Thermoresponsive polymers in controlled drug delivery and gene delivery

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Maria Teresa Calejo

Abstract

In this thesis, emphasis is given to the study of temperature-responsive systems and their use in drug and gene delivery applications.

Studies on polymer-surfactant systems were conducted, using amino acid-based surfactants. In these studies, ethyl (hydroxyethyl) cellulose (EHEC) was the investigated polymer, while the interactions with six different surfactants were analyzed. Three of these were anionic and lysine-based, having a gemini-like structure and different alkyl chain length. In addition, three cationic arginine-based surfactants were investigated. Of these, two had a gemini structure, while the third was monomeric.

The polymer-surfactant systems were characterized in terms of their rheological properties, *i.e.* complex viscosity, gel points, gel properties and thermodynamic features in form of cloud points. It was shown that the mixtures were generally low viscous at room temperature, and that the viscosity increased at higher temperatures, in agreement with the formation of a polymer network stabilized by hydrophobic associations and electrostatic repulsions. Sol-gel transitions were also observed, suggesting that the gels could be employed as pharmaceutical formulations undergoing gelation *in situ*, once injected.

Importantly, surfactants with long alkyl chains and gemini-like structure were the most efficient, which implies that very low amounts are needed in order to induce the sol-gel transition at elevated temperatures.

The biocompatibility of the EHEC-surfactant systems was evaluated by performing *in vitro* experiments on a human cell line (HeLa cells). These studies revealed that the higher toxicity of the long-chain/gemini-like surfactants was significantly compensated by their superior efficiency at low concentrations.

Experiments were further carried out with the intention of developing a microparticulate system for controlled drug delivery. An emulsification-solvent evaporation method was employed according to a planned experimental design that aimed at evaluating the process parameters on the size properties of the microparticles. Chitosan, a hydrophobically-modified chitosan derivative (HM-chitosan) and poly lactic-*co*-glycolic acid (PLGA) were

investigated as the polymeric matrix. The blank microparticles were characterized in terms of size and size distribution. Following the multivariate analysis, specific parameters were chosen to produce the drug-loaded microparticles. Naltrexone, an opioid antagonist, was used as the model drug. The microparticles were evaluated in terms of their morphological properties (scanning electron microscopy), and the drug encapsulation efficiency and loading capacity were determined. *In vitro* drug release experiments were carried out. The results from this study showed that HM-chitosan microparticles were recovered in a high yield and that the encapsulation efficiency was higher as compared to the use of the other polymers. Importantly, this polymer also demonstrated the higher capacity to retain the drug, which was slowly released for at least 50 days.

Finally, four cationic temperature-responsive block copolymers were evaluated as gene carriers. These polymers had a structure based on poly (*N*-isopropylacrylamide)-block-poly((3-acrylamidopropyl) trimethylammonium chloride) (PNIPAAM-*b*-PAMPTMA(+)), and were distinct in terms of the length of the blocks. Complexes between polymer and plasmid DNA were prepared at different polymer/DNA ratios, and used to transfect HeLa cells. Because the plasmid DNA coded for green fluorescent protein (GFP), the expression of this protein was followed by flow cytometry. Cell viability studies were simultaneously carried out in order to evaluate the cytotoxic effects of the complexes. The results showed that polymers with longer PNIPAAM or shorter PAMPTMA(+) were the most effective carriers. A series of physicochemical experiments were carried out (dynamic light scattering, zeta potential and turbidity), so as to gain insights into copolymer structure-activity relationships. It was shown that the most effective carriers had a compact PNIPAAM core that collapsed at 37 °C surrounded by a positively charged corona.

List of papers

The following papers are included in this thesis. Throughout the text they will be referred to by the indicated roman numbers.

Paper I:

Maria Teresa Calejo, Anna-Lena Kjøniksen, Eduardo F. Marques, Maria J. Araújo, Sverre Arne Sande, Bo Nyström (2012), Interactions between ethyl(hydroxyethyl) cellulose and lysine-based surfactants in aqueous media. *European Polymer Journal*, 48(9), 1622-1631.

Paper II:

Maria Teresa Calejo, Ana Maria S. Cardoso, Eduardo F. Marques, Maria J. Araújo, Anna-Lena Kjøniksen, Sverre Arne Sande, Maria C. Pedroso de Lima, Amália S. Jurado, Bo Nyström (2013), *In vitro* cytotoxicity of a thermoresponsive gel system combining ethyl(hydroxyethyl) cellulose and lysine-based surfactants. *Colloids and Surfaces B: Biointerfaces*, 102, 682-686.

Paper III:

Maria Teresa Calejo, Anna-Lena Kjøniksen, Aurora Pinazo, Lourdes Pérez, Ana Maria S. Cardoso, Maria C. Pedroso de Lima, Amália S. Jurado, Sverre Arne Sande, Bo Nyström (2012), Thermoresponsive hydrogels with low toxicity from mixtures of ethyl(hydroxyethyl) cellulose and arginine-based surfactants. *International Journal of Pharmaceutics*, 436, 454-462.

Paper IV:

Maria Teresa Calejo, Anna-Lena Kjøniksen, Atoosa Maleki, Bo Nyström, Sverre Arne Sande (2013), Microparticles based on hydrophobically-modified chitosan as drug carriers, submitted to *European Journal of Pharmaceutical Sciences.*

Paper V:

Maria Teresa Calejo, Ana Maria Cardoso, Anna-Lena Kjøniksen, Aurora Pinazo, Kaizheng Zhu, Catarina M. Morais, Sverre Arne Sande, Ana Luísa Cardoso, Maria C. Pedroso de Lima, Amália Jurado, Bo Nyström (2012), Temperature-responsive cationic block copolymers as nanocarriers for gene delivery, *International Journal of Pharmaceutics*, 448, 105-114.

Abbreviations

6Lys6, 8Lys8, 10Lys10	Lysine-based surfactants with gemini-like structure and different chain length (6, 8 or 10 carbon atoms, respectively)
$C_6(LA)_2$, $C_9(LA)_2$	Gemini lauroylarginine surfactants with different spacer length (6 or 9 carbon atoms, respectively)
cac	Critical aggregation concentration
cmc	Critical micelle concentration
DDSs	Drug delivery systems
CP	Cloud point
CTAB	Cetyl trimethylammonium bromide
d_f	Fractal dimension
EE	Encapsulation efficiency
EHEC	Ethyl(hydroxyethyl) cellulose
GP	Gel point
LAM	N^{α}-lauroyl-L-arginine methyl ester (or the monomeric arginine-based surfactant)
LCST	Lower critical solution temperature
MESC	Minimum effective surfactant concentration inducing gelation of EHEC semidilute solutions
N/P	Polymer nitrogen-to-DNA phosphate (+/-) charge ratio
NTX	Naltrexone
PEG	Polyethylene glycol
PLGA	Poly lactic-*co*-glycolic acid
PDMAEMA	Poly(*N*,*N*'-dimethyl aminoethyl methacrylate)
PNIPAAM	Poly(*N*-isopropylacrylamide)
$PNIPAAM_n$-*b*-$PAMPTMA(+)_m$	Poly (N-isopropylacrylamide)$_n$-block-poly((3-acrylamidopropyl) trimethylammonium chloride)$_m$ (n:m=48:6, 48:10, 48:20, 65:20)
S	Gel strength parameter
SALS	Small-angle light scattering
SDS	Sodium dodecyl sulfate

1 Introduction

1.1 Drug delivery systems

In classical drug delivery formulations, the drug is typically administered by the oral, subcutaneous, intramuscular, intravenous and topical routes, and the plasma drug concentration corresponds directly to the administered dose. In other words, the drug is released relatively fast, often requiring several administrations for a therapeutic effect [1, 2]. A fast drug release also produces high plasma levels, causing adverse effects and compromising patient compliance [2, 3].

Controlled drug delivery systems (DDSs) have been developed in order to circumvent the main limitations of conventional formulations. Advanced systems based on lipids such as liposomes and solid lipid nanoparticles [4-6] have been widely studied over the years. The high versatility and stability of polymers, also make these very useful materials in drug delivery applications, and a number of polymer-based systems such as micro and nanoparticles [7-9], hydrogels [10-12] and films [13] have been extensively investigated as substitutes for the classical formulations.

