depot at time t K_{HC} : release rate constant for Hixson-Crowell model t : time
Korsmeyer-Peppas	$Q_t/Q_\infty = K_Kt^n$	Q_t/Q_∞ : fraction of drug released at time t ($\leq$ 60 % data used for calculation) K_K : release rate constant for Korsmeyer-Peppas model n : release exponent t : time

2.5. Peptide mass balance determination

At the end of the 3 months *in vitro* release test of peptide-loaded formulations, the mass balance was determined. The release medium was filtered through a 0.2 µm membrane before being removed. The hydrogel particles retained on the filter were dissolved in 0.5 mL acetic acid 0.3 M and 1 mL methanol. This solution as well as the remaining CS / G1-P / Peptide depot (see Figure IV-7) were transferred into a 100 mL volumetric flask. The depot was dissolved in 5 mL acetic acid 0.3 M and 25 mL methanol by magnetic stirring and sonication. The flask was then filled to the mark with release medium. The samples were filtered through a 0.2 µm filter and the peptide concentration was determined by HPLC.

2.6. Rheological measurements on CS / G1-P / EY formulations

Rheological measurements were performed using a Haake Rheostress1 rheometer (Thermo Fisher Scientific, Karlsruhe) with a circulating environmental system for the control of temperature. The sample volume was about 0.5 mL, introduced between a cone-plate geometry (2° cone angle, 35 mm diameter). A solvent trap was used to minimize water evaporation during all tests. The viscoelastic properties of the CS / G1-P / EY thermogelling solutions were assessed by measuring the storage modulus (G') and loss modulus (G"), representing the elastic behavior and the viscous behavior of the material, respectively. The

value of the strain amplitude was set at 1 % to ensure that all measurements were carried out within the linear viscoelastic range. Two kinds of tests were performed: *(i)* temperature sweep tests were carried out at a constant frequency of 1 Hz, with a temperature increase from 7 to 50°C and a heating rate of 1°C/min to determine gelation temperatures; *(ii)* time sweep tests were carried out at constant angular frequency of 1 Hz and constant temperature of 37.00 ± 0.20°C to determine gelation times. The sol / gel transition temperature (T_{gel}) or time (t_{gel}) correspond to the intersections of the curves of G' and G".

2.7. Peptide stability in release medium

The stability of the peptide in release medium ± 10 µg/mL lysozyme was determined by incubating three test solutions at 25, 50 and 100 µg/mL at 37°C. At defined time points, the peptide concentration of the test solutions was determined by HPLC against freshly prepared standard solutions and the delta to the initial concentration was calculated.

2.8. CS molecular weight decrease

The degradation of CS was studied at physiological pH in a PBS buffer pH 7.4 (1.03 g/L Na_2HPO_4, 0,32 g/L KH_2PO_4, 8.06 g/L NaCl, 0.25 g/L sodium azide) ± 10 µg/mL lysozyme at 37°C. 1.5 mL of CS / G1-P solution was injected into 25 mL PBS buffer pre-warmed to 37°C to form a solid depot. At t = 0, 2 and 24 days of incubation at 37°C, the buffer was removed from the sample. The remaining depot was dissolved in 20 mL of mobile phase and filtered through a 0.2 µm filter. The molecular weight of the polymer was determined by GPC, using a Water 2695 series liquid chromatograph coupled with a 2414 refractive index detector. 100 µl samples were injected into a series of two columns (Viscotek A6000M General Mixed Aqueous Columns, length: 300 mm, diameter: 7.8 mm, particle size: 13 µm) at an oven temperature of 30°C. The mobile phase consisted of 0.3 M acetic acid, 0.3 M sodium acetate and 1 % ethylene glycol. The system was calibrated using Varian polysaccharide standards (from 667 to 708000 Da). Relative weight average molecular weight (Mw) was calculated with a Chromeleon 6.8 Datasystem (Dionex Corporation, Sunnyvale, CA, USA). The molecular weights of the samples were calculated relative to the Mw at t = 0. All measurements were performed in duplicate.

2.9. Statistical analysis

Data were recorded as mean ± standard deviation. The significance of differences between groups was assessed using a t test when two groups were compared. ANOVA followed by Tukey's tests were applied for comparisons involving 3 or more groups. All significance tests were performed in Minitab 16 (Minitab, State College, PA), and a p value less than 0.05 indicated a statistically significant difference.

3. Results and discussion

3.1. *In vitro* release of water soluble low Mw model compounds

CS / G1-P *in situ* gelling systems were tested for *in vitro* release using three water soluble low molecular weight model compounds, namely MB, DSA and EY. Influence of several factors, like test method, charges of the model compounds and drug loading, on the release behavior were evaluated.

3.1.1. Influence of the *in vitro* release test method

The release behavior of MB from the CS / G1-P solution appears slightly affected by *in vitro* release method used, i.e. formation of a hydrogel before addition of the release medium (method I) or injection of CS / G1-P / MB solution into the release medium followed by sol / gel transition in the medium (method II) (Figure IV-1). The hydrogel formed before addition of the medium released MB statistically significantly faster only during the first 2 hours than the solution that underwent sol / gel transition in the medium (respectively 38 % versus 30 %). After this time point, the MB releases were comparable for the two methods. This slight difference might be explained based on the gelation mechanism (as described in Chapter II and Chapter III). CS / G1-P solution turned into a stronger gel in PBS than in saturated water vapor atmosphere (Chapter III, Figure III-4), due to the diffusion of the excess gelling agent in the buffered medium during the sol / gel transition, allowing enhanced CS – CS attractive interactions. Thus, it is hypothesized that in method I, the CS / G1-P / MB hydrogel network formed at 37°C might have being subjected to further strengthening upon addition of release medium, associated with enhanced G1-P and MB diffusion in the medium. Since method II was considered to be closer to *in vivo* conditions, where gelation takes place locally after injection, this method was selected for further investigations.

