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Aos meus Pais

Maria Altina e António Ângelo

We should be taught not to wait for inspiration to start a thing.
Action always generates inspiration.
Inspiration seldom generates action.
Frank Tibolt

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RESUMO

As ciclodextrinas (CD) e os copolímeros em bloco desempenham um papel fulcral, como excipientes, no desenvolvimento de sistemas de libertação de fármacos. São utilizados individualmente para melhorar as propriedades físico-químicas dos fármacos, nomeadamente solubilidade em água, estabilidade e permeabilidade, através da formação de complexos de inclusão ou de micelas poliméricas. No entanto, em conjunto podem desempenhar novas funções. A inserção das cadeias poliméricas no interior das ciclodextrinas seguida de auto-organização (*self-assembly)*, pode levar à formação de um gel supramolecular adequado à libertação controlada de fármacos. Esta tese desenvolveu-se neste contexto, tendo como objectivo explorar o uso de geles supramoleculares como forma farmacêutica injetável capaz de alojar fármacos por longos períodos de tempo no local do implante. Numa primeira fase, foram revistos os métodos de preparação e avaliados os principais tipos de geles de ciclodextrinas injectáveis. Os geles foram obtidos através de diferentes processos, nomeadamente: auto-agregação de *poly(pseudo)rotaxanes* e ligações tipo escada (*zipper-like*) por reorganização das ciclodextrinas funcionalizadas e hóspedes macromoleculares funcionalizados. Foi feita uma análise cuidadosa dos mecanismos e das variáveis envolvidas nos processos de gelificação. Analisaram-se ainda as aplicações mais recentes destes geles em sistemas de libertação de fármacos e as suas aplicações em medicina regenerativa (Capítulo 2). Numa segunda fase foi estudada a capacidade do copolímero composto por poli(óxido de etileno)-poli(óxido de propileno)-poli(óxido de etileno), designado Pluronic F127, para formar geles supramoleculares na presença de uma quantidade adequada de αCD, e passíveis de serem administrados de forma minimamente invasiva (Capítulo 3). Os geles apresentaram boa estabilidade física, tixotropia e seringabilidade, e foram capazes de uma libertação controlada da vancomicina ao longo de vários dias. Concomitantemente, em estudos *in vitro* mostraram actividade contra culturas de *Staphylococcus aureus*. Estas propriedades fazem com que estes geles supramoleculares de Pluronic-αCD-vancomicina sejam adequados para o tratamento de infecções como osteomielites.

A prevalência de doenças osteodegenerativas bem como de fracturas, maioritariamente devido ao aumento da esperança média de vida e ao aumento da prática de desporto, levam à procura de substitutos para auto e alo-enxertos, bem como de *scalffolds* carregados com proteínas morfogénicas bastante dispendiosas. Um sistema alternativo foi desenvolvido, usando uma outra família de copolímeros de poli(óxido de etileno)-poli(óxido de propileno), poloxamina

908, o único polímero sintético, descrito até agora, com capacidade osteogénica intrínseca. A adaptação de *poly(pseudo)rotaxanes* de poloxamina-αCD com um osteoindutor sintético (sinvastatina) permitiu obter geles supramoleculares capazes de induzir a diferenciação, *in vitro*, de células estaminais mesenquimatosas em osteoblastos (Capítulo 4). Estes geles supramoleculares apresentaram boa citocompatibilidade e promoveram a proliferação das células estaminais mesenquimais durante a primeira semana de cultura, seguindo-se de diferenciação em osteoblastos caracterizada pela deteção da atividade da fosfatase alcalina (ALP). O carácter inovador dos geles supramoleculares de poloxamina-αCD-sinvastatina levou-nos a proteger a propriedade intelectual destes novos geles por meio de uma patente PCT.

Na parte final desta tese, foi descrita uma estratégia alternativa para desenvolver geles supramoleculares, utilizando polímeros de αCD (poly-αCD) solúveis em água, e preparados por polimerização com epicloridrina, em vez de estruturas cristalinas individualizadas de αCD. As interações, entre o poly-αCD e o Pluronic F127, poloxamina T908 e polietileno-glicol (PEG), foram analisadas pormenorizadamente de forma a identificar as proporções adequadas de cada componente para a obtenção de geles com um comportamento injetável e capazes de uma libertação prolongada da vancomicina (Capítulo 5).

ABSTRACT

Cyclodextrins (CDs) and block copolymers play a key role as excipients for drug delivery systems with advanced features. Separately, both can overcome poor physicochemical properties of drug molecules, such as insolubility in water, instability and limited permeability by forming, respectively, inclusion complexes or polymeric micelles. However, when mixed, novel features can be obtained. Threading of CDs along adequate polymer chains and subsequent self-assembly can led to supramolecular gels suitable for controlled release. This thesis emerged in this context and aims to explore pharmaceutical applications of supramolecular gels as syringeable drug depots that can remain for prolonged time in the implantation site. First, preparation and performance of main types of syringeable CD gels, prepared via self-aggregation of poly(pseudo)rotaxanes and via zipper-like assembly of CD-functionalized and guest-functionalized macromolecules are revisited, with a careful analysis of the mechanisms and variables involved in the gelling processes and the most recent applications in the drug delivery and regenerative medicine fields (Chapter 2). Then, the ability of an approved poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) copolymer, Pluronic F127, to form supramolecular gels in the presence of αCD suitable for being administered with minimally invasive maneuvers was explored (Chapter 3). The gels exhibited good physical stability, thixotropy and syringeability and were able to sustain vancomycin release for several days being active against *Staphylococcus aureus* in *in vitro* cultures. These properties make Pluronic-αCD-vancomycin supramolecular gels suitable for osteomielytis treatment.

The prevalence of osteodegenerative diseases as well as accidental fractures, mainly due to the more prolonged life expectation and the popularization of sports practice, are prompting the search for substitutes to the autologous and donor grafts as well as to the scalffolds loaded with expensive bone morphogenic proteins. An alternative system was designed using another family of poly(ethylene oxide)-poly(propylene oxide) block copolymers, the poloxamine group, which has been reported the only synthetic polymer with intrinsic osteogenic capability so far. Integration of poly(pseudo)rotaxanes of poloxamine-αCD with the synthetic osteoinducive drug (simvastatin) resulted in supramolecular gels able to induce *in vitro* differentiation of mesenchymal stem cells to ostoeblasts (Chapter 4). The supramolecular gels showed good cytocompatibility and proliferative effects on mesenchymal stem cells in the first week followed by differentiation to osteoblasts, as characterized by alkaline phosphatase activity (ALP). The

novelty and inventive step of the poloxamine-αCD-simvastatine supramolecular gels prompted us to protect the intellectual property of this type of gels by means of a PCT patent.

Finally, in this Thesis an alternative strategy to design supramolecular gels is present using, instead of individualized pristine αCDs, water-soluble polymers of αCD (poly-αCD) prepared by reaction with epiclorohidrin. Interactions between poly-αCD and Pluronic F127, poloxamine T908 and polyethylene glycol were analyzed in detail in order to identify adequate proportions of the components that can provide gels that behave as syringeable and can sustain vancomycin release for long time (Chapter 5).

LIST OF PUBLICATIONS

S.M.N. Simões, F. Veiga, J.J. Torres-Labandeira, A.C.F. Ribeiro, M.I. Sandez-Macho, A. Concheiro, C. Alvarez-Lorenzo. Syringeable Pluronic-α-cyclodextrin supramolecular gels for sustained delivery of vancomycin. European Journal of Pharmaceutics and Biopharmaceutics, 80(1), 103-112, 2012.

S.M.N. Simões, F. Veiga, J.J. Torres-Labandeira, A.C.F. Ribeiro, A. Concheiro, C. Alvarez-Lorenzo. Poloxamine-cyclodextrin-simvastatin supramolecular systems promote osteoblast differentiation of mesenchymal stem cells. Macromolecular Bioscience, 2013, DOI: 10.1002/mabi.201300017 (*In press*).

S.M.N. Simões, F. Veiga, J.J. Torres-Labandeira, A.C.F. Ribeiro, A. Concheiro, C. Alvarez-Lorenzo. Syringeable self-assembled cyclodextrin gels for drug delivery. Current Topics in Medicinal Chemistry, 2013 *(Accepted for Publication)*.

S.M.N. Simões, F. Veiga, A.C.F. Ribeiro, P. Taboada, A. Concheiro, C. Alvarez-Lorenzo. Supramolecular gels of poly-α-cyclodextrin and PEO-based copolymers for controlled drug release (*Submitted* in Langmuir).

PATENT

S.M.N. Simões, F. Veiga, A. Concheiro, C. Alvarez-Lorenzo. Poloxamine hydrogels and their use for the regeneration or bone repair. (Application number P201330135, Spain, February 2013).

OTHER MANUSCRIPTS NOT INCLUDED IN THE THESIS

S.M.N. Simões, A. Figueiras, F. Veiga. Modular hydrogels for drug delivery. Journal of Biomaterials and Nanobiotechnology, 3(2), 185-199, 2012.

A. Figueiras, A. Vieira, S.M.N. Simões, F. Veiga. Mucoadhesive Buccal Systems as a Novel Strategy for Anti-Inflammatory Drugs Administration. Anti-Inflammatory & Anti-Allergy Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry), 10(3), 190-202, 2011.

LIST OF ABBREVIATIONS

αCD	*alpha*-cyclodextrin
βCD	*beta*-cyclodextrin
γCD	*gamma*-cyclodextrin
π-A isotherms	Surface pressure - area isotherm
ALP	Alkaline phosphatase activity
ANNs	Artificial neural networks
ANOVA	Analysis of variance
AMPD	2-Amino-2-methyl propanediol
BCA	Bicinchoninic acid (Protein assay kit)
BMMSCs	Bone marrow derived mesenchymal stem cells
BMPs	Bone morphogenic proteins
BSA	Bovine serum albumin
C	Coagulation
CDs	Cyclodextrins
CFU	Colony-forming unit
CMC	Critical micellar concentration
CPT	Camptothecin
D	Diffusion coefficients
DNA	Deoxyribonucleic acid
D-MEM	Dulbecco´s modified Eagle medium
DMSO	Dimethyl sulfoxide
DOSY	Diffusion-ordered spectroscopy
DTT	Dithioerythritol
ELISA	Enzyme-linked immuno sorbent assay
EMA	European Medicines Agency
F127	Pluronic F127
FDA	Food and Drug Administration
FTIR	Fourier transform infrared spectroscopy
G′	Storage or elastic modulus
G″	Loss or viscous modulus

G-CSF	Granulocyte colony-stimulation factor
GMP	Good manufacturing practice
H	Hemorrhage
hADSCs	Human adipose-derived stem cells
Hep	Heparin
HET-CAM	Hen's egg test-chorioallantoic membrane
(HMG)-CoA	Hydroxyl-3-methyl-glutaryl coenzyme A
HP-αCD	Hydroxypropyl-α-cyclodextrin
HP-βCD	Hydroxypropyl-β-cyclodextrin
HP-γCD	Hydroxypropyl-γ-cyclodextrin
HPLC	High-performance liquid chromatography
HPMC	Hydroxypropyl methylcellulose
ICCVAM	Interagency coordinating committee on the validation of alternative methods
IS	Irritation scores
L	Lysis
LD_{50}	Median lethal dose
MβCD	Methyl-β-cyclodextrin
MD	Modified dextran
MIC	Minimum inhibitory concentration
MMP	Metalloproteinases
MSCs	Mesenchymal stem cells
MTT	[(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] Cell Proliferation Kit I
PAMAM	Poly(amino amine)
PBS	Phosphate buffered saline
pDNA	Plasmatic DNA
PEG	Poly(ethylene glycol)
PEG_4	4-arm star shaped poly(ethylene glycol)
PEG_8	8-arm star shaped poly(ethylene glycol)
PEO	Poly(ethylene oxide)
PHB	(R)-3-hydroxybutyrate
PLs	Poly(3-lysine)s

Pluronic R	Pluronic reverse
PNIPAAm	Poly(N- isopropylacrylamide)
Poly-βCD	βCD polymer
Poly-αCD	αCD polymer
Poly-γCD	γCD polymer
PPO	Poly(propylene oxide)
PPG	Poly(propylene glycol)
R^2	Correlation coefficient
rhBMP	Recombinant human bone morphogenic proteins
RSD	Relative standard deviation
SAOS-2	Sarcoma osteogenic
S. aureus	*Staphylococcus aureus*
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscope
Si-MPs	Mesoporous silica particles
SLS	Static light scattering
SWNT	Hybrids of single-walled carbon nanotubes
T908	Tetronic 908
TPA	3-Trimethylsilylpropionic acid
Tri-HCl buffer	Tris(hydroxymethyl)aminomethane buffer
UV	Ultraviolet
wt.	Weight
w/v	Weight/ volume

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Chapter 1

GENERAL INTRODUCTION

1.1 SUPRAMOLECULAR SYSTEMS

In recent years, supramolecular polymer chemistry has emerged as a fascinating subject within macromolecular research field. Supramolecular systems are composed of two or more molecular entities held together and organized by means of intermolecular non-covalent binding interactions, such as hydrogen bonding, hydrophobic attractions, van der Waals forces, and electrostatic interactions, which build up larger functional architectures [1, 2]. Supramolecular structures involving macrocyclic compounds have attracted tremendous interest not only as models for understanding natural supramolecular self-assembly and molecular recognition, but also as precursors for designing novel nanomaterials for electronics, biomedical and pharmaceutical application and targeted delivery of therapeutic agents or bioactive materials [3-5]. Advances of the non-covalent organization of compounds, compared to the organization of compounds in a covalent fashion on polymeric scaffolds include the responsive nature of the self-assembly process, the easy supramolecular synthesis and the possibility to incorporate a multiple array of different bioactive molecules.

A physical interaction that has been exploited recently to design physically crosslinked gels is the inclusion complex formation between cyclodextrins (CDs) and lipophilic guest molecules [6]. CDs are cyclic oligosaccharides composed of D-glucose units coupled via α-1,4-glucosidic linkages [7, 8] and with a hydrophobic inner cavity having a depth of ca. 7.0 Å (Figure 1.1). αCD, βCD and γCDs consist of six, seven and eight glucopyranose units, respectively. Various molecules can be inserted into the cavities of CDs to form supramolecular inclusion complexes, and such systems have been studied extensively as models for understanding the mechanism of molecular recognition [4, 9, 10]. Harada and Kamachi [11] reported the first example of an inclusion complex formed via self-assembly of αCD and poly(ethylene glycol). Since this initial discovery of CD-polymer inclusion complexes, many other linear polymeric guests possessing either hydrophilic or hydrophobic properties have been reported to form inclusion complexes with various types of CDs [12-14] (Figure 1.1b). Since then, overwhelming interest has been focused on the studies of supramolecular structures of the polyrotaxanes and the polypseudorotaxanes formed by CDs threaded on a polymer chain [14-19] and their applications as biomaterials [20-23]. These systems have been shown particularly suitable to encapsulate labile pharmaceuticals, such as bioactive proteins, antibiotics and BMPs (bone morphogenetic proteins) for bone generation [24-27].

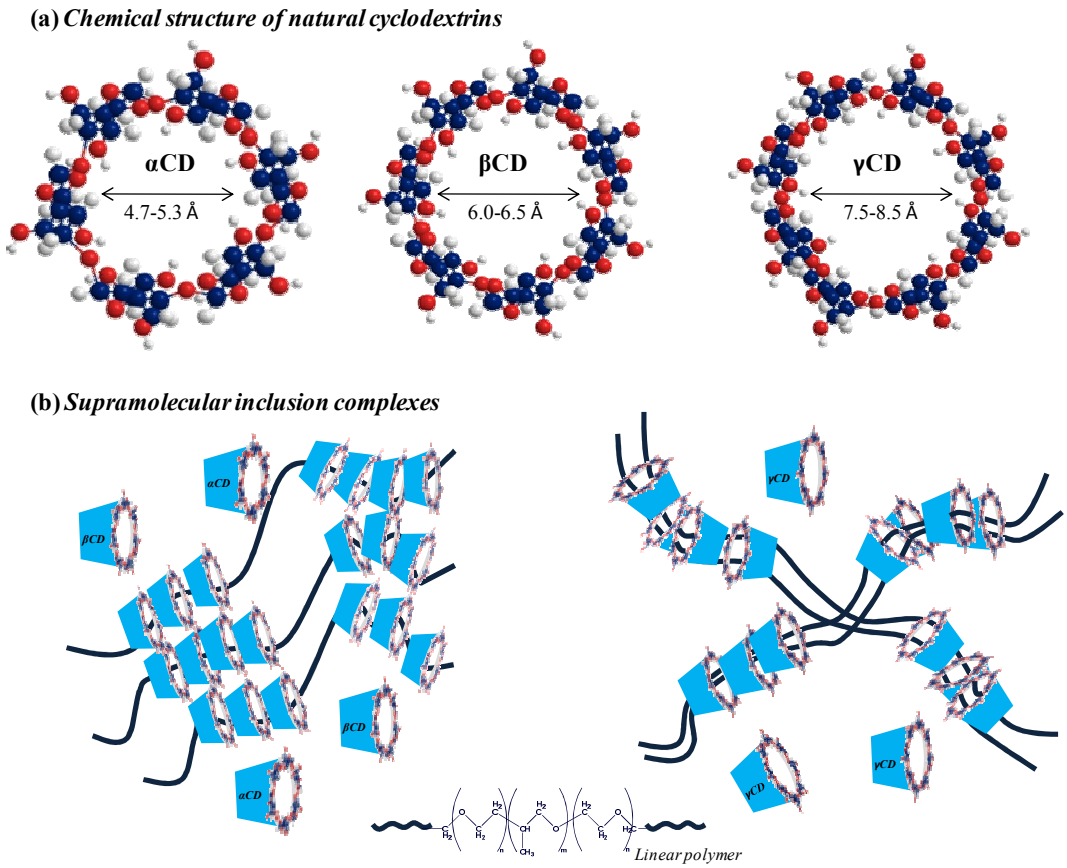


Figure 1.1. Structures of natural cyclodextrins (a), schematic representation of self-assembly of cyclodextrins with a linear polymer (b).

1.2 *IN SITU* GELLING SYSTEMS

For many biomedical applications, injection of so-called *in situ* forming hydrogels is preferred over surgical implantation of flexible or rigid polymeric matrices. Syringeable systems represent an attractive approach for non-invasive prosthetic implantation and for administering depots for sustained delivery drugs, both for human and animal therapeutics [22, 28, 29]. *In situ* gelling systems are administered as a liquid, after which a gel is formed at the site of injection [29-31]. Physically crosslinked gels are particularly suitable as *in situ* gelling devices. Due to their reversible nature, many physically crosslinked gels behave fluid-like, when are subjected to shear stress in the needle and syringe (Figure 1.2) [32, 33]. These injectable, shear-thinning hydrogels often exhibit relatively low mechanical strengths. To minimize leakage of the gelling solution into the surrounding tissue, it is important that gelation at the site of injection occurs within a few minutes [34]. The relevance of injectable gels lies in their economical elaboration, patient-friendly application, and the avoidance of surgical maneuvers that have an inherent risk of infection. For all these reasons, the development of injectable gels for drug delivery and regenerative medicine is gaining an increase commercial interest and, as a consequence, it generates high interest in the research and development activities of pharmaceutical industries.

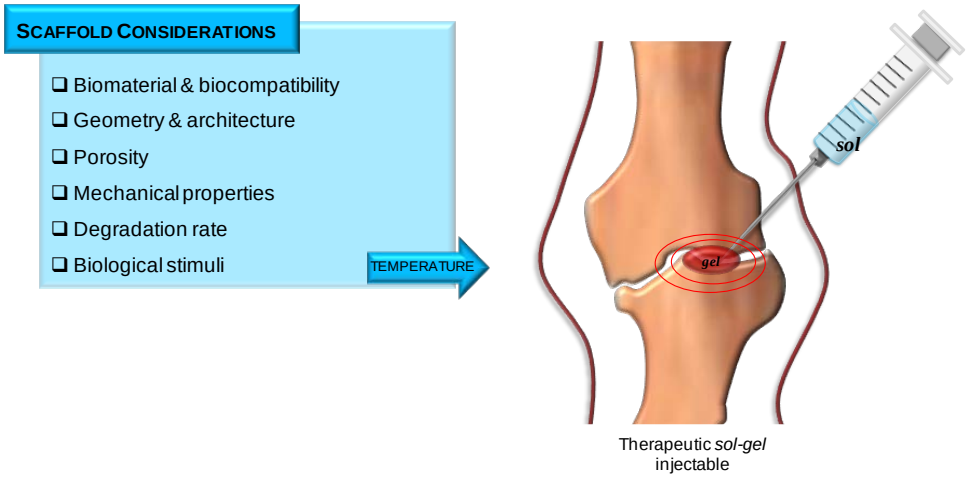


Figure 1.2. Schematic illustration of a therapeutic sol-gel injectable *in situ* in a bone knee joint and scaffolds properties for engineering tissue.

1.3 BONE PATHOLOGIES

The field of tissue engineering integrates the latest advances in molecular biology, biochemistry, engineering, material science, and medical transplantation. Researchers in the developing field of regenerative medicine have identified bone tissue engineering as an attractive translational target [35]. Clinical problems requiring bone regeneration are diverse, and no single regeneration approach will likely resolve all defects. Infections of bones and joints are still difficult problems with several therapeutic considerations. The basic principle of treatment of bone infection is to remove all nonliving tissues and to fill spaces because these are regions where bacteria can hide from the body's immune defense system. To eradicate infection the body needs to deliver antibodies and infection-fighting cells to the bacteria-infected areas [36, 37]. Conventional treatment of chronic infection includes periodic drug administration via oral and other systemic routes, all of which may lead to fluctuations of local drug levels [38, 39]. Various studies have established that local antibiotics delivery provides high concentration of drug at the site of infection with a low systemic toxicity [40-42]. For localized chronic infection treatment (osteomyelitis) and bone defects repair, local drug delivery may be more acceptable to the patients, reducing the period of hospitalization and the cost of treatment.

Recent advances in the field of bone regenerative medicine include the use of sophisticated biocompatible scaffolds (Figure 1.2), new postnatal multipotent cell populations, and appropriate cellular stimulation. In particular, synthetic polymer scaffolds allow fast and reproducible construction, with biocompatible characteristics [25, 43]. Cellular stimulation has been widely achieved by using bone morphogenetic proteins (BMPs), specifically BMP-2 and BMP-7 [44, 45]. These proteins induce osteogenic differentiation *in vitro*, as well as bone defect healing *in vivo* being approval by the FDA. Specific interactions with the scaffold are the key in BMP-scaffold immobilization for prolonged release able to creates the most osteogenic microenvironment [46, 47]. Transition into clinical studies has had only mild success and relies on large doses of BMPs for bone formation. Advances within the field of bone regeneration are likely to overcome these challenges and lead to more clinically relevant therapies (Figure 1.3). A major limitation of recombinant proteins is the rapid local metabolization and their high cost. This fact is prompting the search of more affordable and stable osteogenic/osteoinductive synthetic molecules (like simvastatin) able to the biochemical stimuli that mesenchymal stem cells (MSCs) receive in their native bone niche for their differentiation to bone precursors [48-50].

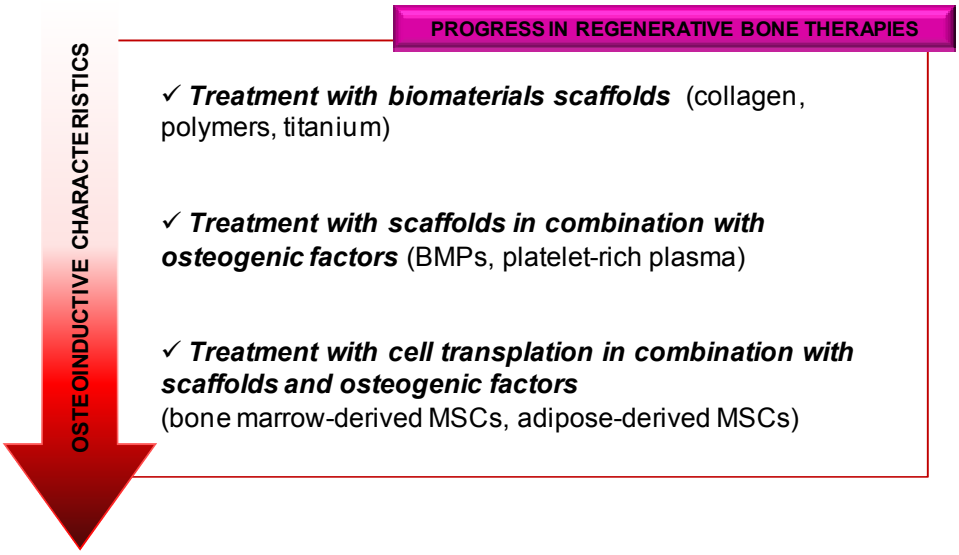


Figure 1.3. Progress in regenerative bone therapies.

Chronic osteomyelitis could be also treated by the implantation of an antibiotic delivery device that can release a high amount of antibiotic for an extended period. In general, oral antibiotics only lead to relative low local concentrations. Intravenous antibiotic regimens enable the attainment of constant levels of drug in the blood stream [42]. However, the infected necrotic focus within the bone is often surrounded by sclerotic, avascular bone, and is therefore almost unreachable with systemic antibiotics. Thus, intravenous antibiotics usually lead to significant relapse rates during or after their administration, and harmful side-effects are common since serum levels remain high during a long period of time (at least 6 weeks) [52-54]. Local administration is thought to increase antibiotic concentrations where it is most needed, without increasing systemic side-effects [42]. Carriers like beads and fillers that are made of non-degradable poly(methyl methacrylate) (PMMA) are the most used for osteomyelitis treatment, but as drawback, they have to be chirurgical implanted and removed afterwards [51]. Minimally invasive treatments are preferred. Several injectable gels have been used to deliver drugs and to carry cells in order to bone engineering [29, 34, 55].

1.4 AIM AND OUTLINE OF THESIS

The aim of the work presented in this thesis was design and characterize novel syringeable supramolecular gels able to be used in the treatment of bone infections and actively participate in the regeneration of bone tissue. The supramolecular gels were based on host-guest inclusion complexes between α-cyclodextrin (αCD) and poly(ethylene oxide)-poly(propylene oxide) (PEO-PPO) block copolymers, and found to be suitable for the controlled and sustained delivery of antibiotics and statins.

Chapter 2 gives a literature overview of syringeable cyclodextrin gels that offer the possibility of being administered with minimally invasive maneuvers to form depots that can remain for prolonged time in the implantation site being suitable for drug delivery. Two different types of cyclodextrin-based supramolecular systems are discussed.

Chapter 3 reports on the formation of poly(pseudo)rotaxanes of Pluronic® F127 with αCD and the subsequent supramolecular assemblies that lead to gel formation. The effect of the proportion of both components on the temperature-sensitiveness, the rheological properties and the vancomycin release behavior were studied. Furthermore, the syringeablility of the gels and the antibacterial activity were evaluated.

Chapter 4 describes osteoinductive systems based on poloxamine-cyclodextrin-simvastatin supramolecular networks. The solubility and stability of simvastatin hydroxyl acid and the poly(pseudo)rotaxanes formation were analyzed. The cytocompatibility of the gels was tested *in vitro* on osteoblasts and applying the HET-CAM assay. Moreover, the proliferation and differentiation of mesenchymal stem cells into osteoblasts and the consequent alkaline phosphatase activity were evaluated in order to establish the osteogenic/osteoinductive activity of ternary poloxamine-cyclodextrin-simvastatin supramolecular systems.

In **Chapter 5**, an alternative strategy is present to design supramolecular gels based on α-cyclodextrin-polymers (poly-αCD) and PEO-based copolymers. The synthesis of poly-αCD was optimized to render high molecular weight hydrosoluble polymers. The interactions with the copolymers were evaluated in detail by means of NMR and their incidence on vancomycin release characterized.

Finally, in **Chapter 6**, main findings of this thesis are summarized and suggestions are given to further development of supramolecular gels technology for *in vivo* applications.

1.5 REFERENCES

[1] Harada, A.; Hashidzume, A.; Yamaguchi, H.; Takashima, Y. *Polymeric rotaxanes*. Chemical Reviews, 2009, 109(11), 5974-6023.

[2] Appel, W.P.J.; Nieuwenhuizen, M.M.L.; Meijer, E.W. Multiple Hydrogen-Bonded Supramolecular Polymers, in *Supramolecular polymer chemistry*. Wiley-VCH Verlag GmbH & Co. KGaA., 2001, 1-28.

[3] Petkau-Milroy, K.; Brunsveld, L. *Supramolecular chemical biology; bioactive synthetic self-assemblies*. Organic & Biomolecular Chemistry, 2013, 11(2), 219-232.

[4] Liao, X.; Chen, G.; Jiang, M. *Hydrogels locked by molecular recognition aiming at responsiveness and functionality*. Polymer Chemistry, 2013, 4(6), 1733-1745.

[5] Yoon, H.J.; Jang, W.D. *Polymeric supramolecular systems for drug delivery*. Journal of Materials Chemistry, 2010, 20(2), 211-222.

[6] Connors, K.A. *The stability of cyclodextrin complexes in solution*. Chemical Reviews, 1997, 97(5), 1325-1358.

[7] Brewster, M.E.; Loftsson, T. *Cyclodextrins as pharmaceutical solubilizers*. Advanced Drug Delivery Reviews, 2007, 59(7), 645-666.

[8] Szejtli, J. *Introduction and general overview of cyclodextrin chemistry*. Chemical Reviews, 1998, 98(5), 1743-1754.

[9] Li, J.; Loh, X.J. *Cyclodextrin-based supramolecular architectures: syntheses, structures, and applications for drug and gene delivery*. Advanced Drug Delivery Reviews, 2008, 60(9), 1000-1017.

[10] Kawabata, R.; Katoono, R.; Yamaguchi, M.; Yui, N. *Bundling two polymeric chains with γ-cyclodextrin cavity contributing to supramolecular network formation*. Macromolecules, 2007, 40(4), 1011-1017.

[11] Harada, A.; Kamachi, M. *Complex formation between poly(ethylene glycol) and α-cyclodextrin*. Macromolecules, 1990, 23(10), 2821-2823.

[12] Tonelli, A.E. *Nanostructuring and functionalizing polymers with cyclodextrins*. Polymer, 2008, 49(7), 1725-1736.

[13] Li, J.; Ni, X.; Leong, K. *Block-selected molecular recognition and formation of polypseudorotaxanes between poly(propylene oxide)-poly(ethylene oxide)-poly(propylene oxide) triblock copolymers and α-cyclodextrin*. Angewandte Chemie International Edition, 2003, 42(1), 69-72.

[14] Chan, S.C.; Kuo, S.W.; Chang, F.C. *Synthesis of the organic/inorganic hybrid star polymers and their inclusion complexes with cyclodextrins*. Macromolecules, 2005, 38(8), 3099-3107.

[15] Wenz, G.; Han, B.H.; Müller, A. *Cyclodextrin rotaxanes and polyrotaxanes*. Chemical Reviews, 2006, 106(3), 782-817.

[16] Li, J.; Li, X.; Zhou, Z.; Ni, X.; Leong, K.W. *Formation of supramolecular hydrogels induced by inclusion complexation between Pluronic and α-cyclodextrin*. Macromolecules, 2001, 34(21), 7236-7237.

[17] Girardeau, T.E.; Zhao, T.; Leisen, J.; Beckham, H.W.;Bucknall, D.G. Solid inclusion complexes of α-cyclodextrin and perdeuterated poly(oxyethylene). Macromolecules, 2005, 38(6), 2261-2270.

[18] Wei, H.; Yu, H.; Zhang, A.; Sun, L.; Hou, D.; Feng, Z. *Synthesis and characterization of thermosensitive and supramolecular structured hydrogels*. Macromolecules, 2005, 38(21), 8833-8839.

[19] Liu, K.L.; Zhang, Z.; Li, J. *Supramolecular hydrogels based on cyclodextrin-polymer polypseudorotaxanes: materials design and hydrogel properties*. Soft Matter, 2011, 7(24), 11290-11297.

[20] Li, J. *Self-assembled supramolecular hydrogels based on polymer-cyclodextrin inclusion complexes for drug delivery*. NPG Asia Materials 2010, 2(3), 112-118.

[21] Li, J.; Zhao, F.; Li, J. *Polyrotaxanes for applications in life science and biotechnology*. Applied Microbiology and Biotechnology, 2011, 90(2), 427-443.

[22] Li, Y.; Rodrigues, J.; Tomas, H. *Injectable and biodegradable hydrogels: gelation, biodegradation and biomedical applications*. Chemical Society Reviews, 2012, 41(6), 2193-2221.

[23] Higashi, T.; Tajima, A.; Motoyama, K.; Arima, H. *Cyclodextrin/poly(ethylene glycol) polypseudorotaxane hydrogels as a promising sustained-release system for lysozyme*. Journal of Pharmaceutical Sciences, 2012, 101(8), 2891-2899.

[24] Shoichet, M.S. *Polymer scaffolds for biomaterials applications*. Macromolecules, 2009, 43(2), 581-591.

[25] Van Vlierberghe, S.; Dubruel, P.; Schacht, E. *Biopolymer-based hydrogels as scaffolds for tissue engineering applications: A review*. Biomacromolecules, 2011, 12(5), 1387-1408.

[26] Lee, S.S.; Huang, B.J.; Kaltz, S.R.; Sur, S.; Newcomb, C.J.; Stock, S.R.; Shah, R.N.; Stupp, S.I. *Bone regeneration with low dose BMP-2 amplified by biomimetic supramolecular nanofibers within collagen scaffolds*. Biomaterials, 2013, 34(2), 452-459.

[27] Lee, S.H.; Shin, H. *Matrices and scaffolds for delivery of bioactive molecules in bone and cartilage tissue engineering*. Advanced Drug Delivery Reviews, 2007, 59(4–5), 339-359.

[28] Packhaeuser, C.B.; Schnieders, J.; Oster, C.G.; Kissel, T. *In situ forming parenteral drug delivery systems: an overview*. European Journal of Pharmaceutics and Biopharmaceutics, 2004, 58(2), 445-455.

[29] Kretlow, J.D.; Klouda, L.; Mikos, A.G. *Injectable matrices and scaffolds for drug delivery in tissue engineering*. Advanced Drug Delivery Reviews, 2007, 59(4-5), 263-273.

[30] Van Tomme, S.R.; Storm, G.; Hennink, W.E. *In situ gelling hydrogels for pharmaceutical and biomedical applications*. International Journal of Pharmaceutics, 2008, 355(1–2), 1-18.

[31] He, C.; Kim, S.W.; Lee, D.S. *In situ gelling stimuli-sensitive block copolymer hydrogels for drug delivery*. Journal of Controlled Release, 2008, 127(3), 189-207.

[32] Ni, X.; Cheng, A.; Li, J. *Supramolecular hydrogels based on self-assembly between PEO-PPO-PEO triblock copolymers and α-cyclodextrin*. Journal of Biomedical Materials Research Part A, 2009, 88A(4), 1031-1036.

[33] Van Tomme, S.R.; van Steenbergen, M.J.; De Smedt, S.C.; van Nostrum, C.F.; Hennink, W.E. *Self-gelling hydrogels based on oppositely charged dextran microspheres*. Biomaterials, 2005, 26(14), 2129-2135.

[34] Temenoff, J.S.; Mikos, A.G. *Injectable biodegradable materials for orthopedic tissue engineering*. Biomaterials, 2000, 21(23), 2405-2412.

[35] Cowan, C.M.;Soo, C.;Ting, K.;Wu, B., *Evolving concepts in bone tissue engineering*, in Current Topics in Developmental Biology. 2005, Academic Press. p. 239-285.

[36] Dinh, P.; Hutchinson, B.K.; Zalavras, C.; Stevanovic, M.V. *Reconstruction of osteomyelitis defects*. Seminars in Plastic Surgery, 2009, 23(2), 108-118.

[37] Lew, D.P.; Waldvogel, F.A. *Osteomyelitis*. The Lancet, 2004, 364(9431), 369-379.

[38] Trampuz, A.; Widmer, A.F. *Infections associated with orthopedic implants*. Current Opinion in Infectious Diseases, 2006, 19(4), 349-356.

[39] Trebse, R.; Pisot, V.; Trampuz, A. *Treatment of infected retained implants*. Journal of Bone & Joint Surgery, British Volume, 2005, 87-B(2), 249-256.

[40] Meyer, J.D.; Falk, R.F.; Kelly, R.M.; Shively, J.E.; Withrow, S.J.; Dernell, W.S.; Kroll, D.J.; Randolph, T.W.; Manning, M.C. *Preparation and in vitro characterization of gentamycin-impregnated biodegradable beads suitable for treatment of osteomyelitis*. Journal of Pharmaceutical Sciences, 1998, 87(9), 1149-1154.

[41] Gitelis, S.; Brebach, G.T. *The treatment of chronic osteomyelitis with a biodegradable antibiotic-impregnated implant*. Journal of Ortophaedic Surgery 2002, 10(1), 53-60.

[42] Kluin, O.S.; van der Mei, H.C.; Busscher, H.J.; Neut, D. *Biodegradable vs non-biodegradable antibiotic delivery devices in the treatment of osteomyelitis*. Expert Opinion on Drug Delivery, 2013, 10(3), 341-351.

[43] Zhang, W.; Abukawa, H.; Troulis, M.J.; Kaban, L.B.; Vacanti, J.P.; Yelick, P.C. *Tissue engineered hybrid tooth-bone constructs*. Methods, 2009, 47(2), 122-128.

[44] Qing, W.; Guang-Xing, C.; Lin, G.; Liu, Y. *The osteogenic study of tissue engineering bone with BMP-2 and BMP-7 gene-modified rat adipose-derived stem cell*. Journal of Biomedicine and Biotechnology, 2012, 2012, 7.

[45] Claude, L.; Mahmoud, R. *Effect of BMP-2 and BMP-7 homodimers and a mixture of BMP-2/BMP-7 homodimers on osteoblast adhesion and growth following culture on a collagen scaffold*. Biomedical Materials, 2008, 3(1), 015008.

[46] Jiang, J.; Fan, C.Y.; Zeng, B.F. *Experimental construction of BMP-2 and VEGF gene modified tissue engineering bone in vitro*. International Journal of Molecular Sciences, 2011, 12(3), 1744-1755.

[47] Gugala, Z.; Davis, A.R.; Fouletier-Dilling, C.M.; Gannon, F.H.; Lindsey, R.W.; Olmsted-Davis, E.A. *Adenovirus BMP-2 induced osteogenesis in combination with collagen carriers*. Biomaterials, 2007, 28(30), 4469-4479.

[48] Crombleholme, T.M. *Adenoviral-mediated gene transfer in wound healing*. Wound Repair and Regeneration, 2000, 8(6), 460-472.

[49] Lo, K.W.H.; Ashe, K.M.; Kan, H.M.; Laurencin, C.T. *The role of small molecules in musculoskeletal regeneration*. Regenerative Medicine, 2012, 7(4), 535-549.

[50] Rey-Rico, A.; Silva, M.; Couceiro, J.; Concheiro, A.; Alvarez-Lorenzo, C. *Osteogenic efficiency of in situ gelling poloxamine systems with and without bone morphogenetic protein-2*. European Cells and Materials, 2011, 21, 317-340.

[51] Guglielmo, B.J.; Luber, A.D.; Paletta, D.; Jacobs, R.A. *Ceftriaxone therapy for Staphylococcal osteomyelitis: A review*. Clinical Infectious Diseases, 2000, 30(1), 205-207.

[52] Hermsen, E.D.; Hanson, M.; Sankaranarayanan, J.; Stoner, J.A.; Florescu, M.C.;Rupp, M.E. *Clinical outcomes and nephrotoxicity associated with vancomycin trough concentrations during treatment of deep-seated infections*. Expert Opinion on Drug Safety, 2010, 9(1), 9-14.

[53] Spellberg, B.; Lipsky, B.A. *Systemic antibiotic therapy for chronic osteomyelitis in adults*. Clinical Infectious Diseases, 2012, 54(3), 393-407.

[54] Neut, D.; van de Belt, H.; van Horn, J.R.; van der Mei, H.C.; Busscher, H.J. *Residual gentamicin-release from antibiotic-loaded polymethylmethacrylate beads after 5 years of implantation*. Biomaterials, 2003, 24(10), 1829-1831.

[55] Peng, K.T.; Chen, C.F.; Chu, I.M.; Li, Y.M.; Hsu, W.H.; Hsu, R.W.W.; Chang, P.J. *Treatment of osteomyelitis with teicoplanin-encapsulated biodegradable thermosensitive hydrogel nanoparticles*. Biomaterials, 2010, 31(19), 5227-5236.

Chapter 2

SYRINGEABLE SELF-ASSEMBLY CYCLODEXTRIN GELS FOR DRUG DELIVERY

2.1 ABSTRACT

The design of syringeable cyclodextrin (CD) gels is a developing area in the drug delivery and tissue engineering fields, since they offer the possibility of being administered with minimally invasive maneuvers to form depots that can remain for prolonged time in the implantation site. Two different supramolecular systems can be obtained exploiting the capability of CDs to form inclusion complexes. (i) The threading of free CDs on certain blocks or side chains of copolymers leads to polypseudorotaxanes, which can assembly via regular stacking of the threaded CDs. The resultant assemblies can be reversible broken under a certain shear stress and reformed at rest, exhibiting thixotropy that enables the flow through the syringe and the gel recovery in the implantation site. (ii) CDs grafted to polymer chains can develop their ability to form inclusion complexes with complementary guest moieties in other polymeric structures. The result is a ladder- or zipper-like arrangement, which can be also broken and reformed under certain stress conditions. Both types of CD-supramolecular gels can load and stabilize a variety of drugs via interaction with available polymer functional groups or with the CDs that are not participating in other complexes. Moreover, since the complex formation depends on various external and internal variables of the body, the syringeable CD gels can also provide stimuli-responsive drug release. This review focuses on the two main types of syringeable CD gels, prepared via self-aggregation of poly(pseudo)rotaxanes and via zipper-like assembly of CD-functionalized and guest-functionalized macromolecules, and analyzes the mechanisms and variables involved in the gelling processes and the most recent applications in the drug delivery field.

2.2 SYRINGEABLE HYDROGELS

Advances in chemistry of polymers and novel cross-linking approaches have led to the current availability of a wide variety of architectures and compositions. In the last decades chemically cross-linked networks have attracted an enormous attention because of the possibilities of finely tuning their mechanical features and degradation/erosion patterns in order to fulfill the demands of fields as diverse as oil refinery, aerospace industry, environmental remediation, agriculture, packaging, dentistry, pharmacy and tissue engineering [1]. Hydrophilic cross-linked networks, named hydrogels, have been shown very convenient as platforms for modified release of drugs and other active substances [2-5]. The tie-junctions (cross-links) among the polymer chains regulate the mesh size of the network, and thus the feasibility that a molecule can diffuse through it. Moreover, since the meshes are randomly ordered, the degree of tortuousness also determines the diffusion rate. The tie-junctions can be permanent or erodible; in this latter case, the biodegradation rate contributes to the drug release pattern too. Additionally, changes in mesh size driven by the polymers responsiveness to the microenvironment can be exploited as a way to achieve an additional control of the release profiles [6-8]. Excellent reviews on the applications of chemically cross-linked hydrogels on the biomedical field can be found elsewhere [5, 9-11].

Despite the outstanding features reported for the chemically cross-linked networks, the fact that the chains are covalently bounded each other may become a drawback for their administration using minimally invasive maneuvers. Compared to conventional chirurgical implantation techniques, administration through a syringe allows placing the formulation into hardly accessible sites (such as ocular structures, bone defects, or tumor tissues), notably reduce the risk of infections and facilitate a prompt recovery of the patient [12-16]. Syringeable systems that form depots able to fill a given space in the body and remain in it for prolonged time are particularly attractive as medicated implants. Three main strategies can be followed to develop polymer-based syringeable depot formulations, as follows (Figure 2.1). One approach (Figure 2.1a) consists in forming the covalently cross-linked network *in situ* after the injection of the polymer precursors, namely monomers or oligomers with the cross-linker agent and the initiators or catalyzers of polymerization. This the case of hydrogels formed by photopolymerization of (meth)acrylates [17-19]. However, the conditions for the reaction to take place and the remnant unreacted monomers as well as the by-products generated during the polymerization might affect the stability and biological activity of encapsulated pharmaceuticals

and the toxicity of the system [20-33]. The application of Michael addition reactions between thiols and acrylates or vinyl sulfones might notably improve the applicability of this approach [24-27]. First hydrogels prepared by Michael addition for pharmaceutical applications involved the reaction between multifunctional PEG acrylate and PEG dithiol or dithioerythritol (DTT) for sustained release of human growth hormone [24, 28, 29]. By using Michael addition, biomimetic scaffolds have been obtained by incorporation of thiol-bearing biomolecules, such as peptide sequences that can be cleaved by metalloproteinases (MMPs) secreted by the encapsulated cells [27] or thiol-modified hyaluronic acid degradable by the enzyme hyaluronase [30, 31]. The gelation and degradation time and the mechanical properties of the hydrogels and, consequently, the release rate of encapsulated hormones or growth factors and/or cell adhesion and survival can be finely tuned varying the degrees of substitution of the molecules bearing reactive groups [25, 32, 33].