The modern DDSs aim at delivering appropriate amounts of the drug to the intended site, at an appropriate rate, while also minimizing local and systemic toxic effects [1, 14]. DDSs can also protect the drug from physiological degradation and elimination and increase its circulation time [15]. This is especially relevant for pharmaceuticals such as proteins and peptides that get easily deactivated and destroyed when administered orally, and are rapidly eliminated after intravenous injection [1, 16].

The poor water solubility of hydrophobic drugs can be a challenge that compromises the use of many investigated compounds [17]. When this is the case, using nanoscale delivery systems can significantly help to improve the drug solubility and increase their bioavailability [17, 18]. For instance, low solubility drugs (*e.g.* paclitaxel, tamoxifen) have been incorporated into nanoparticles [19, 20] and liposomes [21], in order to improve their efficacy.

DDSs also have the possibility of targeting a specific site. The absorption of the drug at the target tissue or organ can also be considerably improved, contributing to the maintenance of therapeutically appropriate doses [1]. From a commercial point of view, the use of controlled delivery technology may additionally be advantageous since formulating marketed drugs into new pharmaceutical forms can result in patent extension [15].

1.1.1 Pharmaceutical and biomedical applications of DDSs

In the challenging field of cancer therapy, DDSs can offer an essential contribution to improve the efficacy of the treatment. The literature concerning the design and properties of new systems that are able to deliver the cytotoxic drugs to tumor sites is therefore very extensive [22-24]. The motives for the high interest of DDSs in cancer therapy can be explained briefly as follows. First of all, DDSs can alter the typical pharmacokinetics and biodistribution profiles of the chemotherapeutic drugs, thereby improving their pharmacological properties [22]. The fact that the cytotoxic drugs are protected within a carrier, allows the presence of high drug concentrations while minimizing the systemic toxicity [22, 23]. Furthermore, DDSs in cancer therapy provide the possibility to reach the tumor site, using passive or active targeting strategies. Passive targeting takes advantage of the leakiness of tumor blood vessels (the so-called 'enhanced permeability and retention effect' or EPR effect), by using nano-sized DDSs that can be taken up and accumulated in tumor tissues. Active targeting is more specific and requires functionalization of the carriers with surface ligands that bind to receptors overexpressed on the tumors or associated endothelium [22, 23]. With respect to the next generations of DDSs for cancer treatment, scientists seem to agree that developing combinatorial or multifunctional 'smart' carriers may provide the best results. Another promising strategy is to develop personalized and tailor-made systems [22-24].

In many other applications, the main role of the DDS is to release the loaded drug at a slow rate, allowing for the reduction of the number of administrations. Systems resulting in a long-lasting effect have been developed for the treatment of tuberculosis [25], retroviral infection [26], Parkinson's [27, 28] and other neurodegenerative diseases [29], glaucoma [30] and also for tissue engineering applications [31]. Controlled delivery systems based on the opioid

antagonist naltrexone have similarly been proposed for the treatment of alcohol and drug addiction [32, 33]. By controlling the drug release rate, it is possible to ensure that the released amounts are within the therapeutic window for sufficiently long periods of time. In polymer-based systems, this can be achieved by modifying the polymer properties such as architecture, composition and molecular weight [34]. From these DDSs, the drug can be released to the external medium by one or more mechanisms, including diffusion, matrix swelling, chemical degradation, dissociation or in response to external stimuli [16, 34].

Gene therapy is considered an encouraging and progressing field for the treatment and prevention of genetic-based disorders [35, 36]. In gene delivery, DDSs have been used in replacement of viral systems to deliver nucleic acids into the cell compartments, where the induction of gene expression or the silencing of specific genes results in a therapeutic effect [35, 36]. The carrier systems provide the nucleic acids with protection during blood circulation, condense them to an appropriate size and facilitate cell uptake. Once in the intracellular medium, the carrier also helps to deliver the payload into the target compartment [35, 37]. Both cationic lipids and cationic polymers have been used to deliver the nucleic acids into the cell compartments. These systems and their mechanisms of action have been widely investigated in gene therapy and it has been shown that the chemical and supramolecular structure of the carrier has an important effect on the delivery efficiency [36]. The subject of gene delivery and its challenges will be briefly introduced in section 1.5.

In this thesis, emphasis will be given to polymer-based formulations and their potential applications as drug delivery systems for conventional drugs and nucleic acids. Because this project was firstly focused on the production of *in situ*-gelling systems for the sustained release of conventional drugs, an overview of hydrogels as DDSs will be presented. The use of stimuli-responsive systems in pharmaceutical and biomedical applications will similarly be described in the following sections. Particular attention will be paid to the temperature-responsive ethyl(hydroxyethyl cellulose) (EHEC) that in combination with ionic surfactants produced low toxicity *in situ* gelling systems (Paper I-III). The use of polymer-based microparticles as controlled drug release systems will be addressed, since a depot formulation was also developed, with naltrexone being the encapsulated drug (Paper IV). Poly(N-isopropylacrylamide) (PNIPAAM), which was further investigated for gene delivery applications (Paper V), will be briefly introduced.

1.2 Polymeric hydrogels

Polymeric hydrogels are three-dimensional networks of polymer chains that are loosely cross-linked, enabling the system to entrap a high amount of water and causing the matrix to swell [38, 39]. In the last years, these materials have been used in numerous applications, with special emphasis on the biomedical and pharmaceutical fields. In tissue engineering and regenerative medicine, hydrogels have shown to be promising materials for the repair of cartilage [40, 41], bone [42, 43] and soft tissue [44, 45], where they form scaffolds for cell growth and proliferation. The applicability in biomedical devices, such as films, sponges and biosensors is also recognized [46]. In drug delivery, hydrogels have been widely used as depot systems, for instance through the oral [47], topical and transdermal [48, 49], gastrointestinal [50, 51], ocular [52], nasal [53, 54] and vaginal [55, 56] administration routes.

Due to the high porosity of the hydrogels, high drug amounts can be loaded into the formulation [57]. Drug release from hydrogels takes place mostly by diffusion, even though it can also depend on matrix swelling and chemical reactions (*e.g.* polymer chain cleavage by hydrolytic or enzymatic degradation) [58]. Importantly, it considerably depends on the polymer composition (type of monomers and chain length), cross-link density, and type/intensity of the external stimuli [11, 58]. Having this in mind, drug-loaded hydrogels with specific properties can be tailor-made in order to meet specific criteria, such as a slow release rate [58]. A very interesting concept is the achievement of a delayed drug release, through chemical or physical interactions with the hydrogel matrix. In one approach, the charged drug can be formulated into an oppositely charged hydrogel, in order to promote the establishment of electrostatic interactions. Other strategies are to covalently link the drug to the polymer chains, thereby allowing a delayed release following hydrolytic or enzymatic degradation of the polymer-drug bonds; and to use specific polymers or monomers with high affinity for particular drugs [11].

The attractiveness of hydrogels in biomedical and pharmaceutical applications for human use is closely related to their properties. In general, hydrogels are considered biocompatible, thereby not causing significant toxic effects once administered [11, 38, 58]. The biocompatibility of a hydrogel can also be increased by including specific polymers in the composition, particularly those that exhibit similar properties to the extracellular matrix, *e.g.* the

hydrophilic polyethylene glycol (PEG) and some polysaccharides [57]. The fact that the hydrogels can be formulated so as to mimic the viscoelastic properties of human tissues is actually one of their most appealing features. In a similar way, biodegradable hydrogels can be produced by using polymers that can be enzymatically or hydrolytically cleaved in the host environment [57]. Moreover, the physicochemical characteristics of the hydrogels can be changed in order to fulfill the requirements of the intended use (*e.g.* adherence and deformation capacity) [10, 11].

1.2.1 Hydrogel classification according to the cross-linking mechanism

Hydrogels can be classified in different ways, namely according to the nature of the monomers (ionic or neutral), and according to the polymer structure. Quite often, hydrogels are also classified in relation to the type of cross-links involved, since this can have a significant effect on the swelling behavior, mechanical strength and deformation properties of the resulting hydrogels. In this classification, two types of cross-links are commonly described: the covalent or chemical, and the non-covalent or physical cross-links [38].