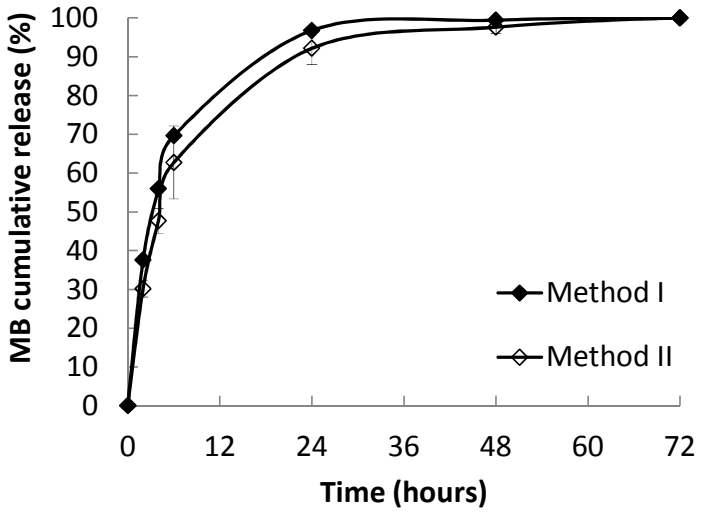

Figure IV-1: Cumulative release of MB from CS / G1-P hydrogels using *in vitro* release method I or II (mean ± SD, n = 3).

3.1.2. Influence of the charge of the low Mw model compounds

Due to the polycationic nature of CS, electrostatic interactions (attractive or repulsive) may occur between $-NH_3^+$ groups of CS and negatively or positively charged substances. Evaluation of the influence of the electrical charge of the model compounds loaded into the CS / G1-P system was performed by comparing the *in vitro* release profiles of MB and DSA (Figure IV-2). Under the experimental conditions (pH of 7.4), MB is positively charged (pKa of 3.8) whereas DSA is negatively charged (pKa of ~ 4). Addition of the gelling agent to the CS solution substantially reduced the release of MB and DSA from 98 % and 76 % respectively to about 30 % after 2 h. The release behavior of MB and DSA appear almost similar, showing a high burst (> 50 %) in the first 6 h, followed by a sustained release over 1 to 2 days. The high initial burst can be explained by the lag time between the injection in the release medium and the complete gelation, while the following sustained release can be attributed to the diffusion of the compounds through the polymeric matrix. These results confirm that the hydrogel network is able to effectively retard the release of the model compounds by physical entrapment. Furthermore, although the release after 6 h and 24 h was slightly affected by the model compounds (with about 7 % more MB released at these time points than DSA), the overall release rate of the monovalent anion DSA was comparable to the one of the monovalent cationic MB. Thus, single ionic interactions between DSA and CS

did not seem to affect substantially the release mechanism, in agreement with previous reports on CS-based thermogelling systems [21,22].

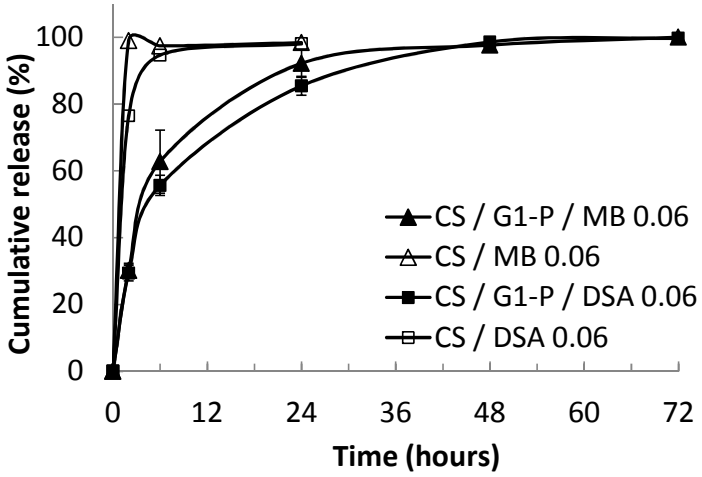

Figure IV-2: Release profiles of MB and DSA from CS / G1-P hydrogels (mean ± SD, *n* = 3) versus pure CS solutions (*n* = 1).

The cumulative release of EY (a multivalent anion under the experimental conditions) from the CS / G1-P hydrogel is shown in Figure IV-3. As observed previously, the hydrogel considerably reduced the EY burst (7.3 % in the first 2 h instead of 63.3 % without G1-P). Furthermore, the depot induced a sustained release over 11 days (versus 3 days without gelling agent). The release pattern was triphasic, comprising an initial burst (20 % release in 6 h), followed by a linear phase from 1 to 4 days up to 78 % release, and a third phase of slower release after 4 days. As for MB and DSA, the initial burst can be assigned to the immediate release of EY during the gelation process and to the rapid diffusion of the EY accumulated on the surface of the hydrogel. Compared to the release profiles observed for the DSA-loaded hydrogels, the sustained release of negatively charged EY from CS / G1-P hydrogel over more than one week suggests that interactions take place between the molecule and the polymeric network. This hypothesis will be further discussed later based on the outcome of the CS / G1-P / EY system rheological characterization.

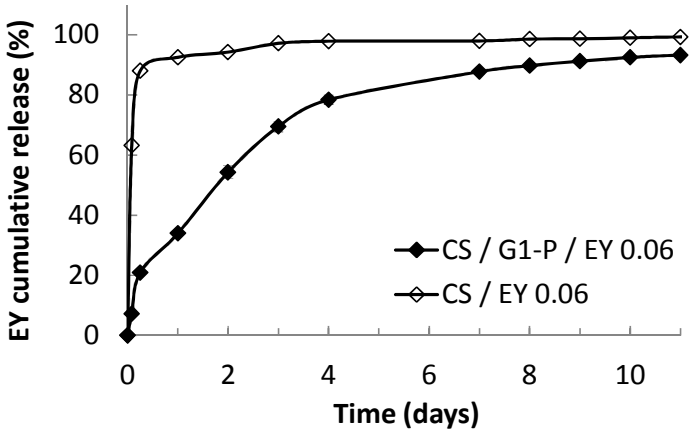

Figure IV-3: Release profile of EY from CS / G1-P hydrogel (mean ± SD, *n* = 3) versus pure CS solution (*n* = 1).