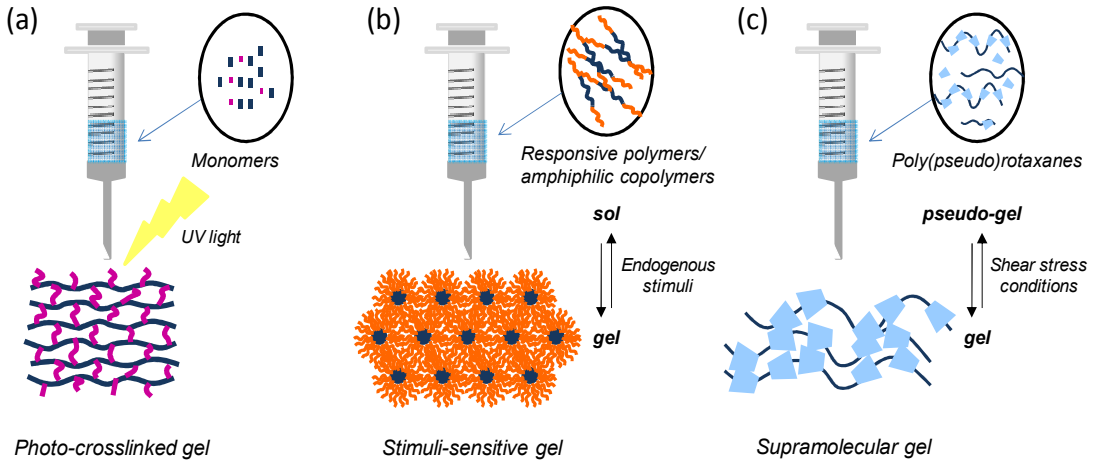


Figure 2.1. Three strategies useful to develop polymer-based syringeable depots formulations.

The two other strategies (Figure 2.1b and 2.1c) to develop syringeable hydrogels rely on the use of polymers that can lead to physically cross-linked networks. One of them involves stimuli-responsive polymers that, once in aqueous medium, can undergo phase transitions which are macroscopically evidenced as notable changes in the viscoelastic features of the dispersion [7, 34-36]. Namely, these polymers modify their aggregation state as a function of pH, temperature,

ionic strength or biomolecules concentration, among other variables of the implantation site [37]. Thus, the formulation is administered as a solution under conditions in which the polymer chains are solvated and mostly individualized and, therefore, the dispersion exhibits a quite low viscosity and can easily flow through needles. Once in the implantation site, the formulation transforms into a viscoelastic gel due to the establishment of ionic or hydrophobic interactions, hydrogen bonds, or biomimetic assemblies among the polymer chains [35]. The formation of these new "physical" bonds among the chains leads to the reinforcement of the polymeric structure, being able to stand the physiological mechanical stress and the natural mechanisms of clearance for a certain period of time. Moreover, the increase in the viscosity makes the regulation of drug release rate possible. Examples of the outstanding performance as drug depots of *in situ* gelling polymers have been recently reported [38, 39]. It is well known that aqueous solutions of certain amphiphilic block copolymers at high concentrations (ca. 20 wt.%) can exhibit reversible sol-to-gel transition behavior in response to temperature. For instance, triblock copolymers of poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO, poloxamer, Pluronic) [40] or poly(ethylene glycol)-poly(lactice-co-glycolide)-poly(ethylene glycol) (PEG-PLGA-PEG) [41] self-assembly in aqueous medium into a physical hydrogel with a packed micellar structure when the temperature is raised above a critical value. A variety of stimuli-sensitive sol-gel transition hydrogels also responding to pH or other variables has been prepared [34, 35]. The polymers can be combined with drugs and/or cells in the sol state at room temperature or acid pH, and the mixture can be injected in a desired tissue site using a syringe [10, 42]. Once triggered by the increase of temperature or a change in pH, *in situ* gelling occurs quite rapidly enabling an efficient encapsulation of the active substances [36]. Nevertheless, since the interactions among the chains are relatively weak, the gel erodes in few hours or days. Moreover, any change in the variable that triggers the gelling process may make the depot to lose its consistency, and thus to not be able to regulate the release of the The third strategy (Figure 2.1c) consists in the design of physically cross-linked networks in which the interactions are stronger but dynamic; namely structured gels that can flow when a certain pressure is applied (e.g. pushing the plunger of a syringe) and that restore their consistency at rest [43]. The ability of cyclodextrins (CDs) to form reversible inclusion complexes with a variety of molecules and macromolecules makes them particularly useful for this application (Figure 2.2). When dispersed in a polymer solution, these cyclic oligosaccharides consisting of 1,4-glycosidically linked α-D-glucose units may interact with certain moieties of the polymer forming back-bone polypseudorotaxanes or side chain complexes [44] (Figure 2.2a and 2.2b).

Hydrogel formation results from the balance between the aggregation of CDs forming part of inclusion complexes with different polymer chains (i.e., polypseudorotaxane formation leads to polymer coacervation) and the hydration of hydrophilic polymer segments (which maintain the aggregates dispersed in solution) [45]. If the polymer chains are functionalized with grafted CDs or with small molecules suitable as guests for CD-dimers or polymers, hydrogels can be obtained by mixing with the complementary guest-polymer or CD-polymer, respectively, by means of a zipper mechanism (Figure 2.2c and 2.2d).

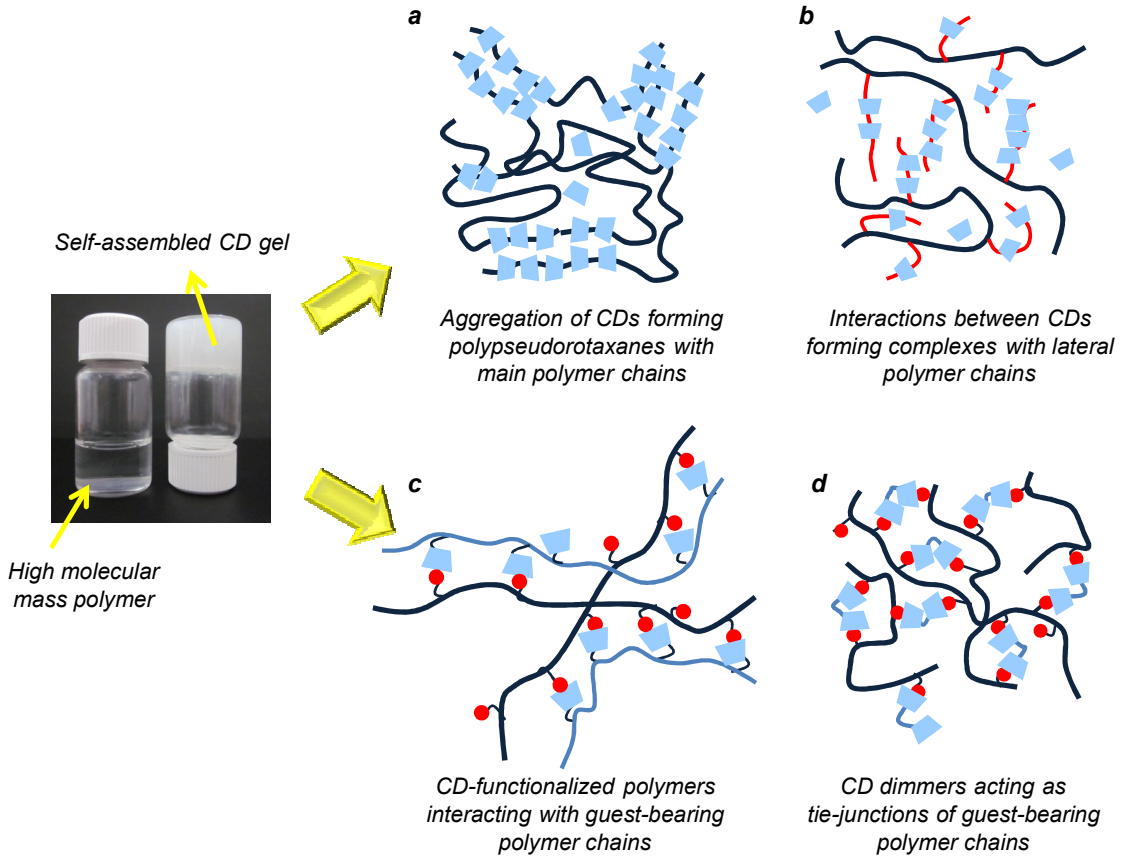


Figure 2.2. Schematic representation of self-assembled cyclodextrin gels that rely on interactions of free CDs with polymer backbones (a) or grafted chains (b) to form poly(pseudo)rotaxanes that self-aggregate, or on CD-functionalized polymers (c) or small CD-dimers (d) that interact with polymers bearing pendant guests suitable to form inclusion complexes, leading to zipper-like assemblies.

In any of the three approaches described above (Figure 2.1), the development of therapeutically useful formulations that exhibit optimal kinetics of *in vivo* formation/regeneration of the gel and that provide adequate drug release profiles is still a challenging task. Furthermore, the list of biocompatible materials suitable for syringeable gels is quite short and, therefore, an intense research is being carried out for synthesizing new polymers and gelling modulators [42, 46, 47]. The design of adequate combinations of already approved materials that can lead to synergic or even novel performances is more preferably from the regulatory point of view [42, 48, 49]. Pharmaceutical grades of natural α, β and γCDs and the derivatives 2-hydroxypropyl-βCD (HP-βCD), sulfobutyl-βCD, randomly methylated βCD and hydroxypropyl-γCD (HP-γCD) have been already used as components of approved drug products and are regarded as safe [50], although the toxicity is strongly dependent on the route of administration [51-53]. CDs interact with certain components of biomembranes and extract phospholipids and cholesterol [54, 55], forming new lipid-containing compartments outside the membrane [51, 56]. The extraction may result in hemolytic activity and nephrotoxicity, which ranks in the order βCD > αCD > γCD [57, 58] (Figure 2.3). The complexes formed by βCD and cholesterol can accumulate in the kidney and produce renal tubule damage [59]. Nevertheless, the LD_{50} values of α, β and γCDs upon intravenous administration are still as high as 1.0 g/kg, 0.79 g/kg and more than 4.0 g/kg, respectively, and therefore at low concentrations they can be considered non toxic. HP-βCD has an even more favorable safety profile [52]. The gain in knowledge about the effects of CDs on the body and their increasing commercial availability at relatively low prices are notably contributing to pave the way to the development of syringeable self-assembled CD gels for drug delivery. Moreover since the gelling process takes place at room temperature without using organic solvents, removal of non-reacted substances is not needed, and no significant changes in bioelimination/biodegradability of the non-covalently interacting components are expected, CD-based physical gels may be clinically advantageous compared to other approaches. The present review focuses on the two main mechanisms to obtain those gels: self-aggregation of poly(pseudo)rotaxanes and zipper-like assembly of CD-functionalized polymers with guest-functionalized macromolecules.

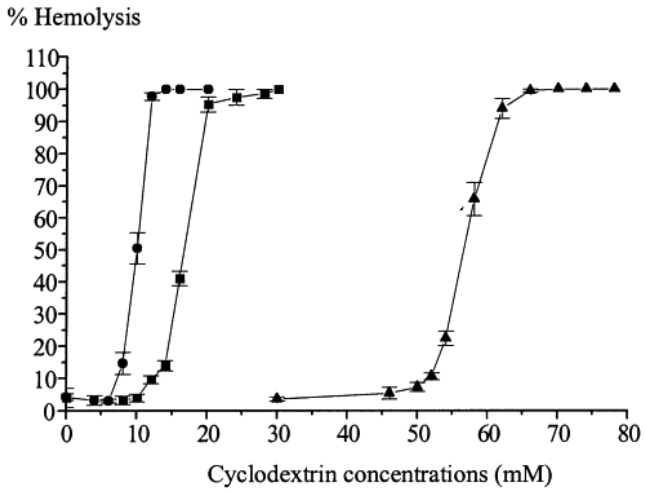


Figure 2.3. Hemolytic effects of α (●), β (■) and γCD (▲) on dog erythrocytes incubated in PBS (pH 7.4) at 37 ºC. Reproduced from [58] with permission of Revue de Médecine Vétérinaire.

2.3 CDs IN PHYSICALLY CROSS-LINKED HYDROGELS

CDs have been traditionally exploited in drug formulation as solubilizers, stabilizing agents, taste maskers, and absorption enhancers of guest molecules that can totally or partially penetrate in the CD cavity and are stabilized by means of hydrophobic and/or van der Waals interactions [60-62]. Nevertheless, CDs can also form non-inclusion complexes with poorly soluble drugs and can even self-assemble in aqueous medium rendering nanosize aggregates able to encapsulate drugs [63-65]. As CD concentration rises above 1 wt.%, the aggregation number rapidly increases (Figure 2.4). Moreover, formation of CD-guest complexes facilitates the assembling and can lead to formation of micellar-type CD aggregates that solubilize poorly soluble drugs that do not readily form inclusion complexes [63, 64, 66]. Aggregation of drug-CD complexes is temperature-dependent since the non-covalent forces involved in the process, such as hydrogen bonding, van der Waals forces or hydrophobic interactions, become weaker at high temperature, resulting in smaller complex aggregates [67].

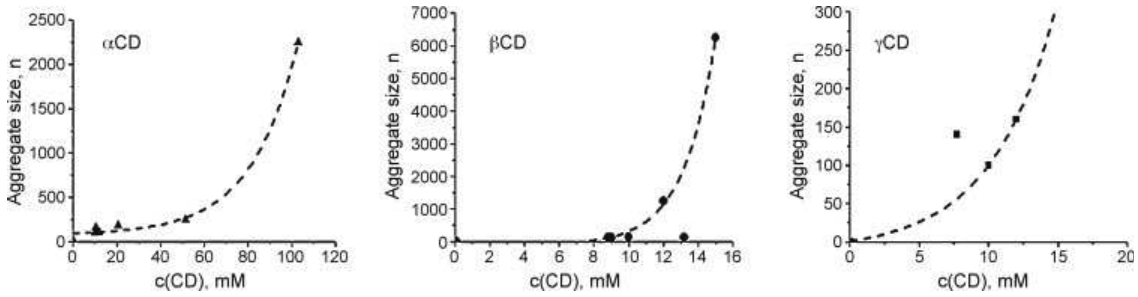


Figure 2.4. Average size of native CD aggregates (*n* represents the number of molecules) versus CD concentration, as observed by light scattering. Reproduced from [63] with permission of Elsevier.

Such capability of CDs to perform a variety of functions determines that their incorporation into polymeric physical gels notably influences the loading of hydrophobic drugs and the release rate of both hydrophobic and hydrophilic species. CDs increase the network/water drug partitioning, while the network structure protects the CD inclusion complexes from rapid dilution once in contact with the physiological fluids. Particularly, the effects of CDs on drug diffusion depend on:

(i) the proportion of drug that can be dissolved by means of inclusion complexes, i.e., the greater the concentration of diffusible species, the faster the release is. Thus, physically dispersed CDs have the potential to enhance drug release rate by increasing the concentration of diffusible species within the matrix if the drug is loaded at doses above saturation;

(ii) the hydrodynamic volume of the complexes compared to that of free drug molecules, i.e., the larger the complexes, the slower the diffusion is. If the complexes are greater than the mesh size, the release can be significantly retarded, as decomplexation should occur for the release to proceed. CDs reduce the free drug concentration slowing down the release rate when drug concentration is below their solubility coefficient or if the drug forms highly stable complexes [6]. An example of this behavior is that of hydroxypropyl methylcellulose (HPMC) gels containing HPβCD or MβCD for nasal release of melatonin [68]. When CDs were

added at a low concentration (1%) an enhancement of nasal drug penetration was observed, but at higher concentrations (5-10%) nasal penetration diminished.

(iii) the hindrance that free CDs can exert on the movement of the other diffusible species [6, 69-71];

(iv) the interaction of CDs with the polymer chains, which makes the scenario to become more complex, with microdomains dense in polymer and CDs that can make the diffusion difficult (Figure 2.5) [70].

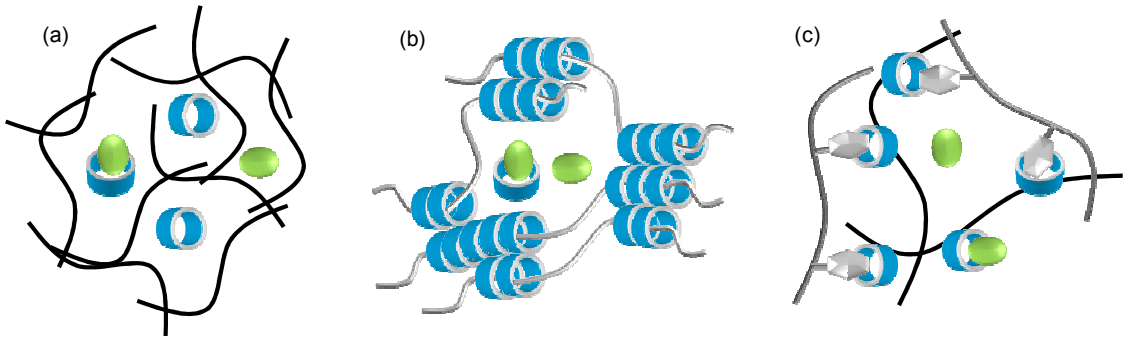


Figure 2.5. Different states in which a CD can be found in a physically cross-linked hydrogel: (a) movable CD species; (b) poly(pseudo)rotaxanes with the threaded CDs interacting each other; and (c) CDs grafted to polymer chains acting as tie-junctions of other chains.

2.4 FREE CDS AS GELLING AGENTS: Poly(pseudo)rotaxanes that self-aggregate

2.4.1 Gel mechanism

Supramolecular self-assemblies are attracting great attention because they do not only serve as models for understanding molecular recognition, but also provide structures useful for designing novel materials [72]. CDs can form necklace-like supramolecular complexes with certain polymers with a stoichiometry well beyond 1:1 [73]. The polymers are threaded by CD units which stack along the polymer axis, forming polypseudorotaxanes [74-76]. If the polymer ends are conveniently blocked using bulky moieties and the CDs cannot move away the polymer, a polyrotaxane is obtained. Intermolecular interactions among the CDs placed in adjacent poly(pseudo)rotaxanes may result in the formation of superstructures that notably modify the rheological properties of the system [77-78] (Figure 2.5b). The correlation between the cross-sectional area of the polymer chains and the internal diameters of the CD cavities plays a key role in the formation of the poly(pseudo)rotaxanes [79]. The hydrophobic cavity of CDs has a depth of ca. 7.0 Å and an internal diameter of ca. 4.5, 7.0, and 8.5 Å for α, β, and γCD, respectively. For example, linear polyethyleneglycol (PEG or PEO) chains with a molecular weight greater than 200 can be threaded by αCDs to form supramolecular self-assembly complexes [80]. Two PEG chains can be simultaneously threaded through γCDs, forming double-stranded inclusion complexes [81]. On the other hand, one PEG chain is too slim to form stable complexes with βCD, while two PEG chains are too thick to fit in the βCD cavity [44]. In contrast, βCD can selectively thread onto propyleneglycol (PPG) chains or onto the poly(propylene oxide) blocks, PPO, of amphiphilic copolymers such as PEO-PPO-PEO [82]. Interestingly, experiments carried out with reverse poloxamers, namely PPO-PEO-PPO block copolymers, revealed that αCD can slid over the end PPO blocks to selectively form the complex with the EO units [83, 84]. This means that the enthalpic driving force of the αCD/PEO complexes overcomes the energy barrier of sliding αCD over the bulky PPO blocks (Figure 2.6). Similarly, γCDs can thread onto *Bombyx mori* silk protein in spite of it contains bulky amino acids on both its N- and C-termini [85]. The versatility of conformations that poly(pseudo)rotaxanes can adopt endow them with tunable performances to fit to a broad range of biomedical applications, as already widely covered in literature [73, 86-89].

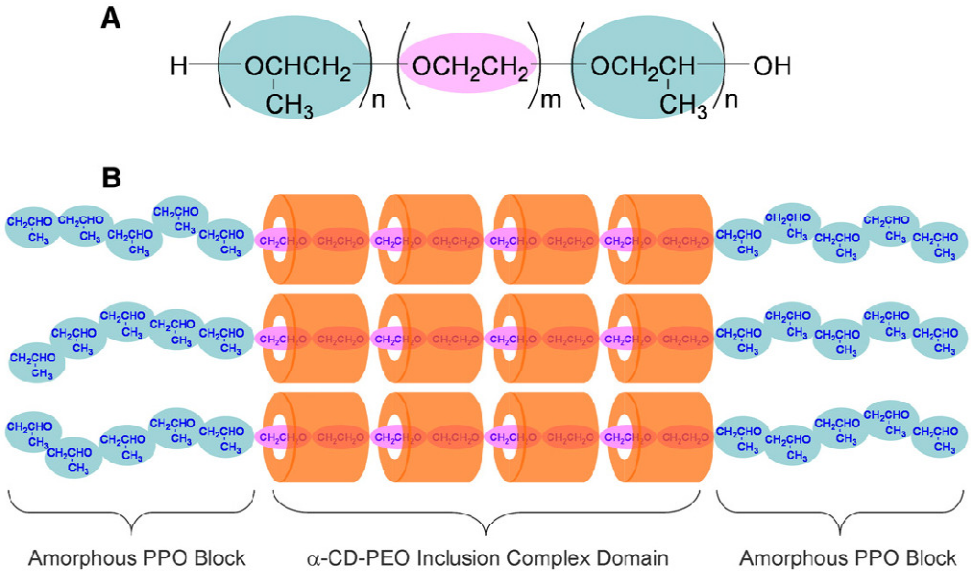


Figure 2.6. The structure of PPO–PEO–PPO triblock copolymer (A) and schematic representation of the structure of the αCD–PPO–PEO–PPO polypseudorotaxanes where the thinner middle PEO blocks form inclusion complex domains with αCD, while the flaking thicker PPO blocks are uncomplexed and remain amorphous (B). Reproduced from [88] with permission of Elsevier.

In the particular case of designing syringeable gels, the concentrations of both the polymer and the CDs have to be adequately tuned to lead to a continuous network, instead of the assembled nanoaggregates (micelles, nanoparticles) that result from diluted systems [90]. When a concentrated aqueous solution of αCDs is mixed with PEG or PEO-block copolymers, the mixture rapidly becomes turbid. The cooperative threading of a number of αCDs molecules onto the hydrophilic polymeric chain causes the dehydration of the polymer chains, as pioneering observed by Harada and coworkers [44, 91]. The stoichiometry of the polypseudorotaxanes is 2 EO units per one αCD, and the αCDs stack forming nanocylinders with a crystalline channel type structure in which PEG is included [91]. This process is entropically unfavorable, but enthalpically favorable due to hydrogen bond formation between αCDs that arrange head-to-head and tail-to-tail along the nanocylinder, which points out the importance of the cooperative effects on the likelihood of polypseudorotaxane formation. The complex formation of PEG becomes faster as the polymer molecular weight increases up to 1000 Da, but beyond that

molecular weight, the rate progressively decreases as the chains are larger [91]. Similarly to the αCD-PEG polypseudorotaxane, PPG chains and PPO blocks can form polypseudorotaxanes with βCD or γCD units each covering two propylene oxide units, which may have crystalline structure [79]. Modification of the PPG structure with short lateral chains does not interfere in the complex formation, but the presence of aromatic rings prevents the complexes with βCD and also makes the interactions with γCD more difficult [79]. Association of the threaded CDs from adjacent polypseudorotaxanes may lead to phase separation or to a three-dimensional network, depending on the polymer and its concentration [92, 93]. Interestingly the inter-polypseudorotaxane interactions are minimized when hydrophilic derivatives of the natural CDs are used; namely, hydroxypropyl-CDs thread onto the polymers but form hydrosoluble polypseudorotaxanes that do not alter the turbidity/viscosity of the system [94, 95].

Association of CD nanocylinders into microcrystals may act as tie-junction for gel formation [96]. Relatively high concentrations of both the CD and the polymer and/or the use of high molecular weight polymers facilitate the formation of precipitated domains, which serve as cross-linking points. Since the inter-polypseudorotaxane interactions are reversible, the supramolecular gels behave as thixotropic, and the viscosity greatly diminishes as the system is subjected to shear stress. As a consequence, the gels transform into a formulation injectable through a fine needle. The viscosity of the gel restores towards its original value when left at rest, forming a depot in the injection site. Compared to the αCD-PEG systems that require several hours in recover the initial viscosity, αCD-PEO-PPO-PEO gels form and reform faster, probably because of the additional contribution of the hydrophobic interactions among PPO blocks (Figure 2.7) [93, 95]. Although the research on these syringeable gels for drug delivery is still quite recent, the already available information highlights the remarkable possibilities that polypseudorotaxane gels can offer for drug loading and controlled, even targeted, release as shown below.

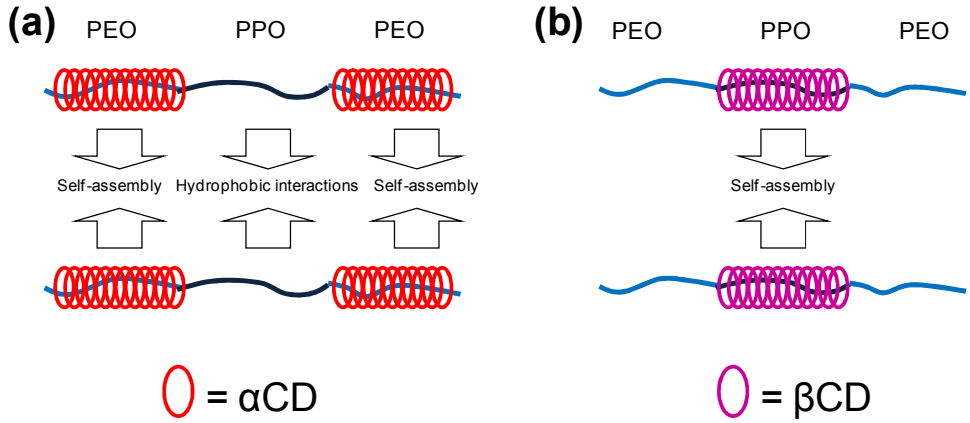


Figure 2.7. Structures of the complexes formed by Pluronic copolymers with αCD (a) and βCD (b). Adapted from [88] and [95] with permission of Elsevier**.**

2.4.2 Applications

Incorporation of labile active substances to polypseudorotaxane-based gels is notably facilitated by the mild conditions under which the gels are prepared. For example, insulin has been shown to remain stable when encapsulated in αCD or γCD-PEG gels [97]. Moreover, pegylated-insulin forms polypseudorotaxanes with αCD or γCD in a similar manner as PEG does, and the resulting gels can sustain the release of the peptide hormone [98, 99]. To avoid pegylation of the protein, the gels can be formed by adding CDs to PEG/insulin solutions (100 mg PEG and 5.74 mg insulin per mL, 0.5 mL). αCDs (145 mg/mL, 3.35 mL) rendered gels in less than one hour, while γCDs (232 mg/mL, 2.23 mL) required 12 hours [97]. Freeze-dried αCD and γCD gels rapidly swelled in water up to about 3.6 and 1.3 times their mass, respectively, and sustained insulin release in PBS pH 7.4 for several hours. Subcutaneous administration of the gels to rats evidenced more marked decrease in glucose serum concentration and more prolonged insulin levels, compared to the insulin solution formulation (Figure 2.8). The area under the serum insulin level-time plot was significantly increased, compared to that of insulin alone, suggesting that the potential use of the gels as an injectable sustained release system for insulin.

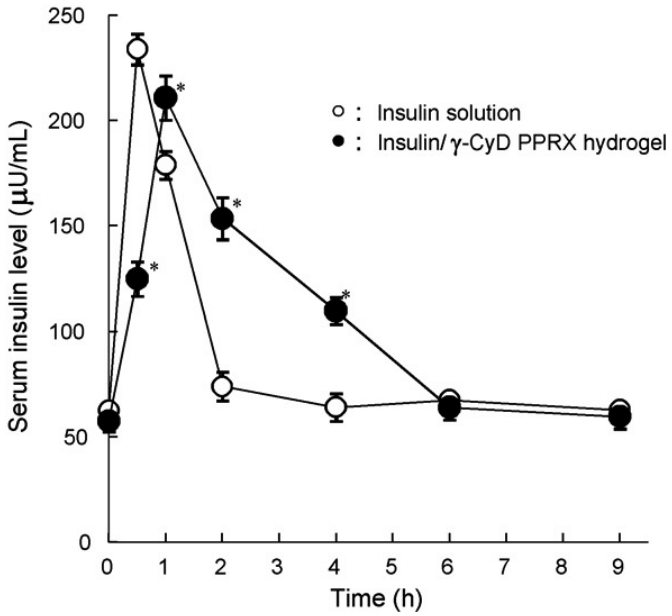


Figure 2.8. Serum levels of insulin after subcutaneous administration to rats of an insulin solution and an insulin/γCD-PEG gel formulation. The insulin gel was diluted with 232 mg/mL γCD solution. Each point represents the mean ± S.E. of nine experiments (*$p < 0.05$ versus insulin). Reproduced from [97] with permission of Elsevier.

In the case of the temperature-sensitive PEO/PPO block copolymers, notable changes of the sol-gel transition temperature and of the viscoelasticity of the gel phase may occur after the addition of CDs [91, 100, 101]. PEO-PPO-PEO form gels at 37 ºC, but the relatively high concentrations required can lead to safety concerns [102, 103]. Thus, approaches for increasing gel strength while reducing Pluronic concentration should render safer systems with improved drug release performance. Threading of αCD onto the PEO blocks largely changes the hydrophobicity of the copolymer and significantly lowers its gelation concentration, as observed for PEO-poly[(R)-3-hydroxybutyrate](PHB)-PEO block copolymers [104]. For example, incorporation 5% w/v αCD to 6.5% w/v Pluronic F127 ($EO_{100}PO_{69}EO_{100}$) can form a supramolecular gel in few hours, but increasing the concentration of Pluronic and αCD, the gel can be obtained in few minutes (Figure 2.9). Besides the associations between the polypseudorotaxanes of complexed PEO blocks, the hydrophobic interaction between the PPO blocks (Figure 2.7) also play a role in the formation and stability of the gels [93]. In fact, gels prepared with 13% w/v Pluronic F127 showed temperature-responsive viscoelasticity and thixotropy and were able to sustain the

release of vancomycin for several days, a drug that does not interact with αCD nor with the copolymer. These gels, which could be easily drawn from 1 mL syringes, were active against *Staphylococcus aureus* in *in vitro* cultures and thus promising as depots for the local treatment of infections. Gels formed adding αCD to $EO_{10}PO_{44}EO_{10}$ were able to slowly release bovine serum albumin (BSA) for one week, while those prepared from $EO_{113}PO_{57}EO_{113}$, $EO_{13}PO_{30}EO_{13}$, $EO_{11}PO_{16}EO_{11}$, and $EO_{76}PO_{30}EO_{76}$, resulted to be too unstable for long-term sustained delivery [88].

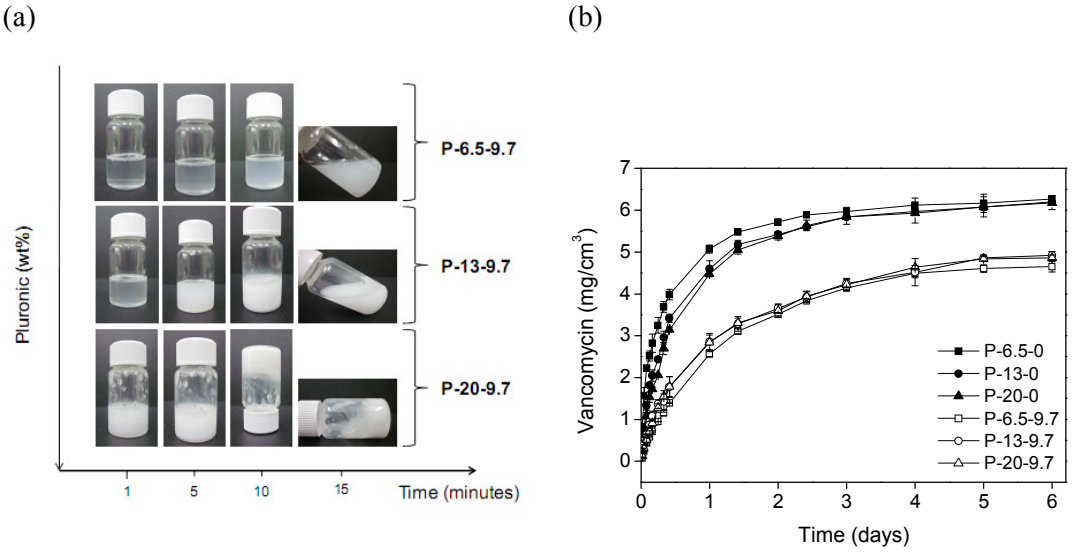


Figure 2.9. Appearance of Pluronic (6.5, 13 and 20%) with 9.7% αCD gels in the first minutes after preparation (a), and vancomycin released at 37 ºC from Pluronic formulations prepared with or without 9.7% αCD (b). Adapted from [93] with permission of Elsevier.

An injectable antitumor formulation has been prepared with Pluronic F127 bearing heparin conjugated to some PEO blocks. The copolymer self-associated as micelles able to host camptothecin (antitumoral drug) in the cores and granulocyte colony-stimulating factor (G-CSF; an hematopoietic growth factor) bound to the heparin moieties. Then, an aqueous solution of αCD (5-7 wt.%) was added to the micelles dispersion (5 wt.%) to induce supramolecular gelation, co-encapsulating both active substances (Figure 2.10). The gels were formed in few minutes and released both active substances in a sustained way for several weeks, preserving its

therapeutic activity [105]. Using a similar strategy, doxorubicin hydrochloride was solubilized in cinnamic acid hydrophobically-modified methoxy-PEG micelles and subsequently gels were formed by incorporation of αCD [106]. As mentioned above (Figure 2.6), αCDs can also form supramolecular aggregates with reverse Pluronic 10R5 (PPO_8-PEO_{22}-PPO_8) block copolymer, resulting in gels with a wide range of viscosities [95].

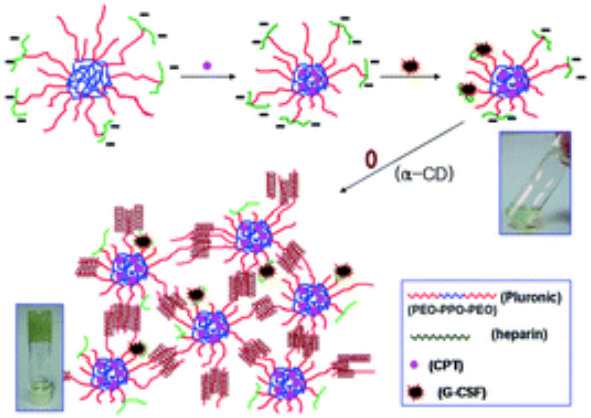


Figure 2.10. Formation of a supramolecular hydrogel/micelle composite from αCD and CPT-loaded Hep-F-127 micelle/G-CSF complexes in an aqueous system. Reproduced from [105] with permission of the Royal Society of Chemistry.

Differently to the effects observed for αCD, polypseudorotaxanes of PEO-PPO-PEO with βCD usually lead to poorly soluble aggregates [95]. Gel formation can be regulated by tuning the solubility and the crystallization process of the threaded βCDs [107]. For example, mixing equal amounts of a reverse Pluronic PEO-PPO-PEO with βCD in water at 25 ºC has been shown to result in weak gels formed by loosely aggregated and heterogeneously distributed particles and some circular structures having thick walls and cavities, which may represent insolubilized βCD particles covered with reverse Pluronic (Figure 2.11a and 2.11d). Incubation at 65 ºC enhanced βCD solubility and thus promoted polypseudorotaxanes formation, resulting in slightly more uniform and viscous gels (Figure 2.11b and 2.11e). Incorporation of citric acid to βCD enabled a complete solubilization of this CD, which became to perform as the αCD. Citric acid altered hydrogen bonding in the βCD, increasing the solubility and thus the amount of βCD available for forming complexes. Nevertheless, the alteration of the hydrogen bonds diminished the gel

formation rhate. Thus, citric acid partially restricted polypseudorotaxanes crystallization, which resulted in a fine, net-like gel structure (Figure 2.11c and 2.11f) with higher gel strength, thixotropy and stability [107].

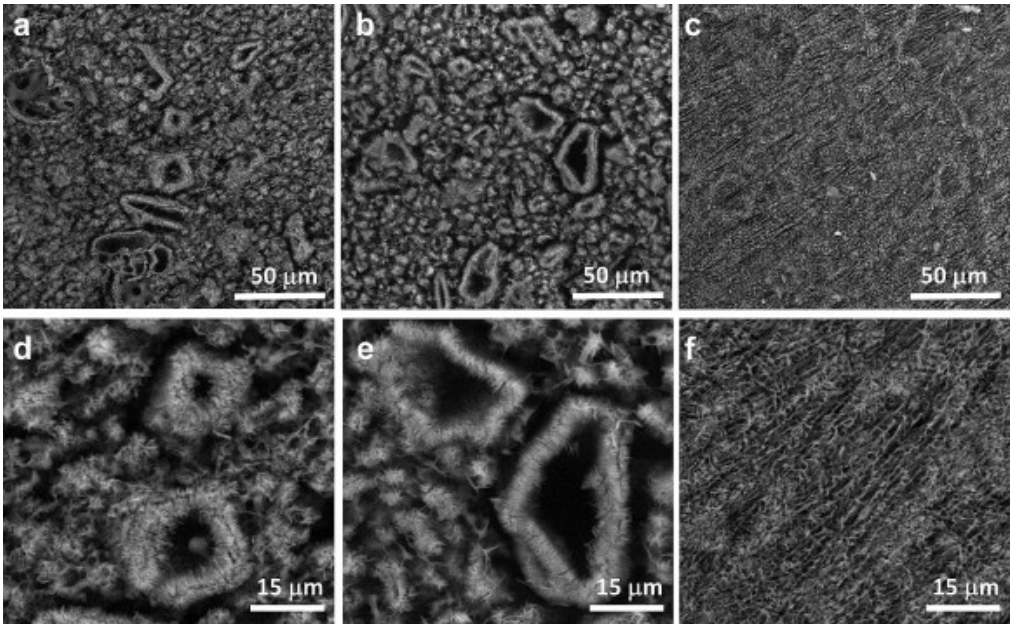


Figure 2.11. SEM micropictures of freeze-fractured cross-sections of the βCD/reverse Pluronic polypseudorotaxane gels prepared in water at 25 ºC (a and d), in water at 65 ºC (b and e), or in citric acid solution (140 mg/mL) at 25 ºC (c and f). Reproduced from [107] with permission of Elsevier.

Incorporation of hydrophilic derivatives of βCD to PEO-PPO-PEO solutions makes the copolymer more hydrophilic, raises the critical micellar concentration and decreases the number of micelles and CD cavities available to host drug molecules. This results in a lower ability to solubilize hydrophobic drugs in the micelles and also in the CDs. For example, addition of 5 wt.% HP-βCD or randomly methylated-βCD increased by 4-6 ºC or up to 15 ºC, respectively, the gelation temperature of 15 wt.% Pluronic F127 solutions, and significantly decreased G′ (storage) and G″(loss) moduli of the gels [94]. Recently, Artificial Neural Networks (ANNs) modeling has been demonstrated to be an useful tool for designing injectable intratumoral formulations of the anticancer drug β-lapachone [108]. The modeling required first the

experimental evaluation of the effects of the interactions between Pluronic, randomly methylated-βCD and β-lapachone on the features of the ternary system, covering a wide range of concentrations of both Pluronic and randomly methylated-βCD. With this input of information, the software generated a model that, after validation, allowed the identification of the composition that renders gels at body temperature with optimal dose of drug solubilized and controlled released features. Intratumoral injection of the β-lapachone ternary system in a mouse xenograft tumor model significantly reduced tumor volume, while increased apoptosis and DNA damage without visible toxicity to liver or kidney [109].

Novel reduction-sensitive supramolecular gels have been prepared from polypseudorotaxanes of αCD with copolymers of monomethyl ether PEG and poly(amido amine) (PAMAM), grafted by means of a disulfide bond (mPEG-g-SS-PAMAM). The disulfide bonds endowed the gels with sensitiveness to the redox conditions of the medium. In the absence of a reducing agent, the gel provided drug sustained release. Incorporating a low loading-level of reducing agent did not inhibit the formation of hydrogel. By contrast, in the presence of high concentration of DTT, the supramolecular gel disintegrated and the release rate accelerated. These polypseudorotaxanes may exploit the reduction-sensitivity to regulate the drug release profile in extracellular medium [110].

Syringeable polypseudorotaxanes have been also tested for gene therapy. Micrometer aggregates were prepared starting from an Starburst® poly(amido amine) (PAMAM) dendrimer, which is a spherical dendritic polymer with 16 primary amino groups on the surface that are positively charged at physiological pH. A few αCDs and PEG chains were chemically grafted to the dendrimer (Figure 2.12), and then polyplexes with plasmid DNA (pDNA) were prepared [111]. Although the pristine dendrimer did not interact with CDs, the incorporation of PEG chains facilitated polypseudorotaxane formation when αCDs or γCDs were added to the system. In the polypseudorotaxanes, the CDs were linearly aligned in the crystalline phase forming hexagonal and tetragonal columnar channels, respectively (Figure 2.12). The polypseudorotaxanes exhibited a high encapsulation efficacy and *in vitro* and *in vivo* gene transfer activity. Importantly, the nature of the CD determined the pDNA release rate *in vitro*, being faster from polypseudorotaxanes prepared with γCD. The tetragonal columnar channel crystal structure has more space between the γCD molecules, compared to the hexagonal columnar channel crystal structure formed by αCDs. Consequently, water molecules could penetrate more easily in the γCD polypseudorotaxanes, accelerating the dissolution process. The

volume of release medium also affected to the release, as the dethreading of CDs from polypseudorotaxanes became faster as the volume of the medium increased. Overall, these findings point out PEG-αCD-dendrimer polypseudorotaxanes as suitable non-viral vectors for delayed and sustained gene expression *in vivo* [111].

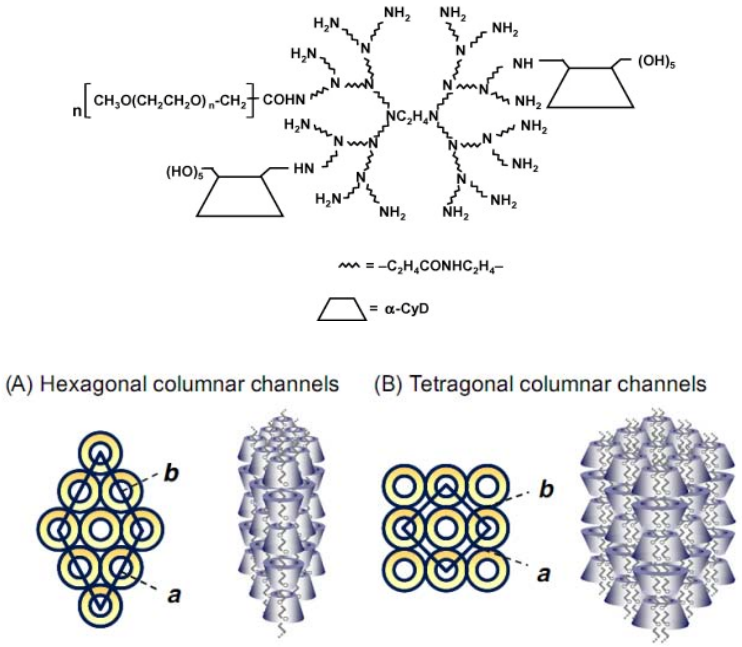


Figure 2.12. Structure of a PEG-αCD-dendrimer and scheme of crystal packing structures of αCD (A) and γCD (B) poly(pseudo)rotaxanes. Reproduced from [111] with permission of Elsevier.