Chemical gels, *i.e.* those prepared by the introduction of covalent cross-links into the network, are generally regarded as 'irreversible' gels. Hydrogels such as these can essentially be produced by irradiation or chemically-induced polymerization between components with reactive functional groups or by cross-linking agents. The chemical cross-linking of the matrix can take place between polymers or copolymers, or between polymers and monomers or macromers in solution [57]. One well-known example of a chemical gel is produced by chemically cross-linking poly(*N*-isopropylacrylamide) (PNIPAAM) with *N,N'*-methylenebis(acrylamide). This example will be discussed in more detail in section 1.3.2.

In physical gels, the polymer chains are not covalently linked, but are instead found entangled or associated by hydrophobic associations, electrostatic interactions or by the establishment of hydrogen bonds [12]. Polymers that undergo gelation by hydrophobic bonding are typically amphiphilic and hence composed by hydrophobic blocks and hydrophilic segments. Often, hydrophobic side-chains are grafted to a hydrophilic backbone, or an amphiphilic block copolymer is produced [11]. The physical gelation of these copolymers sometimes depends on temperature changes (see section 0 for information on

stimuli-responsive hydrogels). In this case, the increase in temperature triggers the association of the hydrophobic domains that respond in this way so as to minimize the contact with the bulk water [11].

A sol-gel transition can also be observed when interactions take place between two oppositely charged polymers, or between a polymer and a small molecule of opposite charge [11]. One well-known example of the latter is the gelation of alginate in the presence of multivalent cations such as Ca^{2+}. Here, each divalent cation binds two glucuronic acid sites, linking the alginate chains together (forming cross-linking points), which results in the formation of a low flexibility network, *i.e.* in the formation of a hydrogel. Alginate hydrogels have been widely used in biomedical and pharmaceutical applications [12, 59, 60].

Hydrogen bonds can also act as physical cross-linkers in block copolymers and homopolymers, forming interactions between polymer chains with compatible geometries [11, 61]. In this case, the intermolecular association of polymer chains through hydrogen bonds can lead to the formation of crystallites, which ultimately contributes to the formation of the hydrogels [61]. Some examples of polymeric hydrogels stabilized by hydrogen bonding are dextran [61], polyvinyl alcohol [62] and blends of natural polymers such as gelatin-agar and hyaluronic acid-methylcellulose [11]. Semidilute pectin solutions also form gels at low temperatures due to intermolecular hydrogen bonding [63].

Physically cross-linked hydrogels are often considered biocompatible and biodegradable. In contrast, the presence of external cross-linkers in chemical gels, renders them more toxic and difficult to degrade [57]. In spite of this clear advantage of the physical gels, particularly in what concerns their presence in formulations for human use, these are also more prone to dilutions at the administration site, and variables such as gelation time, pore size and degradation time are more difficult to control independently [11].

Selected examples of gel systems that respond to temperature changes, such as the triblock copolymers PEO-PPO-PEO (Pluronic) and PLGA-PEG-PLGA will be briefly described in section 0, under the topic '*Temperature-responsive polymers*'.

1.3 Stimuli-responsive polymer systems

In recent years, stimuli-responsive or 'smart' polymers have attracted great attention for drug delivery applications. These polymers respond to small changes in environmental conditions, such as temperature, pH, light, ionic strength, electric or magnetic fields, or the presence of enzymes or specific ligands [64]. In addition, some polymer systems can combine more than one stimuli-responsive mechanisms, for example by responding to both pH and temperature changes [65, 66].

In drug delivery, polymers that respond to pH variations or temperature changes are particularly interesting materials, since this ability to undergo reversible phase transition or conformational changes depending on the external environment can be used to promote the drug loading, and to modulate the rate and site of drug release [64].

Some specific stimuli-responsive polymer systems will be discussed next, with particular emphasis being given to the applications of pH- and temperature-responsive polymers. The structures of some stimuli-responsive polymers are given in Figure 1.

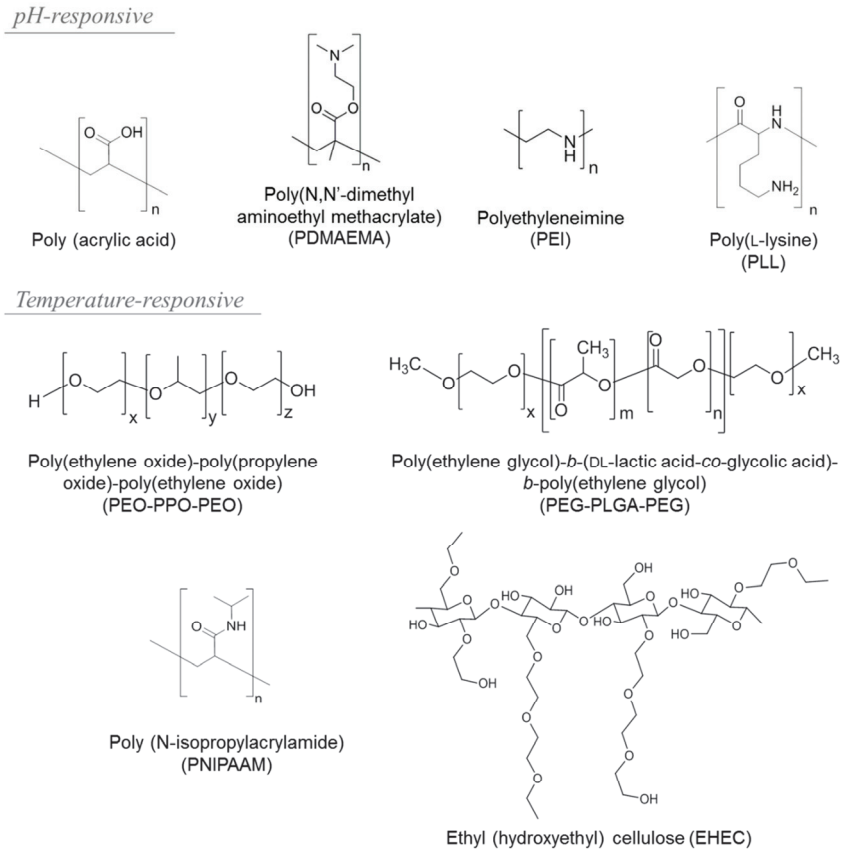


Figure 1. Chemical structures of some polymers that respond to pH and temperature with applications in drug delivery.

pH-responsive polymers

Polymers that respond to pH typically contain ionizable groups that can accept or donate protons in response to the environmental pH. In this case, changing the pH across the pK_a threshold of the polymer, results in a rapid change of its net charge and in the hydrodynamic volume of the polymer chains. In other words, the presence of a highly charged structure causes the matrix to swell as a result of the high charge repulsion, and as the polymer shifts from a collapsed state to an expanded state [67]. The osmotic pressure created by the presence of the mobile counterions also helps to explain the increase in the hydrodynamic volume of the polymer chains [68].

It is particularly interesting to take advantage of the pH differences in the human body (*e.g.* along the gastrointestinal tract) to achieve a targeted drug delivery by using polymers that respond to pH changes. In addition, in anti-cancer drug delivery, the release of the drugs can also be triggered by the acidic extracellular pH of the tumors, which contributes to a higher efficiency and lower toxicity to the surrounding tissues [69, 70].

The polymer composition of a drug delivery system should thus be carefully considered, having in mind the specific aim. Poly (acrylic acid) (Figure 1), has been used in drug delivery systems that target the intestine [71]. Due to the presence of ionizable COOH groups, this polyelectrolyte is protonated and thus uncharged at low pH and negatively charged at high pH. The presence of a protonated structure at low pH (as in the gastric environment) eliminates the electrostatic repulsion forces and contributes to the formation of a compact structure. The formation of a low flexibility network is also promoted by the formation of hydrogen bonds [68]. When the pH is increased above the pK_a of the polymer, the negative charges are dominant, and the high charge repulsion causes the network to swell or dissolve and release the drug. Accordingly, the drug can be released from poly(acrylic acid) matrices as a result of the higher pH (7.4) in the intestinal environment [68, 71].