3.1.3. Influence of drug loading

Six CS / G1-P formulations with EY loading varying from 0.03 to 1.2 wt.% were investigated in order to define the impact of drug loading on the *in vitro* release behavior, as displayed in Figure IV-4. The release profiles of the formulations loaded with 0.03 to 0.3 wt.% EY were similar, showing a triphasic release from 0 to 11 days, as described previously for CS / G1-P / EY 0.06. In contrast, the formulation with 0.6 wt.% EY showed a slightly lower burst (10 % released in 6 h) and the one with 1.2 wt.% EY exhibited a more pronounced burst of 30 % during the first 6 hours, followed by a phase of constant release and a third phase of slower release. The higher burst value for CS / G1-P / EY 1.2 might be attributed to the increased portion of EY which was immediately accessible by the release medium at the surface of the depot.

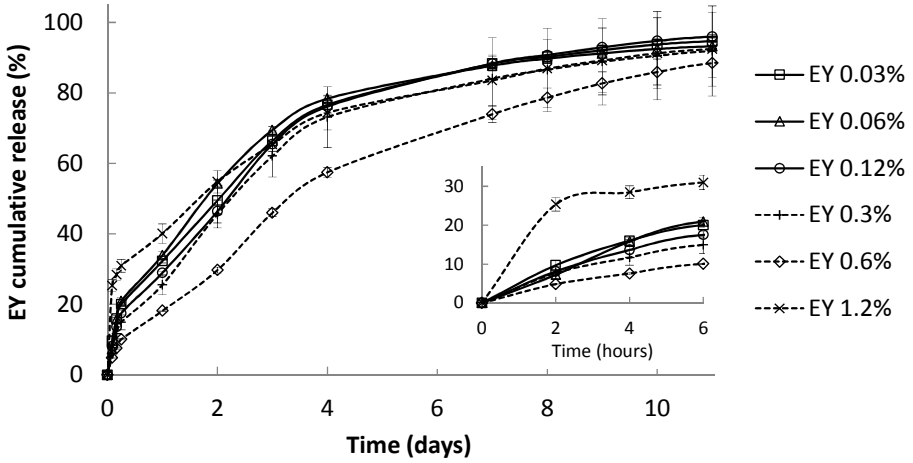

Figure IV-4: Cumulative release of CS / G1-P / EY hydrogels with 0.03 to 1.2 wt.% EY (mean ± SD, *n* = 3).

3.1.4. Release kinetic from CS / G1-P / EY hydrogels

To study the release mechanism and predict the release kinetics of EY from the hydrogel, different mathematical models (i.e. zero order, first order, Higuchi, Hixson-Crowell and Korsmeyer-Peppas) were applied to the data obtained for the *in vitro* release of CS / G1-P / EY 0.12, according to the equations displayed in Table IV-4. The release constant and regression coefficient (R^2) values calculated by these models are shown in Table IV-5. The data obtained show that the overall EY release best fitted in first order model, with a R^2 value of 0.995 (Figure IV-5), suggesting a diffusion controlled release mechanism [23,24]. These results are in alignment with the ones of Peng *et al* [5] on CS / glycerophosphate hydrogel, where it was found that the release kinetic of the highly soluble venlafaxine hydrochloride was best described with the first-order model and that the release was diffusion controlled.

Table IV-5: Release parameters of EY from CS / G1-P hydrogel

Model	**Release constant (K) or exponent (n)**		**Regression coefficient R^2**
Zero order	K_0 (day^{-1})	8.943	0.871
First order	K_1 (day^{-1})	0.126	0.995
Higuchi	K_H ($day^{-1/2}$)	0.695	0.942
Hixson-Crowell	K_{HC} (day^{-1})	0.275	0.969
Korsmeyer-Peppas	n	0.521	0.982

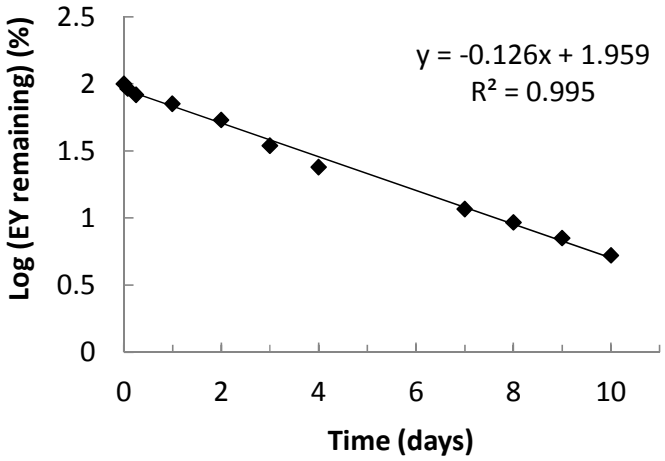

Figure IV-5: First order release profile of EY from CS / G1-P / EY 0.12 hydrogel.

3.1.5. Rheological behavior of CS / G1-P / EY

To examine the influence of EY loading on the thermogelling behavior of the CS / G1-P solution, the changes in gelation temperature and gelation time with the EY content were investigated. In Figure IV-6 (a), a clear sol-to-gel transition can be observed for the reference CS / G1-P solution without EY, which exhibits a typical solution-like behavior with G' < G" below 39°C and a gel behavior with G' > G" above this gelation temperature. Addition of 0.03 wt.% EY led to a sol-gel behavior from 5 to 28.7°C, with G' ≈ G", and to the formation of a weak gel upon further temperature increase. For higher EY loadings (0.3 and 1.2 wt.%), the formulations exhibited a weak gel behavior (G' > G") over the whole temperature range. Corroborating these results, the time sweep tests at 37°C (Figure IV-6 (b)) show a decrease of the gelation time from 8.8 to 0.5 min along with an increase of EY concentration from 0 to 0.03 wt.%. A further increase of the EY loading to 0.3 and 1.2 wt.% led to weak gel behavior from the beginning of the test. Formation of ionic bridges between CS molecules via the use of negatively charged compounds is a well-known process for the preparation of CS hydrogels for pharmaceutical and biomedical applications, especially by using tripolyphosphate or citrate as cross linker [9,25,26]. Due to the multivalent anionic nature of EY at the studied pH, this compound can also behave as a cross linker, binding reversibly the polycationic CS chains through ionic interactions. This ionic crosslinking leads to the formation of a weak network, as demonstrated by the viscoelastic behavior of the EY-loaded hydrogels versus blank CS / G1-P solution. However, the CS / G1-P / EY systems could still be easily injected through 23 G needles.