2.5 POLY-CDS AS GELLING AGENTS: Zipper-like structures

2.5.1 Gel mechanism

Formation of CD hydrogels by means of zipper-like junctions requires starting from oligomers or polymers containing many CD units. Linear and branched CD polymers can be prepared in different ways (Figure 2.13) [112]: (1) grafting of CDs to preformed polymers [113]; (2) cross-linking of CDs with bi- or multifunctional reagents, such as epichlorohydrin, biepoxides and diisocyanates [114-116]; and (3) polymerization of monomer derivatives of CDs bearing reactive double bonds (e.g. acrylic substituted CD) suitable for reaction with other acrylic/vinyl monomers [117]. The CD polymers preserve the capability of CDs to form inclusion complexes, which may be even enhanced due to cooperative effects [65].

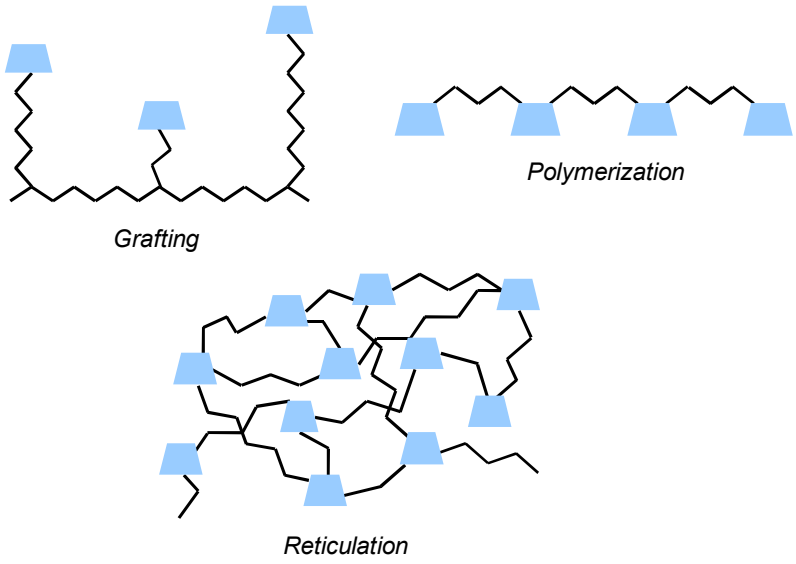


Figure 2.13. Structures of CD polymers obtained by grafting (immobilized CD), polymerization (chain CD polymer) or cross-linking reactions (network CD polymer). Adapted from [112] with permission of Elsevier.

The zipper mechanism is based on the host-guest complexes that can be formed when CD oligomers or polymers enter into contact with multifunctional guests or polymers bearing guest moieties (Figure 2.2c and 2.2d). The CDs recognize the guest moieties and form complexes that

act as tie-junctions, bringing together two or more polymer chains. Such a CD-mediated cross-linking largely increases the viscosity or the gel-like behavior of the system and can be considered as a situation in between physical (i.e., reversible) and chemical (i.e., highly stable upon dilution) cross-linking. This phenomenon has been described for blends of: i) a polymer bearing pendant βCD and a polymer with hydrophobic 4-tert-butyl anilide side chains [118]; ii) CD-chitosan conjugates and adamantyl-grafted chitosan or PEGs [119]; iii) CD-poly(acrylamide) and poly(acrylamide) possessing aromatic rings [120]; and iv) βCD polymers (made with epichlorhydrine) and poly(N-isopropylacrylamide) containing either an adamantyl or a dodecyl group [121, 122]. The strength of the gel and several other properties depend on the number and the strength of host-guest complexes formed and also on the nature of the polymer chains. If a polyelectrolyte is used as backbone (e.g. hyaluronic acid, chitosan, polyacrylic acid), the properties of the zipper gels depend on the balance between attractive interactions due to the inclusion complexes and repulsive electrostatic interactions among the polymer chains. This balance may be strongly modified by external parameters (ionic strength, pH, temperature) as well as by intrinsic factors (degree of substitution of polymers, association constant of the host–guest complex) [113]. A similar situation occurs for polymers that are sensitive to certain internal or external variables of the body. Therefore, there are no general rules for finding the optimal conditions for gel formation, but each system has to be analyzed in separate. As for the polypseudorotaxane gels, zipper-like gels are spontaneously formed in aqueous medium when the components are mixed, without adding any other solvent or catalyst, which avoids environmental and safety concerns. The therapeutic substances, including gene material and cells, can be incorporated in the solution of one of the components (the host or the guest) before the mixing with the complementary component. Particularly, hydrophobic drugs can be solubilized in polymeric host bearing CDs in excess for the gel formation. These systems are also thixotropic, and thus they can be administered as injectable matrices to be implanted in specific sites of the body.

Attempts to explain the mechanism behind zipper-like gels relied on associating polysaccharides having pendant βCDs (host polymer) with others functionalized with adamantine (guest polymer) [119, 123, 124]. Adamantine derivatives form deep and snug-fitting complexes with βCDs, leading to very high association constants (Ka ~ 10^4–10^5 M^{-1}) [62]. Mixing of chitosan or hyaluronic acid functionalized with βCDs or adamantine (0.03-0.08 degree of substitution) provided viscoelastic gels with total polymer concentration only 1.5-fold

higher than the critical overlap concentration of the initial polysaccharides. In this case, the addition of salts has to be avoided to prevent phase separation. The networks are considered to be the result of double chain strands connected by fourfold junction points (see Figure 2.2c). In diluted regime, the mixture of polysaccharides functionalized with βCDs and adamantine results in formation of double chain complexes (also called railway or ladder complexes). As the concentration in polysaccharides rises, the ladder complexes form a reversible network in which the crossed double-chains interchange their partners, leading to three-dimensional junctions. The theory developed to explain the rheological features of this kind of gels is based on the two types of cross-links: (1) strong junctions where two double chains interchange their partners, which dramatically quench the motions of the chains; and (2) weak junctions between a CD unit in one double-chain fragment and an adamantine molecule in another double chain, which can easily dissociate [113]. The presence of competitive molecules in the medium (free guest or host) remarkably weakens the more rigid pairs; namely, those placed in chains where the backbone spacer between the neighboring βCDs or adamantine moieties is stiffer. Dissociation notably decreases the high elastic energy penalty. Further details of this association model can be found elsewhere [113]. The effects of poly-βCDs obtained by cross-linking with epichlorohydrin on gelling of alkylated derivatives of alginate have been also evaluated at various temperatures. At low concentration, poly-βCDs strengthened the gels as they acted as tie-junctions among the hydrophobically modified chains. In contrast, gels became weaker when an excess of poly-βCDs was added, since individual host chains formed complexes with just one hydrophobic unit in the alginate chain and did not act as cross-linkers [125].

Gels can be also formed by adding neutral dimeric βCDs to a solution of guest polymer, such as adamantin-grafted chitosan, although the architecture of the dimer may notably determine the likelihood of cross-linking [126-128]. Dimers prepared with two spacers (called βCD duplexes) significantly enhanced the viscosity, while dimers with only one linkage between the two βCDs did not cause thickening effects. These findings correlate with the fact that the rigid dimer can form 11-times stronger complexes with adamantine, because the two C8 hydrophobic chains strengthen the hydrophobic interactions with the guest. The viscosity decreased using dimers with more hydrophilic spacers, and remarkably increased when tetramers were used (Figure 2.14) [128]. The findings of these works are in agreement with the data reported for βCD-derivatized 8-arm star-shaped poly(ethylene glycol) (PEG_8) when interacted with cholesterol-derivatized linear bifunctional PEG (PEG_2), 4-arm star-shaped PEG (PEG_4) or 8-arm star-

shaped PEG (PEG_8). The 8-arm polymer-based mixtures yielded tight viscoelastic networks, but their storage and loss moduli significantly deviated from those predicted by the Maxwell model. The βCD/cholesterol hydrogels were thermoreversible, showing viscoelastic behavior at low temperature, but liquid-like behavior when heated due to a reduced number of complexes and concomitant faster chain relaxation processes [129].

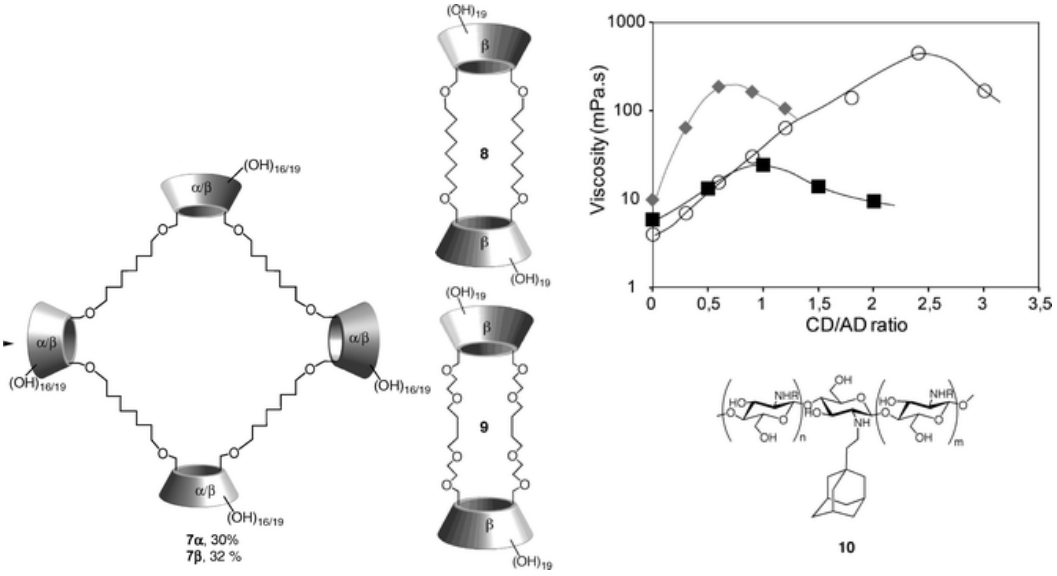


Figure 2.14. Variation of the viscosity of solutions of adamantine-grafted chitosan 10 (2.5-3 g/L in acetate buffer at 25 °C) as a function of the [CD]/[adamantine] ratio; i.e., increasing the concentration of βCD tetramer 7 (◆), hydrophobic βCD dimer 8 (○) and hydrophilic βCD dimer 9 (■). Adapted from [128] with permission of the Royal Society of Chemistry.

2.5.2 Applications

Poly-βCDs spontaneously self-assembled with dextran chains bearing grafted alkyl side moieties and formed gels for sustained drug release (Figure 2.15). The βCD polymer was obtained by means of cross-linking with epichlorohydrin, with a final content in βCDs of ca. 70% [130]. Mixing aqueous solutions of poly-βCDs with lauryl-grafted dextran (4% degree of substitution) at 2.5-7.5 wt.% led to an instantaneous phase separation. The gel phase settled in the bottom of the vial was very rich in both polymers. Gels formed with 50% in each polymer,

namely with a βCD/lauryl chain molar ratio slightly greater than 1, had G′ and G″ values about 400-500 Pa and 1200-1400 Pa, respectively [131, 132]. Lower βCD/lauryl chain molar ratio rendered weak gels, while a decrease in total content in both polymers (0.1-1 wt.%) resulted in stable nanogel particles [64, 133]. The presence of salts or proteins in the medium did not negatively affect to the gel features. Rheological experiments carried out before and after the passage of the gels through a syringe connected to a 18-gauge needle confirmed the reversibility of the physical cross-links, being restored between 30 s and 2 min after the stress had been removed [131]. The gels loaded benzophenone and tamoxifen forming complexes with poly-βCD before mixing with dextran. Empty cavities (i.e., those that were not occupied by drug molecules) were available to host the lauryl chains and served as junctions between the polymers. Benzophenone made the gels weaker because its greater affinity for βCDs compared to tamoxifen, but the release of benzophenone was more sustained due to a complex balance among drug diffusion in the gel, competition between drug and alkyl chains for the CD cavities, and partition of the drug between the gel phase and the supernatant [131]. Tamoxifen was released following a zero-order release profile during four days; then the gel disintegrated. The estimated amount of gel to be formed *in situ* to achieve *in vivo* anti-proliferative effects was found to be compatible with an injection [130]. In addition, it is possible to create pH-responsive gels through combinations of polymers bearing ionizable guests and/or poly-βCDs also having ionizable groups, as shown for dextran bearing 2-carboxycyclohexyl carboxyl groups and neutral or positively charged poly-βCDs [134].

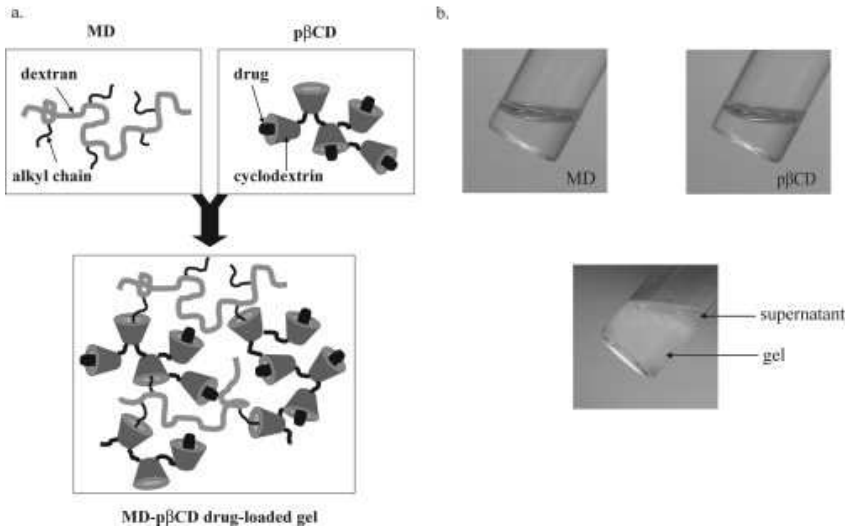


Figure 2.15. Schematic representation of the formation of MD-pβCD gels (a) and photographs after 5 seconds to mixing of two polymeric aqueous solutions of a hydrophobically modified dextran by grafting alkyl side chains (MD) and a β-cyclodextrin polymer (pβCD) (b). Some alkyl chains of MD are complexed into CD cavities of the pβCD, leaving also free CDs for the inclusion of hydrophobic drugs. Reproduced from [131] with permission of John Wiley and Sons.

Stimuli-responsive supramolecular assemblies have been prepared from series of α- and βCD-conjugated poly(3-lysine)s (CDPLs) that served as host molecules for polyfunctional guest moieties containing hydrophobic and anionic groups [135]. Inclusion complexes and intermolecular ionic interactions determined a fast aggregation; e.g., within 100 ms in the case of βCDPL and 3-trimethylsilylpropionic acid (TPA). This rapidly assembling system shows a clear dependence on pH and temperature, which significantly affect the gelling of the supramolecular structure (Figure 2.16). At low pH, each TPA can simultaneously interact with two CDs, but repulsive interactions between cationic amino groups of poly(3-lysine)s prevent intermolecular aggregation and the systems remain as transparent solutions. In the pH region 5.0-8.0 inclusion complexes and ionic attractions between the cationic groups of CDPLs and the anionic end-groups of TPAs occur and a gel is formed. At more alkaline conditions, the poly(3-lysine) backbone becomes neutral, the repulsive ionic interactions between negative carboxylate groups of TPAs prevent aggregation, and solutions are again transparent. These systems also showed temperature- and surfactant-responsiveness [136]. Glucose- and redox-sensitivity have

been attained with supramolecular gels that combine βCD-grafted alginate with ferrocene-modified Pluronic F127 [137].

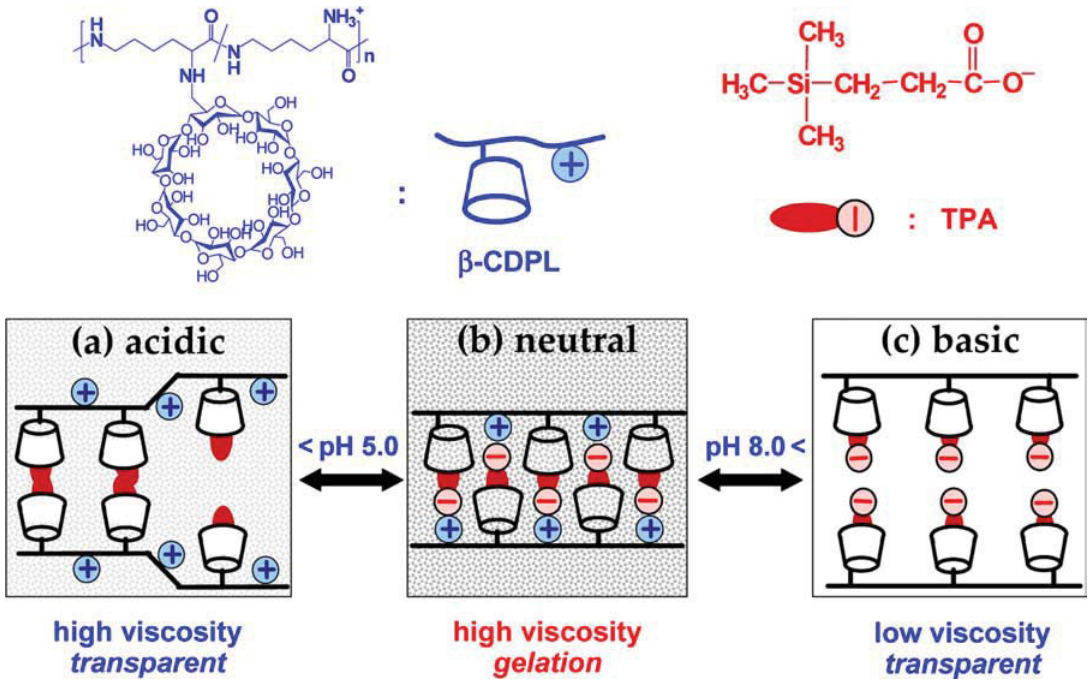


Figure 2.16. pH-reversible properties of a supramolecular assembly based on specific host–guest complexes between βCD-conjugated poly(3-lysine)s (CDPL) and 3-trimethylsilylpropionic acid (TPA) molecules: intermolecular interactions among hydrophobic TPA molecules and cationic repulsive interactions between amino groups (a), supramolecular aggregation via cooperative intermolecular interactions between amino groups along PL chain and carboxyl groups of TPA (b), and anionic repulsive interactions between charged carboxyl groups of TPA (c). Reprinted with permission from [136]. Copyright 2005 American Chemical Society.

Hybrids of single-walled carbon nanotubes (SWNTs) and cyclodextrins have been explored as chemically-responsive hydrogels [138]. Water soluble SWNTs were prepared by chemical adsorption of pyrene-modified βCDs. The surface of the hybrids was thus decorated with empty βCDs available to form inclusion complexes with a variety of guests (Figure 2.17). When poly(acrylic acid) carrying 2 mol% of dodecyl groups was added, host-guest interaction

occurred with the participation of βCDs from various SWNTs and a gel was rapidly formed. Adding competitive guests (sodium adamantane) or hosts (αCDs), the gel transformed again in a solution. This approach may open novel applications of SWNTs in the biomedical field.

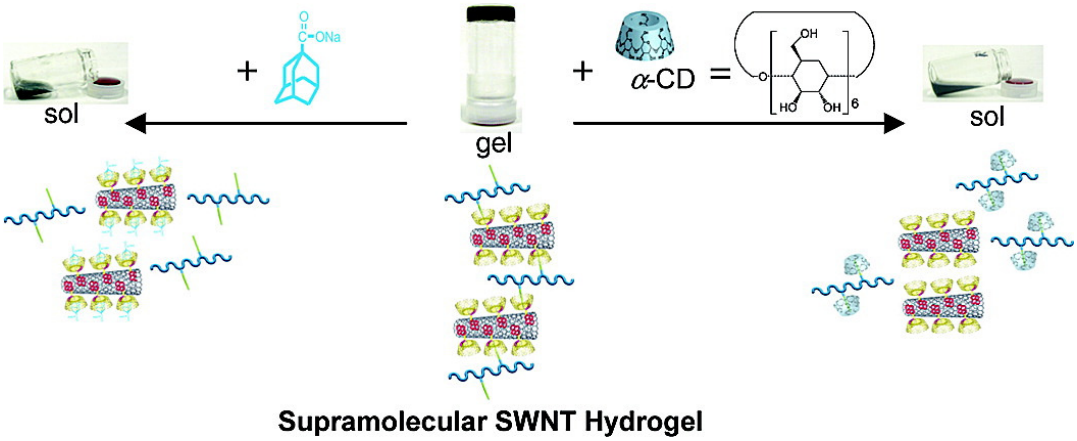


Figure 2.17. Hybrids of single-walled carbon nanotubes (SWNTs) and β-cyclodextrins (βCDs) were formed by π-π interaction between pyrene modified βCDs and SWNTs. A chemically-responsive supramolecular SWNT hydrogel was obtained when poly(acrylic acid) carrying dodecyl groups was added and host-guest interactions occurred. Competitive interactions with sodium adamantane or αCDs reverted the system to the sol state. Reproduced from [138]. Copyright 2007 American Chemical Society.

Photoresponsive gels have been prepared using CD-covered silica particles. Mesoporous silica particles (Si-MPs) provide a rigid framework with a porous reservoir that can encapsulate a large amount of guest molecules and, if adequate gates are inserted, they can exert a precise control of drug release [139]. CD-covered Si-MPs were designed to host calcein, to have photocleavable linkers and CDs as gatekeepers, and to form host-guest gels that exhibit photoresponsive release characteristics [139]. The particles can release their content under UV irradiation. Addition of six-arm PEGs with dodecyl end groups triggered gel formation via host-guest interactions with the CDs covering the Si-MPs. As shown above, the gel reverted to a solution when αCDs were added to the medium. The CD-covered Si-MPs may be suitable

components of injectable implants that can release the drug "on demand" under the action of an externally applied light source.

Finally, host-guest interactions are also being explored as a way to build modular constructs of poly-CD networks with poly-guest-networks, which spontaneously adhere in solution. For this purpose, polyacrylamide-based polymers are preferably as backbone components for both the host and the guest moieties because the well-known absence of interactions between polyacrylamide gels and other molecules, for example proteins, polysaccharides and DNA, which could interfere in the binding process [117]. Acrylamido-αCD, -βCD and -γCD monomers were used as components of the host networks, while guest-polymer networks were prepared by copolymerization of acrylamide, acrylate, or acrylamide derivatives bearing a guest moiety (e.g., hexyl, cyclohexyl, dodecyl, or cyclododecyl chains) and using N,N'-methylenebis(acrylamide) as cross-linker. Pieces of αCD-networks were shown to rapidly adhere to pieces of gels bearing linear alkyl chains, while βCD- and γCD-networks adhered to cyclic alkyl-gels, exhibiting an excellent selectivity only by mixing and shaking in water.

2.6 CONCLUSIONS

The ability of CDs to form inclusion complexes with a variety of small and large molecules can be successfully exploited to develop reversible cross-linked networks, where CDs play a main role in the selectivity and strength of the tie-junctions. Free CDs threaded onto certain blocks or side chains of polymers, forming polypseudorotaxanes, can assembly as crystalline aggregates that communicate a strong viscosity to the system at rest. Similarly, CD dimmers or polymers can interact with guest-bearing macromolecules rendering 3D networks. In both situations, the gelling process takes place at room temperature without using organic solvents or catalysts, and the junctions can be reversibly broken under mild shear conditions, such as those that one can exert with the plunger of a syringe. Moreover the CDs and/or the polymer moieties that do not participate in the junctions remain available for hosting therapeutic substances. Therefore, the syringeable CD gels offer a variety of possibilities regarding control of drug release through diffusion, affinity and/or stimuli-driven mechanisms. These features together with an excellent *in vivo* compatibility ensure a promising future for self-assembling CD gels in the biomedical field.

2.7 REFERENCES

[1] Magomedov, G.; Kozlov, G.V.; Zaikov, G.E. *Structure and properties of crosslinked polymers*. 1st ed.; Smithers Rapra Technology, Shawbury, UK, 2011.

[2] Van Vlierberghe, S.; Dubruel, P.; Schacht, E. *Biopolymer-based hydrogels as scaffolds for tissue engineering applications: A Review*. Biomacromolecules, 2011, 12(5), 1387-1408.

[3] Qiu, Y.; Park, K. *Environment-sensitive hydrogels for drug delivery*. Adv. Drug Deliver. Rev., 2001, 53(3), 321-239.

[4] Vermonden, T.; Censi, R.; Hennink, W.E. *Hydrogels for protein delivery*. Chem. Rev., 2012, 112(5), 2853-2888.

[5] Kopecek, J., *Hydrogels: From soft contact lenses and implants to self-assembled nanomaterials*. J Polym. Sci. A Polym. Chem., 2009, 47(22), 5929-5946.

[6] Alvarez-Lorenzo, C.; Concheiro, A. I*ntelligent drug delivery systems: polymeric micelles and hydrogels*. Mini-Rev. Med. Chem., 2008, 8(11), 1065-1074.

[7] Bajpai, A.K.; Shukla, S.K.; Bhanu, S.; Kankane, S. *Responsive polymers in controlled drug delivery*. Prog. Polym. Sci., 2008, 33(11), 1088-1118.

[8] Wanakule, P.; Roy, K., *Disease-responsive drug delivery: the next generation of smart delivery devices*. Curr. Drug Metab., 2012, 13(1), 42-49.

[9] Caldorera-Moore, M.E.; Liechty, W.B.; Peppas, N.A. *Responsive theranostic systems: integration of diagnostic imaging agents and responsive controlled release drug delivery carriers*. Acc. Chem. Res., 2011, 44(10), 1061-1070.

[10] Mastropietro, D.J.; Omidian, H.; Park, K., *Drug delivery applications for superporous hydrogels*. Expert Opin. Drug Deliv., 2012, 9(1), 71-89.

[11] Hoare, T. R.; Kohane, D. S. *Hydrogels in drug delivery: Progress and challenges*. Polymer, 2008, 49(8), 1993-2007.

[12] Li, Y.; Rodrigues J.; Tomas, H. *Injectable and biodegradable hydrogels: gelation, biodegradation and biomedical applications*. Chem. Soc. Rev., 2012, 41(6), 2193-2221.

[13] Gutowska, A.; Jeong B.; Jasionowski, M. *Injectable gels for tissue engineering*. Anat. Rec., 2001, 263(4), 342-349.

[14] Packhaeuser, C.B.; Schnieders, J.; Oster C.G.; Kissel, T. *In situ forming parenteral drug delivery systems: an overview*. Eur. J. Pharm. Biopharm., 2004, 58(2), 445-455.

[15] Hatefi, A.; Amsden, B. *Biodegradable injectable in situ forming drug delivery systems*. J. Control. Release, 2002, 80(1–3), 9-28.

[16] Kretlow, J.D.; Klouda, L.; Mikos, A.G. *Injectable matrices and scaffolds for drug delivery in tissue engineering*. Adv. Drug Deliver. Rev., 2007, 59(4–5), 263-273.

[17] Hiemstra, C.; Zhou, W.; Zhong, Z.; Wouters, M.; Feijen, J. *Rapidly in situ forming biodegradable robust hydrogels by combining stereocomplexation and photopolymerization*. J. Am. Chem. Soc., 2007, 129(32), 9918-9926.

[18] Tai, H.; Howard, D.; Takae, S.; Wang, W.; Vermonden, T.; Hennink, W.E.; Stayton, P.S.; Hoffman, A.S.; Endruweit, A.; Alexander, C.; Howdle, S.M.; Shakesheff, K.M. *Photo-cross-linked hydrogels from thermoresponsive PEGMEMA-PPGMA-EGDMA copolymers containing multiple methacrylate groups: mechanical property, swelling, protein release, and cytotoxicity*. Biomacromolecules, 2009, 10(10), 2895-2903.

[19] Anseth, K.S.; Metters, A.T.; Bryant, S.J.; Martens, P.J.; Elisseeff, J.H.; Bowman, C.N. *In situ forming degradable networks and their application in tissue engineering and drug delivery*. J. Control. Release, 2002, 78(1-3), 199-209.

[20] Lin, C.C.; Metters, A.T. *Bifunctional monolithic affinity hydrogels for dual-protein delivery*. Biomacromolecules, 2008, 9(3), 789-795.

[21] Tillet, G.; Boutevin, B.; Ameduri, B. *Chemical reactions of polymer crosslinking and post-crosslinking at room and medium temperature*. Prog. Polym. Sci., 2011, 36(2), 191-217.

[22] Williams, C.G.; Malik, A.N.; Kim, T.K.; Manson, P.N.; Elisseeff, J.H. *Variable cytocompatibility of six cell lines with photoinitiators used for polymerizing hydrogels and cell encapsulation*. Biomaterials, 2005, 26(11), 1211-1218.

[23] Omlor, G.W.; Nerlich, A.G.; Lorenz, H.; Bruckner, T.; Richter, W.; Pfeiffer, M.; Gühring, T. *Injection of a polymerized hyaluronic acid/collagen hydrogel matrix in an in vivo porcine disc degeneration model*. Eur. Spine J., 2012, 21(9), 1700-1708.

[24] Elbert, D.L.; Pratt, A.B.; Lutolf, M.P.; Halstenberg, S.; Hubbell, J.A. *Protein delivery from materials formed by self-selective conjugate addition reactions*. J. Control. Release, 2001, 76(1-2), 11-25.

[25] Hiemstra, C.; van der Aa, L.J.; Zhong, Z.; Dijkstra, P.J.; Feijen, J. *Rapidly in situ-forming degradable hydrogels from dextran thiols through Michael addition*. Biomacromolecules, 2007, 8(5), 1548-1556.

[26] Mather, B.D.; Viswanathan, K.;Miller, K.M.; Long, T.E. *Michael addition reactions in macromolecular design for emerging technologies*. Prog. Polym. Sci., 2006, 31(5), 487-531.

[27] Lutolf, M.P.; Raeber, G.P.; Zisch, A.H.; Tirelli, N.; Hubbell, J.A. *Cell-responsive synthetic hydrogels*. Adv. Mater., 2003, 15(11), 888-892.

[28] Metters, A.; Hubbell, J. *Network formation and degradation behavior of hydrogels formed by Michael-type addition reactions*. Biomacromolecules, 2004, 6(1), 290-301.

[29] van de Wetering, P.; Metters, A.T.; Schoenmakers, R.G.; Hubbell, J.A. *Poly(ethylene glycol) hydrogels formed by conjugate addition with controllable swelling, degradation, and release of pharmaceutically active proteins*. J. Control. Release, 2005, 102(3), 619-627.

[30] Zheng S.X.; Liu, Y.; Palumbo, F.S.; Luo, Y.; Prestwich, G.D. *In situ crosslinkable hyaluronan hydrogels for tissue engineering*. Biomaterials, 2004, 25(7-8), 1339-1348.

[31] Cai, S.; Liu, Y.; Zheng S. X.; Prestwich, G.D. *Injectable glycosaminoglycan hydrogels for controlled release of human basic fibroblast growth factor*. Biomaterials, 2005, 26(30), 6054-6067.

[32] Hiemstra, C.; van der Aa, L.J.; Zhong, Z.; Dijkstra, P.J.; Feijen, J. *Novel in situ forming, degradable dextran hydrogels by Michael addition chemistry: synthesis, rheology, and degradation*. Macromolecules, 2007, 40(4), 1165-1173.

[33] Phelps, E.A.; Enemchukwu, N.O.; Fiore, V.F.; Sy, J.C.; Murthy, N.; Sulchek, T.A.; Barker, T.H.; García, A.J. *Maleimide cross-linked bioactive PEG hydrogel exhibits improved reaction*

kinetics and cross-linking for cell encapsulation and in situ delivery. Adv. Mater., 2012, 24(1), 64-70.

[34] Venkatesh, M.P.; Liladhar, P.K.; Kumar, T.M.P.; Shivakumar, H.G. *In situ gels based drug delivery systems*. Current Drug Therapy, 2011, 6(3), 213-222.

[35] Van Tomme, S.R.; Storm, G.; Hennink, W.E. *In situ gelling hydrogels for pharmaceutical and biomedical applications*. Int J Pharm., 2008, 355(1-2), 1-18.

[36] Huynh, C.T.; Nguyen, M.K.; Lee, D.S. *Injectable block copolymer hydrogels: achievements and future challenges for biomedical applications*. Macromolecules, 2011, 44(17), 6629-6636.

[37] Ajazuddin, A.A.; Khan, J.; Giri, T.K.; Tripathi, D.K.; Saraf, S.; Saraf, S. *Advancement in stimuli triggered in situ gelling delivery for local and systemic route*. Expert. Opin. Drug Deliv., 2012, 9(12), 1573-1592.

[38] Jeong, B.; Kim, S.W.; Bae, Y.H. *Thermosensitive sol-gel reversible hydrogels*. Adv. Drug Deliver. Rev., 2002, 54(1), 37-51.

[39] Kempe, S.; Mäder, K. *In situ forming implants - an attractive formulation principle for parenteral depot formulations*. J. Control. Release, 2012, 161(2), 668-679.

[40] Dumortier, G.; Grossiord, J.; Agnely, F.; Chaumeil, J. *A review of Poloxamer 407 pharmaceutical and pharmacological characteristics*. Pharm. Res., 2006, 23(12), 2709-2728.

[41] Lee, S.J.; Han, B.R.; Park, S.Y.; Han, D.K.; Kim, S.C. *Sol-gel transition behavior of biodegradable three-arm and four-arm star-shaped PLGA-PEG block copolymer aqueous solution*. J Polym. Sci. A Polym. Chem., 2006, 44(2), 888-899.

[42] Yu, L.; Ding, J. *Injectable hydrogels as unique biomedical materials*. Chem. Soc. Rev., 2008, 37(8), 1473-1481.

[43] Li, J.; Ni, X.; Leong, K.W. *Injectable drug-delivery systems based on supramolecular hydrogels formed by poly(ethylene oxide)s and α-cyclodextrin*. Biomed. Mater. Res. Part A, 2003, 65A(2), 196-202.

[44] Harada, A. *Preparation and structures of supramolecules between cyclodextrins and polymers.* Coord. Chem. Rev., 1996, 148, 115-133.

[45] Ren, L.; He, L.; Sun, T.; Dong, X.; Chen, Y.; Huang, J.; Wang, C. *Dual-responsive supramolecular hydrogels from water-soluble PEG-grafted copolymers and cyclodextrin*. Macromol. Biosci., 2009, 9(9), 902-910.

[46] Overstreet, D.J.; Dutta, D.; Stabenfeldt, S.E.; Vernon, B.L. *Injectable hydrogels*. J. Polym. Sci. B, 2012, 50 (13), 881-903.

[47] Ruel-Gariépy, E.; Leroux, J.C. *In situ-forming hydrogels - review of temperature-sensitive systems*. Eur. J. Pharm. Biopharm., 2004, 58(2), 409-426.

[48] He, C.; Kim, S.W.; Lee, D.S. *In situ gelling stimuli-sensitive block copolymer hydrogels for drug delivery*. J. Control. Release, 2008, 127(3), 189-207.

[49] Mansour, H.M.; Sohn, M.; Al-Ghananeem, A.; DeLuca, P.P. *Materials for pharmaceutical dosage forms: molecular pharmaceutics and controlled release drug delivery aspects*. Int. J. Mol. Sci., 2010, 11(9), 3298-3322.

[50] Hincal, A.A.; Eroğlu, H.; Bilensoy, E. *Regulatory status of cyclodextrins in pharmaceutical products,* In: Cyclodextrins in pharmaceutics, cosmetics, and biomedicine. John Wiley & Sons, Inc., Hoboken, 2011, 123-130.

[51] Irie, T.; Uekama, K. *Pharmaceutical applications of cyclodextrins. III. Toxicological issues and safety evaluation*. J. Pharm. Sci., 1997, 86(2), 147-162.

[52] Gould, S.; Scott, R.C. *2-Hydroxypropyl-β-cyclodextrin (HP-β-CD): A toxicology review*. Food Chem. Toxicol. 2005, 43(10), 1451-1459.

[53] Luke, D.R.; Tomaszewski, K.; Damle, B.; Schlamm, H.T. *Review of the basic and clinical pharmacology of sulfobutylether-β-cyclodextrin (SBECD)*. J. Pharm. Sci., 2010, 99(8), 3291-3301.

[54] Ohtani, Y.; Irie, T.; Uekama, K.; Fukunaga, K.; Pitha, J. *Differential effects of α-, β- and γ-cyclodextrins on human erythrocytes*. Eur. J. Biochem, 1989, 186(1-2), 17-22.

[55] Thompson, D.O. *Cyclodextrins-enabling excipients: their present and future use in pharmaceuticals*. Crit. Rev. Ther. Drug Carr. Syst., 1997, 14(1), 1-104.

[56] Irie, T.; Uekama, K. *Cyclodextrins in peptide and protein delivery*. Adv. Drug Deliver. Rev., 1999, 36(1), 101-123.

[57] Brewster, M.E.; Loftsson, T. *Cyclodextrins as pharmaceutical solubilizers*. Adv. Drug Deliver. Rev., 2007, 59(7), 645-666.

[58] Arikan, S.; Yigit, A.A.; Zengin, N. *Effect of pre-haemolytic concentrations of α, β, and γ cyclodextrins on the osmotic fragility of dog erythrocytes*. Rev. Med. Vet., 2004, 155(10), 500-503.

[59] Frijlink, H.; Eissens, A.; Hefting, N.; Poelstra, K.; Lerk, C.; Meijer, D.F. *The effect of parenterally administered cyclodextrins on cholesterol levels in the rat*. Pharm. Res., 1991, 8(1), 9-16.

[60] Davis, M.E.; Brewster, M.E. *Cyclodextrin-based pharmaceutics: past, present and future*. Nat. Rev., 2004, 3(12), 1023-1035.

[61] Liu, L.; Guo, Q.X. *The driving forces in the inclusion complexation of cyclodextrins*. J. Incl. Phenom. Macrocycl. Chem., 2002, 42(1-2), 1-14.

[62] Rekharsky, M.V.; Inoue, Y. *Complexation thermodynamics of cyclodextrins*. Chem. Rev., 1998, 98(5), 1875-1918.

[63] Messner, M.; Kurkov, S.V.; Jansook, P.; Loftsson, T. *Self-assembled cyclodextrin aggregates and nanoparticles*. Int. J. Pharm., 2010, 387(1-2), 199-208.

[64] Messner, M.; Kurkov, S.V.; Flavià-Piera, R.; Brewster, M.E.; Loftsson, T. *Self-assembly of cyclodextrins: the effect of the guest molecule*. Int. J. Pharm., 2011, 408(1-2), 235-247.

[65] Fülöp, Z.; Kurkov, S.V.; Nielsen, T.T.; Larsen, K.L.; Loftsson, T. *Self-assembly of cyclodextrins: formation of cyclodextrin polymer based nanoparticles*. J. Drug Del. Sci. Tech., 2012, 22(3), 215-222.

[66] Messner, M.; Kurkov, S.V.; Palazón, M.M.; Fernández, B.Á.; Brewster, M.E.; Loftsson, T. *Self-assembly of cyclodextrin complexes: effect of temperature, agitation and media composition on aggregation*. Int. J. Pharm., 2011, 419(1-2), 322-328.

[67] Bikádi, Z.; Kurdi, R.; Balogh, S.; Szemán, J.; Hazai, E. *Aggregation of cyclodextrins as an important factor to determine their complexation behavior*. Chem. Biodivers., 2006, 3(11), 1266-1278.

[68] Babu, R.J.; Dayal, P.; Singh, M. *Effect of cyclodextrins on the complexation and nasal permeation of melatonin*. Drug Deliv., 2008, 15(6), 381-388.

[69] Pose-Vilarnovo, B.; Rodríguez-Tenreiro, C.; Rosa dos Santos, J.F.; Vázquez-Doval, J.; Concheiro, A.; Alvarez-Lorenzo, C.; Torres-Labandeira, J.J. *Modulating drug release with cyclodextrins in hydroxypropyl methylcellulose gels and tablets*. J. Control. Release, 2004, 94(2-3), 351-363.

[70] Alvarez-Lorenzo, C.; Rosa dos Santos, J.F.; Sosnik, A.; Torres-Labandeira, J.J.;Concheiro, A., *Hydrogels with cyclodextrins as highly versatile drug delivery systems*, In: Handbook of hydrogels: properties, preparation & applications. Nova Science Publishers, Inc., New York, 2009, 61-102.

[71] Loftsson, T.; Stefánsson, E. *Cyclodextrins in eye drop formulations: enhanced topical delivery of corticosteroids to the eye*. Acta Ophthalmol. Scand., 2002, 80(2), 144-150.

[72] Li, J. *Self-assembled supramolecular hydrogels based on polymer-cyclodextrin inclusion complexes for drug delivery*. NPG Asia Mater., 2010, 2(3), 112-118.

[73] Loethen, S.; Kim, J.M.; Thompson, D.H. *Biomedical applications of cyclodextrin based polyrotaxanes*. Polym. Rev., 2007, 47(3), 383-418.

[74] Okada, M.; Kamachi, M.; Harada, A. *Preparation and characterization of inclusion complexes of poly(propylene glycol) with methylated cyclodextrins*. J. Phys. Chem. B, 1999, 103(14), 2607-2613.

[75] Kidowaki, M.; Zhao, C.; Kataoka, T.; Ito, K. *Thermoreversible sol-gel transition of an aqueous solution of polyrotaxane composed of highly methylated α-cyclodextrin and polyethylene glycol*. Chem. Commun., 2006(39), 4102-4103.

[76] Kikuzawa, A.; Kida, T.; Akashi, M. *Unique inclusion complex formation between skeleton-modified cyclodextrin and polymers*. Macromolecules, 2008, 41(10), 3393-3395.

[77] Karaky, K.; Brochon, C.; Schlatter, G.; Hadziioannou, G. *pH-Switchable supramolecular "sliding" gels based on polyrotaxanes of polyethyleneimine-block-poly(ethylene oxide)-block-polyethyleneimine block copolymer and α-cyclodextrin: synthesis and swelling behaviour*. Soft Matter, 2008, 4(6), 1165-1168.

[78] Tamura, K.; Hatanaka, K.; Yoshie, N. *Preparation of a topologically linked branch polymer containing cyclodextrin*. Polym. Int., 2007, 56(9), 1115-1121.

[79] Harada, A.; Okada, M.; Li, J.; Kamachi, M. *Preparation and characterization of inclusion complexes of poly(propylene glycol) with cyclodextrins*. Macromolecules, 1995, 28(24), 8406-8411.

[80] Li, J.; Harada, A.; Kamachi, M. *Sol-gel transition during inclusion complex formation between α-cyclodextrin and high molecular weight poly(ethylene glycol)s in aqueous solution*. Polym. J., 1994, 26(9), 1019-1026.

[81] Harada, A.; Li, J.;Kamachi, M. *Double-stranded inclusion complexes of cyclodextrin threaded on poly(ethylene glycol)*. Nature, 1994, 370(6485), 126-128

[82] Tsai, C.C.; Leng, S.; Jeong, K.U.; Van Horn, R.M.; Wang, C.L.; Zhang, W.B.; Graham, M.J.; Huang, J.; Ho, R.M.; Chen, Y.; Lotz, B.; Cheng, S.Z.D. *Supramolecular structure of β-cyclodextrin and poly(ethylene oxide)-block-poly(propylene oxide)-block-poly(ethylene oxide) inclusion complexes*. Macromolecules, 2010, 43(22), 9454-9461.

[83] Li, J.; Ni, X.; Leong, K. *Block-selected molecular recognition and formation of polypseudorotaxanes between poly(propylene oxide)-poly(ethylene oxide)-poly(propylene oxide) triblock copolymers and α-cyclodextrin*. Angew. Chem. Int. Ed., 2003, 42(1), 69-72.

[84] Yang, C.; Ni, X.; Li, J. *Synthesis of polyrotaxanes consisting of multiple α-cyclodextrin rings threaded on reverse Pluronic PPO-PEO-PPO triblock copolymers based on block-selected inclusion complexation*. Eur. Polym. J., 2009, 45(5), 1570-1579.

[85] Rusa, C.C.; Bridges, C.; Ha, S.W.; Tonelli, A.E. *Conformational changes induced in Bombyx mori silk fibroin by cyclodextrin inclusion complexation*. Macromolecules, 2005, 38(13), 5640-5646.

[86] Huang, F.; Gibson, H.W. *Polypseudorotaxanes and polyrotaxanes*. Prog. Polym. Sci., 2005, 30(10), 982-1018.