For other applications, one can specifically trigger the drug release in response to acidic environments. Polybases such as poly(*N*,*N*'-dimethyl aminoethyl methacrylate) (PDMAEMA) (Figure 1) and poly(β-amino esters) have amino groups that are deprotonated at neutral pH but become positively charged by gaining protons at acidic pH [68]. This is particularly relevant in cancer drug delivery, since the extracellular environment of solid tumors is weakly acidic (pH < 6.5), while the pH in the endosomes and lysosomes of cancer cells is even lower (pH 4.0-6.0) [72]. In this context, particles formed by polybases are stable at physiological pH. However, in the tumor tissues or their intracellular compartments, the particles become positively charged and dissolve rapidly as a response to the lower pH, thus releasing the cytotoxic drug [73]. In normal tissue, the slightly acidic environment of the endosomes can also be used to trigger the release of the payload in gene delivery applications [70, 74]. Polyethyleneimine (PEI) and poly(L-lysine) (PLL) (Figure 1), are two well-known carriers in nonviral gene delivery [64].

Temperature-responsive polymers

Due to their unique properties, polymers that respond to temperature changes are among the most investigated in modern drug delivery applications. Specifically, they are characterized by changing their conformation, solubility and hydrophilic/hydrophobic balance as a response to changes in the temperature of the external environment [64, 75].

In drug delivery, *in situ* gelling systems are a particularly appealing concept. By using temperature-responsive polymers, one can for instance produce a low viscosity solution that can easily be injected but that undergoes fast gelation *in situ* as a response to body temperature.

One example of polymers that undergo temperature-induced gelation are the ABA-type triblock copolymers poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO, also known as Pluronics/Poloxamer) [68, 76]. As the temperature is increased, the PPO blocks associate through hydrophobic interactions, which leads to the formation of micelles with a PPO core and a hydrophilic PEO corona. It has been hypothesized that at sufficiently high concentrations, the micelles arrange in a packed, entangled structure (such as cubic crystalline phase), creating a three dimensional structure, *i.e.* a gel [77]. This sol-gel transition strongly depends on the hydrophilic-hydrophobic balance and on polymer concentration. Under specific conditions, the transition can take place close to physiological temperature, allowing *in situ* gelation [68]. At even higher temperatures, the gel becomes an opaque solution (cloud point, CP), due to the shrinkage of the PEO corona and higher interactions with the PPO core [78].

In spite of generally being regarded as biocompatible, PEO-PPO-PEO block copolymers have some important drawbacks that may limit their applicability, such as their weak mechanical strength, rapid dissolution and non-biodegradability. However, specific synthetic strategies have been discussed in order to circumvent these issues [77].

Poly(ethylene glycol)-*b*-poly(lactide-co-glycolide)-*b*-poly(ethylene glycol) (PEG-PLGA-PEG) was designed for an improved durability and biocompatibility [78-80]. As in the case of Pluronics, a sol-gel transition can also be observed when the temperature of PEG-PLGA-PEG solutions is increased. In this case, it is hypothesized that the amphiphilic nature of the copolymer contributes to form micelles. These micelles are formed by a PLGA (hydrophobic) core, and a PEG (hydrophilic) corona. As the temperature is increased, the

micelles associate to form the interconnected network that constitutes the gel. At sufficiently high temperatures, the hydrogen bonds are broken and the increased hydrophobicity of the polymer leads to a high degree of aggregation and to macroscopic phase-separation [81]. Factors such as the molecular weight of the polymer, the length of the blocks, the composition of the hydrophobic block (ratio between lactic and glycolic acid), the polymer concentration and the presence of salts have been shown to influence the gel temperature, the critical gel concentration and the degradation rate of the hydrogel [78, 81]. One important feature of the copolymers based on PEG and PLGA is that they are biodegradable and can last for a longer period of time [81]. In addition, the release rate greatly depends on the nature of the drug. Particularly, it has been shown that hydrophobic drugs are released slowly and for longer periods of time, due to their preferential incorporation into the hydrophobic PLGA core [82].

Some temperature-responsive polymers are characterized by having a lower critical solution temperature (LCST). The LCST can be defined as the critical temperature at which the polymer solution phase separates [68]. Below this temperature, the polymer chains are water-soluble and form hydrogen bonds with the water molecules. However, as the temperature is raised above the LCST, the hydrogen bonds are broken and the hydrophobic associations dominate, causing the polymer in dilute solution to undergo coil-to-globule phase transitions, resulting in macroscopic phase-separation at higher polymer concentrations. In this process, the balance between the ratio of hydrophobic and hydrophilic monomers is of great importance [64, 75, 83]. A figure illustrating the effect of LCST on the phase transitions of temperature-responsive polymers is shown in Figure 2.

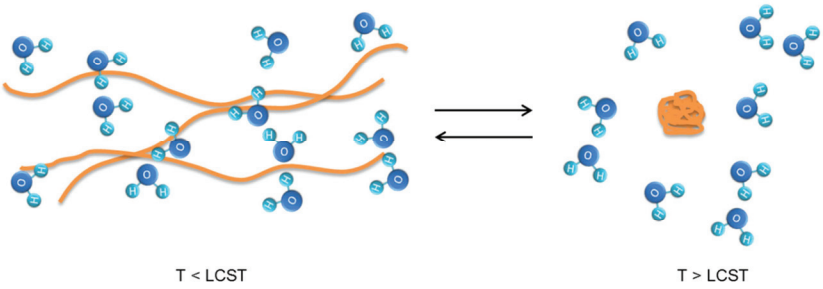


Figure 2. Effect of the lower critical solution temperature (LCST) on the phase separation behavior of thermoresponsive polymers. Below the LCST (left) the polymer chains (orange) are water soluble, due to hydrogen bonding with water molecules. Above the LCST (right) the hydrophobic associations dominate, the polymer aggregates and phase-separation takes place. Figure adapted from ref [83].

A number of review articles describe the wide variety of stimuli-responsive polymers that have been developed so far, including their structure, physicochemical properties and applications. The reading of the review articles by Ruel-Gariépy and Leroux [77], Gil and Hudson [68], Liu and Urban [67] and Talelli and Hennink [83] for instance, is suggested for more detailed information on this matter.

In this thesis, however, most work involved the use of EHEC (in combination with ionic surfactants) and PNIPAAM block copolymers. Major emphasis will hence be given to these systems, as described in the following sections.

1.3.1 Ethyl (hydroxyethyl) cellulose

Ethyl(hydroxyethyl) cellulose (EHEC) is an amphiphilic nonionic cellulose derivative, widely used in the paint industry (where it forms protective colloids, acts as a thickening agent and helps to keep pigments in suspension) and in the building industries (where it is used as a dispersion agent in cement formulations) [84]. EHEC is produced by chemical modification of cellulose, through the substitution of free hydroxyl groups with ethyl and hydroxyethyl groups (Figure 1). This substitution renders the polymer water-soluble [85]. One of the most interesting properties of EHEC is its temperature-responsive behavior, caused by the presence of both hydrophobic and hydrophilic units, typically unevenly distributed along the polymer chains [86, 87]. As the temperature is increased, EHEC becomes less water-soluble, due to the enhanced hydrophobicity of the polymer (and less favorable interactions between the polymer and the solvent). As a consequence, EHEC solutions exhibit a phase separation behavior, as the LCST is reached. One of the phases is therefore enriched with the aggregated EHEC, and the other phase consists mainly of the aqueous solvent [88].

The interactions between EHEC and ionic surfactants have been extensively studied over the years [86-93]. In the presence of ionic surfactants, the phase behavior of EHEC solutions is significantly changed. The first consequence of the presence of ionic surfactants is that they arrange around the hydrophobic domains of the polymer, preventing the formation of large clusters as the temperature is increased. The arrangement of the ionic surfactants around these hydrophobic microdomains creates mixed polymer-surfactant micelles. At moderate surfactant concentrations, the connectivity of the system is increased. In addition, because

the surfactants are charged, the interaction with the polymer chains also endows these with an apparent polyelectrolyte character. In other words, the presence of moderate amounts of the ionic surfactant simultaneously contributes to an enhanced connectivity between EHEC chains, and causes the matrix to swell due to electrostatic repulsion. A temperature-induced viscosification is observed as the polymer becomes more hydrophobic with increasing temperature. The cloud point (CP) is also usually shifted to higher values, due to the improved thermodynamic conditions (increased solubility) created by the presence of the ionic surfactant. Depending on polymer concentration and polymer-to-surfactant ratio, a sol-gel transition may occur at elevated temperatures [87, 88, 90, 94]. At high surfactant concentrations, however, the connectivity of the system will be disrupted due the combinations of an excessive swelling effect and the solubilization of the hydrophobic domains by the surfactant molecules [86, 90].