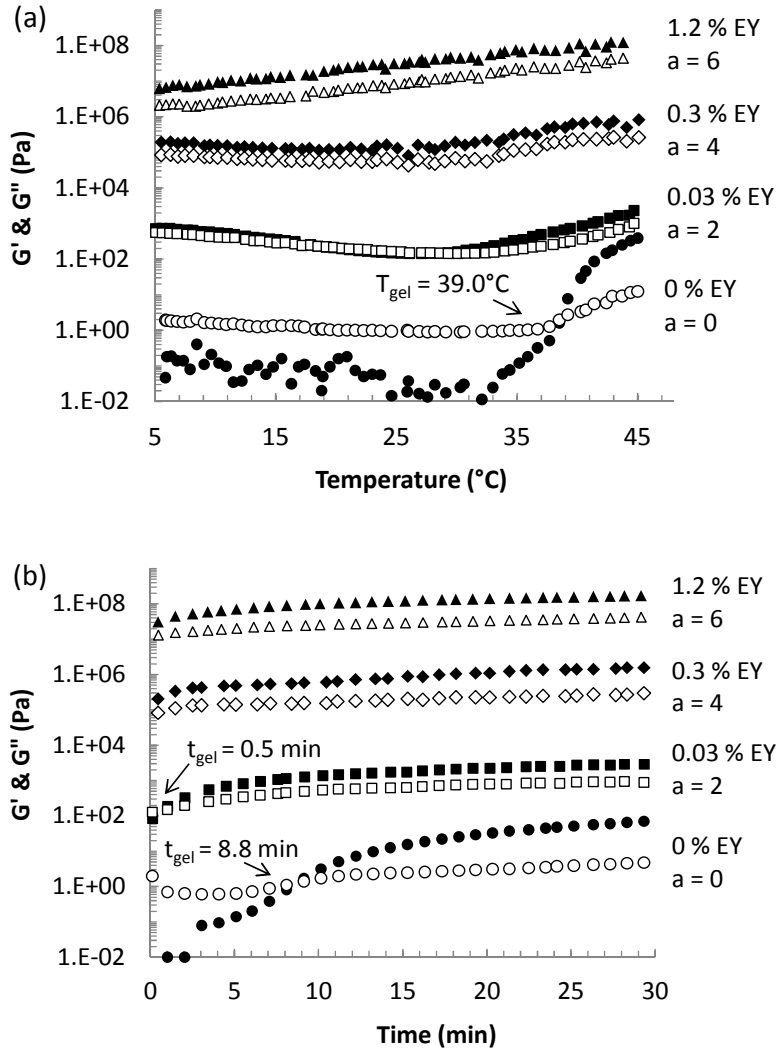

Figure IV-6: (a) Temperature and (b) time dependence at 37°C of storage modulus (G', closed symbols) and loss modulus (G", opened symbols) of CS / G1-P / EY solutions for EY concentrations from 0 to 1.2 wt.%. The data are shifted along the vertical axis by a factor of 10^a to avoid overlapping (n = 1).

3.2. *In vitro* release of poorly water soluble peptide

The successful prolonged delivery of several peptides, like triptorelin acetate, buserelin acetate, insulin and enfuvirtide, over weeks to months from ISFD systems was reported in literature [27-30]. Therefore, as a second part of this study, the application of CS / G1-P *in situ* forming hydrogels for the delivery of a poorly water soluble peptide drug was investigated.

3.2.1. Sustained-release behavior of peptide-loaded CS / G1-P depot

As shown exemplary in Figure IV-7, all CS / G1-P / peptide formulations turned into solid depots after injection into the release medium pH 7.4 at 37°C. In contrast, the peptide-loaded CS solution (without gelling agent) progressively solidified into numerous aggregates, due to the pH of the medium. The CS / G1-P / peptide depots maintained their shape and structural integrity over the 90 days of release test (excluding for CS / G1-P / peptide depots in release medium containing lysozyme, where depots parts detached after 62 days). However, the slight volume reduction of the depots indicates that degradation of the surface of the polymeric matrix occurred with time, notably in the presence of lysozyme. The *in vitro* degradation behavior of CS-based hydrogel in release medium with or without lysozyme is further investigated in part 3.3 of this chapter. The change in the color of the upper part of the depot with time is attributed to degradation of the peptide in contact with the release medium.

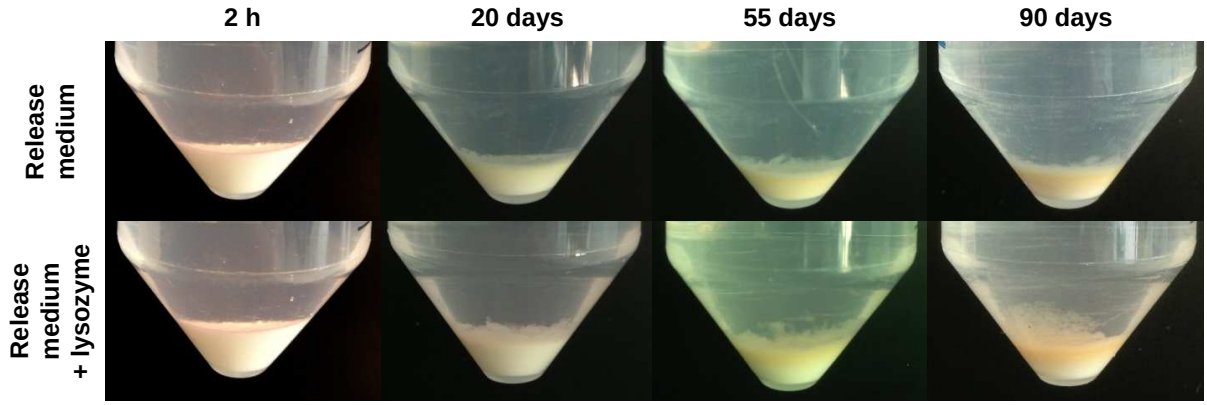

Figure IV-7: Peptide-loaded CS / G1-P depots in release medium pH 7.4 ±10 µg/mL lysozyme at different time points post injection.