[87] Yamashita, A.; Kanda, D.; Katoono, R.; Yui, N.; Ooya, T.; Maruyama, A.; Akita, H.; Kogure, K.; Harashima, H. *Supramolecular control of polyplex dissociation and cell transfection: efficacy of amino groups and threading cyclodextrins in biocleavable polyrotaxanes*. J. Control. Release, 2008, 131(2), 137-144.

[88] Li, J.; Loh, X.J. *Cyclodextrin-based supramolecular architectures: syntheses, structures, and applications for drug and gene delivery*. Adv. Drug Deliver. Rev., 2008, 60(9), 1000-1017.

[89] Li, J.; Zhao, F.; Li, J. *Polyrotaxanes for applications in life science and biotechnology*. Appl. Microbiol. Biotechnol., 2011, 90(2), 427-443.

[90] Yuen, F.; Tam, K.C. *α-cyclodextrin assisted self-assembly of poly(ethylene glycol)-block-poly(N-isopropylacrylamide) in aqueous media*. J. Appl. Polym. Sci., 2013, 127(6), 4785–4794.

[91] Harada, A.; Kamachi, M. *Complex formation between poly(ethylene glycol) and α-cyclodextrin*. Macromolecules, 1990, 23(10), 2821-2823.

[92] Travelet, C.; Schlatter, G.; Hébraud, P.; Brochon, C.; Lapp, A.; Hadziioannou, G. *Formation and self-organization kinetics of α-CD/PEO-based pseudo-polyrotaxanes in water. A specific behavior at 30 °C*. Langmuir, 2009, 25(15), 8723-8734.

[93] Simões, S.M.N.; Veiga, F.; Torres-Labandeira, J.J.; Ribeiro, A.C.F.; Sandez-Macho, M.I.; Concheiro, A.; Alvarez-Lorenzo, C. *Syringeable Pluronic-α-cyclodextrin supramolecular gels for sustained delivery of vancomycin*. Eur. J. Pharm. Biopharm., 2012, 80(1), 103-112.

[94] Nogueiras-Nieto, L.; Alvarez-Lorenzo, C.; Sandez-Macho, I.; Concheiro, A.; Otero-Espinar, F.J. *Hydrosoluble cyclodextrin/poloxamer polypseudorotaxanes at the air/water interface, in bulk solution, and in the gel state*. J. Phys. Chem. B, 2009, 113(9), 2773-2782.

[95] Larrañeta, E.; Isasi, J.R. *Self-assembled supramolecular gels of reverse poloxamers and cyclodextrins*. Langmuir, 2012, 28(34), 12457-12462.

[96] Li, J.; Li, X.; Zhou, Z.; Ni, X.; Leong, K.W. *Formation of supramolecular hydrogels induced by inclusion complexation between Pluronics and α-cyclodextrin*. Macromolecules, 2001, 34(21), 7236-7237.

[97] Hashim, A.I.I.; Higashi, T.; Anno, T.; Motoyama, K.; Abd-ElGawad, H.A.E.; El-Shabouri, M.H.; Borg, T.M.; Arima, H. *Potential use of γ-cyclodextrin polypseudorotaxane hydrogels as an injectable sustained release system for insulin*. Int. J. Pharm., 2010, 392(1-2), 83-91.

[98] Higashi, T.; Hirayama, F.; Misumi, S.; Arima, H.; Uekama, K. *Design and evaluation of polypseudorotaxanes of pegylated insulin with cyclodextrins as sustained release system*. Biomaterials, 2008, 29(28), 3866-3871.

[99] Higashi, T.; Hirayama, F.; Misumi, S.; Motoyama, K.; Arima, H.; Uekama, K. *Polypseudorotaxane formation of randomly-pegylated insulin with cyclodextrins: slow release and resistance to enzymatic degradation*. Chem. Pharm. Bull., 2009, 57(5), 541-544.

[100] Rodriguez-Perez, A.I.; Rodriguez-Tenreiro, C.; Alvarez-Lorenzo, C.; Concheiro, A.; Torres-Labandeira, J.J. *Drug solubilization and delivery from cyclodextrin-Pluronic aggregates*. J. Nanosci. Nanotechnol., 2006, 6(9-10), 3179-3186.

[101] Gaitano, G.G.; Brown, W.; Tardajos, G. *Inclusion complexes between cyclodextrins and triblock copolymers in aqueous solution: A dynamic and static light-scattering study*. J. Phys. Chem. B, 1997, 101(5), 710-719.

[102] Kabanov, A.V.; Batrakova, E.V.; Alakhov, V.Y. *Pluronic® block copolymers as novel polymer therapeutics for drug and gene delivery*. J. Control. Release, 2002, 82(2-3), 189-212.

[103] Millar, J.S.; Cromley, D.A.; McCoy, M.G.; Rader, D.J.; Billheimer, J.T. *Determining hepatic triglyceride production in mice: comparison of poloxamer 407 with Triton WR-1339*. J. Lipid Res., 2005, 46(9), 2023-2028.

[104] Ni, X.; Cheng, A.; Li, J. *Supramolecular hydrogels based on self-assembly between PEO-PPO-PEO triblock copolymers and α-cyclodextrin*. J. Biomed. Mater. Res. Part A, 2009, 88A(4), 1031-1036.

[105] Ma, D.; Zhang, H.B.; Tu, K.; Zhang, L.M. *Novel supramolecular hydrogel/micelle composite for co-delivery of anticancer drug and growth factor*. Soft Matter, 2012, 8(13), 3665-3672.

[106] Long, Y.; Song, H.; He, B.; Lai, Y.; Liu, R.; Long, C.; Gu, Z. *Supramolecular self-assembly of monoend-functionalized methoxy poly(ethylene glycol) and α-cyclodextrin: from micelles to hydrogel*. J. Biomater. Appl., 2012, 27(3), 333-344.

[107] Kuo, W.Y.; Lai, H.M. *Morphological, structural and rheological properties of beta-cyclodextrin based polypseudorotaxane gels*. Polymer, 2011, 52(15), 3389-3395.

[108] Díaz-Rodríguez, P.; Landin, M. *Smart design of intratumoral thermosensitive β-lapachone hydrogels by Artificial Neural Networks*. Int. J. Pharm., 2012, 433(1-2), 112-118.

[109] Seoane, S.; Díaz-Rodríguez, P.; Sendon-Lago, J.; Gallego, R.; Pérez-Fernández, R.; Landin, M. *Administration of the optimized β-lapachone-poloxamer-cyclodextrin ternary system induces apoptosis, DNA damage, and reduces tumor growth in a human breast adenocarcinoma xenograft mouse model*. Eur. J. Pharm. Biopharm., 2013, doi: 10.1016/j.ejpb.2012.12.019.

[110] Yu, J.; Fan, H.; Huang, J.; Chen, J. *Fabrication and evaluation of reduction-sensitive supramolecular hydrogel based on cyclodextrin/polymer inclusion for injectable drug-carrier application*. Soft Matter, 2011, 7(16), 7386-7394.

[111] Motoyama, K.; Hayashida, K.; Higashi, T.; Arima, H. *Polypseudorotaxanes of pegylated α-cyclodextrin/polyamidoamine dendrimer conjugate with cyclodextrins as a sustained release system for DNA*. Bioorg. Med. Chem., 2012, 20(4), 1425-1433.

[112] Morin-Crini, N.; Crini, G. *Environmental applications of water-insoluble β-cyclodextrin-epichlorohydrin polymers*. Prog. Polym. Sci., 2013, 38(2), 344-368.

[113] Auzély-Velty, R. *Self-assembling polysaccharide systems based on cyclodextrin complexation: Synthesis, properties and potential applications in the biomaterials field*. C. R. Chim., 2011, 14(2–3), 167-177.

[114] Renard, E.; Deratani, A.; Volet, G.; Sebille, B. *Preparation and characterization of water soluble high molecular weight β-cyclodextrin-epichlorohydrin polymers*. Eur. Polym. J., 1997, 33(1), 49-57.

[115] Crini, G.; Morcellet, M. *Synthesis and applications of adsorbents containing cyclodextrins*. J. Sep. Sci., 2002, 25(13), 789-813.

[116] van de Manakker, F.; Vermonden, T.; van Nostrum, C.F.; Hennink, W.E. *Cyclodextrin-based polymeric materials: synthesis, properties, and pharmaceutical/biomedical applications*. Biomacromolecules, 2009, 10(12), 3157-3175.

[117] Yamaguchi, H.; Kobayashi, R.; Takashima, Y.; Hashidzume, A.; Harada, A. *Self-assembly of gels through molecular recognition of cyclodextrins: shape selectivity for linear and cyclic guest molecules*. Macromolecules, 2011, 44(8), 2395-2399.

[118] Wenz, G.; Weickenmeier, M.; Huff, J. *Association thickener by host-guest interaction of β-cyclodextrin polymers and guest polymers*, In: Associative polymers in aqueous media. J.E. Glass, ed., American Chemical Society, Washington 2000, 271-283.

[119] Auzély-Velty, R.; Rinaudo, M. *New supramolecular assemblies of a cyclodextrin-grafted chitosan through specific complexation*. Macromolecules, 2002, 35(21), 7955-7962.

[120] Hashidzume, A.; Ito, F.; Tomatsu, I.; Harada, A. *Macromolecular recognition by polymer-carrying cyclodextrins: interaction of a polymer bearing cyclodextrin moieties with poly(acrylamide)s bearing aromatic side chains*. Macromol. Rapid Commun., 2005, 26(14), 1151-1154.

[121] Moya-Ortega, M.D.; Messner, M.; Jansook, P.; Nielsen, T.T.;Wintgens, V.; Larsen, K.L.; Amiel, C.; Sigurdsson, H.H.; Loftsson, T. *Drug loading in cyclodextrin polymers: dexamethasone model drug*. J. Incl. Phenom. Macrocycl. Chem., 2011, 69(3-4), 377-382.

[122] Wintgens, V.; Charles, M.; Allouache, F.; Amiel, C. *Triggering the thermosensitive properties of hydrophobically modified poly(N-isopropylacrylamide) by complexation with cyclodextrin polymers*. Macromol. Chem. Phys., 2005, 206(18), 1853-1861.

[123] Charlot, A.; Auzély-Velty, R. *Synthesis of novel supramolecular assemblies based on hyaluronic acid derivatives bearing bivalent β-cyclodextrin and adamantane moieties*. Macromolecules, 2007, 40(4), 1147-1158.

[124] Charlot, A.; Auzély-Velty, R. *Novel hyaluronic acid based supramolecular assemblies stabilized by multivalent specific interactions: rheological behavior in aqueous solution*. Macromolecules, 2007, 40(26), 9555-9563.

[125] Burckbuchler, V.; Kjøniksen, A.L.; Galant, C.; Lund, R.; Amiel, C.; Knudsen, K.D.; Nyström, B. *Rheological and structural characterization of the interactions between cyclodextrin compounds and hydrophobically modified alginate*. Biomacromolecules, 2006, 7(6), 1871-1878.

[126] Bistri, O.; Mazeau, K.; Auzély-Velty, R.; Sollogoub, M. *A hydrophilic cyclodextrin duplex forming supramolecular assemblies by physical cross-linking of a biopolymer*. Chem. Eur. J., 2007, 13(31), 8847-8857.

[127] Lecourt, T.; Herault, A.; Pearce, A.J.; Sollogoub, M.; Sinaÿ, P. T*riisobutylaluminium and diisobutylaluminium hydride as molecular scalpels: the regioselective stripping of perbenzylated sugars and cyclodextrins*. . Chem. Eur. J., 2004, 10(12), 2960-2971.

[128] Lecourt, T.; Bleriot, Y.; Auzely-Velty, R.; Sollogoub, M. *Cyclodextrin tetraplexes: first syntheses and potential as cross-linking agent*. Chem. Commun., 2010, 46(13), 2238-2240.

[129] van de Manakker, F.; Vermonden, T.; El Morabit, N.; van Nostrum, C.F.; Hennink, W.E. *Rheological behavior of self-assembling PEG-β-cyclodextrin/PEG-cholesterol hydrogels*. Langmuir, 2008, 24(21), 12559-12567.

[130] Daoud-Mahammed, S.; Couvreur, P.; Amiel, C.; Besnard, M.; Appel, M.; Gref R. *Original tamoxifen-loaded gels containing cyclodextrins: in situ self-assembling systems for cancer treatment*. J. Drug Del. Sci. Tech., 2004, 14(1), 51-55.

[131] Daoud-Mahammed, S.; Grossiord, J.L.; Bergua, T.; Amiel, C.; Couvreur, P.; Gref, R. *Self-assembling cyclodextrin based hydrogels for the sustained delivery of hydrophobic drugs*. J. Biomed. Mater. Res. Part A, 2008, 86A(3), 736-748.

[132] Wintgens, V.; Daoud-Mahammed, S.; Gref, R.; Bouteiller, L.; Amiel, C. *Aqueous polysaccharide associations mediated by β-cyclodextrin polymers*. Biomacromolecules, 2008, 9(5), 1434-1442.

[133] Gref, R.; Amiel, C.; Molinard, K.; Daoud-Mahammed, S.; Sébille, B.; Gillet, B.; Beloeil, J.C.; Ringard, C.; Rosilio, V.; Poupaert, J.; Couvreur, P. *New self-assembled nanogels based on host–guest interactions: characterization and drug loading*. J. Control. Release, 2006, 111(3), 316-324.

[134] Gosselet, N.M.; Wintgens, V.; Amiel, C. *Associative pH sensitive system*. Macromol. Biosci., 2005, 5(4), 306-313.

[135] Choi, H.S.; Yui, N. *Design of rapidly assembling supramolecular systems responsive to synchronized stimuli*. Prog. Polym. Sci., 2006, 31(2), 121-144.

[136] Choi, H.S.; Ooya, T.; Huh, K.M.; Yui, N. *pH-Triggered changes in assembling properties of β-cyclodextrin-conjugated poly(ε-lysine) complexes*. Biomacromolecules, 2005, 6(3), 1200-1204.

[137] Tan, L.; Liu, Y.; Ha, W.; Ding, L.S.; Peng, S.L.; Zhang, S.; Li, B.J. *Stimuli-induced gel-sol transition of multi-sensitive supramolecular β-cyclodextrin grafted alginate/ferrocene modified pluronic hydrogel*. Soft Matter, 2012, 8 (21), 5746-5749.

[138] Ogoshi, T.; Takashima, Y.; Yamaguchi, H.; Harada, A. *Chemically-responsive sol-gel transition of supramolecular single-walled carbon nanotubes (SWNTs) hydrogel made by hybrids of SWNTs and cyclodextrins*. J. Am. Chem. Soc., 2007, 129(16), 4878-4879.

[139] Park, C.; Lee, K.; Kim, C. *Photoresponsive cyclodextrin-covered nanocontainers and their sol-gel transition induced by molecular recognition*. Angew. Chem. Int. Ed., 2009, 48(7), 1275-1278.

Chapter 3

SYRINGEABLE PLURONIC-α-CYCLODEXTRIN SUPRAMOLECULAR GELS FOR SUSTAINED DELIVERY OF VANCOMYCIN

3.1 ABSTRACT

The ability of Pluronic® F127 to form supramolecular gels in the presence of αCD has been explored as a way to design syringeable gel formulations able to sustain drug release while using the lowest proportion of both components. The effects of αCD concentration range (0 to 9.7 % w/v) in copolymer (6.5, 13 and 20%) gel features were evaluated at 4, 20 and 37 ºC. An effective complexation of Pluronic and αCD was evidenced as a change in the surface pressure of the π-A isotherm of Pluronic on a subphase of CD solution and the apparition of new peaks in the X-ray spectra. Once the Pluronic and αCD solutions were mixed, the systems became progressively turbid solutions or white gels. The greater the αCD concentration was, the faster the gel formation. The supramolecular hydrogels were thixotropic and those containing 5% or more αCD had G′ values above G″ at room temperature, but they were still easily syringeable. The values of both moduli increased as temperature raised; the effect being more evident for 13 and 20% w/v copolymer. The gels prepared with low proportions of αCD exhibited phase separation in few days, particularly when stored at 4 or 37 ºC. By contrast, those prepared with 6.5% copolymer were stable for at least two months when stored at 20 ºC. The gels were able to sustain vancomycin release for several days; the higher the αCD proportion, the slower the release was. Furthermore, the drug-loaded gels showed activity against *Staphylococcus aureus*. The results obtained highlight the role of the αCD concentration on the tuning of the rheological features and drug release profiles from Pluronic gels.

3.2 INTRODUCTION

Injectable systems capable of forming polymeric matrices *in situ* represent an attractive approach for minimally invasive and patient-friendly implantation of prosthesis and drug depots, avoiding the risk of infection inherent to surgical maneuvers [1, 2]. Two main types of syringeable systems may be distinguished: i) low-viscosity formulations that undergo a transition to gel under physiological conditions [3], and ii) reversible (highly thixotropic) gels that can flow when a certain pressure is applied (e.g. pushing the plunger of a syringe) and that restore their conformation at rest [4]. Any way, the development of therapeutically useful products is a quite complex issue; namely, it involves the design of formulations that exhibit optimal kinetics of *in-vivo* formation/ regeneration of the gel and that provide adequate drug release profiles. Furthermore, the list of biocompatible materials suitable for injectable gels is quite short and, therefore, an intense research is being carried out for synthesizing new polymers and gelling modulators [5-7] or, more preferably from the regulatory point of view, for designing adequate combinations of already approved materials [5, 8, 9].

Several attempts have been made to modulate the gel temperature and strength of poloxamers (Pluronic® or Lutrol®) aqueous dispersions. This family of amphiphilic poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) triblock copolymers is one of the most typical temperature-responsive materials and some varieties (mainly Pluronic F127) are components of FDA and EMA-approved oral and parenteral products [10]. Self-association of poloxamers in water due to hydrophobic interactions among PPO blocks leads to micelle formation [11]. The number of micelles depends on both the copolymer concentration and the temperature. As temperature increases, the micelles pack to form 3D-aggregates, causing reversible sol-to-gel transitions [12, 13]. Nevertheless, high Pluronic concentration is required to form gels *in vivo* able to remain in the application site and to sustain the release for a long time [10]. This is an important drawback not only from a technological point of view but also from safety issues. In fact, the risk of lipid metabolism alteration resulting in hypertriglyceridemia and hypercholesterolemia notably raises when high doses of Pluronic F127 are used [10]. Thus, approaches for increasing gel strength while reducing Pluronic concentration should render more safe systems with improved drug release performance. The gelling features of Pluronic solutions can be modulated by combining several poloxamers, other polymers or additives [14-21] or forming supramolecular structures with cyclodextrins (CDs) [22-24]. Self-assembled supramolecular hydrogels based on the ability of CDs to form inclusion

complexes are attracting a high attention [22]. CDs can act as reversible cross-linkers of quite diverse polymer chains, rendering networks that can host the drug at the same time they are being formed. The process takes place at room temperature without using organic solvents, and removal of non-reacted substances is not needed afterwards (green chemistry). Since no covalent links between the CDs and the polymers are involved, significant changes in bioelimination/ biodegradability of the components are not expected. Most information available on supramolecular gels focuses on thixotropic networks formed between polyethylene glycol (PEG) and αCD. A minimum length of PEG chain of 200 Da is required for the formation of stable inclusion complexes with stoichiometry 2EO:1αCD [25]. The increase in viscosity caused by the network formation has been shown as a very promising way to regulate diffusion of therapeutic substances, namely large molecular weight macromolecules such as proteins [24, 26]. αCD could aid the formation of Pluronic gels at low copolymer concentrations due to interactions between αCD units that are forming inclusion complexes with different PEO blocks [27, 4]. Such a behavior is the opposite to that observed when PPO blocks of Pluronics are threaded through βCD or its derivatives, which results in necklace-like polypseudorotaxanes that exhibit an increase in gel temperature due to the shield of the hydrophobic blocks [28]. Nevertheless, most research carried out with Pluronic:αCD systems dealt with copolymer varieties of lower molecular weight, much shorter PEO blocks and, consequently, higher gel temperature than Pluronic F127 [27, 29]. Interestingly, it was found that αCD can thread onto PEO chains even in the case of reverse Pluronics by sliding over the end PPO blocks [30, 31]; the resultant polypseudorotaxane being promising for gene delivery [32].

The aim of this work was to elucidate the possibilities of combining Pluronic F127 and αCD to render syringeable supramolecular gel depots for controlled release of vancomycin. Vancomycin is a tricyclic glycopeptide useful for treating serious infections, including osteomyelitis, caused by Gram-positive bacteria such as *Staphylococcus aureus* [33, 34], but it is safer for osteoblasts and skeletal cells than other commonly used antimicrobial agents [35, 36]. Furthermore, vancomycin does not interfere in bone fracture healing [37]. Pluronic F127-αCD systems may offer an alternative to the systemic treatment of osteomyelitis if they are able to provide vancomycin levels well above the minimum inhibitory concentration for time enough to eradicate the infection [38]. Previous experiments carried out with 25% Pluronic gels saturated with vancomycin showed that after subcutaneous implantation local levels above the

minimal inhibitory concentration (1-4 mg/L) for most *S. aureus* isolates were achieved [39]. Vancomycin hydrochloride molecules can be entrapped into the Pluronic F127-αCD gel and, since vancomycin is too large to form inclusion complexes with αCD, no interference in the copolymer-αCD supramolecular gel formation is expected. Compared to PEG-αCD systems that show a marked drop in viscosity during injection and require even hours in being restored [4], the self-associative features of PPO in Pluronic may enable a faster recovery of the supramolecular structure at body temperature. That property may avoid premature clearance of the formulation from the injection site. The effect of the concentration of Pluronic F127 and αCD on the syringeability, thixotropy, viscoelasticity, temperature-responsiveness and vancomycin release properties was analyzed in detail. The physical stability of the formulations during storage was also taken into account. To the best of our knowledge the effect of temperature on the aggregation of Pluronic/αCD polypseudorotaxanes and the evolution of the physical networks has not been studied yet. The information obtained should be helpful to identify Pluronic F127-αCD mixtures that render syringeable systems with the minimal content in both pharmaceutically acceptable components that still enables sufficient control of drug release.

3.3 MATERIALS AND METHODS

3.3.1 Materials

Pluronic® F127 (EO_{100}-PO_{69}-EO_{100}, 12600 Da) was from Sigma-Aldrich (St. Louis, MO, USA), vancomycin HCl from Roig Farma (Barcelona, Spain), and αCD from Wacker (Burghausen, Germany). Purified water with a resistivity above 18.2 $M\Omega.cm^{-1}$ was obtained using reverse osmosis (MilliQ®, Millipore, Barcelona, Spain). Other reagents were analytical grade.

3.3.2 π-A isotherms

The pressure-area profiles of Pluronic F127 on water or αCD solution (0.001-0.1%, 450 mL) were recorded with the accuracy of ± 0.1 mN/m, using a Wilhelmy plate made from chromatography paper (Whatman Chr1, UK) as a pressure sensor, in a single barrier NIMA 611

(UK) surface balance with total area 550 cm^2 at 15 $cm^2 \cdot min^{-1}$ compression rate. Prior to experiments, the trough was cleaned with chloroform and ethanol and rinsed with water. The temperature was kept at 25 ^oC. Pluronic F127 solution in chloroform (70 µl, 0.10 mg/mL) was deposited by means of a syringe (Hamilton, USA) and allowed to stand for at least 10 min in order to ensure complete evaporation of the solvent. The monolayer stability was verified by monitoring the change in surface pressure while holding the area constant. The π-A isotherms of αCD solution (0.001-0.1%) included in the subphase were previously recorded at different times to ensure the attainment of the equilibrium before the addition of Pluronic F127 to the air-water interface. Additional tests were carried out by preparing a Pluronic F127-αCD solution in ethanol which was diluted with chloroform (1:10) in order to render Pluronic F127-αCD 3:1 weight ratio (i.e., copolymer and αCD concentrations 0.10 and 0.033 mg/mL, respectively). The π-A isotherms were recorded on water as described above.

3.3.3 Gel preparation

Pluronic F127 solutions in water were prepared according to the cold method [40]. Briefly, a weighed amount of copolymer was added to water under stirring, and the dispersions were kept at 4 ºC for further 12-24 h until a clear solution was obtained. Separately, αCD solutions containing vancomycin were prepared in water. Pluronic and αCD solutions were mixed at different volume ratios to obtain 6.5, 13 or 20% w/v copolymer and 0 (control gels), 2.5, 5.0, 7.0 and 9.7% w/v αCD systems. The detailed composition of each system is given in Table 3.1. After vortexing the mixed solutions, replicates of each system were stored at 4, 25, or 37 ºC.

3.3.4 X-ray diffraction and FTIR

X-ray diffraction was used to characterize the crystalline structure of the aggregates in the systems formed by 13% Pluronic F127 and 5% αCD. The gels were dried at 50 ºC for 5 days and then scanned from 5º to 30º at a speed of 0.4º per minute in a Philips PW 1710 using Ni-filtered Cu Ka radiation. Infrared spectra of the sample dispersed in KBr were recorded on a Bruker IFS 66V FTIR spectrometer (Germany) in the 400-4000 cm^{-1} range, under resolution of 4 cm^{-1}.

3.3.5 Gel appearance and syringeability

Changes in turbidity and phase separation were evaluated though visual inspection of the systems while keeping stored at 4, 25, and 37 ºC for several days after preparation. Gel formation was monitored applying the inverted-tube test. The syringeability of formulations stored for 8 days at 4 ºC was determined in duplicate using a TA-TX Plus Texture Analyzer (Stable Micro Systems Ltd., Surrey, UK). In brief, formulations were transferred into fine-dosage polypropylene syringes (1 mL, Omnifix®-F, B. Braun Melsungen AG, Melsungen, Germany). Half of the content of each syringe was emptied using the texture analyzer in compression mode displacing the plunger at a constant rate of 2 mm/s. The syringeability was estimated as the work required for the process (i.e., the area under the force vs. displacement curve).

3.3.6 Rheological properties

Viscoelastic behavior of Pluronic-αCD systems previously stored at 4 ºC for 5 days was evaluated at 10 (the lowest temperature affordable with the available equipment), 25 and 37 ºC in a Rheolyst AR-1000N rheometer (TA Instruments, New Castle, UK) equipped with an AR2500 data analyzer, and fitted with a Peltier plate. The storage (G′) and the loss (G″) moduli were recorded at 0.5 Pa in the 0.5-50 rad/s angular frequency interval using a cone-plate geometry (diameter 6 cm, angle 2º). The dependence of G′ and G″ on temperature was evaluated at 1 rad/s and 0.1 Pa in the 10 to 50 ºC range. Viscosity and thixotropy of the formulations were recorded applying a flow cycle consisting in a continuously increasing shear rate ramp at 20 ºC from 0.05 to 10 s^{-1} for 10 min, a conditioning step at 37 ºC for 10 s, and a continuously decreasing shear rate ramp at 37 ºC from 10 to 0.05 s^{-1} for 40 min.

3.3.7 Vancomycin release

Vancomycin release from the gels was evaluated using Franz-Chien vertical diffusion cells. The donor compartment was filled with 1 gram of the Pluronic-αCD formulation, while the receptor compartment was filled with water (5.5 mL) and kept at 37 ºC. The compartments were separated by a cellulose acetate membrane filter (0.45 μm, Albet®, Barcelona, Spain). The

surface available for diffusion was 0.785 cm^2. At various times, 0.7 mL aliquots were withdrawn from the receptor compartment, the absorbance measured at 280 nm (Agilent 8453, Waldbronn, Germany) and the amount of drug released determined. During the first three hours of the test, the aliquots were immediately returned to the corresponding cell. Beyond that time, the sample aliquots were replaced by fresh medium. Diffusion coefficients were estimated by fitting the Higuchi (1962) equation:

$$\frac{Q}{A} = 2C_0\left(\frac{Dt}{\pi}\right)^{1/2}$$

where Q is the amount of vancomycin (mg) released by time t (s), A is diffusion area (cm^2), C_0 is the initial concentration of vancomycin in the formulation (mg/mL), and D is the diffusion coefficient (cm^2/s) [41]. Statistical analysis of the dependence of D values on the gel composition was carried out by multiple regression using Statgraphics Plus 5.1 software (Statpoint Technologies, Inc., Warrenton, Virginia USA).

3.3.8 Antibacterial activity

Staphylococcus aureus ATCC 25923 was cultured on TSA plates and incubated at 37 ºC for 24 h. Then, the microorganisms were removed from the isolation medium and suspended in PBS pH 7.4 to a final concentration of $7.8 \cdot 10^8$ CFU/mL. *S. aureus* was then seeded on Muller-Hinton Agar plates. Aliquots of each formulation were directly placed in the centre of the plates, except in the case of those prepared without αCD, which were previously embedded in sterile paper discs (6 mm in diameter). Then the plates were incubated at 37 ºC and the growth of bacteria on the surface was visually inspected at 24 and 48 h. Formulations without vancomycin were similarly evaluated.

3.4 RESULTS AND DISCUSSION

3.4.1 π-A isotherms

Supramolecular complexes between Pluronic varieties and αCD have been largely studied by NMR techniques [30, 42]. Thus, as an initial step we wanted to elucidate if a simpler alternative technique, such as the record of π-A isotherms, which requires minimum amounts of components and can be completed in a few minutes, could also reveal the association of Pluronic F127 and αCD. The π-A isotherms of the block copolymer were firstly recorded on water and on αCD aqueous solutions. We have previously observed that, using this technique, strong changes in the isotherms occur during the interaction of Pluronic F127 with hydroxypropyl-βCD and methyl-βCD. These CDs are surface-active by themselves [43] and we observed that they can indeed form monolayers at the air-water interface [28]. The interactions with the βCD derivatives cause the drainage of some Pluronic F127 unimers towards the bulk (owing to an increase in the hydrophilicity of the macromolecule as the PPO block is threaded by CDs), a change in the CD concentration at the water surface, and the appearance of polypseudorotaxanes in the interface [28]. Although it has been previously reported that αCD is not surface active [43], we observed a small increase in the surface pressure when 0.001-0.1% αCD solutions were compressed, which indicate that they can move to the interface (Figure 3.1). To the best of our knowledge, the π-A isotherm analysis of αCD interactions with poloxamers has been only carried out with Pluronic L61 (PEO_3-PPO_{30}-PEO_3) [44]. This copolymer did not show interaction with αCD, differently to what was observed with βCD derivatives. That finding can be attributed to the short PEO blocks, which have a length below that found as the minimum (200 Da) to form stable inclusion complexes [25]. The length should be not a problem in the case of Pluronic F127, which possesses PEO blocks of roughly 4200 Da. In fact we observed that, compared to Pluronic F127 isotherm recorded on water, the presence of αCD (either in the subfase or in the deposition solution) leads to a small increase in the pressure when the area is large and the copolymer is expanded. Under these conditions, the PO and EO units of the copolymer are lying flat on the interface and the increase in pressure can be attributed to the presence of some αCD also on the surface; some may be forming complexes with the PEO blocks, slightly increasing the area occupied per copolymer molecule. As the pressure increases, the EO units are pushed into the aqueous phase. This behavior is seen as a pseudoplateau, which finishes when all EO units are in the subphase [45-47]. In the presence of αCD lower pressures were recorded in this pseudoplateau region (Figure 3.1), which suggests that this cyclodextrin

makes the immersion of EO units in the subphase more favorable. Further compression caused a rapid increase in pressure since the movement of PPO block becomes restricted. It has been reported that, in the absence of cyclodextrin, PEO chains in the subphase are in helicoidal conformation and entangle with those of neighbor copolymer molecules [45]. In the presence of αCD, the PEO chains may be partially inserted in the CD cavities, adopting a more linear conformation. The αCD units of adjacent PEO chains are responsible for the interaction in the subphase. Similar profiles were recorded for the two αCD concentrations tested in the subphase, which indicates that in both cases the interface and the subphase contains sufficient αCD molecules for interacting with Pluronic F127. Furthermore, the profiles were also similar to those recorded when the Pluronic F127-αCD mixture was deposited on water surface. By contrasts, they were quite different to those recorded using the same concentrations of βCD derivatives in the subphase. Hydroxypropyl-βCD and methyl-βCD caused more marked changes in the isotherms, probably because they interact with the PPO block, which is the main responsible for the changes observed when the area is stretched [28].

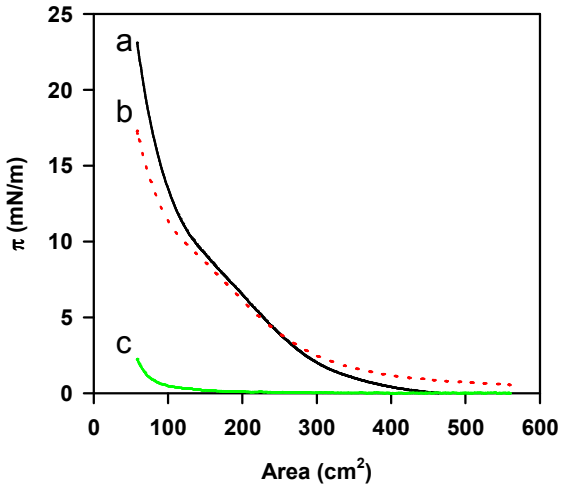


Figure 3.1. π-A isotherms recorded for Pluronic F127 on water (a) or on the αCD solutions (b) and for the αCD solutions used as subphase (c).

To gain an insight into the thermodynamics of the Pluronic F127-αCD interactions, the differences between the pressure recorded for Pluronic F127 on αCD subphase and the sum of the pressures recoded for Pluronic F127 on water and for αCD alone were estimated as follows:

$$\Delta\pi = \pi_{\exp,A} - \pi_{ideal,A} = \pi_{\exp,A} - (\pi_{PF127,A} + \pi_{\alpha CD,A})$$

The negative values (-2.5 mN/m) recorded for areas below 100 mm^2 indicate favorable interaction between both components; i.e., the area occupied by both components when they are together is smaller than the sum of the areas occupied by each component alone [28]. Therefore, despite the changes in pressure are smaller than those previously observed for hydroxypropyl-βCD and methyl-βCD (up to -8 mN/m at 100 cm^2), αCD also causes an area-condensing effect which suggests a net attractive interaction among the PEO blocks immersed in the subphase. This finding is in agreement with the observation that in the bulk the polypseudorotaxanes aggregate to reduce the contact with the aqueous medium [25].

3.4.2 Gel appearance and stability

As soon as the Pluronic and αCD solutions were mixed, the systems became progressively turbid dispersions or white gels. Systems containing the lowest αCD concentration tested (2.5%) did not render gels at any temperature. Addition of greater proportions of αCD (although still far from saturation of EO groups) led to gels or phase separation depending on the time and the temperature at which the systems were stored (Table 3.1). The time required for strong gel formation at room temperature, as estimated using the inverted-tube test, is shown in Figure 3.2. Those values were also corroborated by oscillatory rheometry analysis (Figure 3.3). The greater the αCD concentration was, the faster the gel formation.

Table 3.1. Composition and appearance after 24 h of the Pluronic-αCD formulations loaded with vancomycin.

Formulation code	F127 % w/v	αCD % w/v	αCD:EO molar ratio	Vancomycin.HCl mg/mL	4 ºC	25 ºC	37 ºC
P-6.5	6.5	0	0	0	sol	sol	sol
P-6.5-0	6.5	0	0.000	5.5	sol	sol	sol
P-6.5-2.5	6.5	2.5	0.025	5.5	sol	sol	sol
P-6.5-5	6.5	5	0.050	5.5	gel	gel	sol[b]
P-6.5-7	6.5	7	0.070	5.5	gel	gel	gel
P-6.5-9.7	6.5	9.7	0.097	5.5	gel	gel	gel
P-13	13	0	0	0	sol	sol	sol
P-13-0	13	0	0.000	5.5	sol	sol	sol
P-13-2.5	13	2.5	0.012	5.5	sol[b]	sol	sol
P-13-5	13	5	0.025	5.5	gel	gel	sol[b]
P-13-7	13	7	0.035	5.5	gel	gel	gel
P-13-9.7	13	9.7	0.048	5.5	gel	gel	gel
P-20	20	0	0	0	sol	sol	sol
P-20-0	20	0	0.000	5.5	sol	sol	sol
P-20-2.5	20	2.5	0.008	5.5	sol[a,b]	sol	sol
P-20-5	20	5	0.016	5.5	gel[b]	gel	sol[a,b]
P-20-7	20	7	0.023	5.5	gel[b]	gel	gel
P-20-9.7	20	9.7	0.031	5.5	gel[b]	gel	gel

[a] turbid white solution,
[b] phase separation was observed after 8 days

Interestingly the copolymer concentration only affected to the gelling rate of systems containing 5% αCD. Above this αCD concentration, the time required for gel formation was the same disregarding the copolymer concentration. It has been previously observed that the rate of complex formation of PEG depends on its molecular weight and that for PEG chains as large as each PEO block of Pluronic (roughly 4200 Da) the process requires several minutes [25]. In the case of Pluronic F127-5% αCD systems, the effect of the copolymer concentration on the gel

time could be related to the fact that, after the threading of αCD onto PEO blocks, the polypseudorotaxanes (namely αCD-based nanocylinders with a channel type structure [25]) self-aggregate to minimize the contact with the aqueous medium. The concentration of polypseudorotaxanes should increase as the copolymer concentration raises, facilitating the formation of precipitated domains as observed for PEG-αCD systems [42]. In the particular case of Pluronic F127, because of the presence of the PPO block which does not form inclusion complexes with αCD, the association of the nanocylinders of αCD may act as the tie-junctions for gel formation. It should be noticed that, as previously found also for PEG systems [42], turbidity of the systems occurred more rapidly than gel formation indicating that polypseudorotaxane formation is faster than the association of the threaded αCD to lead to phase separation or to a three-dimensional network.

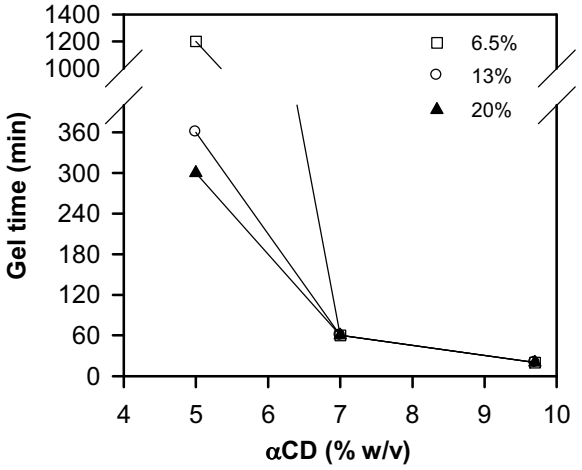


Figure 3.2. Time required for the Pluronic:αCD aqueous systems to become a gel (estimated using the inverted-tube test) when stored at 20 ºC.

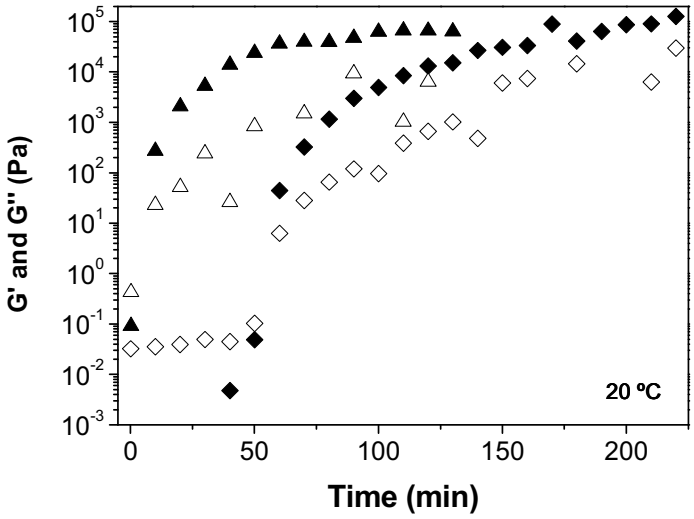


Figure 3.3. Evolution of storage (G′, full symbols) and loss (G″, open symbols) moduli of Pluronic:αCD formulations containing 13% Pluronic F127 and 7% (up triangles) or 9.7% (diamonds) αCD.

The inclusion complex formation between PEO block of the Pluronic and αCD in the gels was confirmed with wide-angle X-ray diffraction studies (Figure 3.4). The major peaks for αCD were observed at 9.78º, 12.08º, 13.7º, 14.48º, 16.02º, 18.26º and 21.84º 2θ while the main reflection of Pluronic F127 appeared at 19.45º and 23.62º 2θ. The X-ray pattern of the gel P-13-5 showed peaks at ca. 7.71º, 12.1º and 13.29º 2θ, which are coincident with those resulting of the hexagonal unit cell found for αCD inclusion complex with EO [48]. In particular the sharp reflection at 19.35º strongly support the channel-type crystalline structure of the obtained polyrotaxanes [4, 25]. The changes in the FTIR spectra (data not shown) were less significant although a new band appeared at 1343 cm^{-1} in the FTIR spectra of the dried gels, which was absent in the αCD spectrum, and may be assigned to the stretching band for the EO block included inside the αCD cavity.

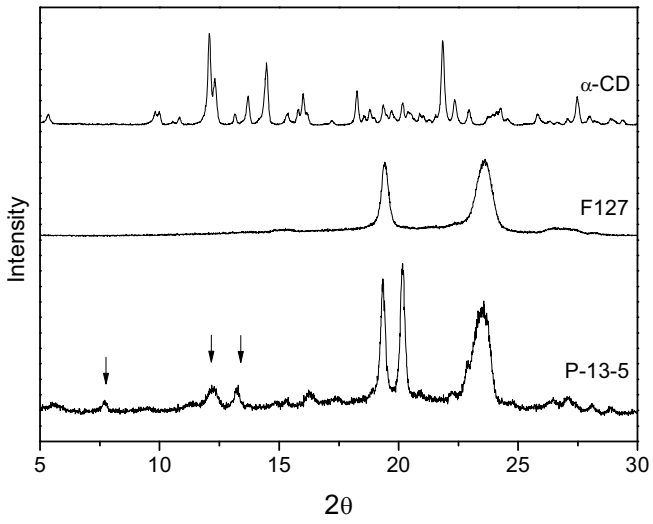


Figure 3.4. X-ray diffraction patterns of αCD, Pluronic F127 and the Pluronic F127 (13%)-αCD (5%) gel.

The αCD:EO molar ratio played a relevant role in the physical stability of the systems (Figure 3.5). The lower the ratio, the faster the trend to phase separation was. Particularly, 16 h after being prepared, systems containing 20% Pluronic F127 stored at 4 ºC led to phase separation when the αCD:EO molar ratio was 0.008 and to gel formation for αCD:EO molar ratios equals to or above 0.016. Eight days latter phase separation was observed in all 20% Pluronic F127 systems stored at 4 ºC (Figure 3.6). By contrast, when stored at 37 ºC such rapid phase separation was only observed for systems containing 5% αCD and Pluronic at any concentration. Gels stability was maintained for more time at 20 ºC than at 4 ºC or 37 ºC; after two months formulations prepared with 6.5% Pluronic and 5-9.7% αCD did not evidence phase separation (Figure 3.5). The effect of temperature on the evolution of the phase separation process may be related to the fact that the interaction (threading) of αCD and PEO is more favorable at low temperature and also the stacking of the αCD nanotubes [42]. In the case of the PEG-αCD systems, water at 5 ºC is not a good solvent for the polypseudorotaxanes and large precipitated domains are formed, while at 70 ºC the system is in good solvent conditions and the formation of polypseudorotaxanes is hindered [42]. The behavior of Pluronic-αCD systems against temperature is expected to be more complex, since self-association of PPO blocks as temperature increases may also play a role in the formation of aggregated domains. It should be noticed that Pluronic F127 concentrations tested are well above the critical micellar

concentration in water (0.02-0.50%) [49]. Therefore, it seems plausible that at 20 ºC the rate of polypseudorotaxane formation and subsequent aggregation is lower than at 4 ºC (driven by the stacking of the αCD nanotubes) or at 37 ºC (driven by the self-aggregation of PPO blocks). If fast gelling is wanted, storage at 4 or 37 ºC is beneficial. Conversely, if stability of the gels is preferred, 20 ºC is a more adequate temperature for storage.