So far, most research on EHEC-ionic surfactant systems has been performed using conventional surfactants such as the anionic sodium dodecyl sulfate (SDS) [86-88, 90] and the cationic cetyltrimethylammonium bromide (CTAB) [86, 92, 93]. However, the pharmaceutical use of such surfactants is limited due to their environmental toxicity, low chemical and biological biodegradability and poor biocompatibility [95].

Only a few articles concerning pharmaceutical applications of EHEC-surfactant systems can thus be found in the literature. In one case, an EHEC-SDS gel system was developed for intranasal insulin administration [96]. The formulation presented a low viscosity at room temperature, facilitating the administration to the nasal cavity, and formed a gel *in situ* as a consequence of the interactions between EHEC and the ionic surfactant. The viscous formulation was found to be mucoadhesive and to improve the nasal absorption of insulin [96]. In another example, an *in situ* gelling formulation was developed from EHEC in combination with different surfactants for the delivery of lidocaine and prilocaine into the periodontal pocket (local anesthetic effect) [97]. This study revealed that the presence of the drug can affect the polymer-surfactant interaction, even though under specific conditions the gelation behavior can still be retained [97]. A gelling system combining EHEC and the ionic long chain alkyl betainate surfactant was also developed, using timolol maleate as the active ingredient for ophthalmic administration in the treatment of glaucoma [98]. The phase behavior significantly depended on surfactant concentration, while the presence of the drug

generally shifted the gel region to higher surfactant concentrations. The gelling system allowed a slower release of the drug in comparison with the non-gelling formulation [98].

Due to the complex interactions taking place between EHEC and the ionic surfactants, several parameters should be carefully considered in order to preserve the thermogelling capacity of the system and to produce a gel with the desired properties.

Perhaps one of the most important factors to keep in mind is surfactant concentration, since low surfactant concentrations might be insufficient to cause charge repulsion, while high surfactant concentrations may cause excessive swelling and result in the disruption of the network [99, 100]. A delicate balance between the hydrophobic associations and the electrostatic repulsion is therefore a pre-requisite to produce the temperature-responsive gel [90, 100], as described by the model of Cabane et al. [94].

Secondly, one should keep in mind that the gel point can in principle be tuned by changing the polymer concentration. For the EHEC/SDS and EHEC/CTAB system [93], it has in fact been shown that increasing the polymer concentration results in a decrease of the gel point. In consistency, increasing the polymer concentration also produces stronger gels. By using different surfactants with different charged head groups, the gel temperatures and the gel strength can also be altered [93]. Karlström et al. [89] demonstrated that the phase behavior of semidilute EHEC solutions is affected by the length of the alkyl chains of the surfactant, the size and charge of the headgroup, and by the type of counterions present.

It is also well-known that the interactions between EHEC and ionic surfactants are significantly affected by the salinity of the solution [100]. In the presence of salts, it has been shown that higher surfactant concentrations are needed in order to increase the viscosity of the solution at high temperature, and that the cloud point decreases with increasing salt concentration [100]. Different salt types can also have a different effect over the phase transitions of EHEC [101]. The effect of salinity on the rheological behavior of EHEC is explained by a screening effect of the charges of the ionic surfactants, which causes an increase of the connectivity of the system. In this context, higher surfactant concentrations are needed so as to restore the thermogelling capacity of the system [99, 100]. This can be an important issue in parenteral formulations, since the presence of salts in the formulation is required to create isotonic conditions.

In drug delivery, it is finally important to emphasize that the presence of the drug itself can affect the rheological properties of the solution and the sol-gel transition [98, 101]. For this reason, adjustments of the formulation components and concentrations may be required in order to produce an *in situ* gelling system optimized for the envisioned aim.

1.3.2 Poly(*N*-isopropylacrylamide)

One of the most extensively studied thermoresponsive polymers is poly(*N*-isopropylacrylamide) (PNIPAAM) (Figure 1). This polymer can exhibit a phase transition at about 32 °C in water, depending on its concentration and molecular weight, and this proximity to human body temperature makes it particularly interesting for pharmaceutical and biomedical applications [102]. PNIPAAM can additionally be copolymerized with other blocks in order to achieve combined properties, for instance in the case of the copolymerization of PNIPAAM with poly(acrylic acid) for both temperature- and pH-responsiveness [103]. The major limitation of PNIPAAM is its low biodegradability, but the copolymerization with other biodegradable polymers can partially help to overcome this issue [104]. By copolymerization with other hydrophilic or hydrophobic blocks, one can also modify the LCST of the polymer, or produce distinct micellar structures for specific applications [105-107].

Below the LCST of PNIPAAM, copolymers of PNIPAAM with hydrophobic segments can form core-shell micelles, where the core is composed of the hydrophobic domain and the shell is composed of the hydrated PNIPAAM. Hydrophobic drugs can therefore be incorporated in the core, while the hydrophilic shell contributes to stabilize the micelle and to increase the circulation time of the drug delivery system [104]. As the temperature is increased above the LCST, the hydrophobic groups of PNIPAAM associate, causing the destabilization and aggregation of the micelles and triggering a fast drug release [104] (Figure 3 a.). This reversible and thermo-sensitive response has been shown for adriamycin-loaded PNIPAAM-*b*-poly(butylmethacrylate) micelles, demonstrating the potential of these polymeric systems when used in combination with localized hyperthermia [108].

Another strategy is to conjugate or copolymerize PNIPAAM with a hydrophilic segment. In this case, PNIPAAM forms the core of the micelles (if the temperature is raised above its

LCST), while the hydrophilic domain is located at the surface, constituting the corona (Figure 3 b.). Another usual consequence of the presence of a permanently hydrophilic segment is the increase of the LCST of the thermo-responsive polymer [83, 104]. One of the most well-studied hydrophilic polymers that have been copolymerized with PNIPAAM is polyethylene glycol (PEG). Below the LCST, PNIPAAM-*b*-PEG is highly hydrophilic and soluble in aqueous solution, while increasing the temperature above the LCST of the polymer causes the PNIPAAM block to aggregate due to its enhanced hydrophobicity. Micelles with a PNIPAAM core and a PEG shell are thereby formed [83, 104]. In drug delivery technology, this has practical advantages, since a hydrophobic drug can be loaded by simply heating the solution to a temperature higher than the LCST of the polymer. In addition, the drug can be released from the micelles by local hypothermia, since the phase transitions are reversible [104, 109].

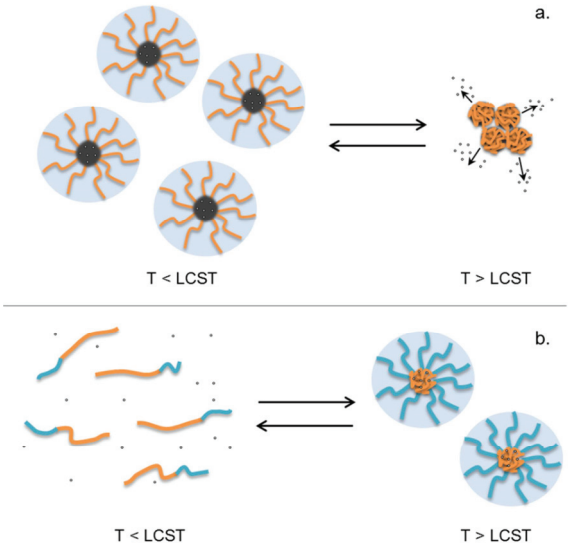


Figure 3. Temperature-induced behavior of PNIPAAM-containing polymers. **a.** In the presence of a permanently hydrophobic segment, micelles are formed below the LCST of the polymer. Under these conditions, the hydrophobic polymer forms the core (black) and PNIPAAM (orange) forms the hydrated outer shell. The increase in temperature further causes the collapse of PNIPAAM and the destabilization of the micelles, which triggers the release of the drug. **b.** In the presence of a hydrophilic polymer segment (blue chains) and below the LCST, the polymer is water soluble. Above the LCST, micelles are formed. These micelles are formed by a PNIPAAM core (orange) and a hydrophilic polymer shell. Figure adapted from refs. [108] and [83].