The release profile of the peptide-loaded CS / G1-P hydrogel is displayed in Figure IV-8. Addition of G1-P to the CS solution significantly reduced the initial burst (2.5 ± 2.1 % release within 6 h), compared with the peptide suspension without gelling agent (60.6 ± 16.8 %). Furthermore, CS / G1-P / peptide2.5 formulation showed a sustained release of the peptide over more than 3 months, whereas 80 % released was achieved in about 3 days for the pure CS-based positive control. Actually, the depots comprising 2.5 % peptide exhibited 47 % release within 90 days, following a biphasic release pattern. It displayed a small burst of about 20 % throughout the first week, followed by a slower sustained release phase. Similar biphasic release profiles have been observed for insulin-loaded CS / glycerophosphate

hydrogels, as reported by Kodaverdi *et al* [31]. It can be assumed that, for the positive control, the considerable increase in the contact surface area between the CS / peptide aggregates in suspension and the release medium allowed an enhanced solvation of the peptide, while the CS / G1-P / peptide2.5 depot slowed down the peptide solvation and diffusion. Furthermore, due to the formation of the depot at the bottom of the tube, peptide diffusion is restricted to one-site, which also contributes to the slower release compared to the CS / peptide aggregates in suspension. In view of the conical shape of the depot, the release is faster at the beginning of the test, due to the dissolution and diffusion of the peptide entrapped on the surface of the hydrogel in contact with the release medium. The decrease of the release rate over time is attributed to slower release medium diffusion rate into the depot as well as increased length of the peptide diffusion pathway from the hydrogel. A small standard deviation in the quantity of peptide released, mostly below ± 3 %, was observed between the triplicate samples through the complete test period, showing that the depot formulations achieve good repeatability of the release over time.

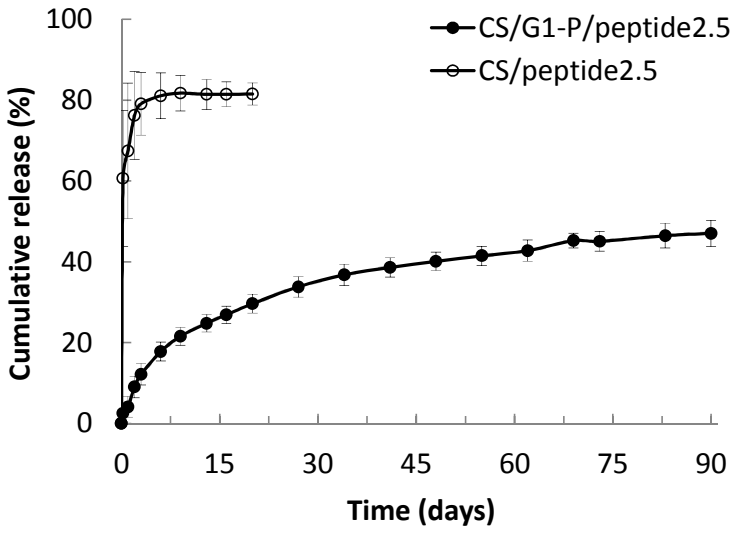

Figure IV-8: Peptide release profiles from CS / G1-P / peptide2.5 hydrogels versus CS / peptide2.5 solutions (mean ± SD, *n* = 3).

3.2.2. Influence of drug loading

As shown in Figure IV-9, doubling the peptide loading between CS / G1-P / peptide2.5 and CS / G1-P / peptide5 formulations did not significantly affect the initial burst (2.5 % and 2.3 % respectively in 6 h) and the overall biphasic release pattern was

maintained. However, during the phase of slower release, the 5 %-loaded hydrogel released the peptide slower than the 2.5 %-loaded hydrogel, leading to a difference of 18 % between the two formulations after 90 days. The reduction of the release rate after increasing the drug load has been observed previously for CS-based hydrogels loaded with peptides or hydrophobic drug substances [1,31,32]. Due to its poor water solubility, the peptide was dispersed in the CS / G1-P hydrogel. Thus, the release medium needed to penetrate the hydrogel and dissolve the peptide before diffusion. Higuchi was the first to develop a mathematical model to describe the rate of release of drug dispersed in matrix systems into a surrounding medium [33]. One form of this model is given in the following equation:

$$Q = \sqrt{2ADC_S t}$$

where Q corresponds to the amount of drug release in time t per unit exposed area, A is the total drug load, D is the diffusivity of the drug in the matrix and C_S is the drug solubility in the matrix [1]. Assuming that all parameters stayed constant except the drug loading, the 2-times increased drug loading would theoretically give rise to a 1.4-fold increase of the amount of released peptide over time. In the case of formulation CS / G1-P / peptide2.5 versus CS / G1-P / peptide5, a ratio of 1.3 – 1.5 between the amounts of released peptide was observed from 1 to 90 days. Furthermore, the release profiles were fitted to several mathematical models to determine the release kinetics of the formulations (Table IV-6) and the R^2 values obtained demonstrate a good fit with the Higuchi square root model.

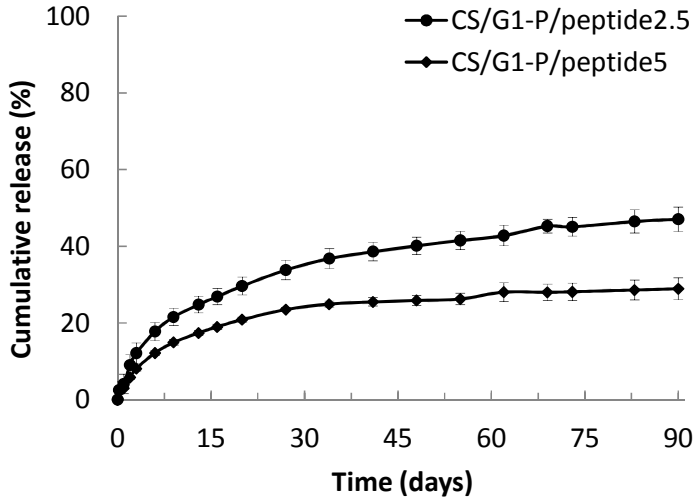

Figure IV-9: Peptide release profiles from CS / G1-P hydrogels as a function of initial loading (mean ± SD, n = 3).