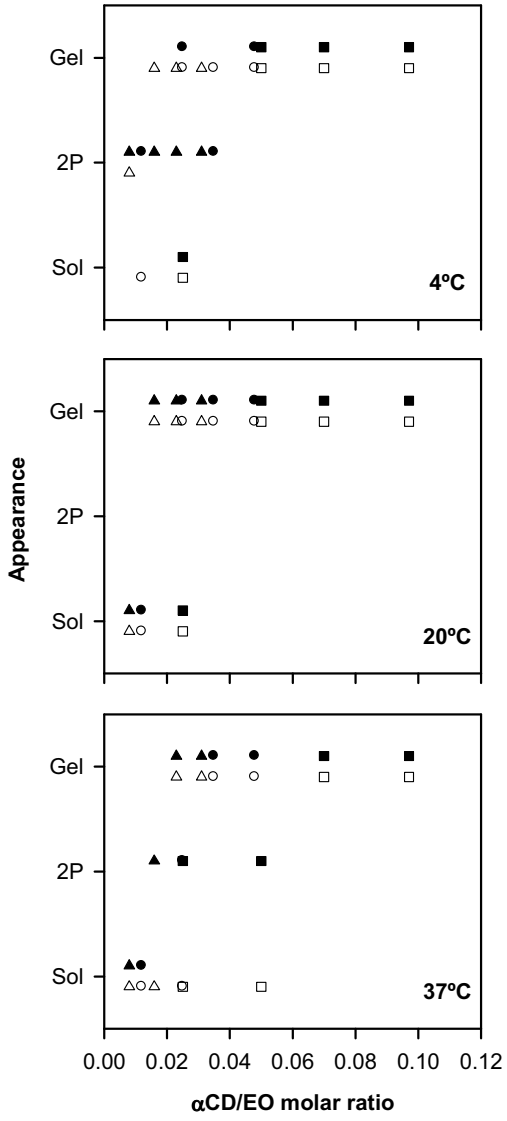


Figure 3.5. Phase diagram of Pluronic:αCD formulations after being stored for 16 hours (open symbols) or 8 days (solid symbols) at 4, 20, or 37 ºC. Pluronic concentration was 6.5% (squares), 13% (circles) and 20% (upper triangles).

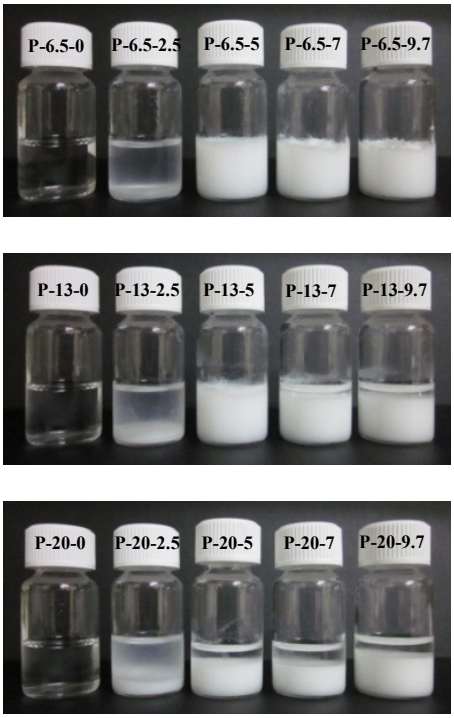


Figure 3.6. Gel appearance of Pluronic-αCD formulations after two months of storage at 4 ºC.

3.4.3 Syringeability and viscoelasticity

Pluronic-αCD formulations stored for 8 days at 4 ºC were easily drawn from 1-mL syringes (Table 3.2). The work required for moving the plunger at a constant rate was quite similar to that required in the case of an empty syringe 14.20 (SD 1.59) N·mm. Only 6.5% Pluronic F127-9.7% αCD system, i.e., the one with the largest αCD:EO molar ratio (0.097), offered higher resistance to flow, although the value was still low and in the range that can be considered easily syringeable.

Table 3.2. Mean values of work (N·mm) required to draw the formulations from 1-mL syringes and the respective standard deviations, indicated in parenthesis.

αCD (% w/v)	Pluronic (% w/v)		
	6.5	13	20
0	11.61 (1.14)	13.27 (1.86)	11.31 (1.78)
2.5	13.95 (0.89)	11.12 (0.58)	8.74 (2.14)
5	9.96 (1.43)	16.00 (1.06)	11.08 (1.03)
7	15.74 (0.22)	10.80 (0.66)	7.71 (0.77)
9.7	22.73 (0.01)	9.93 (1.50)	9.95 (0.58)

Viscoelastic behavior of the Pluronic-αCD formulations containing vancomycin was evaluated after being stored at 4 ºC for 5 days (Figure 3.7). 6.5% Pluronic F127 aqueous dispersions behaved as Newtonian fluids; the storage modulus (G′) being negligible at any temperature tested. Incorporation of 2.5% αCD (αCD:EO molar ratio 0.025) did not alter the rheological profile. By contrast, addition of αCD at 5% (αCD:EO molar ratio 0.050) caused the formulations to become structured gels with storage modulus above the loss modulus; the values of both moduli being practically independent of the angular frequency at any temperature tested. Further increase in αCD concentration (αCD:EO molar ratios 0.070 and 0.097) led to almost one order of magnitude increase in G′ and G″. No noticeable effect of the temperature on the viscoelastic parameters was recorded, which can be related to the fact that 6.5% Pluronic concentration is far from the concentration required for temperature-induced gelling.

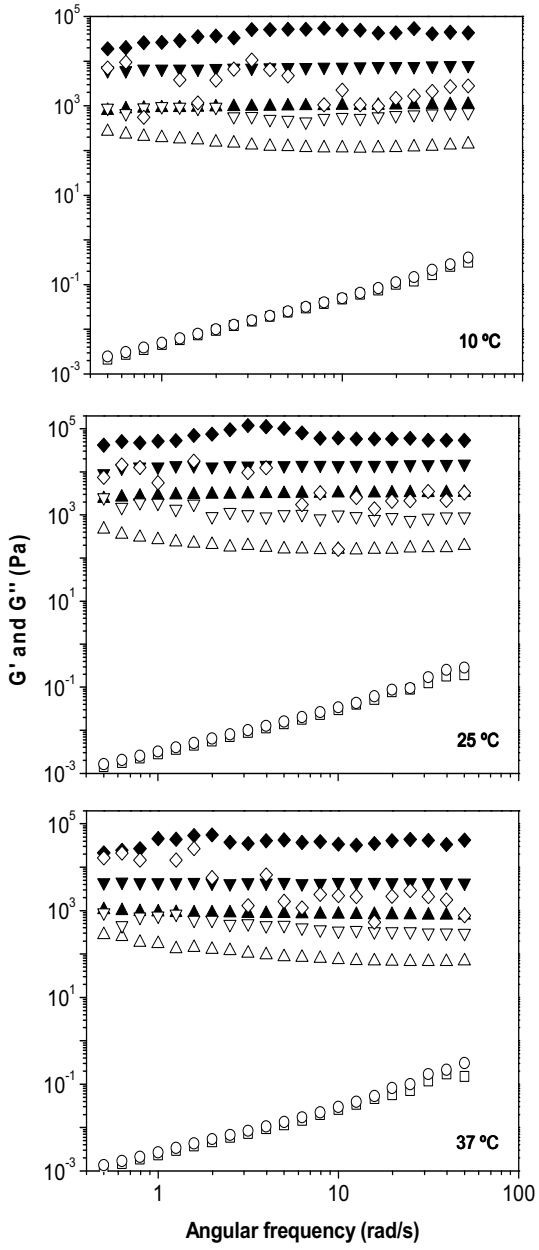


Figure 3.7. Storage (full symbols) and loss (open symbols) moduli of the Pluronic-αCD formulations containing 6.5% copolymer without αCD (squares) or containing 2.5% (circles), 5% (up triangles), 7% (down triangles) or 9.7% (diamonds) αCD, recorded at 10, 25 and 37 ºC. The systems without αCD (squares) or containing 2.5% (circles) had superimposable (small) loss modulus values and did not show perceptible storage modulus.

Viscoelastic parameters of formulations containing 13% Pluronic showed a dependence on αCD similar to that exhibited by 6.5% copolymer formulations, although the effect of temperature was more evident, as also occurred in the case of 20% Pluronic systems (Figure 3.8). 13% Pluronic F127 solely aqueous dispersion exhibited a sol to gel transition at ca. 39 ºC rendering G′ and G″ values in the 10^2 Pa range. Formulations containing 2.5% αCD (αCD:EO molar ratio 0.012) showed a similar rheological profile. A sharp increase in G′ and G″ values was observed for 5% αCD systems (αCD:EO molar ratio 0.025) which resulted in moduli values close to 10^2 Pa already at room temperature, while at 39 ºC a further increase in G′ and G″ up to 10^4 Pa was recorded. Further increase in αCD concentration did not result in greater G′ and G″ values, but a decrease was observed. The gel temperature of 20% Pluronic F127 systems was 28.5 ºC. Incorporation of 2.5% αCD slightly increased the gel temperature up to 30 ºC. Further increase in αCD resulted in networks with greater G′ and G″, particularly at temperature below the gel transition. It is interesting to note that the greatest moduli values were achieved for 13% Pluronic-5% αCD and 20% Pluronic-9.7% αCD; i.e., for αCD:EO molar ratio 0.031-0.035.

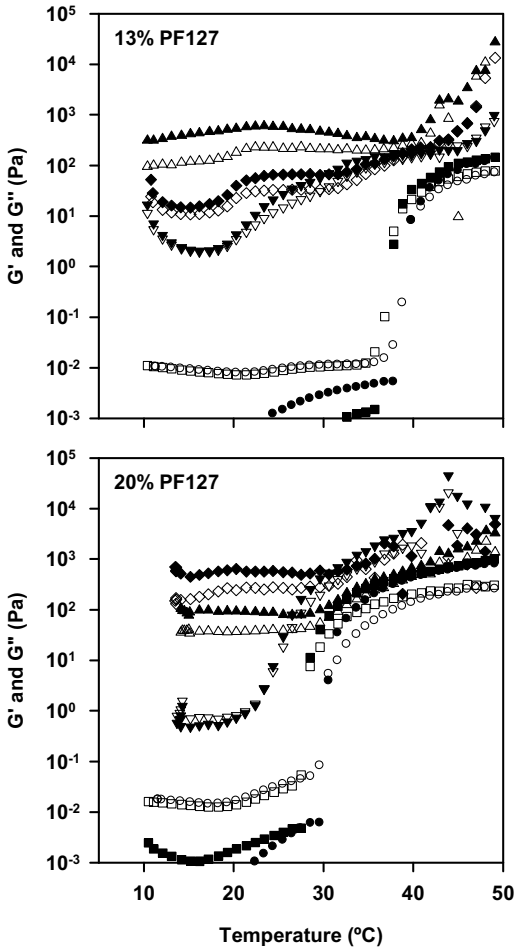


Figure 3.8. Dependence of the storage (G′, solid symbols) and loss (G″, open symbols) moduli of 13% and 20% Pluronic F127 formulations without αCD (squares) or containing 2.5% (circles), 5% (up triangles), 7% (down triangles) or 9.7% (diamonds) αCD.

When subjected to flow experiments, the Pluronic-αCD formulations were found to be thixotropic and reversible (Figure 3.9). The experiments were carried out at 20 ºC applying increasing shear rates and at 37 ºC applying decreasing shear rates in order to mimic the conditions of *in vivo* administration through a syringe. The viscosity of the formulations greatly diminished as the stirring increased. Therefore, even the networks that under oscillatory rheometry behaved as the most viscoelastic, easily disassembly under low shear rate values, which is in agreement with the good syringability observed (Table 3.2). This property makes the formulations to be syringeable even through a fine needle. The drop in viscosity was restored to

different extent as the shearing decreased at 37 ºC. 13% Pluronic systems recovered more rapidly than 6.5% Pluronic ones (Figure 3.9). The thixotropic behavior is explained by the physical nature of gelation.

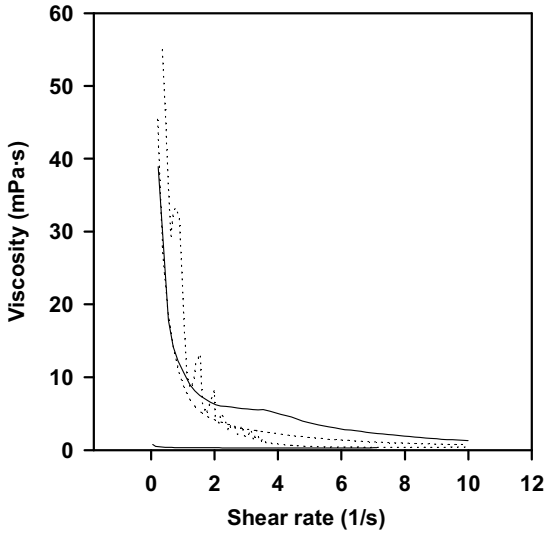


Figure 3.9. Changes in viscosity of 6.5% (continuous lines) and 13% (dotted lines) Pluronic F127 formulations containing 9.7% αCD. For each formulation the upper line corresponds to the increasing shear rate values.

3.4.4 Drug release

Vancomycin hydrochloride was incorporated to the formulations before Pluronic and αCD solutions were mixed. This drug was physically entrapped in the supramolecular gels and did not interfere in the Pluronic-αCD interaction. From the point of view of the application, Pluronic solution and vancomycin/αCD solution could be separately sterilized by filtration through 0.22 μm membranes, owing to their low viscosity at room temperature, and then mixed by transfer of one of the solutions to a syringe preloaded with the other solution. The gel could be then formed inside the syringe used for the administration. Drug diffusion profiles obtained using the Franz-Chien vertical diffusion cells setup are shown in Figure 3.10. All formulations were able to sustain vancomycin release for several days. Nevertheless, those prepared without

αCD released vancomycin quite fast disregarding Pluronic concentration. Small differences in drug diffusion coefficients were obtained when the release profiles from 6.5, 13 and 20% Pluronic were fitted to the Higuchi equation (Table 3.3). The diffusion values were of the same order of magnitude as those previously recorded for 25% Pluronic gels loaded with 20 mg/mL vancomycin ($21 \cdot 10^{-6}$ cm^2/s) [39]. Although no diffusion values were reported, Talasaz et al. (2008) observed that similar vancomycin release rates can be achieved from gels in which Pluronic F127 was partially replaced by hydroxypropyl cellulose or hydroxypropyl methylcellulose, decreasing the copolymer concentration from 18% to 10% [50]. It should be noticed the goodness of the fitting to the Higuchi equation, which is foreseeable for gels exposed to minimal dilution with the release medium as one may expect to occur when the formulations are delivered into minimally irrigated body sites (as the bones).

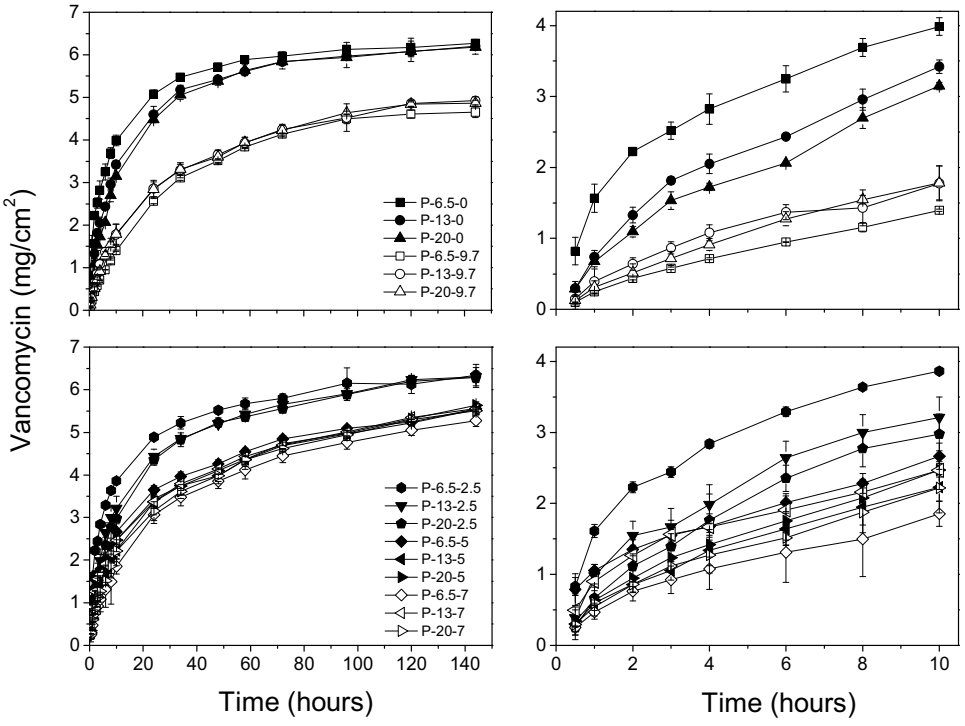


Figure 3.10. Vancomycin released at 37 ºC from Pluronic formulations with and without αCD. The data corresponding to the first 10 h are expanded in the plot on the right.

Vancomycin release rate became even faster in the presence of 2.5% αCD, which is in agreement with the phase separation that occurs without increase in viscosity, as observed in the rheological experiments. By contrast, a brusque decrease in release rate was observed for systems containing 5% or more αCD (Figure 3.10). The greater the αCD concentration, the smaller the diffusion coefficient was (Table 3.3). The effect of αCD was more marked in the case of 6.5% Pluronic F127 systems, as expected from the changes in viscoelasticity. Gel formulation without αCD released 80% drug during the first 24 h, while that prepared with 9.7% αCD released less than 45% in the same period of time. In this last case, 100% release was achieved at day 7.

Table 3.3. Mean diffusion coefficients and the respective standard deviations, indicated in parenthesis, (n=3). The goodness of the fit to the Higuchi equation (obtained with a confidence interval of 98%) can be assessed by the excellent correlation coefficients, R^2.

Formulations	*D* (x10^6 cm^2/s)	R^2
P-6.5-0	10.70 (0.28)	0.972
P-6.5-2.5	12.99 (0.66)	0.987
P-6.5-5	3.78 (0.66)	0.984
P-6.5-7	2.65 (1.59)	0.990
P-6.5-9.7	1.92 (0.09)	0.994
P-13-0	10.98 (0.16)	0.991
P-13-2.5	12.47 (1.08)	0.986
P-13-5	4.46 (1.62)	0.997
P-13-7	3.96 (0.48)	0.986
P-13-9.7	2.95 (0.45)	0.972
P-20-0	7.36 (0.58)	0.989
P-20-2.5	13.28 (1.29)	0.983
P-20-5	4.99 (2.92)	0.989
P-20-7	3.94 (0.45)	0.981
P-20-9.7	3.43 (0.98)	0.984

Multiple regression of the diffusion coefficient values on the proportions of Pluronic and αCD confirmed that, in the evaluated intervals, Pluronic concentration does not significantly affect vancomycin release rate. By contrast, the increase in the diffusion coefficient values at 2.5% αCD and the subsequent decrease at 5% or higher αCD concentrations was shown as a polynomial dependence involving linear, quadratic and cubic effects (Figure 3.11).

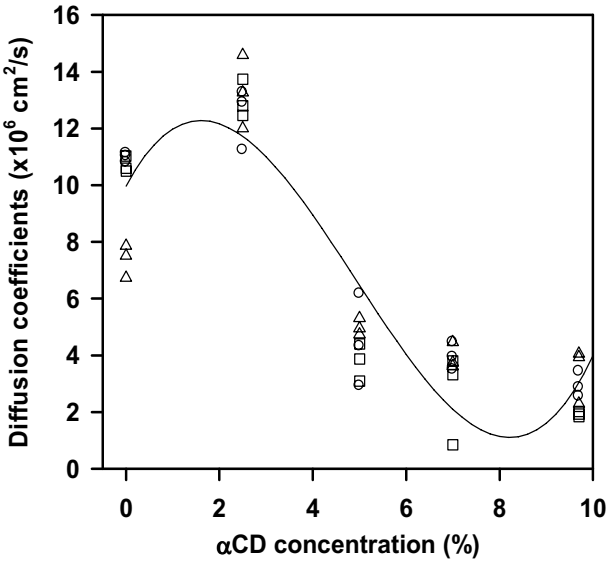


Figure 3.11. Dependence of the diffusion coefficient on the αCD concentration for all Pluronic systems tested (6.5%, squares; 13%, circles; 20%, upper triangles). $D = 0.00000996712 + 0.00000307249\ \alpha CD - 0.00000114176\ \alpha CD^2 + 7.74594 \cdot 10^{-8}\ \alpha CD^3$ ($R^2 = 0.833$; $F_{3,41\ d.f.} = 68.44$; $\alpha < 0.01$).

4.4.5 Antibacterial activity

The antibacterial activity of the vancomycin-loaded supramolecular gels was tested against *Staphylococcus aureus* cultures in vitro. The inhibition zone diameters ranged from to 18 to 30 mm (Figure 3.12). The antibacterial activity indicates that the release of vancomycin is above the MIC. As expected, the gels without antibiotic did not inhibit bacterial growth. Therefore, the possibility of incorporating quite large amounts of antimicrobial drug and to sustain its release may result in suitable formulations for the management of localized infections.

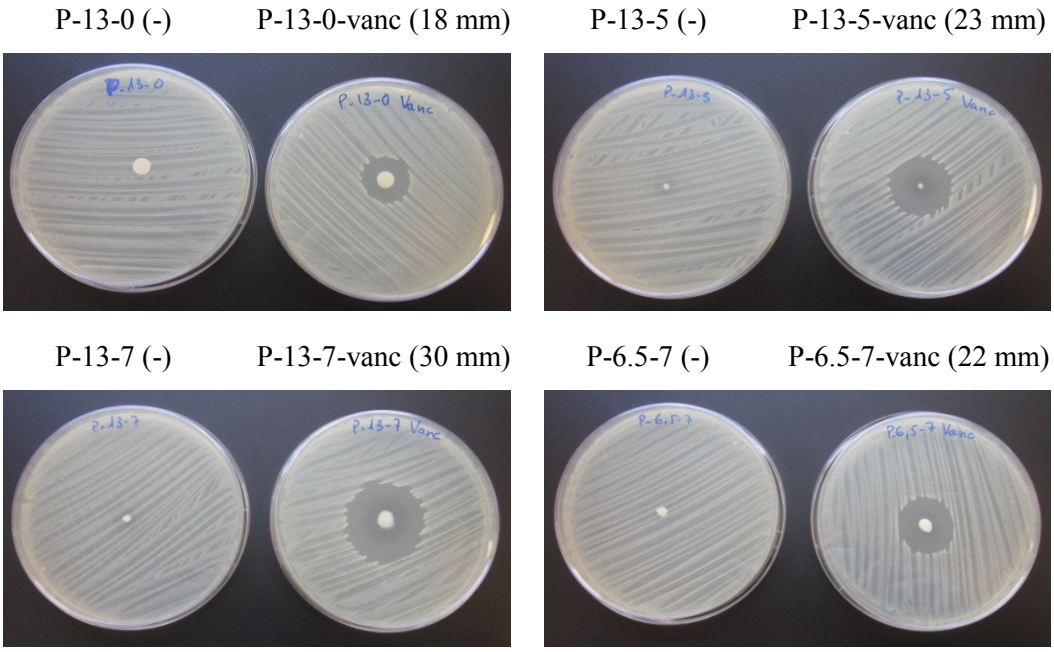


Figure 3.12. Culture plates after incubation for 24 h at 37 ºC, showing zones of inhibition for *Staphylococcus aureus*.

3.5 CONCLUSIONS

Combination of Pluronic F127 with αCD results in supramolecular systems that behave as viscoelastic but syringeable gels when the concentration of αCD is equals to or above 5%. This enables the decrease of Pluronic concentration up to 6.5% while keeping high storage and loss moduli. A strong dependence of the gel rate formation and physical stability on the Pluronic:αCD molar ratio and storage temperature was observed. Pluronic F127-αCD systems containing 6.5% copolymer and 5% or more αCD at 20 ºC were the most physically stable, while the minimum Pluronic F127 concentration that provides thixotropy and temperature-responsiveness was 13%. All gels sustained vancomycin release for several days being active against *S. aureus* in *in vitro* cultures. Therefore, 6.5-13% Pluronic F127 and 5-7% αCD appear as the most adequate concentrations for preparing injectable drug depots for the local treatment of infections. Among them, the formulation containing 13% Pluronic F127 and 5% αCD appears to be particularly promising since it combines fast gel formation, stability for one month, easy syringeability, high viscoelastic behaviour, and control of drug release for several days.

3.6 REFERENCES

[1] Gutowska, A.; Jeong, B.; Jasionowski, M. *Injectable gels for tissue engineering*. Anatomical Record., 2001, 263(4), 342-349.

[2] Packhaeuser, C.B.; Schnieders, J.; Oster, C.G.; Kissel, T. *In situ forming parenteral drug delivery systems: An overview*. Eur. J. Pharm. Biopharm., 2004, 58(2), 445-455.

[3] Schwach-Abdellaoui, K.; Moreau, M; Schneider, M.; Boisramc, B.; Gurny, R. *Controlled delivery of metoclopramide using an injectable semi-solid poly(ortho ester) for veterinary application*. Int. J. Pharmaceut., 2002, 248(1-2), 31-37.

[4] Li J., Ni, X., Leong, K.W. *Injectable drug-delivery systems based on supramolecular hydrogels formed by poly(ethylene oxide)s and α-cyclodextrin*. J. Biomed. Mater. Res. A, 2003, 65A(2), 196-202.

[5] Yu, L.; Ding, J. *Injectable hydrogels as unique biomedical materials*. Chem. Soc. Rev., 2008, 37(8) 1473-1481.

[6] Jeong, B.; Kim, S.W.; Bae, Y.H. *Thermosensitive sol-gel reversible hydrogels*. Adv. Drug Deliv. Rev., 2002, 54(1), 37-51.

[7] Ruel-Gariépy, E.; Leroux, J.C. *In situ-forming hydrogels-review of temperature-sensitive systems*. Eur. J. Pharm. Biopharm., 2004, 58(2), 409-426.

[8] He, C.; Kim, S.W.; Lee, D.S. *In situ gelling stimuli-sensitive block copolymer hydrogels for drug delivery*. J. Control. Release, 2008, 127(3), 189-207.

[9] Mansour, H.M.; Sohn, M.; Al-Ghananeem, A.; DeLuca, P.P. *Materials for pharmaceutical dosage forms: Molecular pharmaceutics and controlled release drug delivery aspects*. Int. J. Mol. Sci., 2010, 11(9), 3298-3322.

[10] Dumortier, G.; Grossiord, J.; Agnely, F.; Chaumeil, J. *A review of poloxamer 407 pharmaceutical and pharmacological characteristics*. Pharm. Res., 2006, 23(12), 2709-2728.

[11] Mortensen, K.; Pedersen, J.S. *Structural study on the micelle formation of poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) triblock copolymer in aqueous solution*. Macromolecules, 1993, 26(4), 805-812.

[12] Bromberg, L.E.; Ron, E.S. *Temperature-responsive gels and thermogelling polymer matrices for protein and peptide delivery*. Adv. Drug Deliv. Rev., 1998, 31(3), 197-221.

[13] Kabanov, A.V.; Batrakova, E.V.; Alakhov, V.Y. *Pluronic® block copolymers as novel polymer therapeutics for drug and gene delivery*. J. Control. Release, 2002, 82(2-3), 189-212.

[14] Cabana, A.; Aït-Kadi, A.; Juhász, J. *Study of the gelation process of polyethylene oxide$_a$-polypropylene oxide$_b$-polyethylene oxide$_a$ copolymer (Poloxamer 407) aqueous solutions*. J. Colloid Interface Sci., 1997, 190(2), 307-312.

[15] Xuan, J.J., Balakrishnan, P., Oh, D.H.; Yeo, W.H.; Park, S.M.; Yong, C.S.; Choi, H.G. *Rheological characterization and in vivo evaluation of thermosensitive poloxamer-based hydrogel for intramuscular injection of piroxicam*. Int. J. Pharm., 2010, 395(1-2), 317-323.

[16] Niu, G.; Du, F.; Song, L.; Zhang, H.; Yang, J.; Cao, H.; Zheng, Y.; Yang, Z.; Wang, G.; Yang, H.; Zhu, S. *Synthesis and characterization of reactive poloxamer 407s for biomedical applications*. J. Control. Release, 2009, 138(1), 49-56.

[17] Desai, S.D.; Blanchard, J. *In vitro evaluation of Pluronic F127-based controlled-release ocular delivery systems for pilocarpine*. J. Pharm. Sci., 1998, 87(2), 226-230.

[18] Vadnere, M.; Amidon, G., Lindenbaum, S.; Haslam, J.L. *Thermodynamic studies on the gel-sol transition of some pluronic polyols*. Int. J. Pharm., 1984, 22(2-3), 207-218.

[19] Wang, P.; Johnston, T.P. *Kinetics of sol-to-gel transition for poloxamer polyols*. J. Appl. Polym. Sci., 1991, 43(2), 283-292.

[20] Choi, H.G.; Jung, J.H.; Ryu, J.M.; Yoon, S.J.; Oh, Y.H.; Kim, C.K. *Development of in situ-gelling and mucoadhesive acetaminophen liquid suppository*. Int. J. Pharm., 1998, 165(1), 33-44.

[21] Choi, H.G.; Lee, M.K.; Kim, M.H.; Kim, C.K. *Effect of additives on the physicochemical properties of liquid suppository bases*. Int. J. Pharm., 1999, 190(1), 13-19.

[22] Li, J. *Self-assembled supramolecular hydrogels based on polymer-cyclodextrin inclusion complexes for drug delivery*. NPG Asia Mater., 2010, 2(3), 112-118.

[23] Li, J.; Li, X.; Ni, X.; Wang, X.; Li, H.; Leong, K.W. *Self-assembled supramolecular hydrogels formed by biodegradable PEO-PHB-PEO triblock copolymers and α-cyclodextrin for controlled drug delivery*. Biomaterials, 2006, 27(22), 4132-4140.

[24] Li, J.; Loh, X.J. *Cyclodextrin-based supramolecular architectures: Syntheses, structures, and applications for drug and gene delivery*. Adv. Drug Deliv. Rev., 2008, 60(9), 1000-1017.

[25] Harada, A.; Li, J.; Kamachi, M. *Preparation and properties of inclusion complexes of polyethylene glycol with α-cyclodextrin*. Macromolecules, 1993, 26(21), 5698-5703.

[26] Li, J. *Cyclodextrin inclusion polymers forming hydrogels*. In: Inclusion Polymers. Adv. Polym. Sic., 2009, 222, 79-113.

[27] Li, J.; Li, X.; Zhou, Z.; Ni, X.; Leong, K.W. *Formation of supramolecular hydrogels induced by inclusion complexation between pluronics and α-cyclodextrin*. Macromolecules, 2001, 34(21), 7236-7237.

[28] Nogueiras-Nieto, L.; Alvarez-Lorenzo, C.; Sandez-Macho, I.; Concheiro, A.; Otero-Espinar, F.J. *Hydrosoluble cyclodextrin/poloxamer polypseudorotaxanes at the air/water interface, in bulk solution, and in the gel state*. J. Phys. Chem. B, 2009, 113(9), 2773-2782.

[29] Ni, X.; Cheng, A.; Li, J. *Supramolecular hydrogels based on self-assembly between PEO-PPO-PEO triblock copolymers and α-cyclodextrin*. J. Biomed. Mater. Res. Part A, 2009, 88A(4), 1031-1036.

[30] Li, J.; Ni, X.; Leong, K. *Block-selected molecular recognition and formation of polypseudorotaxanes between poly(propylene oxide)-poly(ethylene oxide)-poly(propylene oxide) triblock copolymers and α-cyclodextrin*. Angew. Chem. Int. Ed., 2003, 42(1), 69-72.

[31] Yang, C.; Ni, X.; Li, J. *Synthesis of polyrotaxanes consisting of multiple α-cyclodextrin rings threaded on reverse Pluronic® PPO-PEO-PPO triblock copolymers based on block-selected inclusion complexation*. Eur. Polym. J., 2009, 45(5), 1570-1579.

[32] Yang, C.; Wang, X.; Li, H.Z.; Ding, J.L.; Wang, D.Y.; Li, J. *A supramolecular gene carrier composed of multiple cationic α-cyclodextrins threaded on a PPO-PEO-PPO triblock polymer*. Polymer, 2009, 50(6), 1378-1388.

[33] Lee, S.H.; Lee, J.E.; Baek, W.Y.; Lim, J.O.; *Regional delivery of vancomycin using pluronic F-127 to inhibit methicillin resistant staphylococcus aureus (MRSA) growth in chronic otitis media in vitro and in vivo*. J. Control. Release, 2004, 96(1), 1-7.

[34] Stigter, M.; Bezemer, J.; Groot, K.; Layrolle, P. *Incorporation of different antibiotics into carbonated hydroxyapatite coatings on titanium implants, release and antibiotic efficacy*. J. Control. Release, 2004, 99(1), 127-137.

[35] Antoci, V.Jr.; Adams, C.S.; Hickok, N.J.; Shapiro, I.M.; Parvizi, J. *Antibiotics for local delivery systems cause skeletal cell toxicity in vitro*. J. Clin. Orthop. Relat. Res., 2007, 462, 200-206.

[36] Edin, M.L.; Miclau, T.; Lester, G.E.; Lindsey, R.W.; Dahners, L.E. *Effect of cefazolin and vancomycin on osteoblasts in vitro*. Clin. Orthop. Relat. Res., 1996, 333, 245-251.

[37] Haleem, A.A.; Rouse, M.S.; Lewallen, D.G.; Hanssen, A.D.; Steckelberg, J.M.; Patel, R. *Gentamicin and vancomycin do not impair experimental fracture healing*. Clin. Orthop. Relat. Res., 2004, 427, 22-24.

[38] Peng, K.T.; Chen, C.F.; Chu, I.M.; Li, Y.M.; Hsu, W.H.; Hsu, R.W.W.; Chang, P.J. *Treatment of osteomyelitis with teicoplanin-encapsulated biodegradable thermosensitive hydrogel nanoparticles*. Biomaterials, 2010, 31(19), 5227-5236.

[39] Veyries, M.L.; Couarraze, G.; Geiger, S.; Agnely, F.; Massias, L.; Kunzli, B.; Faurisson, F.; Rouveix, B. *Controlled release of vancomycin from poloxamer 407 gels*. Int. J. Pharm., 1999, 192(2), 183-193.

[40] Schmolka, I.R. *Artificial skin I. Preparation and properties of Pluronic F-127 gels for treatment of burns*. J. Biomed. Mater. Res., 1972, 6(6), 571-582.

[41] Higuchi, W.I. *Analysis of data on the medicament release from ointments*. J. Pharm. Sci., 51(8), 802-804.

[42] Travelet, C.; Schlatter, G.; Hébraud, P; Brochon, C.; Lapp, A.; Hadziioannou, G. *Formation and self-organization kinetics of α-CD/PEO-based pseudo-polyrotaxanes in water. A specific behavior at 30 ºC*. Langmuir, 2009, 25(15), 8723-8734.

[43] Leclercq, L.; Bricout, H.; Tilloy, S.; Monflier, E. *Biphasic aqueous organometallic catalysis promoted by cyclodextrins: Can surface tension measurements explain the efficiency of chemically modified cyclodextrins?* J. Colloid Interface Sci., 2007, 307(2), 481-487.

[44] Klyamkin, A.A.; Topchieva, I.N.; Zubov, V.P. *Monomolecular films of Pluronic-cyclodextrin inclusion complexes at the water-gas interface*. Colloid Polym. Sci., 1995, 273(6), 520-523.

[45] O'Connor, S.M.; Gehrke, S.H.; Retzinger, G.S. *Ordering of poly(ethylene oxide)/poly(propylene oxide) triblock copolymers in condensed films*. Langmuir, 1999, 15(7), 2580-2585.

[46] Haefele, T.; Kita-Tokarczyk, K.; Meier, W. *Phase behavior of mixed langmuir monolayers from amphiphilic block copolymers and an antimicrobial peptide*. Langmuir, 2005, 22(3), 1164-1172.

[47] Kiss, E.; Keszthelyi, T.; Kormány, G.; Hakkel, O. *Adsorbed and spread layers of poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) block copolymers at the air-water interface studied by sum-frequency vibrational spectroscopy and tensiometry*. Macromolecules, 2006, 39(26), 9375-9384.

[48] Chung, J.W.; Kang, T.J.; Kwak, S.Y. *Supramolecular self-assembly of architecturally variant α-cyclodextrin inclusion complexes as building blocks of hexagonally aligned microfibrils*. Macromolecules, 2007, 40(12), 4225-4234.

[49] Barreiro-Iglesias, R.; Alvarez-Lorenzo, C.; Concheiro, A. *Poly(acrylic acid) microgels (carbopol 934)/surfactant interactions in aqueous media. Part I: Nonionic surfactants*. Int. J. Pharm., 2003, 258(1-2), 179-191.

[50] Talasaz, A.H.H.; Ghahremankhani, A.A.; Moghadam, S.H.; Malekshahi, M.R.; Atyabi, F.; Dinarvand, R. *In situ gel forming systems of poloxamer 407 and hydroxypropyl cellulose or hydroxypropyl methyl cellulose mixtures for controlled delivery of vancomycin*. J. Appl. Polym. Sci., 2008, 109(4), 2369-2374.

Chapter 4

POLOXAMINE-CYCLODEXTRIN-SIMVASTATIN SUPRAMOLECULAR SYSTEMS PROMOTE OSTEOBLAST DIFFERENTATION OF MESENCHYMAL STEM CELLS

4.1 ABSTRACT

Osteogenic/osteoinductive systems combine simvastatin, poloxamine Tetronic® 908 (T908) and α-cyclodextrins (αCDs) in a supramolecular network that enhances the solubility/stability of simvastatin hydroxy acid form and synergistically promote osteoblast differentiation. Incorporation of 5% αCD transforms dilute T908 solutions (as low as 2% copolymer) into gels, enhances the osteoinductive activity of T908 and provides simvastatin sustained release for more than one week, which results in higher and more prolonged alkaline phosphatase (ALP) activity. The performance of the intrinsically osteoinductive polypseudorotaxane scaffold can be easily tuned modifying the concentrations of T908, αCD and simvastatin in a certain range of values. Moreover, the use of affordable, stable materials that can be sterilized applying conventional method make the supramolecular gels advantageous candidates as scaffolds to be applied in the critical defect using minimally invasive techniques.

4.2 INTRODUCTION

Prevalence of osteodegenerative diseases as well as accidental fractures, mainly due to the more prolonged life expectation and the popularization of sports practice, are prompting the search for alternatives to the autologous and donor grafts as well as to the scaffolds loaded with expensive recombinant morphogenic proteins [1, 2]. Currently, bone morphogenetic proteins (BMPs), mainly rhBMP-2 and/or rhBMP-7, are clinically used in the form of collagen sponges for non-union, open tibial fractures and spinal fusions [3, 4]. Alternative carriers/depots that can be applied in the critical defect using minimally invasive techniques, while combining stabilization and slow clearance of BMPs from the application site are under study [5-7]. Nevertheless, the rapid local metabolization and the high cost of recombinant proteins make necessary the finding of more affordable and stable osteogenic/osteoinductive synthetic molecules that can mimic the biochemical stimuli that mesenchymal stem cells (MSCs) receive in their native bone niche to differentiate to bone precursors [1, 7].

Statins are potent pro-drugs of hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase inhibitors that block conversion of HMG-CoA to mevalonic acid needed for cholesterol biosynthesis [8], and are currently the first line treatment of hypercholesterolemia [9]. Several reports have pointed out the effect of statins, particularly simvastatin (Figure 4.1a), on osteogenesis in various murine and human cell lines [10-12]. Compared to other synthetic osteoinductive agents, statins offer additional benefits, such as promotion of new blood vessel growth [1, 13] and anti-inflammatory effects [14]. Mundy's group demonstrated that statins induce expression of BMP-2 and stimulate bone formation on the calvaria of mice following daily subcutaneous injections [15]. However, there is still a paucity of information on the doses and the delivery systems suitable for simvastatin to develop osteoinductive activity [16, 17]. Doses required for the osteoinductive effect are greater than those for the hypolipidemic therapy, which may cause relevant side effects if systemically administered. Oral administration is also limited by the low solubility of simvastatin and its tendency to open the lactone group converting in the hydroxy acid form, which hardly penetrates through the gut wall [8, 18]. Thus, local delivery in the bone defect seems to be mandatory.

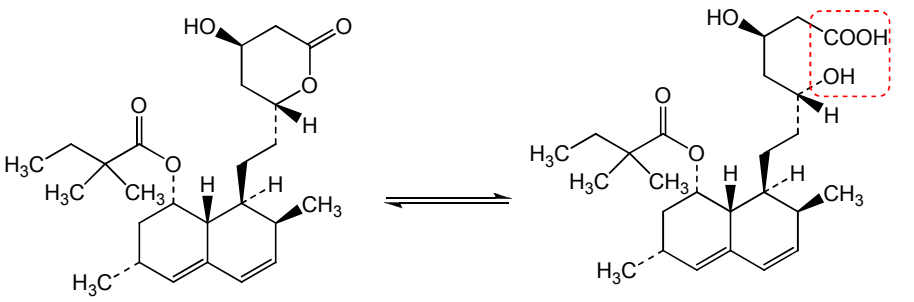
Some of us have recently identified poly(ethylene oxide)-poly(propylene oxide) (PEO-PPO) block copolymers of the poloxamine (Tetronic®) family as able to induce *in vitro* proliferation of MSCs (first week), followed by differentiation to osteoblasts (second to third week) [7]. The unique X-shape structure of these block copolymers, with four arms of PEO-PPO blocks linked

together through an ethylenediamine group (Figure 4.1b), makes them to self-associate as micellar and gel structures as a function of their concentration and the pH and temperature of the medium [18-20]. Certain resemblances of this structure with that of physiological osteoinductive agents as spermine and spermidine may explain that some varieties of poloxamine are the only osteoinductive polymers described so far [7]. The intrinsic osteoinductive activity of poloxamines (only recently also observed for poloxamer Pluronic® F68 variety) [21] offers novel perspectives for bone regeneration using minimally invasive procedures; namely, the preparation of aqueous systems that can be easily administered through a syringe and that undergo a sol-to-gel transition at 37 ºC, becoming viscoelastic osteoinductive scaffolds. Nevertheless, although some poloxamine varieties have shown an excellent cytocompatibility [7], the obtaining of gels able to remain in the injection site (the critical defect in our case) and sustain the release of incorporated active species requires high concentration of the block copolymer, which may cause toxicity problems. This limitation could be overcome with the use of α-cyclodextrins (αCDs) that thread onto PEO chains (forming polypseudorotaxanes) and act as reversible cross-linking points due to interactions among αCD units that are forming inclusion complexes with adjacent PEO blocks, as previously described for other copolymers [22-24]. The result is a viscoelastic gel that decrease the viscosity under a shear stress but recovers its consistency at rest, as the intermolecular interactions diminish and recover, respectively.

The aim of this work was to combine the osteoinductive abilities of simvastatin and poloxamine Tetronic® 908 (T908) in a syringeable formulation based on supramolecular complexes of T908 and αCD. Thus, the first step was to identify suitable combinations of T908 and αCD that can render syringeable gels with the minimum concentration of both components, and to elucidate the concentrations of simvastatin and T908 that can lead to differentiation of MSCs to osteoblasts, in a synergic way without compromising cell viability. The hydrophilic shell-hydrophobic core architecture of T908 aggregates may enable the solubilization and stabilization of such a labile hydrophobic drug as simvastatin [18-20]. Thus, T908 is expected to play a triple role: (i) intrinsic osteoinductive activity, (ii) solubilization/stabilization of simvastatin, and (iii) physical support for preparing formulations that can be syringeable and form depots in the critical defects. Phase diagram, viscoelastic properties, simvastatin solubilization and release rate, biocompatibility, MSCs proliferation and differentiation, and alkaline phosphatase (ALP) activity were characterized in detail. To the best of our knowledge

this is the first time that synergic osteoinductive effects of two synthetic molecules are described. The results obtained clearly highlight the potential of the developed formulations as syringeable scaffolds with osteoinductive activity.

(a)



Simvastatin (lactone form)
Chemical Formula: $C_{25}H_{38}O_5$ (Mw = 418.57)

Simvastatin (hydroxy acid form)
Chemical Formula: $C_{25}H_{40}O_6$ (Mw = 436.58)

(b)

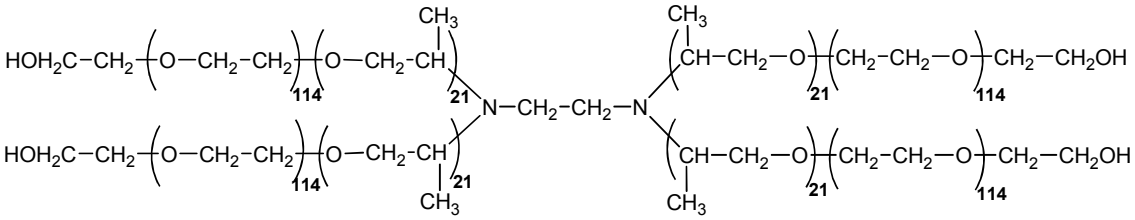


Figure 4.1. Structure of the lactone and hydroxy acid forms of simvastatin (a) and of Tetronic 908 (b).