In gene delivery, PNIPAAM was first used in a block copolymer containing the positively charged PDMAEMA [110]. In this case, the authors discussed that the presence of a positively charged polymer motivates the formation of electrostatic interactions with the negatively charged phosphate groups of DNA, thereby leading to the formation of polymer-DNA complexes. In addition, the copolymer was shown to be able to condense the DNA into small particles, while the overall presence of positive charges on the complexes was said to facilitate cell uptake, ultimately leading to higher transfection efficiencies [110].

Other copolymers have been synthesized for the same purpose. Reports on the use of polyethyleneimine (PEI)-*g*-PNIPAAM [111], trimethyl chitosan-*g*-PNIPAAM [112] and poly(L-lysine)-*g*-PNIPAAM [113] can be found in the literature.

PNIPAAM has also been widely formulated into hydrogels. This can be achieved *e.g.* by cross-linking with a chemical cross-linker such as *N,N'*-methylenebis(acrylamide) [114-116]. Below the LCST, the hydrophilic amide groups of the polymer form hydrogen bonds with the surrounding water molecules and the network swells creating a gel. As the temperature is increased above the LCST, the hydrogen bonds are broken and the hydrophobic associations between the hydrophobic isopropyl groups increase, causing aggregation. In other words, the polymer becomes dehydrated, expelling a high amount of water, and collapses into a stiff and opaque structure [114, 115]. This phase-transition behavior is reversible, and decreasing the temperature below the LCST will therefore result in the hydration and reswelling of the network [114, 115].

It has been shown that the level of cross-linking of PNIPAAM gels can influence the porosity and release rates of loaded drugs [116]. Furthermore, the properties of the hydrogels can be significantly changed by copolymerization of NIPAAM with other monomers. Copolymerization with a hydrophilic monomer, for instance, can induce the formation of a macroporous structure, due to the enhanced swelling and deswelling properties of the polymer [117]. By changing the composition of the copolymer, one can also produce a system that undergoes physical gelation as a response to temperature changes. Self-gelling systems have been produced by synthesizing copolymers of PNIPAAM with hyaluronic acid [118] or alginate [119]. The same strategy can be used to enhance the mechanical strength of the hydrogel and to prevent syneresis (expulsion of water from the gel), as shown for

PNIPAAM-*g*-methylcellulose [120]. Comb-type structures also contribute to induce a fast transition (due to the high mobility of the temperature-responsive PNIPAAM grafted to the chain end), which is an essential feature in *in situ* gelling systems [118].

For all the above mentioned, it is clear that PNIPAAM is a polymer of considerable interest. The phase changes that PNIPAAM undergoes as a response to temperature changes have motivated the scientific community to synthesize a wide range of PNIPAAM copolymers, having in mind specific applications, such as drug delivery, nonviral gene delivery, tissue engineering and cell culture [72, 78]. The possibility to functionalize the polymeric carriers for a targeted delivery is also very attractive. This has been achieved *e.g.* by conjugation with biotin [121, 122] or folate [123, 124] for cancer therapy.

1.4 Microparticles

Microparticles (1-1000 μm [125, 126], typically smaller than 100 μm [127]) are amongst the most well studied and established DDSs, due to their unique properties and advantages in the treatment of many diseases. Their large volume in comparison with nanocarriers allows the easy encapsulation of large amounts of drug, and the encapsulation of large molecules such as proteins and nucleic acids [126]. In addition, these DDSs can show remarkable drug release properties, with long-standing effects being observed. Poly lactic-*co*-glycolic acid (PLGA) microspheres, for instance, have been developed for the sustained release of β-metasone over a period of 11 days [128]; dexamethasone was released from PLGA particles for over a month [129], while in another study paclitaxel was slowly released from the microparticles for more than 110 days, when the DDS was formulated in the presence of appropriate emulsifiers or additives [130].

Many methods have been employed to prepare microparticles, such as emulsification and solvent evaporation/extraction, phase separation (coacervation), spray-drying and *in situ* polymerization [126, 131-133]. The choice of the preparation method should be closely related with the properties of the polymer and drug, the intended site of action, and the intended duration of the therapy [131].

In 1986, Decapeptyl® by Ipsen became the first approved product of sustained-release injectable microspheres (gonadotropin-releasing hormone agonist). A number of other products are nowadays also in the market, to name a few Lupron Depot (leuprolide acetate, from Takeda), Risperdal Consta (risperidone, from Janssen) and Vivitrol (naltrexone, from Alkermes) [133]. These products differ significantly in terms of particle size (up to 300 µm in some cases), polymer composition and molecular weight, and drug release properties [133].

Typically, drug release from microparticles based on biodegradable polymers depends on diffusion, polymer erosion or a combination of both effects, and it is affected by the polymer and drug properties, particle porosity and size, drug loading and drug distribution, and presence of residual solvents [133, 134]. The presence of enzymes and lipids can also have an important effect on the drug release rate *in vivo* [133].

Drug release from microspheres is often described by a two-stage process. In the first stage, an initial burst of expelled drug is observed, due to excessive drug diffusion or drug desorption from the surface of the particles, whereas the second stage is defined by a more constant drug release rate, corresponding to both diffusion and polymer degradation effects [134]. A high burst release can cause toxic effects and decrease the amount of available drug in the system and should therefore be avoided [133].

In this thesis, polymeric microspheres were developed as an additional barrier to drug release from a temperature-responsive hydrogel and with the intention of producing a controlled drug delivery system with a low initial burst release. This system can be compared with the production of PLGA microspheres loaded with transforming growth factor-β_1 (TGF- β_1) and embedded onto PEG-based hydrogels, as described by DeFail et al. [135].

1.5 Gene delivery

In gene therapy, a vector containing nucleic acids is introduced into target cells so as to modify gene expression in a way that prevents, stops or reverses a pathology [136].

Viral vectors have shown to be the most efficient carriers for nucleic acids, particularly DNA. For this reason, scientists have developed genetically modified viruses by introducing foreign genes of interest, while deleting the genes that allow replication, assembling or infection of the virus [137, 138].

In spite of their high efficiency, the use of viral vectors poses a number of concerns. In clinical trials, it has been shown for instance that the viral carriers can induce an excessive immune response due to the presence of viral capsid proteins, or lead to leukemia, due to random integration of the genes into the host chromosome [137]. The production procedure is also quite complex, and the viruses are difficult to produce in a large-scale [137].

Due to these disadvantages, nonviral gene delivery methods have been considered interesting alternatives to the viral carriers [137, 139]. Nonviral carriers are generally recognized to induce low immune response and low toxicity, and they can be easily produced in large scale [137, 139]. In addition, the nonviral carriers can be designed in order to target specific cells or tissues [139]. The major limitation of the nonviral methods is that these are usually less efficient than the viral methods. In spite of this, the carriers can be administered repeatedly, if necessary, and the methods can be changed in order to improve the gene expression [139].

The delivery of genes by nonviral carriers is a very challenging field that differs significantly from the DDSs described in the previous sections, used to systemically or locally deliver the active pharmaceutical ingredients. A number of requirements need to be fulfilled, and several barriers need to be surpassed until the therapeutic genes are integrated into the host's genome. The DNA-carrier system must firstly overcome the extracellular barriers, such as the elimination by renal filtration and by the reticuloendothelial system (RES) in the liver, spleen and lung [137]. In addition, the carrier should be able to protect the nucleic acids from the action of nucleases present in the blood and extracellular matrix. One critical step is crossing the plasma membrane; in this case, the carrier should further facilitate cell uptake, through mechanisms such as endocytosis, pinocytosis or phagocytosis [137, 139]. Once inside the cell, the system should escape the endosomes, in order to avoid enzymatic digestion, and finally it should travel to the nucleus where the transcription can take place after the carrier and the nucleic acid payload have been dissociated [139].