Table IV-6: Release parameters of peptide from CS / G1-P hydrogel

Model	CS / G1-P / peptide2.5			CS / G1-P / peptide5		
	Release constant (K) or exponent (n)		R^2	Release constant (K) or exponent (n)		R^2
Zero order	K_0 (day^{-1})	0.477	0.825	K_0 (day^{-1})	0.286	0.756
First order	K_1 (day^{-1})	0.003	0.881	K_1 (day^{-1})	0.002	0.786
Higuchi	K_H ($day^{-1/2}$)	5.106	0.966	K_H ($day^{-1/2}$)	3.127	0.928
Hixson-Crowell	K_{HC} (day^{-1})	0.009	0.864	K_{HC} (day^{-1})	0.005	0.776
Korsmeyer-Peppas	n	0.477	0.950	n	0.452	0.932

3.2.3. Peptide mass balance determination

At the end of *in vitro* release test after 3 months, only 47 ± 3.2 % and 28.9 ± 2.8 % of the total peptide was released from formulations CS / G1-P / peptide2.5 and CS / G1-P / peptide5 respectively and a significant amount of peptide seemed to remain inside the depots, as shown in Figure IV-7. Therefore, the mass balance of released and remaining peptide in the hydrogel was determined for these two formulations. As displayed in Table IV-7, 16.8 ± 3.7 % and 39.0 ± 2.6 % of the peptide remained in the 2.5 and 5 % peptide-loaded depot respectively, resulting in mass balances of 63.8 and 67.9 %. The incomplete recovery of the peptide from the hydrogels might be caused by degradation in the release medium. This hypothesis will be further discussed in the next paragraph.

Table IV-7: Balance of released and residual peptide during *in vitro* release tests.

Formulation	Released peptide (%)	Residual peptide (%)	Sum (%)
CS / G1-P / Peptide2.5	47.0 ± 3.2	16.8 ± 3.7	63.8 ± 6.9
CS / G1-P / Peptide5	28.9 ± 2.8	39.0 ± 2.6	67.9 ± 5.4

3.2.4. Peptide stability in release medium

The stability of the peptide at different concentrations in release medium (pH 7.4) ± 10 µg/mL lysozyme at 37°C was evaluated by HPLC. The degradation profiles are shown in Figure IV-10. Significant degradation of the peptide was observed over time in the release medium (100, 59.2 and 56.1 % degradation after 76 days for solutions initially containing 25,

50 and 100 µg/mL peptide respectively). Addition of lysozyme increased the degradation rate, since complete degradation occurred in less than 30 days for the 25 µg/mL peptide solution. Thus, the 36.2 and 32.1 % difference between theoretical and recovered peptide from the CS / G1-P / peptide depots, loaded with 2.5 and 5 % peptide respectively, during the release study is attributed to the degradation of the peptide in the release medium.

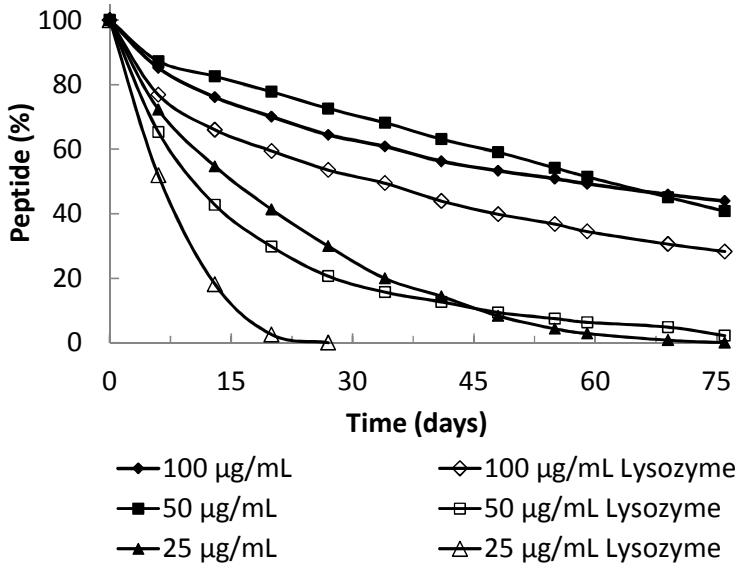

Figure IV-10: Stability of peptide solutions at 25, 50 and 100 µg/mL in release medium (pH 7.4) ± 10 µg/mL lysozyme at 37°C (n = 1).

3.2.5. Influence of enzymatic degradation

Several authors reported the addition of lysozyme, i.e. the CS lytic enzyme, in the release medium to significantly increase the release of drug-loaded CS-based matrices [21,34]. As shown in Figure IV-11, the release profiles of CS / G1-P / peptide5 depots are similar up to 55 days in release medium ± 10 µg/mL lysozyme. After this time point, an increase of the peptide release can be observed in the medium containing lysozyme (35.5 % at 62 h) compared to the medium without enzyme (28.0 %), in conjunction with a standard deviation raise from below ± 3 % to 4.6 – 5.5 %. As displayed in Figure IV-7 and demonstrated by Ganji *et al* [35], enzymatic degradation occurred via the surface of the hydrogel in contact with the release medium in a deacetylation degree-dependent way: the higher the DD, the lower the degradation rate. Since CS with a high DD of 88.7 % was used for the hydrogel formulation, the enzymatic degradation rate of the depot was expected to be quite slow, thereby having no significant effect of the peptide release during the first two

months. After 62 days of exposure to lysozyme, the hydrogels start to loose their structure. The detachment of some parts is observed, thereby increasing the specific surface of the hydrogel in contact with the release medium and resulting in a higher peptide release. Furthermore, as an enhanced peptide degradation rate was observed upon addition of lysozyme in the release medium, the real cumulative release of CS / G1-P / Peptide5 is expected to be slightly higher than the one disclosed in Figure IV-11. Thus, by progressively degrading the polymeric matrix, the presence of lysozyme could accelerate the long term release rate of the peptide from CS / G1-P hydrogels.