4.3 MATERIALS AND METHODS

4.3.1 Materials

Tetronic® 908 (T908; number of EO and PO units indicated in Figure 4.1b) was from BASF Corporation (Ludwigshafen, Germany) and α-cyclodextrin (αCD) from Wacker (Burghausen, Germany). Simvastatin (molecular weight 418.57 Da) was from AK Scientific, Inc. (Union City, USA). Alkaline phosphatase (ALP) substrate and p-nitrophenylphosphate solution were supplied by Sigma-Aldrich (St. Louis Mo, USA); kit BCA by Pierce (Rockford, IL, USA), and Cell Proliferation Kit (MTT) by Roche (Basel, Switzerland). Mesenchymal stem cells StemPRO human adipose-derived stem cells, and MesenPRO RS™ Basal Medium and MesenPRO RS™ Growth Supplement were from Gibco (Invitrogen, Carlsbad, CA, USA). Dulbecco's Modified Eagle medium (D-MEM) Nutrient mixture F-12 Ham without phenol red was from Sigma-Aldrich (USA). Purified water with resistivity above 18.2 MΩ cm^{-1} was obtained by reverse osmosis (MilliQ®, Millipore, Barcelona, Spain). Other reagents were analytical grade.

4.3.2 Gels preparation

T908 and αCD solutions were separately prepared by dissolving the required amount of each component in phosphate buffer saline (PBS) pH 7.4. Then, the solutions were mixed at different volume ratios to cover a wide range of concentrations in both components and thus molar ratios; T908 concentration was chosen to be always below that required by the copolymer solely to form gels itself (Table 4.1). After vortexing the mixed solutions, replicates of each system were stored at 4, 20 and 37 ºC.

4.3.3 Rheological properties

The storage or elastic (G′) and the loss or viscous (G″) moduli of dispersions prepared with T908 (1 to 5, 8, 13, and 20%) without and with αCD (5 or 9.7%) were recorded at 0.5 Pa in the 0.5-50 rad/s angular frequency interval using a cone-plate geometry (diameter 6 cm, angle 2º) at 10, 25 and 37 ºC using a Rheolyst AR-1000N rheometer (TA Instruments, New Castle, UK) equipped with a AR2500 data analyzer, and fitted with a Peltier plate.

4.3.4 Cytocompatibility screening

4.3.4.1 HET-CAM assay

Fertilized broiler chicken eggs (not older than 3 days; Avirojo, Pontevedra, Spain) were incubated at 37 ± 0.3 ºC and 60 ± 2.6% relative humidity (Ineltec CCSP0150 Tona, Barcelona, Spain). Eggs were rotated (five times per day) for 8 days to prevent the attachment of the embryo to one side of the egg. Then, the ICCVAM-recommended hen's egg test-chorioallantoic membrane test (HET-CAM) method protocol was followed [25]. The upper part of the eggshell (air cell) was removed using a rotary saw (Dremel 300, Breda, The Netherlands) and the intact inner membrane of the eggs was moistened with 0.9% (w/v) NaCl solution for 30 min and then detached with a forceps. Aliquots of T908-αCD systems (300 µL at 25 ºC) were placed on the chorioallantoic membrane of the egg and the irritation potential (hemorrhage, vascular lysis and coagulation) was monitored for 5 min. The experiments were carried out in triplicate. Negative (0.9% NaCl solution) and positive (0.1 N NaOH) controls were performed under the same conditions. Irritation scores (IS) were calculated from the time (in seconds) at which hemorrhage (H), lysis (L) or coagulation (C) started, as follows [25]:

$$\text{IS} = \left[\left(\frac{301 - H_{time}}{300}\right) \text{x } 5 + \left(\frac{301 - L_{time}}{300}\right) \text{x } 7 + \left(\frac{301 - C_{time}}{300}\right) \text{x } 9\right]$$

According to the IS values, the materials can be classified as non-irritating (0–0.9), weakly irritating (1–4.9), moderately irritating (5–8.9) or severely irritating (9–21) [25].

4.3.4.2 Osteoblasts viability

SAOS-2 human osteogenic sarcoma cells (HTB-85, LGD Standards, ATCC, Manassas, VA) were cultured in D-MEM supplemented with 10% w/v fetal bovine serum and gentamicine (0.1 mg/mL). A calcein/propidium iodide staining (phosphate buffer solution:calcein:propidium iodide 98:1:1) was carried out to differentiate live and dead cells. Cells (50,000 per well; 0.5 mL) were seeded in Millicell® 8-well glass EZ slide (Millipore) and 80 µL of each sample were added. Confocal microscopy pictures (Leica TCS-SP2, Leica Microsystem GmbH, Mannheim,

Germany) were taken at 24 h and 72 h. MTT assay was carried out according to the Cell Proliferation Kit (Roche), seeding cells (200,000 per well; 1.5 mL) in 24-well plates to which 200 μL of T908 (2, 4, 8 or 13%)/αCD (0, 2.5, 5, 7 or 9.7%) systems were added. Cells cultured in the medium under the same conditions but without adding T908 and αCD were used as controls. The plates were incubated at 37 ºC for 24 h or 72 h [7]. All experiments were performed in triplicate.

4.3.5 Simvastatin solubilization and stability

T908 and αCD dispersions in a wide range of concentrations in PBS (5 mL) were separately poured into vials containing simvastatin in large excess. The vials were shaken at 25 ºC and 50 rpm for 5 days and then the dispersions were filtered thought 0.45 μm cellulose acetate membranes (Albet®, Barcelona, Spain) to remove the non-dissolved drug. The concentration of the dissolved drug was measured by UV spectrophotometry at 239 nm (Agilent 8453, Waldbronn, Germany) [18].

The lactone and hydroxy acid forms of simvastatin in the formulations were analyzed by HPLC using a LiChroCART RP-C18 (3.9 x 150 mm, 5 μM) column kept at 25 ºC, an UV-vis diode array detector (238 nm, L-4500 Merck-Hitachi, Germany) and acetonitrile:28 mM phosphate buffer pH 4 (65:35, 1 mL/min flow) as isocratic mobile phase [18,26]. The method showed a good precision (< 3.5 % RSD; relative standard deviations). The limit of quantification was calculated to be 0.18 μM for simvastatin-lactone form (retention time 6 min) and 0.22 μM for simvastatin-hydroxy acid form (retention time 3 min), respectively. This HPLC method was also applied to evaluate the stability of the drug in formulations stored at 4 ºC for 65 days.

4.3.6 Simvastatin release

Simvastatin was dispersed, at a fix concentration of 50 μM (0.0209 mg/mL), in T908 solutions before the incorporation of αCD. Simvastatin release from the T908-αCD formulations was evaluated using Franz-Chien vertical diffusion cells. The donor compartment filled with 2 mL of the formulation was separated from the receptor containing PBS (6 mL, 37 ºC, magnetic stirring) by means of a cellulose acetate membrane filter (0.45 μm, Albet®, Barcelona, Spain).

The surface available for diffusion was 0.785 cm^2. At various times, 300 μL were withdrawn from the receptor (replaced with the same volume of PBS at 37ºC) and the drug released was quantified (as total amount and the relative content in lactone/hydroxy acid forms) using the HPLC method described above [18,26].

4.3.7 Alkaline phosphatase (ALP) assay

MSCs were cultured in MesenPRO RS medium (Gibco, Invitrogen) supplemented with 1% glutamine and 1% penicillin and streptomycin. Then, they were seeded (30,000 cells/well, 2.5 mL) in 6-well plates. 200 μL of T908 (4% and 8%) containing or not αCD (5%) and without and with simvastatin (0.08 μM and 8.5 μM, prepared as for the release experiments) were placed into the upper compartment of the 6-well plate Transwell permeable supports. Negative and positive controls were carried out with cells in culture medium and cells in osteogenic differentiation medium (10 μM β-glycerophosphate, 100 nM dexametasone and 50 μM ascorbic acid). Plates were incubated at 37 ºC, in a humidified atmosphere with 5% CO_2. The medium was replaced twice a week. Cells were observed at inverted microscopy (x10) to discard possible contamination by bacteria. Each experiment was carried out two independent times, each in duplicate. For the alkaline phosphatase (ALP) assay, cells were lysed at 3, 7 and 12 days by addition of 150 μL Tris HCl buffer 10 mM, pH 7.5, with 0.1% Triton X-100. Samples were exposed to 3 freezing (-80 ºC for 45 minutes)/thawing cycles. Lysates were cleared applying centrifugation at 14,000 rpm for 15 min at 4 ºC. Aliquots (50 μL) were incubated with ALP substrate (150 μL) in 96-well plates at 37 ºC for 30 min and the absorbance read at 405 nm using an ELISA plate reader (BIORAD Model 680 Microplate Reader). A p-nitrophenylphosphate calibration curve was used to determine ALP concentration. Each ALP activity measurement was normalized by the protein concentration measured using the BCA protein assay [7]. Results were reported as nmoles of ALP per min and mg of protein.

Hystochemical staining was performed at days 7 and 12. Briefly, the medium was removed, and the cells were fixed using 4% paraformaldehyde solution for 5 min, washed with phosphate buffer, incubated in darkness with 0.1% naphthol ASMX-phosphate and 0.1% fast violet in 56 mM AMPD (2-amino-2-methyl propanediol) for 10 min, and observed by means of light microscopy.

4.3.8 Cell proliferation

MSCs were cultured as described above. At 3, 7 and 12 days of culture, transwell compartments were removed, and 1.5 mL of culture medium withdrawn. Then, 100 μL MTT solution (5 mg/mL stock in PBS) were added to each well containing 1 mL of remaining culture medium and the plates were incubated during 4 h at 37 ºC. Then, 1 mL MTT solvent (10% SDS in 0.01 M HCl) was added and the wells were incubated overnight at 37 ºC. Aliquots were processed as recommended in the Cell Proliferation Kit (Roche).

4.3.9 Statistical analysis

Osteoblasts viability, simvastatin diffusion coefficients, MSCs proliferation and ALP levels obtained for formulations with a fix proportion of T908 and variable proportions of αCD were analyzed using ANOVA and multiple range test (Statgraphics Centurion XVI 1.15, StatPoint Technologies Inc., Warrenton VA).

4.4 RESULTS AND DISCUSSION

4.4.1 Gels preparation and stability

T908 solely dispersions require near 20% copolymer to undergo sol-to-gel transition at the body temperature [7, 20]. When a small proportion of αCD (5-7%) was added, gels were even formed at room temperature using a T908 concentration as low as 2% (Table 4.1). Interestingly, this phenomenon did not occur with so diluted poloxamer (e.g. Pluronic® F127) [24] dispersions. Therefore, the four arms architecture of poloxamines facilitates the dynamic cross-linking of adjacent unimers through interactions among the threaded αCD units. Few minutes (< 5) after T908 and αCD solutions were mixed, the systems became progressively turbid dispersions and then white gels (Table 4.1), which is in agreement with the time required for polypseudorotaxanes involving PEO blocks as large as those of T908 [22, 24]. At the lowest concentration tested (2.5%), αCD did not render gels at any temperature in the 1 to 13% T908 concentration range, which means that the αCD:EO molar ratio was too low for effective 3D-interaction among various polypseudorotaxanes. As a consequence, the polypseudorotaxanes self-aggregated to minimize the contact with the aqueous medium, and phase separated.

Table 4.1. Composition and appearance of the αCD:T908 aqueous dispersions (observed at 24h after preparation).

T908 (% w/v)	αCD (% w/v)	αCD:EO molar ratio	4 ºC	20 ºC	37 ºC[c)]
1	0	0	Sol	Sol	Sol
1	2.5	0.141	Sol	Sol	Sol
1	5	0.283	Sol[a)]	Gel[b)]	2P
1	7	0.396	Gel	Gel	Gel
1	9.7	0.548	Gel	Gel	Gel
2	0	0	Sol	Sol	Sol
2	2.5	0.071	Sol[a)]	Sol	Sol
2	5	0.141	Gel	Gel[b)]	2P
2	7	0.198	Gel	Gel	Gel
2	9.7	0.274	Gel	Gel	Gel
3	0	0	Sol	Sol	Sol
3	2.5	0.047	Sol[a)]	Sol	Sol
3	5	0.094	Gel	Gel[b)]	2P
3	7	0.132	Gel	Gel	Gel
3	9.7	0.183	Gel	Gel	Gel
4	0	0	Sol	Sol	Sol
4	2.5	0.035	Sol[a)]	Sol	Sol
4	5	0.071	Gel	Gel[b)]	2P
4	7	0.099	Gel	Gel	Gel
4	9.7	0.137	Gel	Gel	Gel
5	0	0	Sol	Sol	Sol
5	2.5	0.028	Sol[a)]	Sol	Sol
5	5	0.057	Gel	Gel[b)]	2P
5	7	0.079	Gel	Gel	Gel
5	9.7	0.110	Gel	Gel	Gel
8	0	0	Sol	Sol	Sol
8	2.5	0.018	Sol[a)]	Sol	Sol
8	5	0.035	Gel	Gel[b)]	2P
8	7	0.049	Gel	Gel	Gel
8	9.7	0.069	Gel	Gel	Gel
13	0	0	Sol	Sol	Sol
13	2.5	0.007	Sol[a)]	Sol	Sol
13	5	0.014	Gel	Gel[b)]	2P
13	7	0.020	Gel	Gel	Gel
13	9.7	0.027	Gel	Gel	Gel

[a)] Turbid white solution; [b)] Gelled after 3 days; 2P: two phases separation.

For larger αCD concentrations, the systems increased the viscosity in a time-dependent manner along the first three days. This finding is in agreement with the behavior observed for polyethyleneglycol (PEG) and poloxamer, and indicates that polypseudorotaxane formation is faster than the association of the threaded αCD that leads to three-dimensional aggregates [27].

The storage temperature of αCD-T908 systems also played a role in gel formation, being more favorable at 4 ºC or 20 ºC than at 37 ºC. In some cases, 5% αCD led to phase separation at 37 ºC (Figure 4.2), which suggests an incomplete threading, as occurred for 2.5% αCD. It has been shown that the interaction of αCDs and the EO groups is more favorable at low temperature [27]. Once the gels were formed at 4 ºC or 20 ºC, they remained stable for at least one month. Importantly, the temperature could then be increased to 37 ºC without triggering phase separation.

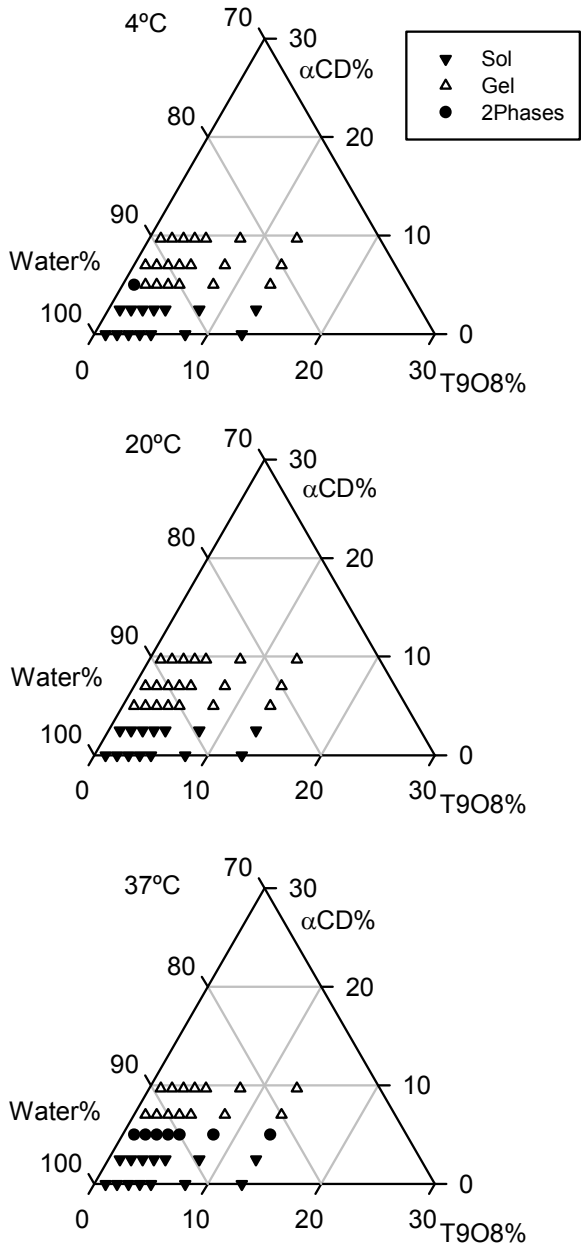


Figure 4.2. Phase diagram of T908:αCD formulations after 5 days of storage at 4, 20 or 37 ºC.

Below the sol–gel temperature, aqueous solutions of poloxamine solely exhibited Newtonian rheological behavior. Conversely, above the gelation temperature they became viscoelastic systems, although following cooling, the gel reverted to the initial low viscosity dispersions. In the 1 to 13% concentration range, T908 solely dispersions in PBS pH 7.4 showed small values

of G″, while G′ was negligible even at 37 ºC (Figure 4.3). Addition of αCD at 5% made G″ to increase almost 5 orders of magnitude, except for 1% T908 which was not affected. Moreover, G′ became larger than G″ and independent of the angular frequency, which is typical of a well-structured three-dimensional network. In the 10 ºC to 37 ºC range evaluated, the increase in temperature did not cause relevant effects on G′ and G″ (Figure 4.4). Adding more αCD up to 9.7%, led to stronger, more viscoelastic networks, even in the case of 1% T908. This finding suggests that not only the αCD:EO molar ratio, but also the total concentration in both components influence the phase diagram and rheological features of the polypseudorotaxanes based on T908 (Table 4.1). It should be noticed that the gels can be heated in autoclave at 120 ºC for 20 min without causing changes in the rheological behavior. Incorporation of simvastatin in the μM range did not alter the rheological properties of the gels.

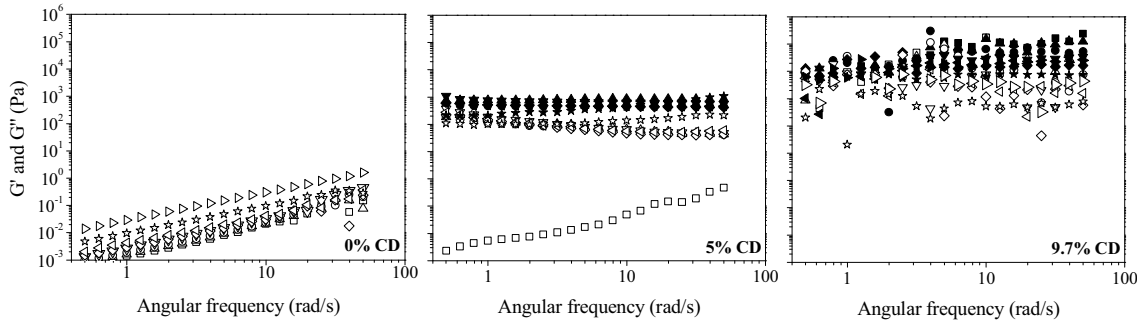


Figure 4.3. Storage (full symbols) and loss (open symbols) moduli of the T908-αCD formulations containing 1% (squares), 2% (up triangles), 3% (circles), 4% (down triangles), 5% (diamonds), 8% (left triangles), 13% (star) and 20% (right triangles) T908 without αCD and with 5% and 9.7% αCD at 37ºC.

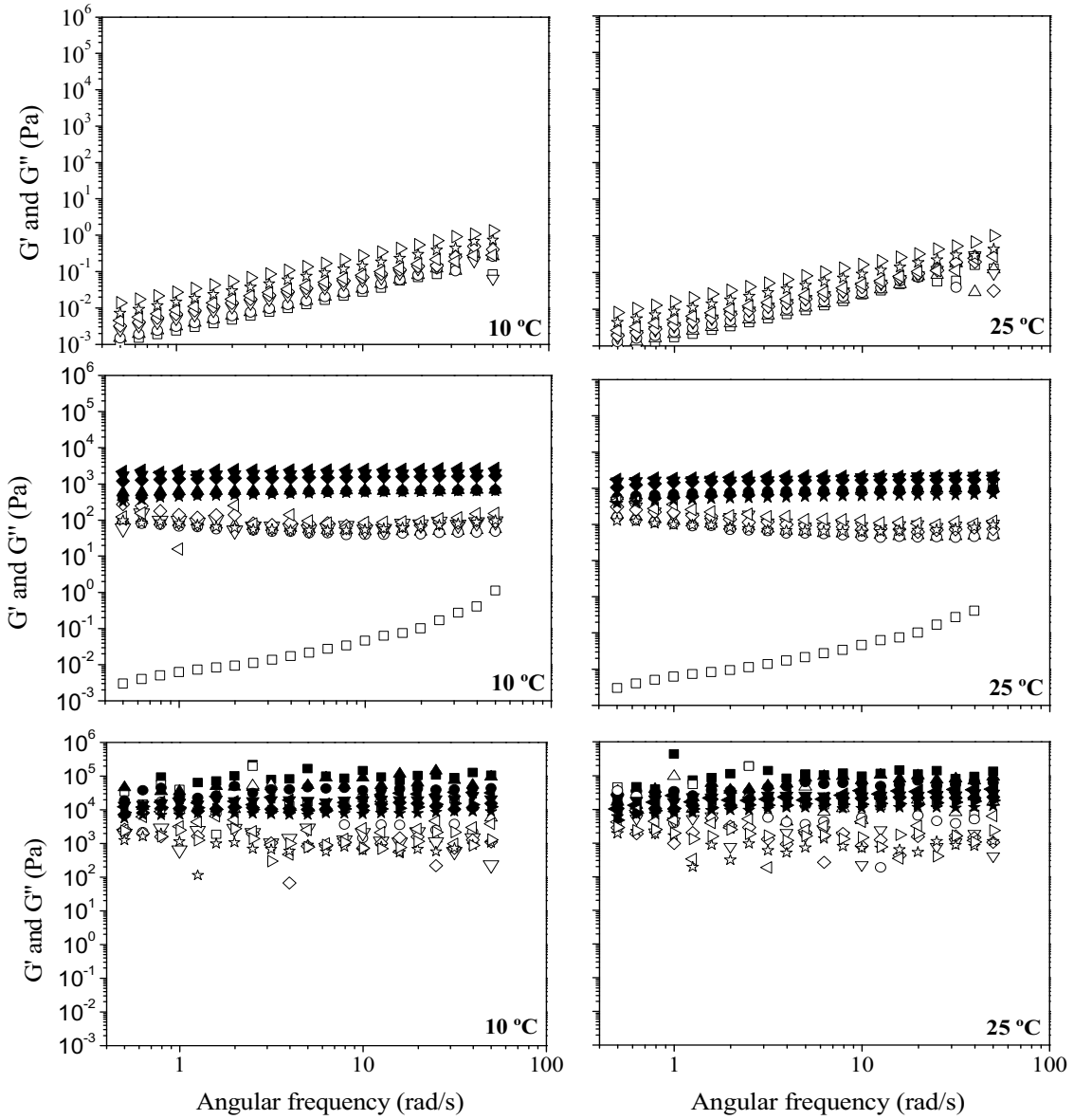


Figure 4.4. Storage (full symbols) and loss (open symbols) moduli of the T908-αCD formulations containing 1% (squares), 2% (up triangles), 3% (circles), 4% (down triangles), 5% (diamonds), 8% (left triangles), 13% (star) and 20% (right triangles) T908 without αCD and with 5% and 9.7% αCD at 10 and 25ºC.

4.4.2 Cytocompatibility screening

4.4.2.1 HET-CAM assay

Since free αCD units have been reported to cause hemolysis at high concentrations [28], T908:αCD formulations were evaluated for biocompatibility applying the HET-CAM assay [29, 30]. Free 5% αCD solutions and T908 solely (data not shown) or with 5% αCD dispersions (Figure 4.5a) did not induce hemorrhage, lysis or coagulation. Thus, their IS was 0.0, as occurred for the negative control (0.9% NaCl; Figure 4.5b). By contrast, the IS of the positive control was 18.9 ± 0.3 (Figure 4.5c), fulfilling the requirement for an acceptable test. The fact that the CDs are threaded onto the copolymer backbone in the form of a polypseudorotaxane would also enable an increase in αCD concentration without detrimental effects on cytocompatibility [31].

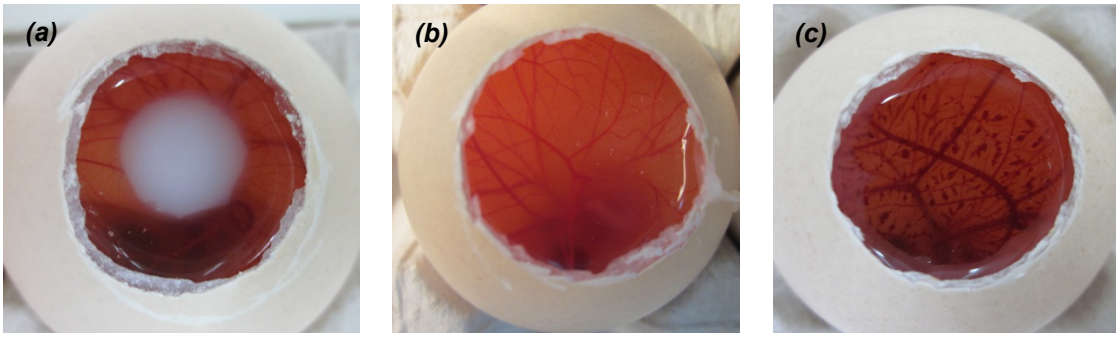


Figure 4.5. HET-CAM test results of 8% T908-5% αCD supramolecular gel (a), negative control (0.9% NaCl; b) and positive control (0.1 N NaOH; c). Only in the possitive control, hemorrhage, vascular lysis and coagulation were evidenced.

4.4.2.2 Osteoblasts viability

Live/dead stain and MTT assays of SAOS-2 cells (human osteoblast cell line) after 24 and 48 h of culture with T908 dispersions (0-13%) corroborated the cytocompatibility of this block copolymer (Figure 4.6 and confocal images as Figure 4.7). Incorporation of αCD caused a progressive decrease in osteoblasts viability; the viability was around 60% for gels containing 5% αCD. Only for those containing 9.7% αCD the viability was lower than 50%.

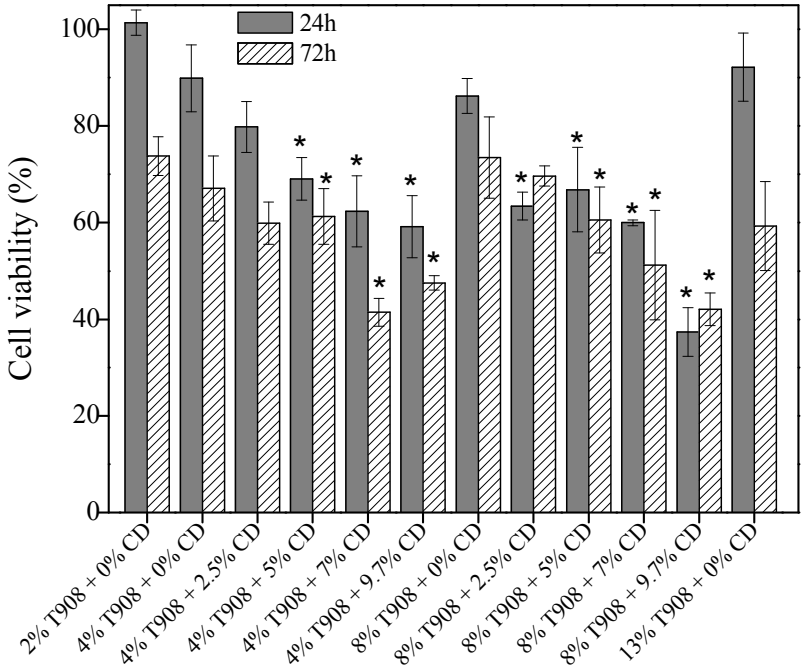


Figure 4.6. Viability of osteoblasts in contact with T908-αCD supramolecular gels. *Significantly lower than for the system without αCD ($p < 0.001$).

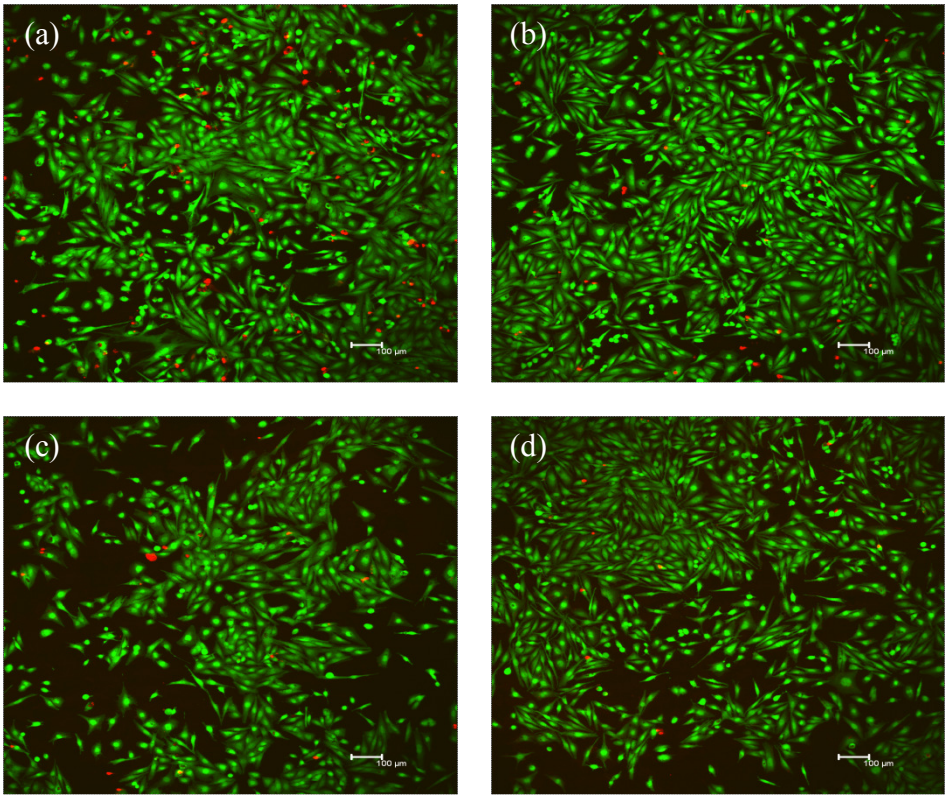


Figure 4.7. Confocal images of live (calcein, green) and dead (propidium iodide, red) osteoblasts after 72 h in contact with 4% T908 (a), 4% T908-5% αCD (b), 8% T908 (c) and 8% T908-5% αCD (d).

4.4.3 Simvastatin solubilization and stability

Simvastatin is a polycyclic compound with a pH-dependent structure and solubility, being poorly soluble in water [8, 19]. At intermediate pH values (pH ≈ 5) simvastatin predominantly exists in the lactonic form, but at both low and alkaline pH it is reversibly hydrolyzed to the hydroxy acid form [26]. Osteogenic activity has been reported for the hydroxy acid form, which should be the predominant one at pH 7.4. Thus, the ability of αCD and T908 separately to solubilize simvastatin was evaluated at this pH. It has been previously shown that simvastatin can form inclusion complexes with the natural cyclodextrins and also with synthetic derivatives such as hydroxypropyl-β-cyclodextrin with a 1:1 stoichiometry [32], remaining the lactone ring outside the cyclodextrin. Such a complex formation notably accelerates the hydrolysis of the ring (Table 4.2) [33]. As previous observed at pH 6.5 [32], αCD showed a relatively low ability to solubilize simvastatin at pH 7.4 (Table 4.2). At the highest αCD concentration tested (9.7%) the amount of simvastatin dissolved increased 5-fold. On the other hand, above the critical micellar concentration (CMC = 1%) [18], an increase in T908 concentration progressively raised simvastatin solubility. Compared to previous studies carried out in HCl 10 mM [18], the solubility factors attained at pH 7.4 were much larger probably because the concomitant effects of (i) a more favored self-aggregation process of the copolymer when the nitrogen atoms are not protonized [18], and (ii) an increase in the intrinsic solubility of the drug in the hydroxy acid form [8], which favors the diffusion inside the micelles. In fact, in the micellar systems at pH 7.4, the predominant form of the drug was also the hydroxy acid one (Table 4.2). Only in the systems prepared with T908 at 10% or more, lactone form was detected but still in a low proportion (Table 4.2). This finding indicates that the protective effect that poloxamines can exert on the lactone ring is much lower at pH 7.4 than at acid pH [18]. Thus, at pH 7.4 it is foreseeable that poloxamines do not negatively affect to the osteogenic/osteoinductive activity of simvastatin. Moreover, monitoring for 65 days of the two forms of simvastatin in T908 dispersions stored at 4 ºC revealed no changes in the lactone/hydroxy acid molar ratio.

Table 4.2. Apparent solubility and lactone/hydroxy acid molar ratio of simvastatin in αCD or T908 solutions in phosphate buffer pH 7.4.

Medium	Simvastatin solubility (SD x10^{-4}) [mg.mL^{-1}]	*fs*	*X* [a)]	*P* [b)]	Simvastatin/ PO (mg/g)	Lactone/ hydroxy acid molar ratio[c)]
H_2O	0.0018 (1.3)	_	_	_	_	1:1
Phosphate buffer pH 7.4	0.0032 (5.3)	_	_	_	_	0.9:1
2.5% αCD	0.0082 (3.2)	2.56	_	_	_	0:1
5% αCD	0.0104 (6.4)	3.25	_	_	_	0:1
7% αCD	0.0125 (7.7)	3.91	_	_	_	0:1
9.7% αCD	0.0167 (7.9)	5.22	_	_	_	0.01:1
1% T908	0.0063 (4.9)	1.97	0.000	2.50	3.173	0:1
2% T908	0.0080 (2.5)	2.50	0.037	3.44	2.012	0:1
3% T908	0.0100 (2.6)	3.13	0.024	4.56	1.669	0:1
4% T908	0.0123 (4.4)	3.84	0.021	5.83	1.534	0:1
5% T908	0.0144 (4.4)	4.50	0.019	7.00	1.441	0:1
8% T908	0.0220 (8.8)	6.88	0.017	11.22	1.373	0:1
10% T908	0.0259 (4.6)	8.09	0.016	13.39	1.296	0.1:1
13% T908	0.0395 (13.6)	12.34	0.019	20.94	1.518	0.1:1
20% T908	0.2468 (18.2)	77.13	0.077	136.11	6.172	0.2:1

[a)] number of moles that can be solubilized by 1 mol of copolymer in the micellar state; [b)] micelle/water partition coefficient, i.e. the ratio of the drug concentration in the micelle to the drug concentration in water.

5.4.4 Simvastatin release

For comparative purposes, T908 dispersions and T908-αCD systems were prepared with a fix concentration of simvastatin (50 μM = 0.0209 mg/mL). Since the drug was completely solubilized in the gels, T908 dispersions were transparent while T908-αCD systems maintained the homogenous whitish appearance. The greater the concentration in T908, the slower the release process was (Figure 4.8). Incorporation of simvastatin to the T908 dispersions did not alter the subsequent gelling process with αCD, which can be explained by the fact that simvastatin and αCD interact with different sites of the copolymer; namely the PPO and the PEO blocks, respectively. The gelation led to more sustained release profiles; T908-αCD

formulations controlled simvastatin release for more than one week. Diffusion coefficients were calculated from the slope of the fitting to the Higuchi equation [34]:

$$\frac{Q}{A} = 2C_0\left(\frac{Dt}{\pi}\right)^{1/2}$$

where Q is the amount of drug (mg) released at time t (s), A is diffusion area (cm^2), C_0 is the initial concentration of simvastatin in the formulation (mg/mL), and D is the diffusion coefficient (cm^2/s).

A negative correlation between the content in αCD and the diffusion coefficient values (Table 4.3) was found. In general, addition of 5% αCD decreased 2-fold the diffusion coefficient, while 9.7% αCD led to 3- to 4-fold decrease compared to the values recorded for T908 solely formulations. This is in agreement with the increase in viscosity that occurred when the polypseudorotaxanes were formed. Tie-junctions among the αCDs threaded on the PEO blocks may notably hinder the diffusion of simvastatin molecules trapped close to the inner PPO blocks.

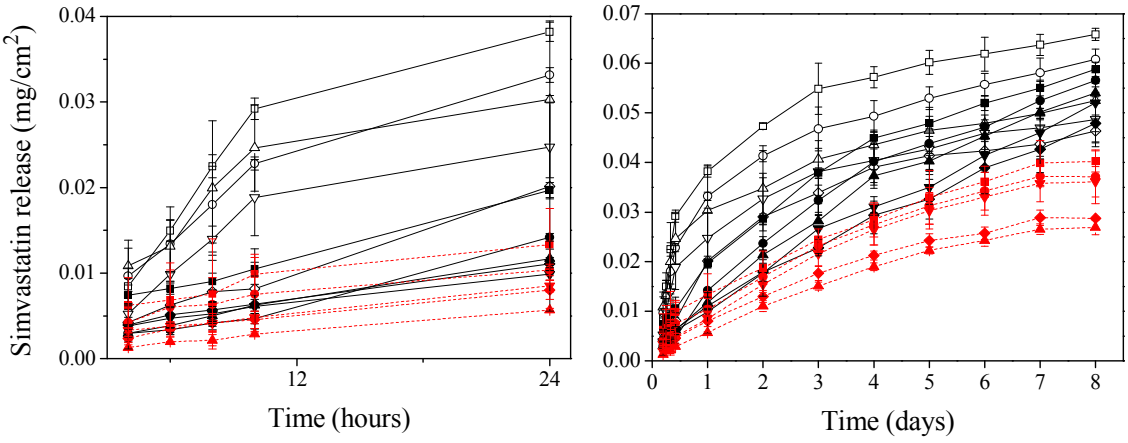


Figure 4.8. Simvastatin release at 37 ºC from Tetronic 908 formulations without αCD (open symbols), with 5% αCD (black solid symbols) and 9.7% αCD (red solid symbols with dashed lines). T908 was evaluated at 1% (squares), 2% (circles), 4% (up triangles), 8% (down triangles) and 13% (diamonds). 100% release would correspond to 0.064 mg/cm^2. The plot on the right expands the first 24 h depicted in the plot on the left.

In sum, taken into account the rheology, cytocompatibility and release data, 5% αCD and 4-8% T908 systems seem to be the most convenient for preparing syringeable scaffolds for sustained drug release: 5% αCD ensures gel formation at any T908 concentration without compromissing cell viability, while T908 concentration above 8% did not enhance further the consistency of the polypseudorotaxane gels. Separately, those concentrations of αCD and T908 led to one order of magnitud increase in the solubility of simvastatin, which is also maintained in the hydroxy acid form in the gels. Thus, subsequent studies are mainly focussed on these systems (Table 4.4).

Table 4.3. Diffusion coefficient values obtained from the slope of the fitting to the Higuchi equation [34] of simvastatin release data plotted in Figure 4.8 ($R^2 > 0.95$). Mean values and, in parenthesis, standard deviations (n=3).

Content in T908 (%)	Content in αCD (%)		
	0	5	9.7
1	$21.7 \cdot 10^{-6}$ ($2.2 \cdot 10^{-6}$)	$11.4 \cdot 10^{-6}$ ($0.2 \cdot 10^{-6}$) *	$4.1 \cdot 10^{-6}$ ($0.7 \cdot 10^{-6}$) *,**
2	$15.8 \cdot 10^{-6}$ ($0.5 \cdot 10^{-6}$)	$11.2 \cdot 10^{-6}$ ($0.2 \cdot 10^{-6}$) *	$4.1 \cdot 10^{-6}$ ($1.1 \cdot 10^{-6}$) *,**
4	$9.6 \cdot 10^{-6}$ ($1.6 \cdot 10^{-6}$)	$8.2 \cdot 10^{-6}$ ($0.9 \cdot 10^{-6}$)	$2.7 \cdot 10^{-6}$ ($0.2 \cdot 10^{-6}$) *,**
8	$11.3 \cdot 10^{-6}$ ($1.4 \cdot 10^{-6}$)	$6.9 \cdot 10^{-6}$ ($1.5 \cdot 10^{-6}$) *	$4.2 \cdot 10^{-6}$ ($0.9 \cdot 10^{-6}$) *
13	$11.6 \cdot 10^{-6}$ ($2.6 \cdot 10^{-6}$)	$4.6 \cdot 10^{-6}$ ($0.6 \cdot 10^{-6}$) *	$2.7 \cdot 10^{-6}$ ($0.3 \cdot 10^{-6}$) *

*Statistically significant different to the formulation without αCD ($p<0.05$; ANOVA, multiple range test);
**Statistically significant different to the formulation with 5% αCD ($p<0.05$; ANOVA, multiple range test).

4.4.5 Alkaline phosphatase (ALP) assay

Recent *in vitro* and *in vivo* studies have demonstrated the capability of simvastatin to elicit osteoblasts proliferation, new bone formation and angiogenesis [11, 15, 35, 36]. However, strong discrepancies on the optimal concentration to attain such effects can be found. Concentrations of 2 μM or higher could cause cell grow inhibition [36]. Some studies reported that 1 μM simvastatin provided the highest viability [35, 36], while others found cytotoxic effects on human adipose-derived stromal cells (hADSCs) [37]. Both 0.01 and 1 μM simvastatin

could induce the osteogenic differentiation of human bone marrow derived mesenchymal stem cells (BMMSCs), although the two concentrations were found to inhibit the cells proliferation [38]. It was also reported that 2 μM simvastatin stimulated primary culture bone marrow stromal cells to differentiate to osteoblasts, inhibiting adipogenic differentiation [39]. Therefore, as a first step, we evaluated MSCs proliferation and osteoblast differentiation in presence of simvastatin at 0.01 to 5 μM in the cellular medium (0.08 to 42.5 μM in PBS solution; Table 4.4).

Table 4.4. Concentrations of simvastatin and T908 in the formulations and in the medium used for *in vitro* studies on cell proliferation and osteoblasts differentiation.

Simvastatin in the formulation (μM)	Formulation (%, w/v)	Concentration in cellular medium	
		Simvastatin (μM)	Formulation
0	4% T908 4% T908 – 5% αCD 8% T908 8% T908 – 5% αCD	0	0.5% T908 0.5% T908 – 0.6% αCD 1% T908 1% T908 – 0.6% αCD
0.08	No formulation 4% T908 4% T908 – 5% αCD 8% T908 8% T908 – 5% αCD	0.01	No formulation 0.5% T908 0.5% T908 – 0.6% αCD 1% T908 1% T908 – 0.6% αCD
0.85	No formulation	0.1	No formulation
8.5	No formulation 4% T908 4% T908 – 5% αCD 8% T908 8% T908 – 5% αCD	1	No formulation 0.5% T908 0.5% T908 – 0.6% αCD 1% T908 1% T908 – 0.6% αCD
42.5	No formulation	5	No formulation

Compared to the negative control, simvastatin led to a similar cell proliferation in the first 3 days, but to a minor decrease on days 7 and 12, although with values above those of the positive control (Figure 4.9a). ALP activity was maximum at day 7 for 0.1, 1 and 5 μM simvastatin, reaching values similar to those found with the positive control (Figure 4.9b). At day 12 the protein activity decreased in the presence of simvastatin, except in the medium containing the

lowest concentration tested (0.01 μM). Thus, based on our data and in the literature, 0.01 and 1 μM simvastatin concentrations were chosen for further studies with supramolecular gels of T908-αCD, which were prepared with 0.08 and 8.5 μM simvastatin in order to render 0.01 and 1 μM concentrations after the dilution in the cellular medium.