In recent years, researchers have shown the potential of cationic polymers as nonviral gene carriers [35, 140, 141]. Advantages such as the low immunogenicity, high structure versatility and

ease of production scale-up have been pointed out [140, 142]. In addition, cationic polymers can condense DNA molecules to a smaller size as compared to cationic liposomes, which is an essential property for an efficient transfection [36]. Review articles on the use of polymers (*e.g.* poly(L-lysine), PEI, poly(amidoamine) dendrimers, chitosan) as nonviral gene carriers can be found in the literature [36, 137, 139, 143, 144]. Despite their advantages, the use of cationic polymers in gene therapy is still considered to be in its early stages [37].

In this challenging field, stimuli-responsive polymers that transform their physicochemical properties in response to extra- and intracellular stimuli have received increasing attention [145]. Stimuli such as pH, redox potential, temperature and enzymatic activity have been shown to modulate the gene delivery ability of a polymeric carrier, while another possibility is to use artificial stimuli (*e.g.* light, ultrasound, magnetic field) to externally trigger the gene delivery pathways [145].

Two of the most important requirements of a gene carrier is that it forms tight complexes with the DNA (protecting it from the degradation by nucleases, condensing it to an appropriate size and endowing it with a positive charge suitable for internalization) and that, once in the nucleus, it dissociates from the payload so that gene expression can take place [146]. The use of stimuli-responsive polymers is considered particularly advantageous in this context, since by applying a physical stimuli, such as light and temperature, complex association or dissociation can be controlled [146]. In addition, by using stimuli-responsive carriers, one can control the site, timing and duration of the gene expression [146].

2 Aim of the project

The major objectives of this project were to investigate different formulations based on temperature-responsive polymers in terms of their properties and applicability in drug and gene delivery.

The specific objectives of this project can be described as follows.

First of all, it was the aim of this work to develop a new injectable temperature-responsive hydrogel for drug delivery purposes. Such gel should be low toxic, exhibit low viscosity at (or below) room temperature and undergo gelation as the temperature is raised to 37 °C. In view of this purpose and having in mind the temperature-responsive properties of EHEC, the specific goals were:

i. To study the rheological profile of EHEC in the presence of ionic amino acid-based surfactants;
ii. To explore the potential of this binary formulation as an *in situ* gelling system;
iii. To investigate the cytotoxicity of the components of the formulation and to discuss the applicability of the system in drug delivery.

Because it was hypothesized that the drug release capacity of the hydrogels could be improved through the combination of the gels with other controlled release systems, it was furthermore the aim of this work to develop polymer-based microparticles with enhanced stability properties.

Another goal of this thesis was to investigate the potential use of temperature-sensitive polymers in gene delivery applications. In this case, the previous knowledge of the thermosensitive behavior of PNIPAAM allowed us to draw additional specific aims, including:

iv. To study the efficiency of PNIPAAM block copolymers to interact and carry plasmid DNA into the nucleus of cells;
v. To evaluate the effect of the polymer composition on the transfection efficiency;
vi. To investigate the influence of the properties of the polymer-DNA complexes on the gene delivery potential.

3 Experimental considerations

3.1 Materials

The polymers and surfactants used in this work are indicated in sections below. All other chemicals were of analytical grade and were used as received. Details on the materials used in each study can be found in papers I-V.

3.1.1 Polymers

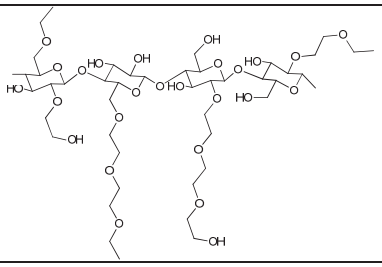
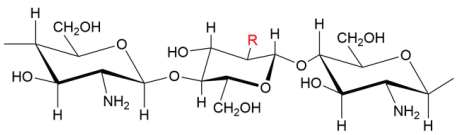
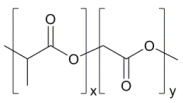
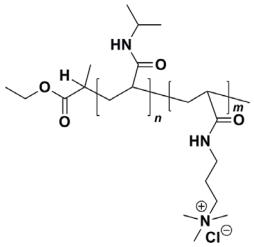
A number of different polymers were used in this work. A summary of these polymers is given in Table 1, where some of the most important properties are also indicated.

Ethyl(hydroxyethyl) cellulose (EHEC), mentioned in Papers I-III was gently offered by Akzo Nobel Surface Chemistry AB, Stenungsund, Sweden.

In Paper IV, chitosan from crab shells was from Sigma (USA) and poly(lactic-*co*-glycolic acid) (PLGA) was from Aldrich (USA). The hydrophobically-modified chitosan (HM-chitosan) was prepared according to a previously described procedure [147, 148], by reaction of the amino groups of the polymer chains with a C_{12} aldehyde.

In Paper V, four block copolymers based on poly(*N*- isopropylacrylamide)$_n$-*block*-poly((3-acrylamidopropyl)trimethylammonium chloride)$_m$ (PNIPAAM$_n$-*b*-PAMPTMA(+)$_m$) were studied. These block copolymers were distinct with respect to the length of the two blocks, where n = 48 or 65 and m = 6, 10 or 20. These polymers were synthesized by Dr. Kaizheng Zhu, Department of Chemistry, University of Oslo and the synthesis details can be found in the article by Bayati et al [149].

Table 1. Polymers used in this work.

POLYMER	SPECIFIC PROPERTIES	MOLECULAR STRUCTURE	PAPER NUMBER
EHEC	M_n = 80,000 DS_{ethyl} = 1.9/anhydroglucose unit MS_{EO} = 1.3/ anhydroglucose unit M_w/M_n = 2		**I, II, III**
Chitosan HM-chitosan	DD=75-85% M_W = 300-500 kDa DD=84% M_W = 400,000 C_{12}-imine: 5 mol%	 (Chitosan: R= H or R= -CO-CH_3 HM-chitosan: R= NH_2 or R= NH-CO-CH_3 or R= N=CH-$C_{11}H_{23}$)	**IV**
PLGA (RESOMER® RG 502)	Ester terminated Lactide:glycolyde 50:50 M_W = 7,000-17,000	 (x: number of lactic acid units, y: number of glycolic acid units)	
$PNIPAAM_{48}$-*b*-$PAMPTMA(+)_6$ $PNIPAAM_{48}$-*b*-$PAMPTMA(+)_{10}$ $PNIPAAM_{48}$-*b*-$PAMPTMA(+)_{20}$ $PNIPAAM_{65}$-*b*-$PAMPTMA(+)_{20}$	M_W = 6,770 Cloud point* = 35 °C M_W = 7,600 Cloud point* = 36 °C M_W = 9,690 Cloud point* = 37 °C M_W = 11,600 Cloud point* = 34 °C	 (*n:m*= 48:6, 48:10, 48:20, 65:20)	**V**

M_n – Number average molecular weight; DS_{ethyl} – Degree of substitution of ethyl groups; MS_{EO} – Molar substitution of ethylene oxide groups; M_w/M_n – Polydispersity index; DD – Degree of deacetylation
* determined for a polymer concentration of 0.3-0.7 wt%, depending on the maximum concentration used in the transfection experiments.

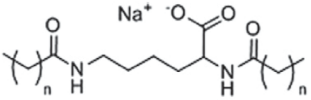
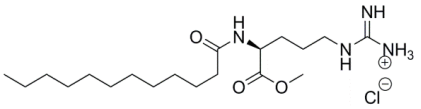
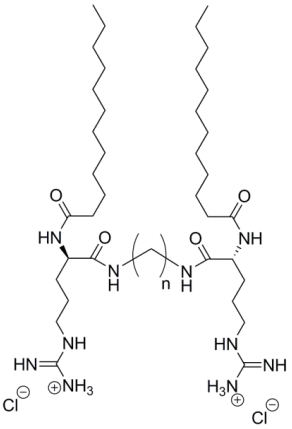
3.1.2 Surfactants

A list of the surfactants used in papers I-III is shown in Table 2. All surfactants were amino acid-derived. It was expected that by using amino acid-based surfactants, low toxicity levels would be observed, rendering them suitable for pharmaceutical applications.