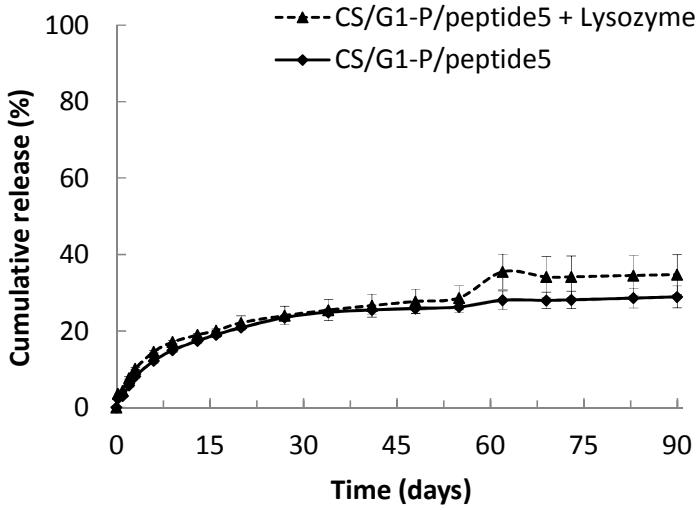

Figure IV-11: Peptide release profiles from CS / G1-P / peptide5 hydrogels in release medium ± 10 µg/mL lysozyme (mean ± SD, *n* = 3).

3.3. CS degradation under *in vitro* conditions

The degradation behavior of CS / G1-P depots incubated in release medium ± 10 µg/mL lysozyme at 37°C, was determined by measuring the Mw of CS by GPC. The results, reported in Figure IV-12, revealed no significant degradation of the polymer over 24 days for the depot submerged in release medium. In contrast, in the presence of lysozyme, the Mw reduced by about 20 % during the same period. Consistently with literature, these results suggest that the polymer underwent very limited hydrolysis in pure release medium, while progressive enzymatic degradation occurred in the presence of lysozyme, due to the cleavage of the β-(1–4) glycosidic linkages between the glucosamine and N-acteylglucosamine units [19]. Thus, the slow degradation behavior of the CS-based depot

in release medium with lysozyme is in line with the slightly enhanced peptide release observed over time in Figure IV-11.

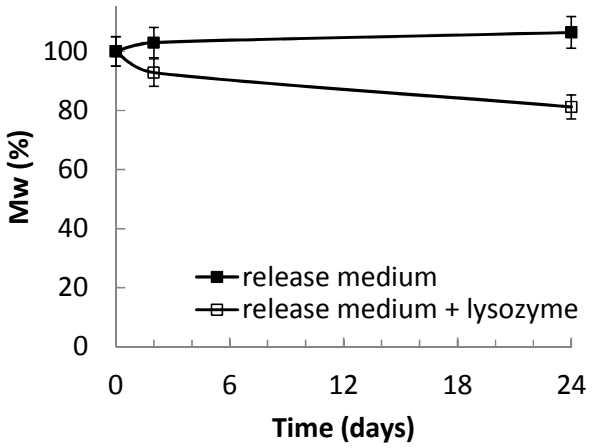

Figure IV-12: Molecular weight of CS in CS / G1-P hydrogels incubated in release medium ± 10 µg/mL lysozyme (*n* = 2, SD = 5 %).

4. Conclusion

Evaluation of the release profiles obtained with different types of low Mw hydrophilic compounds indicates that CS / G1-P thermosensitive hydrogels were able to retard their release for one to several days by physical entrapment. No decrease in release was observed by loading the hydrogel with the monoanionic DSA compared to the monocationic MB. Thus, reversible ionic bindings between MB and CS did not seem to influence the diffusion controlled release mechanism. However, as confirmed by the viscoelastic behavior of CS / G1-P / EY systems, EY behaves as a cross linker between CS molecules due to its dianionic nature. Consequently, the *in vitro* release of this low Mw compound displayed only a mild initial burst and was extended over more than one week. As shown in this study, the incorporation of a poorly water soluble peptide by dispersion in the CS / G1-P solution was feasible. The depot system demonstrated a continuous sustained delivery of the model peptide over at least 3 months, compared to only few days for the dispersion in a pure CS solution. The release could be divided into two phases: an initial burst attributed to the dissolution and diffusion of the peptide during the gelation process and entrapped directly on the surface of the hydrogel, followed by a sustained diffusion of the peptide through the CS-based matrix. At the end of test period, the peptide was not fully released and recovered, due to degradation in the release medium. After 2 months, lysozyme slightly increased the peptide release rate compared to the hydrogel in release medium without enzyme, demonstrating that the progressive polymeric carrier degradation could influence the long term release rate of the peptide. In conclusion, CS / G1-P *in situ* forming hydrogels showed promising results for the controlled and sustained release of model compounds over days to months, depending on the physico-chemical properties of the compounds.

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Conclusion and perspectives

Parenteral injectable formulations which turn into depots *in situ* by temperature modulation are interesting technologies for various pharmaceutical applications. The aim of the present work was to develop an injectable ISFD system for the controlled delivery of drug substances. CS-based thermogelling systems were selected for their interesting characteristics, including the absence of organic solvents or toxic crosslinking agents, the biocompatibility and biodegradability of the system and its use for various applications such as drug delivery, cell encapsulation and tissue engineering.

In the first part of this work, the recent progresses on standard CS / glycerophosphate thermogelling systems were reviewed. This allowed getting a better understanding of (*i*) the sol / gel transition mechanism upon temperature increase, (*ii*) the impact of the formulations parameters on the hydrogel properties, (*iii*) the drug delivery mechanisms and (*iv*) the potential ways to improve the standard system (e.g. use of different gelling agents or of chemically modified CS, addition of carrier particles or of a second biodegradable polymer in the system). The remaining issues were also highlighted, in particular the lack of stability upon storage of the CS-based thermogelling solutions.

The synergistic mechanisms underlying the temperature-induced gelation of the CS / gelling agent systems were then further investigated through comprehensive rheological studies, completed by ^{31}P-NMR and pH measurements, using different polyol-phosphates and polyol-free compounds as alternative to glycerophosphate. These investigations emphasized the key roles of both the phosphate and the polyol part of the gelling agent. The phosphate group, in its dibasic form, is needed to increase the pH of the solution in the vicinity of physiological pH, in order to reduce the charge repulsion between the polymeric chains, while the polyol moiety is essential to prevent the interactions between CS molecules at low temperatures. This protective effect is due to the water-structuring properties of glycerol and glucose, resulting in the formation of a protective hydration shell around the CS chains. Furthermore, the stability of the hydration layer was found to be dependent on the polyol part size, since it increased when using glucose-phosphate compared to glycerophosphate.