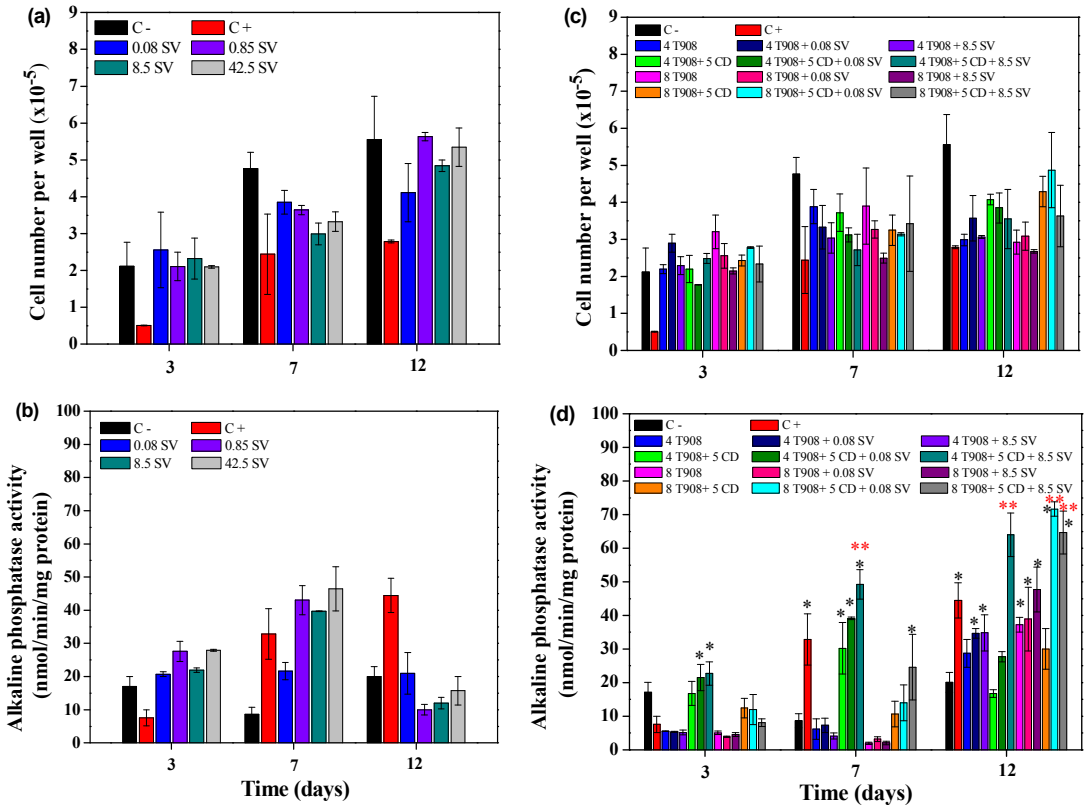
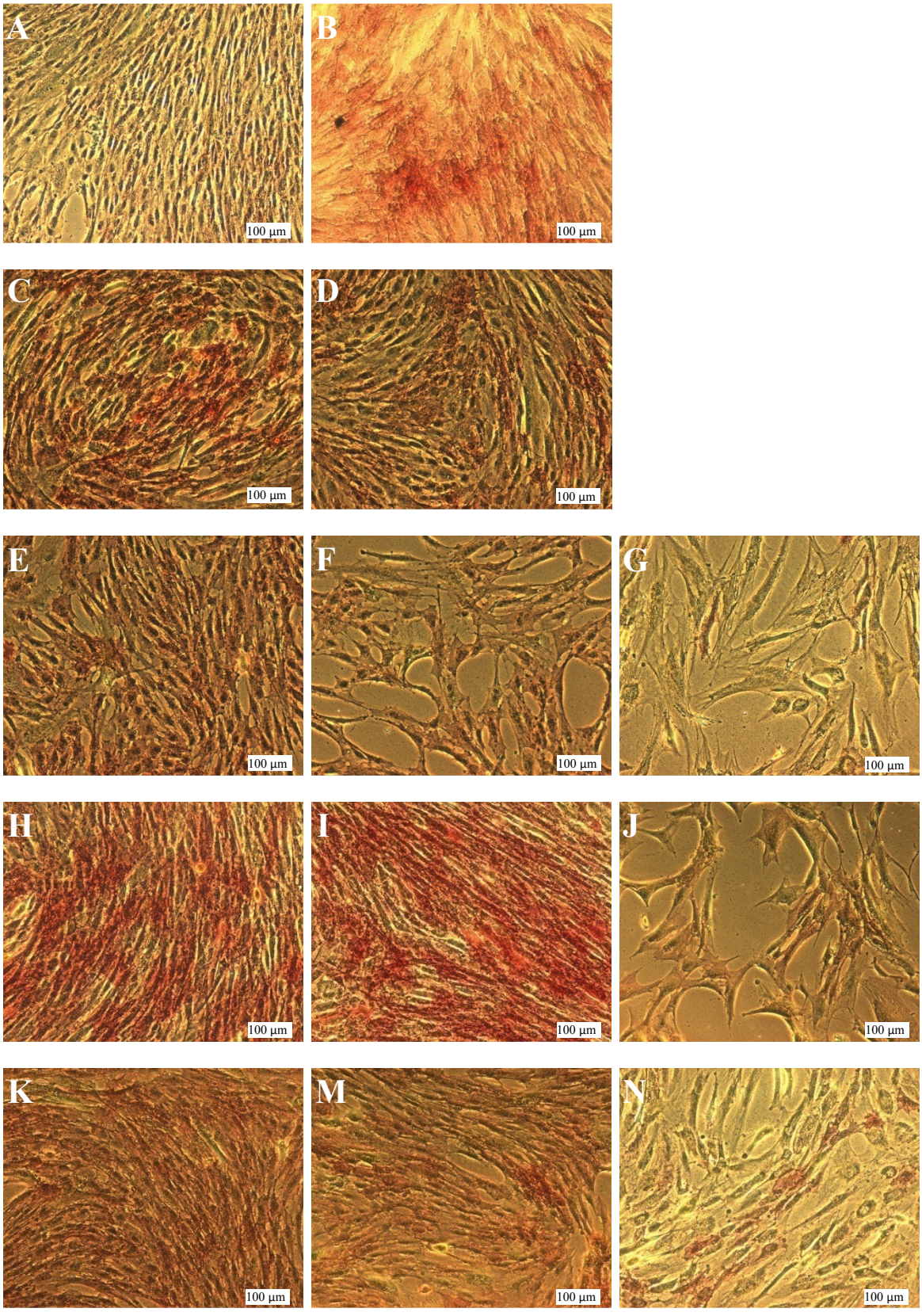


Figure 4.9. Time evolution of viable mesenchymal stem cell number (a, c) and ALP activity of mesenchymal stem cells (b, d) in culture medium (negative control), in osteogenic medium (positive control) and in culture medium to which (a, b) solutions of 0.08, 0.85, 8.5 and 42.5 μM simvastatin (final drug concentrations in the culture medium were 0.01, 0.1, 1 and 5 μM, respectively) or (c, d) formulations of T908 with and without αCD and simvastatin (drug concentrations indicated in Table 4.4) were added. *Significantly greater than negative control ($p< 0.01$). ** Significantly greater than positive control ($p< 0.01$).

The T908-αCD systems identified as the most suitable from the rheological and the cytocompatibility data (i.e., those prepared with 4 or 8% copolymer and 5% αCD) were the ones chosen for the osteogenicity studies. The supramolecular gels exhibited good cytocompatibility and proliferative effects on MSCs in the first week (Figure 4.9c) and caused differentiation to osteoblasts later (Figure 4.9d). The number of cells remained in between those attained with the negative control and the osteogenic (positive) control medium (Figure 4.9c). Compared to T908 solely at 4 or 8%, incorporation of 5% αCD and/or simvastatin did not cause detrimental effects on cell proliferation. Moreover, the ALP activity was significantly larger (ANOVA and multiple range test, $p < 0.05$) for the systems containing αCD compared to T908 solely gels at days 3 and 7, and the incorporation of simvastatin 8.5 μM (i.e., 1 μM in the culture medium) increased even more the ALP activity at days 7 and 12 compared to the supramolecular systems prepared without the drug (Figure 4.9d). In agreement with a previous report [12], in the presence of T908 dispersions solely or combined with simvastatin (without αCD) the osteogenic/osteoinductive effects appeared one week later than in the case of the osteogenic (positive) control medium. By contrast, αCD favored an earlier and prolonged differentiation of the MSCs to osteoblasts, which could be related to a more sustained delivery of both T908 and simvastatin (as registered in Figure 4.8). The ALP values obtained are in the range of those previously reported for gels with rhBMP-2 [7]. Differentiation of MSCs to osteoblasts was also confirmed by means of ALP staining, using an inverted microscope (Figure 4.10). In the negative control medium MSCs formed homogeneous monolayers, with a high proliferation index (Figure 4.10A). In the osteoinductive medium (positive control; Figure 4.10B), the polygonal shape of osteoblasts was evident since day 7 with a strong staining. In the case of the T908 formulations at day 12 (Figure 4.10E-Q), in the absence of αCD the number of cells was higher. Cells cultured in the presence of simvastatin showed more clearly the characteristic shape of osteoblasts. The staining was potentiated in the presence of αCD. Therefore, the ternary poloxamine-cyclodextrin-simvastatin supramolecular systems exhibit synergistic osteogenic/osteoinductive effects. Although poloxamines and αCDs are not biodegradable, it is expected that as the polypseudorotaxane scaffold erodes and the copolymer and the αCDs reach systemic circulation, they could be rapidly excreted in the urine as occurs for other hydrophilic polymers and cyclodextrins. Nevertheless, *in vivo* studies would be necessary to further clarify the clinical applicability of the developed polypseudorotaxane-based scaffolds.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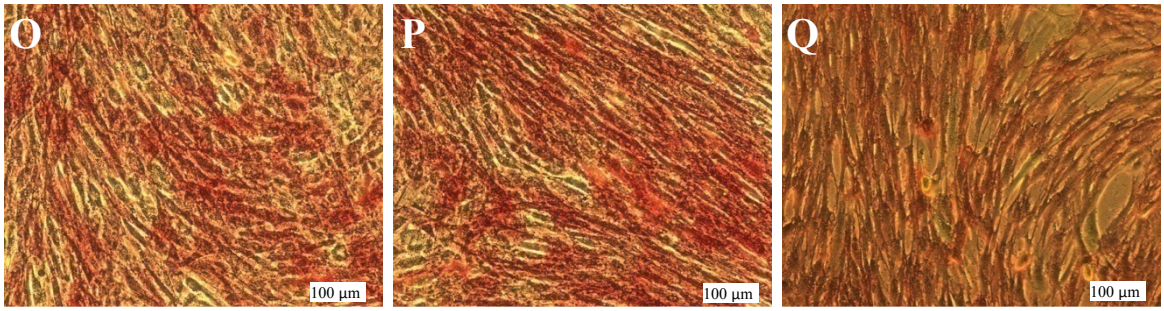

Figure 4.10. ALP staining of cells after 12 days in control medium (A), osteogenic medium (B), 0.08 or 8.5 µM simvastatin in PBS (C, D), 4 % T908 with 0, 0.08 or 8.5 µM simvastatin (E, F, G), 4 % T908 – 5 % αCD with 0, 0.08 or 8.5 µM simvastatin (H, I, J), 8 % T908 with 0, 0.08 or 8.5 µM simvastatin (K, M, N) and 8 % T908 – 5 % αCD with 0, 0.08 or 8.5 µM simvastatin (O, P, Q).

4.5 CONCLUSIONS

Polypseudorotaxanes of poloxamine T908 with αCD enable the preparation of syringeable viscoelastic gels using minimal amounts of both components, which leads to good compatibility with both MSCs and osteoblasts and enhances the intrinsic osteogenic/osteoinductive effect of this block copolymer. T908-αCD systems can stand moist heat sterilization (autoclaving) without relevant changes in the rheological properties, which is an important aspect for the development of injectable scaffolds. Moreover, the polypseudorotaxane-based gels can solubilize simvastatin, stabilize the hydroxy acid form at pH 7.4, and provide sustained drug release for more than one week. The release rate can be easily tuned by means of the content in αCD. Such a controlled release of simvastatin hydroxy acid form allows polypseudorotaxane-based gels to attain faster and more prolonged ALP activity than that displayed by T908 solely formulations. The remarkable osteoinductive effects attained with simvastatin-loaded T908-αCD polypseudorotaxanes make them suitable syringeable synthetic scaffolds with intrinsic ability to promote osteoblast differentiation.

4.6 REFERENCES

[1] Lo, K.W.H.; Ashe, K.M.; Kan, H.M.; Laurencin, C.T. *The role of small molecules in musculoskeletal regeneration*. Regen. Med., 2012, 7(4), 1-15.

[2] Melches, F.P.W.; Domingos, M.A.N.; Klein, T.J.; Malda, J.; Bartolo, P.J.; Hutmacher, D.W. *Additive manufacturing of tissues and organs*. Prog. Polym. Sci., 2012, 37(8), 1079-1104.

[3] McKay, W.F.; Peckham, S.M.; Badura, J.M. *A comprehensive clinical review of recombinant human bone morphogenetic protein-2 (INFUSE® Bone Graft)*. Int. Orthop., 2007, 31(6), 729-734.

[4] White, A.P.; Vaccaro, A.R.; Hall, J.A.; Whang, P.G.; Friel, B.C.; McKee, M.D. *Clinical applications of BMP-7/OP-1 in fractures, nonunions and spinal fusion.* Int. Orthop., 2007, 31(6), 735-741.

[5] Seeherman, H.J.; Bouxsein, M.; Kim, H.; Li, R.; Li, X.J.; Aiolova, M.; Wozney, J.M. *Recombinant human bone morphogenetic protein-2 delivered in an injectable calcium phosphate paste accelerates osteotomy-site healing in a nonhuman primate model*. J. Bone Joint Surg. Am., 2004, 86(9), 1961-1972.

[6] Bessa, P.C.; Casal, M.; Reis, R.L. *Bone morphogenetic proteins in tissue engineering: the road from laboratory to clinic, part II (BMP delivery)*. J. Tissue Eng. Regen. M., 2008, 2(2-3), 81-96.

[7] Rey-Rico, A.; Silva, M.; Couceiro, J.; Concheiro, A.; Alvarez-Lorenzo, C. *Osteogenic efficiency of in situ gelling poloxamine systems with and without bone morphogenetic protein-2*. Eur. Cell. Mater., 2011, 21, 317-340.

[8] Serajuddin, A.T.M.; Ranadive, S.A.; Mahoney, E.M. *Relative lipophilicities, solubilities, and structure-pharmacological considerations of 3-hydroxy-3-methylglutaryl-coenzyme a (HMG-COA) reductase inhibitors pravastatin, lovastatin, mevastatin, and simvastatin*. J. Pharm. Sci., 1991, 80(9), 830-834.

[9] Schachter, M. *Chemical, pharmacokinetic and pharmacodynamic properties of statins: an update*. Fundam. Clin. Pharmacol., 2005, 19(1), 117-125.

[10] Sugiyama, M.; Kodama, T.; Konishi, K.; Abe, K.; Asami, S.; Oikawa, S. *Compactin and simvastatin, but not pravastatin, induce bone morphogenetic protein-2 in human osteosarcoma cells*. Biochem. Biophys. Res. Commun., 2000, 271(3), 688-692.

[11] Maeda, T.; Matsunuma, A.; Kawane, T.; Horiuchi, N. *Simvastatin promotes osteoblast differentiation and mineralization in MC3T3-E1 Cells*. Biochem. Biophys. Res. Commun., 2001, 280(3), 874-877.

[12] Pagkalos, J.; Cha, J.M.; Kang, Y.; Heliotis, M.; Tsiridis, E.; Mantalaris, A. *Simvastatin induces osteogenic differentiation of murine embryonic stem cells*. J. Bone Miner. Res., 2010, 25(11), 2470-2478.

[13] Kureishi, Y.; Luo, Z.; Shiojima, I.; Bialik, A.; Fulton, D.; Lefer, D.J.; Sessa, W.C.; Walsh, K. *The HMG-CoA reductase inhibitor simvastatin activates the protein kinase Akt and promotes angiogenesis in normocholesterolemic animals*. Nat. Med., 2000, 6(9), 1004-1010.

[14] Davignon, J.; Laaksonen, R. *Low-density lipoprotein-independent effects of statins*. Curr. Opin. Lipidol., 1999, 10(6), 543-559.

[15] Mundy, G.; Garrett, R.; Harris, S.; Chan, J.; Chen, D.; Rossini, G.; Boyce, B.; Zhao, M.; Gutierrez, G. *Stimulation of bone formation in vitro and in r rodents by statins*. Science, 1999, 286(5446), 1946-1949.

[16] Park, J.B. *The use of simvastatin in bone regeneration*. Med. Oral Patol. Oral Cir. Bucal, 2009, 14(9), 485-488.

[17] Jeon, J.H.; Thomas, M.V.; Puleo, D.A. *Bioerodible devices for intermittent release of simvastatin acid*. Int. J. Pharm., 2007, 340(1-2), 6-12.

[18] Gonzalez-Lopez, J.; Alvarez-Lorenzo, C.; Taboada, P.; Sosnik, A.; Sandez-Macho I.; Concheiro, A. *Self-associative behavior and drug-solubilizing ability of poloxamine (Tetronic) block copolymers*. Langmuir, 2008, 24(19), 10688-10697.

[19] Gonzalez-Lopez, J.; Sandez-Macho, I.; Concheiro, A.; Alvarez-Lorenzo, C. *Poloxamines and poloxamers as polymeric micellar carriers for simvastatin: interactions at the air−water interface and in bulk solution*. J. Phys. Chem. C, 2010, 114(2), 1181-1189.

[20] Alvarez-Lorenzo, C.; Rey-Rico, A.; Sosnik, A.; Taboada, P.; Concheiro, A. *Poloxamine-based nanomaterials for drug delivery*. Front. Biosci. (Elite Ed), 2010, 2(2), 424-440.

[21] Doğan, A.; Yalvaç, M.E.; Şahin, F.; Kabanov, A.V.; Palotás, A.; Rizvanov, A.A. *Differentiation of human stem cells is promoted by amphiphilic pluronic block copolymers*. Int. J. Nanomed., 2012, 7(1), 4849-4860.

[22] Harada, A.; Li, J.; Kamachi, M. *Preparation and properties of inclusion complexes of polyethylene glycol with α-cyclodextrin*. Macromolecules, 1993, 26(21), 5698-5703.

[23] Li, J.; Li, X.; Ni, X.; Wang, X.; Li, H.; Leong, K.W. *Self-assembled supramolecular hydrogels formed by biodegradable PEO-PHB-PEO triblock copolymers and α-cyclodextrin for controlled drug delivery*. Biomaterials, 2006, 27(22), 4132-4140.

[24] Simões, S.M.N.; Veiga, F.; Torres-Labandeira, J.J.; Ribeiro, A.C.F.; Sandez-Macho, M.I.; Concheiro, A.; Alvarez-Lorenzo, C. *Syringeable Pluronic-α-cyclodextrin supramolecular gels for sustained delivery of vancomycin*. Eur. J. Pharm. Biopharm., 2012, 80(1), 103-112.

[25] NICEATM-ICCVAM. *In vivo test methods for detecting ocular corrosives and severe irritants*. Available from URL:http://iccvam.niehs.nih.gov/methods/ocutox/ivocutx.htm, accessed on October 2012.

[26] Alvarez-Lueje, A.; Valenzuela, C.; Squella, J.A.; Núñez-Vergara, L.J. *Stability study of simvastatin under hydrolytic conditions assessed by liquid chromatography*. J. AOAC Int., 2005, 88(6), 1631-1636.

[27] Travelet, C.; Schlatter, G.; Hébraud, P.; Brochon, C.; Lapp, A.; Hadziioannou, G. *Formation and self-organization kinetics of α-CD/PEO-based pseudo-polyrotaxanes in water. A specific behavior at 30 ºC.* Langmuir, 2009, 25(15), 8723-8734.

[28] Leroy-Lechat, F.; Wouessidjewe, D.; Andreux, J.P.; Puisieux, F.; Duchêne, D. *Evaluation of the cytotoxicity of cyclodextrins and hydroxypropylated derivatives*. Int. J. Pharm., 1994, 101(1-2), 97-103.

[29] Valdes, T.I.; Kreutzer, D.; Moussy, F. *The chick chorioallantoic membrane as a novel in vivo model for the testing of biomaterials*. J. Biomed. Mater. Res., 2002, 62(2), 273-282.

[30] Baiguera, S.; Macchiarini, P.; Ribatti, D. *Chorioallantoic membrane for in vivo investigation of tissue-engineered construct biocompatibility*. J. Biomed. Mater. Res. B Appl. Biomater., 2012, 100B(5), 1425-1434.

[31] Loethen, S.; Kim, J.M.; Thompson, D.H. *Biomedical applications of cyclodextrin based polyrotaxanes*. Polym. Rev., 2007, 47(3), 383-418.

[32] Süle, A.; Szente, L.; Csempesz, F. *Enhancement of drug solubility in supramolecular and colloidal systems*. J. Pharm. Sci., 2009, 98(2), 484-494.

[33] Ungaro, F.; Giovino, C.; Catanzano, O.; Miro, A.; Mele, A.; Quaglia, F.; La Rotonda, M.I. *Use of cyclodextrins as solubilizing agents for simvastatin: Effect of hydroxypropyl-β-cyclodextrin on lactone/hydroxyacid aqueous equilibrium*. Int. J. Pharm., 2011, 404(1-2), 49-56.

[34] Higuchi, W.I. *Analysis of data on the medicament release from ointments*. J. Pharm. Sci., 1962, 51(8), 802–804.

[35] Chen, P.Y.; Sun. J.S.; Tsuang, Y.H.; Chen, M.H.; Weng, P.W.; Lin, F.H. *Simvastatin promotes osteoblast viability and differentiation via Ras/Smad/Erk/BMP-2 signaling pathway*. Nutr. Res., 2010, 30(3), 191-199.

[36] Zhou, Y.; Ni, Y.; Liu, Y.; Zeng, B.; Xu, Y.; Ge, W. *The role of simvastatin in the osteogenesis of injectable tissue-engineered bone based on human adipose-derived stromal cells and platelet-rich plasma*. Biomaterials, 2010, 31(20), 5325-5335.

[37] Kupcsik, L.; Meurya, T.; Flury, M.; Stoddart, M.; Alini, M. *Statin-induced calcification in human mesenchymal stem cells is cell death related*. J. Cell. Mol. Med., 2009, 13(11-12), 4465-4473.

[38] Baek, K.H.; Lee, W.Y,; Oh, K.W.; Tae, H.J.; Lee, J.M.; Lee, E.J.; Han, J.H.; Kang, M.I.; Cha, B.Y.; Lee, K.W.; Son, H.Y.; Kang, S.K. *The effect of simvastatin on the proliferation and differentiation of human bone marrow stromal cells*. J. Korean Med. Sci., 2005, 20(3), 438-444.

[39] Song, C.; Guo, Z.; Ma, Q.; Chen, Z.; Liu, Z.; Jia, H.; Dang, G. *Simvastatin induces osteoblastic differentiation and inhibits adipocytic differentiation in mouse bone marrow stromal cells*. Biochem. Biophys. Res. Commun., 2003, 308(3), 458-462.

Chapter 5

SUPRAMOLECULAR GELS OF POLY-α-CYCLODEXTRIN AND PEO-BASED COPOLYMERS FOR CONTROLLED DRUG RELEASE

5.1 ABSTRACT

The aim of this work was to develop syringeable supramolecular gels of α-cyclodextrin-polymer (poly-αCD) with poly(ethylene oxide) (PEO)-based copolymers, suitable to form depots for controlled release of vacomycin. A series of water-soluble poly-αCD was synthesized from αCD by crosslinking with epichlorohydrin in alkaline medium. The chemical composition of the polymers was characterized by NMR (αCD content > 53%) and the molecular weight by means of static light scattering (SLS). Supramolecular assemblies occurred by mixing poly-αCD (20-40% w/v) with PEO-based polymer (i.e., PEG, Pluronic® F127 or Tetronic® 908) (10-15%). Phase separation was observed and the αCD content in each phase was determined by means of the phenol-sulfuric acid colorimetric method. Formation of poly-αCD/PEO-based polymer supramolecular complexes was confirmed by diffusion-ordered NMR spectroscopy (DOSY) and X-ray difractometry. The supramolecular assemblies showed good cytocompatibility against SAOS-2 cells and in the HET-CAM tests. The gels were able to sustain vacomycin release at 37 ºC for several days. These results open new possibilities in the design of novel controlled delivery systems for treatment of bone infections.

5.2 INTRODUCTION

Spontaneous association of either a few or many components forming discrete oligomolecular or extended polymolecular assemblies enables the design of new architectures with novel features [1, 2]. Self-assembly systems combining polymers and pristine cyclodextrins (CDs) have been widely studied [3-6]. Polymers of CD (CD-polymers) are receiving increasing attention in pharmaceutical and biomedical fields [3, 7-10]. For example, randomly cross-linked epichlorohydrin-CD polymers can be tailored to yield either water-soluble [11] or insoluble polymers [12, 13] with the CDs incorporated into the backbone [14] or as pendant units [15, 16]. Compared to pristine CDs, CD-polymers can cooperatively participate in the formation of inclusion complexes, resulting in higher stability constants [17, 18]. Moreover, CD-polymers can form nanoparticles or gels in aqueous media when associated with different hydrophilic polymers bearing complementary substituents (e.g. adamantane) that fit into the CD cavities [19, 20]. These type of self-assembly systems have been shown useful as drug carriers that can load the active ingredients (e.g. benzophenone and tamoxifen) in the remnant CD cavities and control the release in the physiological environment [8, 21]. Most publications focused on the synthesis of βCD-polymers since βCD forms complexes with the widest range of drugs [9, 18, 21, 22].

The aim of this work was to evaluate the feasibility of obtaining supramolecular gels combining poly-αCD with poly(ethylene oxide) (PEO)-based copolymers, applying a variety of complementary techniques, and to elucidate the suitability of the supramolecular gels as syringeable depots for controlled release of vacomycin. The ability of pristine αCDs to thread along PEO chains forming poly(pseudo)rotaxanes is already well know [23-26]. However, the capability of poly-αCD to form complexes with PEO chains has not been explored yet. Interactions between poly-αCD and PEO-based copolymers may result in a novel family of 3D-poly(pseudo)rotaxanes, in which the polymeric links of the αCDs should act as tie-junctions among various poly(pseudo)rotaxane units. Thus, the first step of the work was to synthesize water-soluble high molecular weight αCD-polymers. Aqueous systems comprising poly-αCD, vancomycin (optionally), and Pluronic® F127, Tetronic® 908 or polyethylene glycol PEG 6000 were then prepared. *In situ* gelling dispersions based on PEO-PPO block copolymers are being increasingly evaluated as drugs or growth factors delivery systems suitable to promote the regeneration of injured tissues using minimally invasive procedures [25-28]. Complex formation with poly-αCD may allow forming gels

with greater consistence using lower amounts of block copolymer, and still adequate for being administered using minimally invasive protocols. Vancomycin was incorporated to the supramolecular gels because of its efficiency against Gram-positive bacteria responsible for most osteomyelitis associated to trauma, surgery or implantation [29, 30]. This glycopeptide antibiotic does not form inclusion complexes with αCD and, therefore, quantification of the diffusion coefficient may provide information about the effect of the poly-αCD/block copolymer interactions on the microviscosity of the gels. Composition, structure, stability and cytocompatibility of the supramolecular gels, and drug release performance were evaluated in detail.

5.3 MATERIALS AND METHODS

5.3.1 Materials

α-Cyclodextrin (αCD, Mw = 973, Cavamax®) was from Wacker (Burghausen, Germany); vancomycin HCl from Roig Farma (Barcelona, Spain); epichlorohydrin (EP), deuterium oxide (D_2O) and Pluronic® 127 (F127, 12600 Da; 200 EO units and 69 PO units) from Sigma Aldrich (St. Louis, MO, USA); Tetronic® 908 (T908, 25000 Da, 456 EO units and 80 PO units) from BASF Corporation (Ludwigshafen, Germany); and polyethylene glycol 6000 (PEG, 136 EO units) from Merck (Frankfurter, Germany). Purified water (resistivity >18 MΩ cm, MilliQ®, Millipore, Madrid, Spain) was obtained by reverse osmosis. All other reagents were of analytical grade.

5.3.2 Synthesis of α-CD polymers (poly-αCD)

Water soluble of poly-αCDs were prepared by crosslinking of αCD with EP in alkaline medium following the method proposed by Renard et al. [11] for poly-βCD. Briefly, 10 g αCD were dissolved in 20 mL NaOH solution of various concentrations (Table 5.1), and left overnight at 30 ºC under mechanical stirring. The desired amount of EP was rapidly added to the solution under vigorous stirring. The reaction was stopped in the vicinity of the gelation point (ca. 3 h) by adding acetone (25 mL), and the mixture was transferred to a decantation

ampoule. After decantation, the denser aqueous phase was recovered. The pH was adjusted to 10 with HCl (6 N). The turbid solution was left under stirring at 50 ºC overnight. After cooling, the solution was neutralized with HCl (6 N) and dialyzed (MWCO 3500 Da) against purified water, and the polymers were recovered by freeze-drying. Poly-αCDs are designed as P*x*αCD*y* where *x* refers to EP/αCD molar ratio and *y* to NaOH/αCD molar ratio (Table 5.1).

One-dimensional ^{1}H NMR analysis of poly-αCDs was carried out in a Bruker DRX-500 operating at 500 MHz at 298 K. The poly-αCD solutions were prepared by dissolving appropriate amounts of polymer in D_2O and then transferred to 5 mm diameter NMR tubes. The chemical shifts (δ) were referenced to the residual water signal (4.7 ppm). The mass fractions of CD and EP in the polymers were calculated from the integral areas of protons.

5.3.3 Poly-αCD molecular weight

Static light scattering (SLS) intensities of 1 to 50 g/L polymer solutions were measured at 25 °C by means of an ALV-5000F (ALV-GmbH, Germany) instrument with vertically polarized incident light (λ= 488 nm) supplied by a CW diode-pumped Nd:YAG solid-state laser (Coherent Inc., Santa Clara, CA) operated at 2 W. The intensity scale was calibrated against scattering from toluene. Measurements were made at the scattering angle of 90° to the incident beam. Solutions were equilibrated at the chosen temperature for 30 min before making a measurement. Experiment duration was in the range 5-15 min and each experiment was repeated at least twice. To eliminate dust, samples were filtered through Millipore Millex filters (Triton free, 0.22 μm pore size).

5.3.4 Supramolecular assemblies

Poly-αCD, T908, F127 and PEO solutions were separately prepared by dissolving the required amount of each component in phosphate buffer pH 7.4. The solutions were mixed at different volume ratios to obtain systems comprising 20-40% w/v poly-αCD and 10-15% w/v PEO-based polymer, vortexed for few minutes, stored at 20 ºC and observed after 24 h.

5.3.5 Quantification of αCD

The phenol-sulfuric acid colorimetric assay [31] was used for quantification of αCD in the two-phases of the supramolecular gels. An aliquot of each phase (0.05-0.10 mL) was diluted in 5 mL phosphate buffer pH 7.4. Then, two milliliters of the diluted solution were poured into a glass test tube with 50 μL of phenol 80% w/w and vortexed. Then 5 mL of concentrated sulfuric acid were added rapidly (the stream of acid being directly poured against the liquid surface in order to obtain good mixing). The tubes were allowed to stand for 30 minutes at room temperature. The absorbance of the yellow-orange color was measured in a UV spectrophotometer at 486 nm (Agilent 8453, Waldbronn, Germany). The blank was prepared replacing the αCD solution by phosphate buffer pH 7.4. All solutions were prepared in triplicate.

5.3.6 X-ray diffractometry

X-ray powder diffraction spectra of αCD and poly-αCD were obtained at room temperature on a Philips PW 1710 apparatus (voltage 40 kV, current 30 mA) using Ni-filtered CuKα radiation, from 2 to 65 °2θ at a speed of 0.02° per second. Diffractograms of supramolecular gels were similarly recorded in an Empyrean X-ray diffractometer (voltage 45 kV, current 40 mA). The samples were placed in a glass capillary with 1 mm of diameter and 80 mm in length and the spectra were obtained using a PIXcel3D detector with a scanning from 2 to 65° 2θ at a speed of 0.02° per second.

5.3.7 DOSY-NMR spectroscopy

The diffusion coefficients of the polymers and the supramolecular assemblies were obtained from a single- or double-exponential nonlinear least-squares fitting of the echo attenuation decay. Peak intensities were monitored for all diffusion analyses. Diffusion coefficients were determined by fitting to the equation of Stejskal-Tanner [32, 33]:

$$I(K) = I(0) \cdot exp\,[-K^2 \cdot D\,(\Delta - \delta/3)]$$

In this expression, *I(K)* and *I(0)* are the signal intensities obtained with gradient strengths of *K* (= $\gamma g \delta$, in which γ represents the gyromagnetic ratio, g the gradient strength, δ the gradient pulse width) and *0*, respectively, *D* is the diffusion coefficient, and Δ is the diffusion time during which the diffusion is being monitored [32, 34].

Two-dimensional diffusion ordered spectroscopy (DOSY) studies were acquired in a 750 MHz Varian Inova NMR instrument at 298 K using the bipolar pulse pair and longitudinal eddy current delay (BPP-LED) sequence [35]. Field gradient calibration was performed using the self-diffusion coefficient of water at 298 K. The gradients were applied for 4 ms (δ), and the diffusion time (Δ) was varied from 0 to 450 ms. Gradient settling times were 0 ms, and the eddy current elimination duration was 500 μs. The gradients (g) were increased from 2 to 54 G/cm in 15 steps, resulting in an attenuation of the molecule resonances to approximately 10% of their original intensities. All DOSY experiments were constructed by assuming monoexponential diffusion decay for all chemical shifts [33].

5.3.8 Cytocompatibility screening

5.3.8.1 Osteoblasts viability

SAOS-2 human osteogenic sarcoma cells (HTB-85, LGD Standards, ATCC, Manassas, VA) were cultured in D-MEM supplemented with 10% w/v fetal bovine serum and gentamicine (0.1 mg/mL). MTT assay was carried out according to the Cell Proliferation Kit (Roche, Barcelona, Spain). Cells (200,000 per well; 1.5 mL) were seeded in 24-well plates to which 200 μL of autoclaved poly-αCD solutions at 20, 40 and 80% were added. Supramolecular assemblies of 40% poly-αCD with 10% of T908, F127 or PEG were also tested. Cells cultured in the medium under the same conditions, but without adding polymers were used as controls. The plates were incubated at 37 ºC for 24 h or 72 h. All experiments were performed in triplicate.

5.3.8.2 HET-CAM assay

Fertilized broiler chicken eggs (Avirojo, Pontevedra, Spain) were incubated at 37.0 ± 0.1 ºC and 60.0 ± 0.6% relative humidity (Ineltec CCSP0150 Tona, Barcelona, Spain) for 8 days to being rotated five times per day. Then and according the ICCVAM-recommended hen's egg test-chorioallantoic membrane test (HET-CAM) method [36], the upper part of the eggshell (air cell) was removed using a rotary saw (Dremel 300, Breda, The Netherlands) and the intact inner membrane of the eggs was moistened with 0.9% w/v NaCl solution for 30 min and then detached with a forceps. Aliquots of poly-αCD supramolecular gels (300 µL at 25 ºC) were placed on the chorioallantoic membrane of the egg and the irritation potential was monitored for 5 min. The experiments were carried out in triplicate. Negative (0.9% NaCl solution) and positive (0.1 N NaOH) controls were performed under the same conditions. Irritation scores (IS) were calculated from the time (in seconds) at which hemorrhage, lysis (L) or coagulation (C) started, as follows [36]:

$$\text{IS} = \left[\left(\frac{301 - H_{time}}{300}\right) \text{x } 5 + \left(\frac{301 - L_{time}}{300}\right) \text{x } 7 + \left(\frac{301 - C_{time}}{300}\right) \text{x } 9\right]$$

According to the IS values, the materials can be classified as non-irritating (0–0.9), weakly irritating (1-4.9), moderately irritating (5-8.9) or severely irritating (9-21) [36].

5.3.9 Vancomycin loading

Vancomycin hydrochloride (5.5 mg/mL) was dispersed in 10% w/v of T908, F127 and PEG solutions in phosphate buffer pH 7.4 before mixing with 40% poly-αCD to form the supramolecular gels. Drug solutions (5.5 mg/mL) were also prepared in plain buffer pH 7.4, 20% αCD, and in 20%, 40% and 80% poly-αCD solutions in the same buffer. The drug release from the solutions and the gels was evaluated at 37 ºC using Franz-Chien cells. The donor compartment was filled with 1 gram of formulation, and the receptor compartment with 7 mL of phosphate buffer pH 7.4. The compartments were separated by a cellulose acetate membrane filter (0.45 µm, Albet®, Barcelona, Spain). The surface available for

diffusion was 0.785 cm^2. At various times, 0.7 mL aliquots were withdrawn from the receptor compartment, the absorbance was measured at 280 nm (Agilent 8453, Waldbronn, Germany) and the amount of drug released was determined. During the first three hours of the test, the aliquots were immediately returned to the receptor compartment. Beyond that time, the sample aliquots were replaced by fresh medium. Diffusion coefficients were estimated by fitting the Higuchi equation [37]:

$$\frac{Q}{A} = 2C_0\left(\frac{Dt}{\pi}\right)^{1/2}$$

where Q is the amount of vancomycin (mg) released at time t (s), A is diffusion area (cm^2), C_0 is the initial concentration of vancomycin in the formulation (mg/mL), and D is the diffusion coefficient (cm^2/s) [37].

5.4 RESULTS AND DISCUSSION

5.4.1 Synthesis and characterization of poly-αCD

αCD possesses hydroxyl groups at the 2-, 3- and 6-positions in the glucose unit, whose reactivities depend on reaction variables such as temperature and alkalinity [13, 38]. The hydroxyl groups allow direct substitution reaction and/or chemical modification to different positions yielding a variety of derivatives when react with EP (Figure 5.1). The starting experimental conditions for preparing poly-αCD were similar to those previously described for water-soluble high molecular weight polymers of βCD and γCD [11, 39]. Conditions of poly-αCD reaction are summarized in Table 5.1.

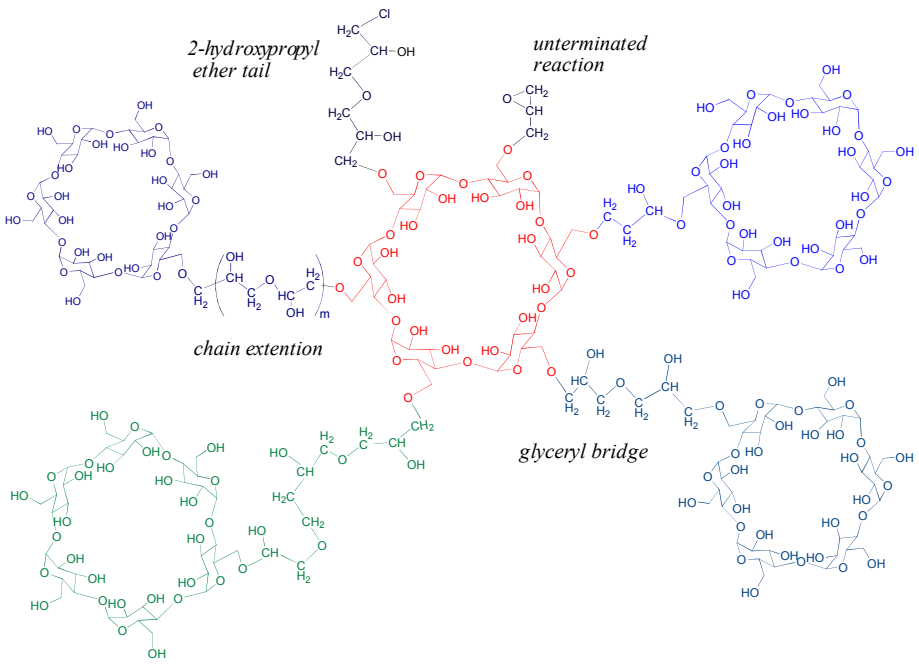

Figure 5.1. Possible structures of poly-αCD obtained through polycondensation with EP. For the sake of clarity, only the reaction on the primary alcohols is shown in the central αCD.

The synthesis was carried out at 30 ºC in order to control the polycondensation rate and to be able to stop the reaction with EP before a three-dimensional insoluble network was formed [11]. The reaction time was fixed at 180 minutes. First, EP/αCD molar ratio was fixed and

the influence of NaOH concentration was investigated. EP/αCD molar ratio above 10 led to insoluble polymers after a few minutes of reaction. For EP/CD molar ratio < 10 and NaOH/αCD molar ratio < 10, highly water soluble polymers (solubility > 80% w/w) were obtained. The synthesis yield varied between 26.5% and 61.5%; the higher yield corresponded to EP/αCD molar ratio equals to 7 and NaOH/αCD molar ratio of 7.3. The polymer obtained under these latter conditions, P7αCD15, resulted to have the largest content in αCD as determined by means of ^{1}H NMR.

Table 5.1. Experimental conditions for poly-αCD synthesis, yield and content in αCD.

Polymer code	NaOH (% w/v)	NaOH/CD (mol/mol)	EP/αCD (mol/mol)	Soluble polymer yield (%)	%CD (^{1}H RMN)
P5αCD33	33	16.1	5	Insoluble	_
P5αCD25	25	12.2	5	Insoluble	_
P5αCD20	20	9.7	5	26.5	59.2
P5αCD15	15	7.3	5	50.3	57.5
P5αCD10	10	4.9	5	29.2	58.8
P7αCD33	33	16.1	7	Insoluble	_
P7αCD25	25	12.2	7	Insoluble	_
P7αCD20	20	9.7	7	51.9	57.1
P7αCD15	15	7.3	7	61.5	72.8
P7αCD10	10	4.9	7	45.1	53.8
P10αCD33	33	16.1	10	Insoluble	_
P10αCD25	25	12.2	10	Insoluble	_
P10αCD20	20	9.7	10	Insoluble	_
P10αCD15	15	7.3	10	Insoluble	_
P10αCD10	10	4.9	10	Insoluble	_

^{1}H NMR spectra of unmodified αCD and poly-αCD (P7αCD15) are shown in Figure 5.2. The spectra showed one peak ca. 5 ppm assigned to the C_1-OH of the glucose unit. Polymerization caused a broadening of the peaks between 4.1 to 3.4 ppm, corresponding to protons from C-OH (2, 3, 4, 5 and 6) of the αCD and hydrogen atoms due to EP. ^{1}H NMR analysis demonstrated that substitution simultaneously occurred at different hydroxyl groups of natural αCD. The determination of the structure of poly-αCD is rather complex due to the polyfunctionality of CDs with three possible positions C_2-OH, C_3-OH and C_6-OH on which the substitution can take place, and by the fact that EP can react with grafted (2-hydroxypropyl)ether residues leading to polytails and polybridges [11, 40] (Figure 5.1). It has been shown that high NaOH concentration leads to substitution mainly on one side, favoring a long 2-hydroxypropyl ether segment while a low NaOH concentration makes the substitution on the two sides of the cavity possible [11, 40]. As shown in Figure 5.2, for NaOH/αCD molar ratio < 10 the reaction with EP occurs at the three hydroxyl groups positions (Figure 5.2b).

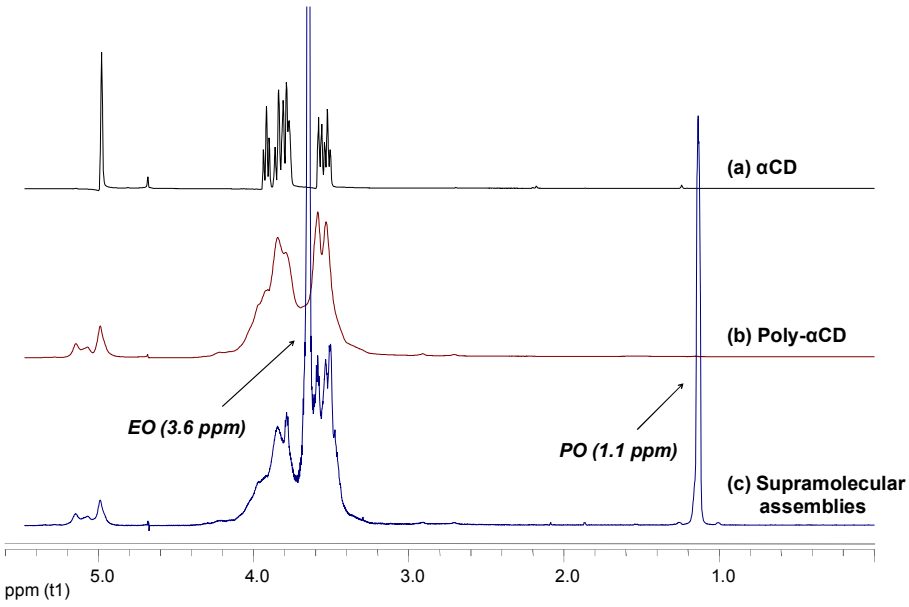

Figure 5.2. ^{1}H RMN spectra of αCD (a), poly-αCD (b) and supramolecular assemblies of 40% poly-αCD with 10% T908 (c) dissolved in D_2O.