The experiments performed throughout this second part of the work allowed to identify Glucose-1-phosphate as a potential alternative gelling agent for the formulation of CS-based thermosensitive systems. Therefore, this compound was selected for further *in vitro* and *in vivo* investigations. While rheology and differential scanning calorimetry

measurements confirmed that sol / gel transition of the system took place at around body temperature and was not fully thermoreversible, the long term storage stability evaluation highlighted an enhanced stability of the CS / G1-P solution at room temperature and at refrigerated conditions compared to the standard system with β-glycerophosphate. Actually, CS solution containing 0.40 mmol/g G1-P is stable for at least 9 months at 2 - 8°C, versus less than 1 month when using β-glycerophosphate as gelling agent. Viscosity and injectability of the CS / G1-P solution was determined under different conditions (varying the shear rate, the needle sizes and/or the injection speeds) and showed that it was easy to inject, even through thin needles of 30 G. Furthermore, the local tolerability study performed over 3 weeks in a murine model confirmed the formation of the CS / G1-P hydrogel *in situ* at the injection site in few minutes. The inflammatory response observed at the interface of the hydrogel and the subcutaneous tissues was a typical foreign body reaction which diminished over time, as reported for standard CS / glycerophosphate hydrogels or for other depot systems like PLGA microparticles.

In vitro release studies were performed on four model compounds, i.e. three low molecular weight hydrophilic substances and a poorly water soluble peptide. Release profiles of methylene blue, drug substance A and eosin Y showed that the CS / G1-P hydrogel was effectively able to sustain the release of the hydrophilic model compounds for one to several days, depending on the compounds charges and mono- or multivalent character. In addition, the release of the hydrophobic peptide could be extended over at least 3 months. The stability of the matrix in the release medium was further evaluated by gel permeation chromatography, which demonstrated an increased degradation rate of CS in the presence of lysozyme, correlated with an enhanced release of the peptide.

Thus, a successful development of an *in situ* forming hydrogel composed of CS and G1-P was demonstrated, showing an acceptable shelf life upon storage at 2 – 8°C, a sol / gel transition under the physiological pH and temperature conditions, acceptable *in vivo* local tolerability and prolonged release of hydrophilic and hydrophobic model compounds. The extended release over at least 3 months achieved *in vitro* for the model peptide should be further confirmed through *in vivo* pharmacokinetic studies. Other potential next steps could include additional physico-chemical characterization of the drug substance-loaded CS / G1-P systems. For example, isothermal titration calorimetry could be used to determine the molecular interactions between CS and negatively charged drug substances. Additional

stability studies should be conducted to confirm the stability of the thermogelling solution containing a dissolved or dispersed active pharmaceutical ingredient. From a methodology point of view, further investigations could be performed towards the development of the *in vitro* release tests, e.g. assessing a flow-through cell system (USP IV) and/or adapting the release medium (pH, temperature, surfactant type and concentration, etc.), in order to achieve ≥ 80 % release in each case and to better mimic *in vivo* conditions. Further work could also include comprehensive studies on formulation parameters and excipients that are expected to influence drug release performance, for example determination of the effect of polymer concentration and characteristics (e.g. Mw, DD) on the release profiles. Finally, it would be of interest to optimize the sterilization process for drug substance-loaded CS / G1-P solutions, to identify another approach for CS / G1-P / drug substance dispersions and to further evaluate preparation of the formulation under aseptic conditions.

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Stéphanie SUPPER

NOVARTIS

Development and characterization of parenteral *in situ* gelling chitosan / glucose-1-phosphate depot system for controlled drug release

Résumé

L'objectif principal de ce travail de thèse est de développer une nouvelle formulation formant un dépôt *in situ* après administration parentérale pour la libération prolongée de principes actifs. Les systèmes à base de chitosane (CS) formant des hydrogels sous l'action de la chaleur corporelle ont été choisis parmi les différentes catégories de formulations injectables se solidifiant *in situ* pour la biocompatibilité et la biodégradabilité reconnue de ce polymère. Après une revue des différents systèmes thermo-gélifiants à base de CS et de leurs utilisations, nous nous sommes intéressés en détail aux mécanismes sur lesquels reposaient la formation des hydrogels de CS / agent gélifiant. Une étude rhéologique approfondie combinée à de la ^{31}P-RMN a permis de mettre en évidence le rôle clé de la partie polyol de l'agent gélifiant dans ce mécanisme. La troisième partie a été consacrée au développement d'un nouveau système associant le CS au glucose-1-phosphate (G1-P). Une étude des propriétés physico-chimiques et de la stabilité de ce système a mis en évidence sa gélification dans les conditions physiologiques et l'amélioration significative de sa stabilité à long terme par rapport au système standard CS / glycérophosphate. Des essais de tolérance locale sous-cutanée réalisés sur un modèle murin ont montré que le système est raisonnablement bien toléré. Enfin, la dernière partie, consacrée à l'étude de la libération *in vitro* de différents composés modèles, a démontré l'aptitude du réseau polymère de CS / G1-P à prolonger la libération des substances incorporées.

Mots-clés : chitosane, délivrance de principe actif, glucose-1-phosphate, hydrogel, thermo-gélifiant.

Résumé en anglais

The aim of this work was to develop a new parenteral *in situ* forming depot (ISFD) system for the controlled delivery of drugs. Chitosan (CS)-based systems that undergo sol / gel transition upon heating at physiological temperature were selected among the different categories of ISFDs due to their well-known biocompatibility and biodegradability. After an overall review of the recent progresses on standard CS-based ISFD systems, the synergistic mechanisms underlying the temperature-induced gelation of the CS / gelling agent systems were investigated through comprehensive rheological studies completed by ^{31}P-NMR measurements. These investigations emphasized the key role of the polyol part of the gelling agent. The next step consisted in developing a new system combining CS and glucose-1-phosphate (G1-P). The physico-chemical characteristics and storage stability of this system were investigated. The results highlighted a sol / gel transition under physiological conditions and improved storage stability compared to the standard CS / glycerophosphate system. Local tolerability studies of the hydrogels in rats showed that the system was reasonably well tolerated. Finally, the last chapter, dedicated to the study of the *in vitro* release behavior of several model compounds, emphasized the ability of the polymeric CS / G1-P network to sustain the release of the incorporated substances.

Keywords : chitosan, drug delivery, glucose-1-phosphate, hydrogel, thermogelling.