5.4.2 Poly-αCD molecular weight

Static light scattering (SLS) was used to determine the mass-weighted molecular weight of P7αCD15. The intensity of scattered light depends on molecular weight of the particle and, thus, light scattering provides information about the weight average molecular weight. Poly-αCD dispersion of concentrations ranging from 1 to 50 $g.L^{-1}$ were measured. The dissymmetry of light scattering from solution at 45º and 135º, I_{45}/I_{135}, in SLS was shown to be 1.03 or less, which is consistent with polymeric chains with relatively small radii of gyration and corresponds to a correction factor less than 3% to the 90º scattering intensity [41, 42]; consequently, scattering intensities measured at 90° were used without correction for intraparticle interference. The basis for analysis of SLS intensities was the Debye equation:

$$K^*c/(I-I_S) = 1/M_W + 2A_2c + \ldots...$$

where I is the intensity of light scattering from solution relative to that from toluene, I_S, c is the concentration (in $g.dm^{-3}$), M_W is the mass-average molar mass of the solute, A_2 is the second virial coefficient (higher coefficients being neglected), and K^* is the appropriate optical constant ($K^* = 4\pi^2(dn/dc)^2 n_{ref}^2/(R_{ref}N_a\lambda^4)$. Values of the specific refractive index increment, dn/dc, were determined to be 0.13413 ± 0.00001 $m^3 \cdot Kg^{-1}$ by analyzing the concentration dependence of the polymer refractive index by using a refractometer RA-510M (Mettler-Toledo, Spain). Other quantities used were the Rayleigh ratio of toluene for vertically polarized light, R_{ref} = $2.57 \cdot 10^{-5}$ $[1+3.68 \cdot 10^{-3}(t-25)]$ cm^{-1} (t in °C) and the refractive index of toluene, $n_{ref} = 1.4969 \cdot [1-5.7 \cdot 10^{-4}(t-20)]$.

The Debye plot of P7αCD15 is shown in Figure 5.3. The red line corresponds to the fit of experimental points to the Debye equation. From the intercept, the mass-weighted molecular mass of the poly-αCD chains was determined to be 454,750 ± 11,850 $g \cdot mol^{-1}$.

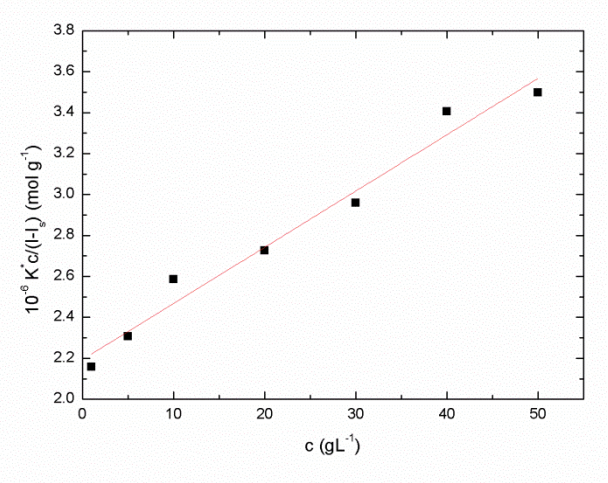

Figure 5.3. Debye plot for aqueous solutions of poly-αCD (P7αCD15) at 25 ºC.

5.4.3 Formation of supramolecular gels

Three different PEO-based polymers were tested regarding their interaction with poly-αCD, namely PEG (136 EO units/molecule), Pluronic F127 (200 EO units/molecule) and Tetronic 908 (456 EO units/molecule). The X-shaped architecture of Tetronic 908 can be considered to be formed by two pluronic chains bound each other by means of an ethylenediamine group.[43] When solutions of these polymers were mixed with P7αCD15 solution, the systems rapidly evolved to turbid dispersions (Figure 5.4), suggesting the formation of poly(pseudo)rotaxanes-like structures. Phase separation was observed after 1 hour. This behavior occurred for systems comprising 20% or 40% poly-αCD and 10% or 15% PEO-based polymers. The αCD:EO molar ratio in each supramolecular system was calculated from the number of moles of αCD given as follows [9]:

$$N_{\alpha CD} = \frac{m_{poly-\alpha CD} \cdot 0.7}{M_{\alpha CD}}$$

where N is the number of αCD cavities (molar), $m_{poly\text{-}\alpha CD}$ is the mass of poly-αCD used in the supramolecular gels, 0.7 is the αCD content in the polymer and M the molar mass of αCD. The molar ratios ranged between 0.06 and 0.18.

It has been previously shown that after incorporation of pristine αCD to PEO copolymers solutions, insoluble poly(pseudo)rotaxanes are initially formed and then the systems increase their consistency as αCDs threaded along adjacent PEO chains self-organize [25, 44, 45]. This seems to be also the case of P7αCD15/PEO-based polymer systems as indicated by the two well-defined peaks in the 3.7-4.0 ppm region of the ^{1}H NMR spectrum of 40% poly-αCD/10% T908 system, which correspond to the interaction between the EO blocks (*-O-CH_2-CH_2* at 3.6 ppm) and the αCD cavities (Figure 5.2c).

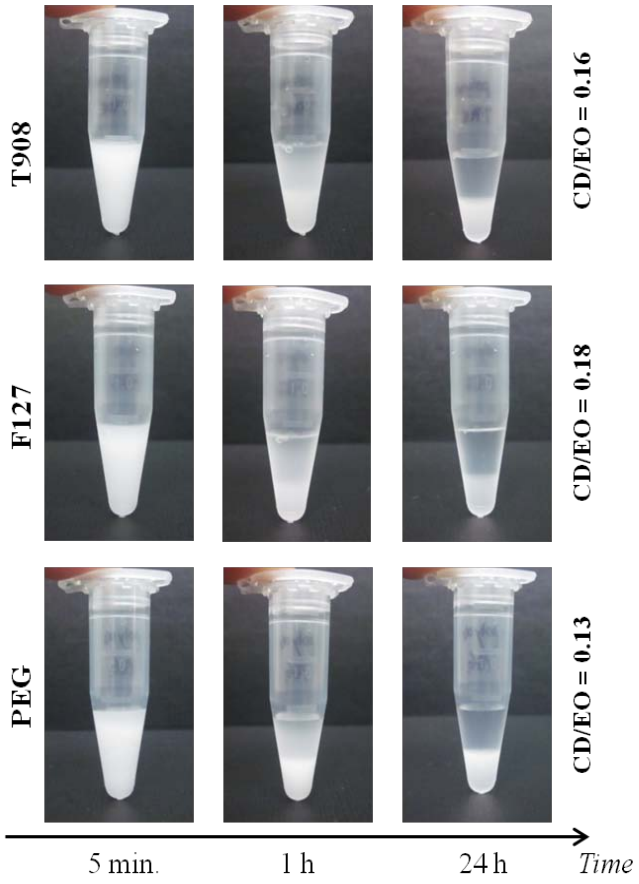

Figure 5.4. Evolution at 25 ºC of the appearance of systems comprising 40% poly-αCD and 10% T908, F127 or PEG (CD/EO molar ratios on the right axis).

The content of αCD in each phase was determined by means of the phenol-sulfuric acid colorimetric method [31]. Most αCD (> 80%) concentrated in the gel phase, particularly in the case of systems prepared with 40% poly-αCD (near 90%) (Figure 5.5).

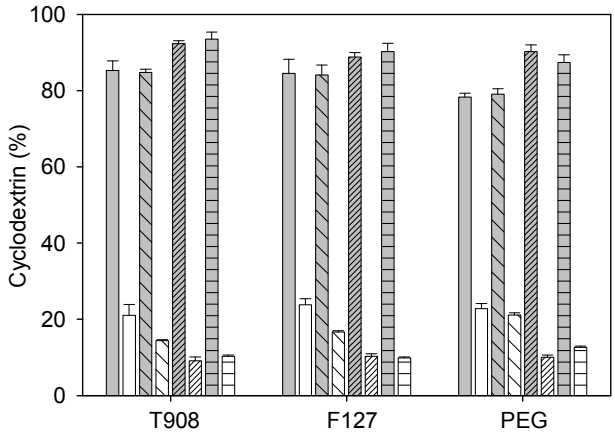

Figure 5.5. Partitioning of αCD between the gel phase (grey bars) and the sol phase (white bars) of the systems comprising 20% poly-αCD/10% PEO-based polymer (non-stripped bar), 20% poly-αCD/15% PEO-based polymer (coarse stripped bar), 40% poly-αCD/10% PEO-based polymer (fine stripped bar), and 40% poly-αCD/15% PEO-based polymer (horizontally stripped bar), as determined by the phenol method after 24 h of preparation of the gels.

To gain an insight into the structure of the complexes of poly-αCD with PEO-based polymers, X-ray analysis was carried out (Figure 5.6). Differently, from the herringbone packing of pristine αCD, powdered poly-αCD was not crystalline. As previously observed, poly(pseudo)rotaxanes of pristine αCD and T908 showed the typical diffractogram of hexagonal columnar channels of the aligned αCDs in the crystalline phase (Figure 5.6c) [24, 26]. Interestingly, freshly mixed poly-αCD/T908 systems after 1 h of storage did not exhibit any peak although they were already turbid. After 24 h, the gel phase showed a broad band (Figure 5.6e) that indicates the formation of poly(pseudo)rotaxanes with more complicated structure than those of pristine αCD/T908, as previously observed for PEG- and αCD-grafted dendrimers [24]. Interactions of PEO-blocks with poly-αCDs can occur through αCDs

placed in the same or in different poly-αCD species, making the attainment of ordered packing structures more difficult.

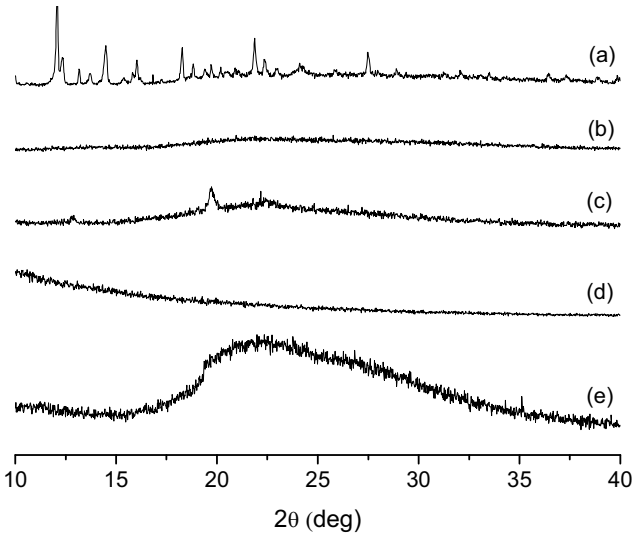

Figure 5.6. Power X-ray diffraction patterns of αCD alone (a), poly-αCD alone (b), 20% αCD + 10% T908 gel (c), 10% poly-αCD + 10% T908 after 1 h of preparation (d), 10% poly-αCD + 10% T908 gel after 24h of preparation (e).

Complex formation of poly-αCD/PEO-based polymers was also investigated by DOSY [45]. This method relies on the differences in the self-diffusion coefficients of diffusing species: a molecule in aggregate or bound state diffuses slower that in free state [46-48]. Free poly-αCD exhibited a lower diffusion coefficients than pristine αCD ($5.59 \cdot 10^{-11}$ $m^2 \cdot s^{-1}$ *vs.* $28 \cdot 10^{-11}$ $m^2 \cdot s^{-1}$), as expected from its larger molecular weight. PEG diffused faster and T908 diffused slower than poly-αCD (Figure 5.7; Table 5.2). The diffusion of the poly-αCD decreased more in presence of the PEO-based polymers, confirming the formation of supramolecular aggregates [34, 48]. Nevertheless, in the supramolecular systems of poly-αCD with PEG, all poly-αCD peaks exhibited similar diffusion coefficient (ca. $4.86 \cdot 10^{-11}$ $m^2 \cdot s^{-1}$), while the PEG peak showed a slightly greater diffusion coefficient ($6.02 \cdot 10^{-11}$ $m^2 \cdot s^{-1}$) suggesting that PEG was only partially threaded by poly-αCDs.

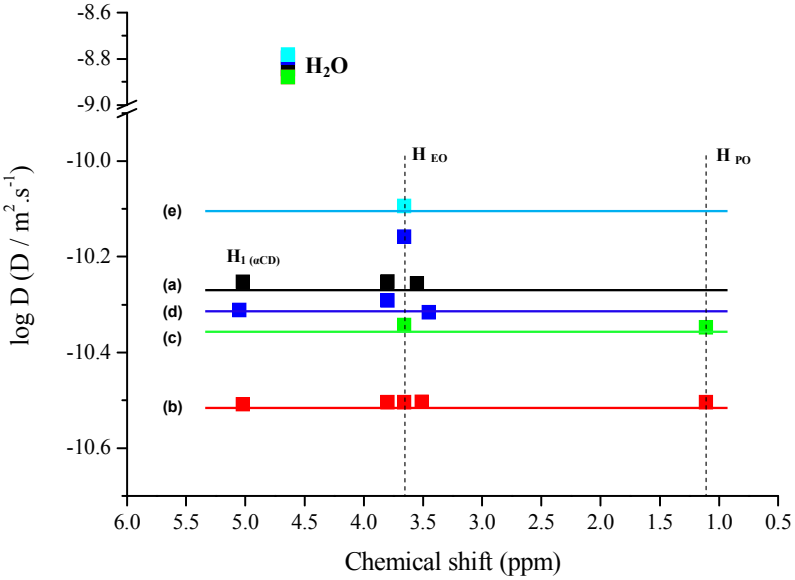

Figure 5.7. 2D DOSY spectra of (a) poly-αCD at 40%, (b) 40% poly-αCD + 10% T908, (c) 10% T908, (d) 40% poly-αCD + 10% PEG and (e) PEG at 10% in D_2O at 289 K.

Table 5.2. Diffusion coefficients determined from NMR-DOSY data at 25 ºC. Mean values and, in parenthesis, standard deviations (n=3).

Compound in D_2O	Concentration % (w/v) in D_2O	D x10^{11} ($m^2.s^{-1}$)	D_{D2O} x10^{11} ($m^2.s^{-1}$)
αCD	0.97	28 [a)]	[b)]
Poly-αCD	3.64	5.59 (0.09)	142.91 (0.14)
T908	0.91	4.05 (0.07)	178.06 (0.09)
Poly-αCD + T908	3.64+0.91	3.11 (0.05)	131.82 (0.15)
PEG	0.91	8.05 (0.06)	212.23 (0.16)
Poly-αCD + PEG	3.64+0.91	4.86 (0.04)	206.56 (0.11)
		6.02 (0.10)	

[a)] From reference [34]; [b)] From reference [49].

5.4.4 Cytocompatibility screening

Preliminary biocompatibility of autoclaved poly-αCD solutions at 20, 40 and 80% and supramolecular assemblies of 40% poly-αCD with 10% of T908, F127 or PEG was assessed by recording the cytocompatibility against SAOS-2 cells (human osteoblast cell line) for 72 h and the compatibility with the chorioallantoic membrane (HET-CAM test). The viability of SAOS-2 cells after 24 h was above 80%, except for 80% poly-αCD and supramolecular systems comprising 40% poly-αCD and 10% PEG (Figure 5.8). At 72 h a decrease in cell viability was observed, although it still remained above 60%. The most cytocompatible systems resulted to be 20% poly-αCD and 40% poly-αCD with 10% T908.

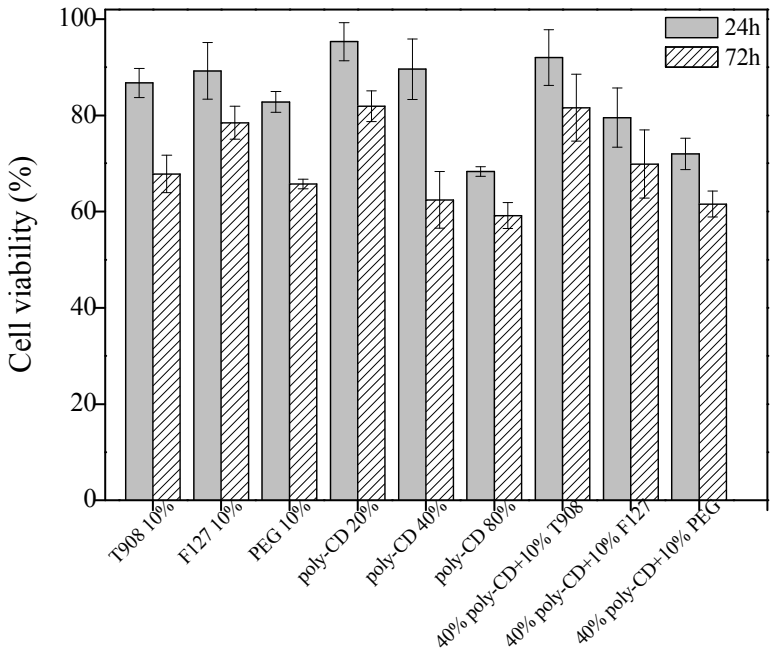

Figure 5.8. Viability of SAOS-2 cells in contact with PEO-based polymer solutions at 10%, poly-αCD solutions at 20, 40 and 80%, and supramolecular assemblies of 40% poly-αCD with 10% of T908, F127 or PEG.

The HET-CAM test is a subrogate fast and cheap test that provides measurable indices of the biocompatibility of a material [50, 51]. The IS values obtained for negative (0.9% NaCl) and positive (NaOH 0.1 N) controls were 0.0 (Figure 5.9a) and 17.8 ± 0.2 (Figure 5.9b), respectively. Free αCD (20% w/v) also led to IS = 0. None of poly-αCD solutions (up to 80%, Figure 5.9c) and supramolecular gels (Figure 5.9d) induced hemorrhage, lysis or coagulation, as previously observed for poly(pseudo)rotaxanes of αCD-T908 [25]. Therefore, the cross-linking of αCD with EP and the gel formation at high concentrations do not have detrimental effects on cytocompatibility.

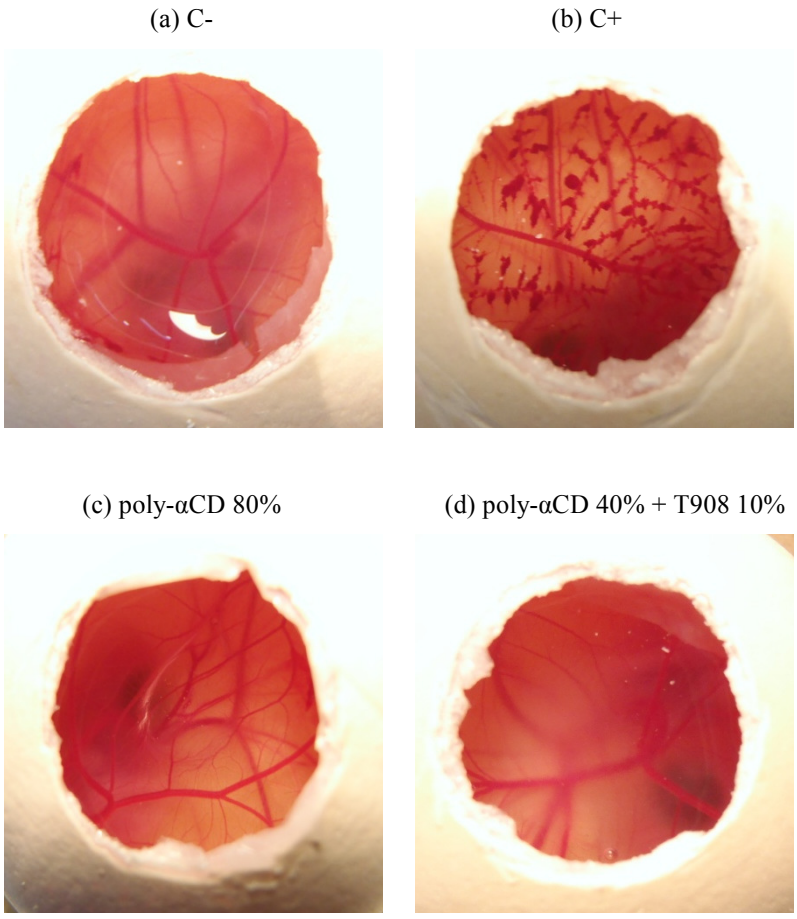

Figure 5.9. HET-CAM test results of negative (a) and positive (b) controls, and poly-αCD 80% dispersions (c) and poly-αCD-T908 40:10% gels (d) prepared in PBS at pH 7.4.

5.4.5 Vancomycin loading and release

Vancomycin hydrochloride easily dissolved either in poly-αCD or PEO-based polymer solutions in phosphate buffer pH 7.4. In the case of the supramolecular gels, the drug was incorporated to the T908, F127 and PEG solutions before mixing with poly-αCD. Drug release from 10% T908, F127 or PEG solutions and from 20% αCD solutions was completed in 24 h (Figure 5.10). Vancomycin diffusion was slower from poly-αCD solutions; the large molecular weight and lower mobility of poly-αCD make the movement of drug molecules difficult. The slowest release was observed for the supramolecular gels, with release profiles that fitted well to the square-root kinetics [37]. Those prepared with 40% poly-αCD and 10% PEO-based polymer sustained the release for several days and showed one order of magnitude lower drug diffusion coefficients than those recorded for the free drug in the buffer (Table 5.3).

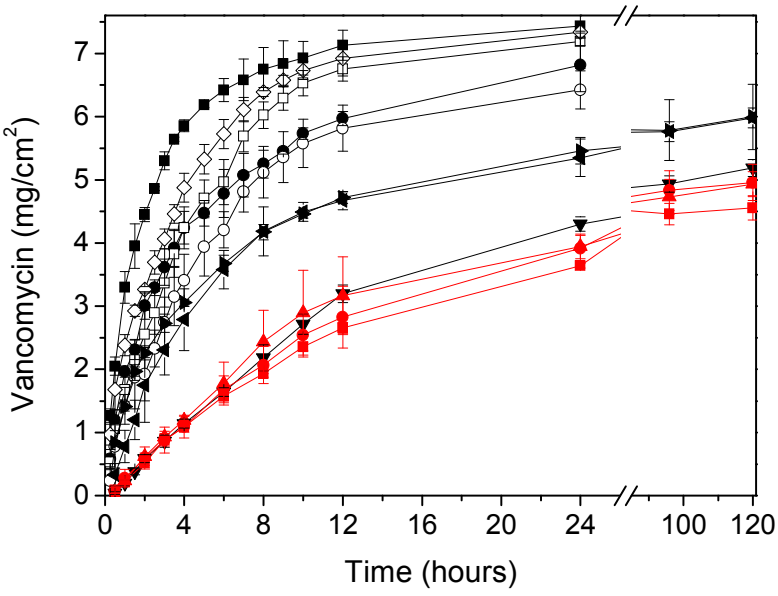

Figure 5.10. Vancomycin release at 37 ºC from PBS pH 7.4 (■), 20% αCD (●), 10% T908 (□), 10% F127 (○), 10% PEG (◊), and 20% (►), 40% (◄) and 80% (▼) poly-αCD dispersions, and 40% poly-αCD with 10% T908 (■), 10% F127 (●) and 10% PEG (▲) supramolecular gels.

Microviscosity values, estimated from the drug diffusion coefficients [52], indicated that supramolecular gels offer 10-fold and 2-fold enhanced resistance against drug diffusion compared to the solvent and the poly-αCD solely systems respectively (Table 5.3).

Table 5.3. Vancomycin diffusion coefficients from PEO-based polymers, αCD and poly-αCD solutions and from poly-αCD/PEO-based polymer supramolecular gels, obtained by fitting the Higuchi equation to the release profiles ($R^2 > 0.973$). Mean values and, in parenthesis, standard deviations (n=3).

Systems	αCD/EO ratio molar	D x10^5 (cm^2/s)	Microviscosity (mPa·s)
PBS	_	6.74 (0.84)	0.707
10% T908	_	4.19 (0.39)	1.14
10% F127	_	3.08 (0.81)	1.55
10% PEG	_	4.42 (0.79)	1.08
20% αCD	_	3.84 (0.67)	1.24
20% poly-αCD	_	2.26 (0.11)	2.11
40% poly-αCD	_	1.39 (0.02)	3.43
80% poly-αCD	_	0.96 (0.07)	4.96
20% poly-αCD + 10% T908	0.080	1.53 (0.09)	3.11
40% poly-αCD + 10% T908	0.160	0.59 (0.02)	8.08
40% poly-αCD + 10% F127	0.184	0.69 (0.08)	6.91
40% poly-αCD + 10% PEG	0.129	0.79 (0.12)	6.04

5.5 CONCLUSIONS

A soluble poly-αCD with high molecular weight (P7αCD15) was synthesized using NaOH/αCD molar ratio < 10 and EP/αCD molar ratio < 10. Mixing of P7αCD15 (20-40%) solutions with PEG, Pluronic F127 or Tetronic 908 solutions resulted in rapidly evolving turbid dispersions, indicating the formation of poly(pseudo)rotaxane structures. Phase separation was observed after 1 hour; αCD being concentrated in the gel phase. The formation of supramolecular aggregates was confirmed by DOSY as a decrease in the diffusion coefficient of poly-αCD. The resultant supramolecular systems exhibited good cytocompatibility and notably reduced vancomycin diffusion rate, rendering sustained profiles for several days under physiological mimicking conditions.

5.6 REFERENCES

[1] Brunsveld, L.; Folmer, B.J.B.; Meijer, E.W.; Sijbesma, R.P. *Supramolecular Polymers*. Chemical Reviews, 2001, 101(12), 4071-4098.

[2] Lehn, J.M. Self-Processes-Programmed Supramolecular Systems, in *Supramolecular Chemistry*. Wiley-VCH Verlag GmbH & Co. KGaA. 2006, 139-197.

[3] van de Manakker, F.; Vermonden, T.; van Nostrum, C.F.; Hennink, W.E. *Cyclodextrin-based polymeric materials: synthesis, properties, and pharmaceutical/biomedical applications.* Biomacromolecules, 2009, 10(12), 3157-3175.

[4] Wenz, G.; Han, B.H.; Müller, A. *Cyclodextrin rotaxanes and polyrotaxanes*. Chemical Reviews, 2006, 106(3), 782-817.

[5] Auzély-Velty, R. *Self-assembling polysaccharide systems based on cyclodextrin complexation: Synthesis, properties and potential applications in the biomaterials field.* Comptes Rendus Chimie, 2011, 14(2–3), 167-177.

[6] Simões, S.M.N.; Veiga, F.; Torres-Labandeira, J.J.; Ribeiro, A.C.F.; Concheiro, A.; Alvarez-Lorenzo, C. *Syringeable self-assembled cyclodextrin gels for drug delivery*. Current Topics in Medicinal Chemistry, 2013, *in press*.

[7] Wintgens, V.; Layre, A.M.; Hourdet, D.; Amiel, C. *Cyclodextrin polymer nanoassemblies: strategies for stability improvement*. Biomacromolecules, 2012, 13(2), 528-534.

[8] Gref, R.; Amiel, C.; Molinard, K.; Daoud-Mahammed, S.; Sébille, B.; Gillet, B.; Beloeil, J.C.; Ringard, C.; Rosilio, V.; Poupaert, J.; Couvreur, P. *New self-assembled nanogels based on host-guest interactions: characterization and drug loading*. Journal of Controlled Release, 2006, 111(3), 316-324.

[9] Daoud-Mahammed, S.; Grossiord, J.L.; Bergua, T.; Amiel, C.; Couvreur, P.; Gref, R. *Self-assembling cyclodextrin based hydrogels for the sustained delivery of hydrophobic drugs*. Journal of Biomedical Materials Research Part A, 2008, 86A(3), 736-748.

[10] Orprecio, R.; Evans, C.H. *Polymer-immobilized cyclodextrin trapping of model organic pollutants in flowing water streams*. Journal of Applied Polymer Science, 2003, 90(8), 2103-2110.

[11] Renard, E.; Deratani, A.; Volet, G.; Sebille, B. *Preparation and characterization of water soluble high molecular weight β-cyclodextrin-epichlorohydrin polymers*. European Polymer Journal, 1997, 33(1), 49-57.

[12] Vélaz, I.; Isasi, J.R.; Sánchez, M.; Uzqueda, M.; Ponchel, G. *Structural characteristics of some soluble and insoluble β-cyclodextrin polymers*. Journal of Inclusion Phenomena and Macrocyclic Chemistry, 2007, 57(1-4), 65-68.

[13] Morin-Crini, N.; Crini, G. *Environmental applications of water-insoluble β-cyclodextrin–epichlorohydrin polymers*. Progress in Polymer Science, 2013, 38(2), 344-368.

[14] Gonzalez, H.; Hwang, S.J.; Davis, M.E. *New class of polymers for the delivery of macromolecular therapeutics*. Bioconjugate Chemistry, 1999, 10(6), 1068-1074.

[15] Renard, E.; Volet, G.; Amiel, C. *Synthesis of a novel linear water-soluble β-cyclodextrin polymer*. Polymer International, 2005, 54(3), 594-599.

[16] Harada, A.; Furue, M.; Nozakura, S. *Cyclodextrin-containing polymers. 1. Preparation of polymers*. Macromolecules, 1976, 9(5), 701-704.

[17] Danel, C.; Azaroual, N.; Chavaria, C.; Odou, P.; Martel, B.; Vaccher, C. *Comparative study of the complex forming ability and enantioselectivity of cyclodextrin polymers by CE and ^{1}H NMR*. Carbohydrate Polymers, 2013, 92(2), 2282-2292.

[18] Concheiro, A.; Alvarez-Lorenzo, C. *Chemically cross-linked and grafted cyclodextrin hydrogels: From nanostructures to drug-eluting medical devices*. Advanced Drug Delivery Reviews, 2013, DOI: 10.1016/j.addr.2013.04.015.

[19] Koopmans, C.; Ritter, H. *Formation of physical hydrogels via host−guest interactions of β-cyclodextrin polymers and copolymers bearing adamantyl groups*. Macromolecules, 2008, 41(20), 7418-7422.

[20] Nielsen, A.L.; Steffensen, K.; Larsen, K.L. *Self-assembling microparticles with controllable disruption properties based on cyclodextrin interactions*. Colloids and Surfaces B: Biointerfaces, 2009, 73(2), 267-275.

[21] Daoud-Mahammed, S.; Ringard-Lefebvre, C.; Razzouq, N.; Rosilio, V.; Gillet, B.; Couvreur, P.; Amiel, C.; Gref, R. *Spontaneous association of hydrophobized dextran and poly-β-cyclodextrin into nanoassemblies.: Formation and interaction with a hydrophobic drug*. Journal of Colloid and Interface Science, 2007, 307(1), 83-93.

[22] Nie, S.; Zhang, S.; Pan, W.; Liu, Y. *In vitro and in vivo studies on the complexes of glipizide with water-soluble β-cyclodextrin–epichlorohydrin polymers*. Drug Development and Industrial Pharmacy, 2011, 37(5), 606-612.

[23] Ni, X.; Cheng, A.; Li, J. *Supramolecular hydrogels based on self-assembly between PEO-PPO-PEO triblock copolymers and α-cyclodextrin*. Journal of Biomedical Materials Research Part A, 2009, 88A(4), 1031-1036.

[24] Motoyama, K.; Hayashida, K.; Higashi, T.; Arima, H. *Polypseudorotaxanes of pegylated α-cyclodextrin/polyamidoamine dendrimer conjugate with cyclodextrins as a sustained release system for DNA*. Bioorganic & Medicinal Chemistry, 2012, 20(4), 1425-1433.

[25] Simões, S.M.N.; Veiga, F.; Torres-Labandeira, J.J.; Ribeiro, A.C.F.; Sandez-Macho, M.I.; Concheiro, A.; Alvarez-Lorenzo, C. *Syringeable Pluronic–α-cyclodextrin*

supramolecular gels for sustained delivery of vancomycin. European Journal of Pharmaceutics and Biopharmaceutics, 2012, 80(1), 103-112.

[26] Harada, A.; Li, J.; Kamachi, M. *The molecular necklace: a rotaxane containing many threaded α-cyclodextrins*. Nature, 1992, 356(6367), 325 - 327.

[27] Doğan, A.; Yalvaç, M.E.; Şahin, F.; Kabanov, A.V.; Palotás, A.; Rizvanov, A.A. *Differentiation of human stem cells is promoted by amphiphilic pluronic block copolymers*. International Journal of Nanomedicine, 2012, 7(1), 4849-4860.

[28] Rey-Rico, A.; Silva, M.; Couceiro, J.; Concheiro, A.; Alvarez-Lorenzo, C. *Osteogenic efficiency of in situ gelling poloxamine systems with and without bone morphogenetic protein-2*. European Cells and Materials, 2011, 21, 317-340.

[29] Jobson, S.; Moise, P.A.; Eskandarian, R. *Retrospective observational study comparing vancomycin versus daptomycin as initial therapy for Staphylococcus aureus infections*. Clinical therapeutics, 2011, 33(10), 1391-1399.

[30] Marculescu, C.E.; Osmon, D.R. *Antibiotic prophylaxis in orthopedic prosthetic surgery*. Infectious Disease Clinics of North America, 2005, 19(4), 931-946.

[31] Dubois, M.; Gilles, K.A.; Hamilton, J.K.; Rebers, P.A.; Smith, F. *Colorimetric method for determination of sugars and related substances*. Analytical Chemistry, 1956, 28(3), 350-356.

[32] Stejskal, E.O.; Tanner, J.E. *Spin diffusion measurements: spin echoes in the presence of a time-dependent field gradient*. Journal of Chemical Physics, 1965, 42(1), 288-292.

[33] Morris, K.F.; Johnson, C.S. *Resolution of discrete and continuous molecular size distributions by means of diffusion-ordered 2D NMR spectroscopy*. Journal of the American Chemical Society, 1993, 115(10), 4291-4299.

[34] Chwalek, M.; Auzely, R.; Fort, S. *Synthesis and biological evaluation of multivalent carbohydrate ligands obtained by click assembly of pseudo-rotaxanes*. Organic & Biomolecular Chemistry, 2009, 7(8), 1680-1688.

[35] Wu, D.H.; Chen, A.D.; Johnson, C.S. *An improved diffusion-ordered spectroscopy experiment incorporating bipolar-gradient pulses*. Journal of Magnetic Resonance, Series A, 1995, 115(2), 260-264.

[36] NICEATM-ICCVAM In vivo test methods for detecting ocular corrosives and severe irritants. See http://iccvam.niehs.nih.gov/methods/ocutox/ivocutox.htm, accessed on February 2013.

[37] Higuchi, W.I. *Analysis of data on the medicament release from ointments*. Journal of Pharmaceutical Sciences, 1962, 51(8), 802-804.

[38] Boger, J.; Corcoran, R.J.; Lehn, J.M. *Cyclodextrin chemistry. Selective modification of all primary hydroxyl groups of α- and β-cyclodextrins*. Helvetica Chimica Acta, 1978, 61(6), 2190-2218.

[39] Wintgens, V.; Amiel, C. *Water-soluble γ-cyclodextrin polymers with high molecular weight and their complex forming properties*. European Polymer Journal, 2010, 46(9), 1915-1922.

[40] Renard, E.; Sebille, B.; Barnathan, G.; Deratani, A. *Polycondensation of cyclodextrins with epichlorohydrin. Influence of reaction conditions on the polymer structure*. Macromolecular Symposia, 1997, 122(1), 229-234.

[41] Casassa, E.F. *Particle scattering factors in rayleigh scattering*. In: Polymer Handbook. J. Brandrup, E.H. Immergut, and E.A. Grulke Editors, Wiley, New York. 1999, 629-636.

[42] Beattie, W.H.; Booth, C. *Table of dissymmetries and correction factores for use in light scattering*. The Journal of Physical Chemistry, 1960, 64(5), 696-698.

[43] Alvarez-Lorenzo, C.; Rey-Rico, A.; Brea, J.; Loza, M.I.; Concheiro, A.; Sosnik, A. *Inhibition of P-glycoprotein pumps by PEO–PPO amphiphiles: branched versus linear derivatives*. Nanomedicine, 2010, 5(9), 1371-1383.

[44] Harada, A.; Kamachi, M. *Complex formation between poly(ethylene glycol) and α-cyclodextrin*. Macromolecules, 1990, 23(10), 2821-2823.

[45] Simoes, S.M.N.; Veiga, F.; Torres-Labandeira, J.J.; Ribeiro, A.C.F.; Concheiro, A.; Alvarez-Lorenzo, C. *Poloxamine-cyclodextrin-simvastatin supramolecular systems promote*

osteoblast differentiation of mesenchymal stem cells. Macromolecular Bioscience, 2013, DOI: 10.1002/mabi.201300017.

[46] Cohen, Y.; Avram, L.; Frish, L. *Diffusion NMR spectroscopy in supramolecular and combinatorial chemistry: an old parameter-new insights*. Angewandte Chemie International Edition, 2005, 44(4), 520-554.

[47] Jia, L.; Guo, C.; Xiang, J.; Wang, N.; Yang, L.; Tang, Y.; Liu, H. *Interaction between PEO–PPO–PEO copolymers and a hexapeptide in aqueous solutions*. Langmuir, 2011, 28(3), 1725-1732.

[48] Jiang, Y.; Wu, J.; He, L.; Tu, C.; Zhu, X.; Chen, Q.; Yao, Y.; Yan, D. *Construction of polyrotaxanes via reversible chain exchange between acylhydrazone bonds*. Chemical Communications, 2008, 21(47), 6351-6353.

[49] Mills, R. *Self-diffusion in normal and heavy water in the range 1-45.deg*. The Journal of Physical Chemistry, 1973, 77(5), 685-688.

[50] Valdes, T.I.; Kreutzer, D.; Moussy, F. *The chick chorioallantoic membrane as a novel in vivo model for the testing of biomaterials*. Journal of Biomedical Materials Research, 2002, 62(2), 273-282.

[51] Loethen, S.; Kim, J.M.; Thompson, D.H. *Biomedical applications of cyclodextrin based polyrotaxanes*. Polymer Reviews, 2007, 47(3), 383-418.

[52] Alvarez-Lorenzo, C.; Duro, R.; Gómez-Amoza, J.L.; Martínez-Pacheco, R.; Souto, C.; Concheiro, A. *Influence of polymer structure on the rheological behavior of hydroxypropylmethylcellulose-sodium carboxymethylcellulose dispersions*. Colloid and Polymer Science, 2001, 279(11), 1045-1057.

Chapter 6

CONCLUSIONS AND FUTURE PERSPECTIVES

As stated in Chapter 1, the aim of this thesis was the design and the characterization of novel syringeable supramolecular gels that can used in the treatment of an infection of bone and can actively participate in the regeneration of bone tissue. In particular, the work focused on supramolecular hydrogels based on the self-assembly of poly(pseudo)rotaxanes between α-cyclodextrin (αCD) and bioeliminable block copolymers suitable for sustained release. Gel formation occurred spontaneously upon mixing of αCD and PEO-PPO block copolymers in aqueous environment. Detailed analysis of the structure, physical-chemical properties, physical stability, drug release performance and biocompatibility were carried out.

Briefly, the most relevant conclusions obtained throughout the work subjacent to the present thesis are the following:

Chapter 1. There is a brief of literature that highlights the biomedical potential of supramolecular systems obtained by means of self-assembly of copolymer or their poly(pseudorotaxanes) formed by threading of CDs along certain blocks of the copolymers. Moreover, relevant advantages can be foreseen for syringeable systems compared to solid prosthesis or scaffolds, namely: feasibility of application using minimally invasive strategies (syringe), they can form depots in the application site for prolonged release of the active substance, and since there is not covalent links among the components, the systems are bioerodible and can be eventually cleared via renal route.

Chapter 2. There are two main procedures to obtain injectable supramolecular systems based on free CDs or polymers containing CDs. (i) Threading of free CDs on certain blocks or side chains of copolymers leads to polypseudorotaxanes, which can assembly via regular stacking of the threaded CDs. (ii) CDs grafted to polymer chains can develop their ability to form inclusion complexes with complementary guest moieties in other polymeric structures, resulting in ladder- or zipper-like arrangements. The resultant assemblies can be reversible broken under a certain shear stress and reformed at rest, exhibiting thixotropy that enables the flow through the syringe and the gel recovery in the implantation site. CDs and/or the polymer moieties that do not participate in the junctions remain available for hosting therapeutic substances. Therefore, the syringeable CD gels offer a variety of possibilities regarding control of drug release through diffusion, affinity and/or stimuli-driven mechanisms. These features together with an excellent *in vivo* compatibility ensure a promising future for self-assembling CD gels in the biomedical field.

Chapter 3. Pluronic F127-αCD interactions were characterized in detail in order to exploit the spontaneous threading of αCDs along the PEO block and subsequent formation of microcrystalline structures for developing injectable supramolecular gels suitable for sustained release of vancomycin. The effects of αCD concentration (0 to 9.7% w/v) and copolymer (6.5, 13 and 20% w/v) on the gel features were evaluated at 4, 20 and 37 ºC. An effective complexation of Pluronic and αCD was evidenced as a change in the surface pressure of the π-A isotherm of Pluronic on a subphase of CD solution and the apparition of new peaks in the X-ray spectra. Compared to Pluronic F127 solely dispersions that require nearly 20% to form gels at 37ºC, thixotropic supramolecular gels can be obtained with lower copolymer concentrations (from 6.5%) just incorporating 5% αCD. A strong dependence of the gel rate formation and the physical stability on the Pluronic:αCD molar ratio and storage temperature was observed. Pluronic F127-αCD systems containing 6.5% copolymer and 5% or more αCD at 20 ºC were the most physically stable, while the minimum Pluronic F127 concentration that provides thixotropy and temperature-responsiveness was 13%. Vancomycin could be easily dissolved in the supramolecular gels without detrimental effects on their features. All gels sustained vancomycin release for several days being active against *Staphylococus aureus* in *in vitro* cultures. In sum, 6.5-13% Pluronic F127 and 5-7% αCD supramolecular gels could be suitable as syringeable vancomycin-sustained release depots for treatment of bone infections.

Chapter 4. Poly(pseudo)rotaxanes of αCD with poloxamines, namely X-shaped block copolymers bearing four arms PEO-PPO connected to a central ethylendiamine group, were characterized for first time in this Thesis. The work was carried out with a poloxamine variety that possesses osteoinductive capability with the aim of developing osteogenic syringeable supramolecular gels that can also solubilize and sustain the release of simvastatin as an ancillary osteogenic synthetic molecule. Incorporation of 5% αCD transformed dilute T908 solutions (as low as 2% copolymer) into gels, enhanced the osteoinductive activity of T908, stabilized the hydroxy acid form at pH 7.4, and provide sustained drug release for more than one week, leading to higher and more prolonged alkaline phosphatase (ALP) activity. The performance of the intrinsically osteoinductive polypseudorotaxane scaffold could be easily tuned modifying the concentrations of T908, αCD and simvastatin in a certain range of values. The presence of αCD favored an earlier and prolonged differentiation of mesenchymal stem cells to osteoblasts due to a more

sustained delivery of both T908 and simvastatin. The remarkable osteoinductive effects attained with simvastatin-loaded T908-αCD polypseudorotaxanes make them suitable syringeable synthetic scaffolds with intrinsic ability to promote osteoblast differentiation.

Chapter 5. A water-soluble high molecular weight αCD-polymer (poly-αCD; P7αCD15) was synthesized using NaOH/αCD molar ratio < 10 and EP/αCD molar ratio < 10. Mixing of P7αCD15 (20-40%) solutions with PEG, Pluronic F127 or Tetronic 908 solutions resulted in rapidly evolving turbid dispersions, indicating the formation of poly(pseudo)rotaxane structures. The formation of supramolecular aggregates was confirmed by DOSY as a decrease in the diffusion of poly-αCD. The supramolecular assemblies exhibited good cytocompatibility and notably reduced vancomycin diffusion rate at 37 ºC for several days, opening new possibilities in the design of novel controlled delivery systems for treatment of bone infections.

Supramolecular structures based on CDs and PEO copolymers offer enormous possibility for developing novel drug delivery systems and scaffold networks. However, translation to the clinical arena still requires further work on scalability of the preparation processes and working in GMP environment in order to obtain reproducible batches of standardized quality and safety. Additional studies in animal models are also mandatory as a step previous to the administration to human begins.

The high prevalence of musculoskeletal disorders, due to the increase in sport practice, and longer expectancy of life, prompts the development of alternatives to the scarce autologous grafts and to expensive recombinant bone morphogenic proteins (BMPs) treatments. In this clinical context, injectable supramolecular gels combining synthetic active ingredients appear as an attractive affordable alternative.