In papers I and II, the following lysine-based surfactants were studied: sodium *N,N'*-dihexanoyl-L-lysinate (6Lys6), sodium *N,N'*-dioctanoyl-L-lysinate (8Lys8) and sodium *N,N'*-didecanoyl-L-lysinate (10Lys10). These anionic double-chained surfactants were prepared by lysine acylation with natural saturated fatty acids as described elsewhere [150] and they were kindly supplied by Professor Eduardo F. Marques from the Department of Chemistry and Biochemistry, Faculty of Science, University of Porto, Porto, Portugal.

In Paper III, three cationic arginine-based surfactants were investigated. Among these, one was a monomeric surfactant, and two had a gemini-like structure (bis(Arg) surfactants). The monomeric surfactant was N^{α} lauroyl arginine methyl ester hydrochloride (LAM) and the gemini were of the type N^{α},N^{ω}-bis(N^{α},-lauroyl arginine)α,ω-alkylenediamides, having 6 or 9 carbon atoms in the spacer (respectively designated $C_6(LA)_2$ or $C_9(LA)_2$). The synthesis procedure for these compounds can be found in previous publications [151, 152]. The three arginine-based surfactants were generously supplied by Professor Aurora Pinazo, Department of Chemical and Surfactant Technology, IQAC-CSIC, Barcelona, Spain.

Table 2. Surfactants used in this work.

SURFACTANT	SPECIFIC PROPERTIES	MOLECULAR STRUCTURE	PAPER NUMBER
6Lys6	M_W = 364 *cmc* (25 °C) ≥ 200 mM	 (n=4, 6Lys6; n=6, 8Lys8; n=8, 10Lys10)	**I, II**
8Lys8	M_W = 420.5 *cmc* (25 °C) = 7.0 mM		
10Lys10	M_W = 475 *cmc* (25 °C) = 0.29 mM		
LAM	M_W = 405.5 *cmc* (25 °C) = 6 mM [ref 153]		
$C_6(LA)_2$	M_W = 1057 *cmc* = 2 x 10^{-3} mM [ref 153]	 (n=6, $C_6(LA)_2$; n=9, $C_9(LA)_2$)	**III**
$C_9(LA)_2$	M_W = 1099 *cmc* = 3 x 10^{-3} mM [ref 153]		

M_W –molecular weight; *cmc* – critical micelle concentration

3.2 Methods

In this thesis, a variety of methods and techniques were used. Rheology was particularly relevant in papers I, III and IV, while cytotoxicity experiments were the focus of paper II and III. Cell viability studies were also employed in paper V. In paper IV, an emulsification-solvent evaporation method was employed to produce polymeric microparticles, according to a planned experimental design. Finally, in paper V, gene delivery studies were carried out. The main points of these techniques and methods will be briefly described in the next lines.

3.2.1 Rheology principles and Rheo-SALS

In papers I and III, the interactions between EHEC and the amino acid-based surfactants were studied by the combined technique Rheo-Small Angle Light Scattering (Rheo-SALS). In Paper IV, the viscosity of chitosan and HM-chitosan was evaluated by rheology. The concepts on which these techniques are based will be briefly presented below.

Rheology is concerned with the study of the deformation and flow of matter, when a mechanical stress is applied, and the studied parameters include the viscosity, elasticity, shear rate, shear strain and shear stress [154-157].

Some simple substances such as water exhibit Newtonian flow features in the sense that the shear stress (σ) is proportional to the shear rate ($\dot{\gamma}$) (where the constant of proportionality is the dynamic viscosity) at constant temperature and pressure.

For most substances, however, this linear relationship does not apply. These complex fluids are called Non-Newtonian, and systems such as foams, emulsions, and polymer melts and solutions can usually be included in this group [154, 158]. Some materials can for example demonstrate a decrease in viscosity at higher shear rates (shear-thinning or pseudoplastic behavior), or an increased viscosity at intermediate shear rates (shear-thickening or dilatant behavior)[158]. In some cases, the mechanical properties are intermediate between ordinary liquids and ordinary solids, and these fluids are hence designated as viscoelastic [154, 155].

Polymer solutions such as those studied in this thesis can exhibit strong viscoelastic effects, and/or shear thinning, shear thickening or time-dependent behavior [154].

The complexity of these systems immediately suggests that they cannot be fully characterized by the viscosity values alone, but rather by a combination of parameters such as the frequency-dependent storage modulus (G') and loss modulus (G'') and the shear-rate dependency of the viscosity [154]. For a complete characterization of the complex fluids, the relation of stress to strain must additionally be measured over a wide range of strains, strain rates and time scales [155]. The ultimate aim is to relate the viscoelastic response with the material structures [155].

In Paper V, the temperature-dependency of the viscosity of chitosan and HM-chitosan aqueous solutions was evaluated over a range of shear rates. In these steady shear measurements, a cone and plate geometry was used (Figure 4). The sample is placed between the stationary plate and the rotating cone. The cone angle is such that in each point on the cone surface, the ratio of angular speed and distance to the plate is constant. In other words, there is a constant shear rate across the entire radius of the cone.[154] The system was equipped with a Peltier temperature control unit.

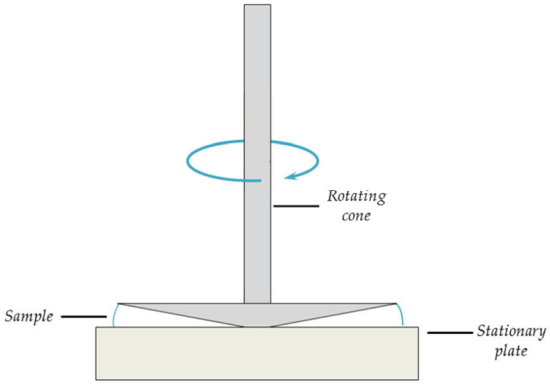


Figure 4. Cone-plate geometry used to measure the viscosity of chitosan and HM-chitosan in Paper IV. Figure adapted from ref [156].

In steady shear measurements using the cone-plate geometry, the shear rate $\dot{\gamma}$ is related with the steady angular rotation speed of the cone Ω and with the cone angle α (usually ≤ 0.10 radians) according to the relationship: $\dot{\gamma} = \Omega / \tan \alpha$ [154].

After a steady shear flow is applied to the polymer solution for a suitable period of time, the shear stress (σ) reaches a steady state. The determination of this value allows us to estimate the shear viscosity η according to: $\eta = \sigma/\dot{\gamma}$ [154].

In papers I and III, a Rheo-SALS with a plate-plate geometry was used. The plate-plate geometry allowed measurements on a very low sample volume (0. 6 ml) due to the possibility to adjust the gap height.

Temperature control was ensured by an external thermostat bath. The sample was applied between the two parallel plates, and the exposed surface of the sample was covered with a few drops of silicone oil, so as to prevent evaporation during the measurements. In this work, the rotating upper glass plate applied an oscillatory shear of small amplitude to the sample. Oscillatory shear experiments are widely used to characterize the viscoelastic behavior of non-Newtonian fluids. In polymer systems, oscillatory shear can also be used to monitor the viscoelastic properties of the networks during gel evolution [159].

In these experiments, the material is subjected to a sinusoidal deformation and the resulting mechanical response is measured over time [160]. The viscoelastic response of a sample such as a polymer solution is quantified by the elastic storage modulus (G') and the viscous loss modulus (G'') [160]. In this work, G' and G'' were estimated over a range of angular frequencies (ω).

In Paper I and III, the acquired rheological data was further used to assess the gel points. According to the method of Winter and Chambon [161], the gel points can be determined by the determination of the frequency-independent value of the viscoelastic loss tangent or damping tan δ (where δ is the phase angle between stress and strain and tan δ = G''/G'). In other words, the gel point can be determined by plotting tan δ versus temperature at different frequencies. The intersection point of the curves indicates the gel point.

The gel point can also be found by plotting the apparent viscoelastic exponents *n'* and *n''* (G' ~ $\omega^{n'}$; G'' ~ $\omega^{n''}$) at different temperatures, and finding the crossover where *n'* = *n''* = *n*. When a gelling system is identified, the structure of the network can be defined by the gel strength parameter (*S*). This parameter is determined by the equation

$G' = \frac{G''}{\tan\delta} = S\omega^n \Gamma(1-n)\cos\delta$, where *S* is the gel strength parameter and $\Gamma(1\text{-}n)$ is the Legendre gamma function. The gel strength depends on factors such as the molecular weight of the polymer and the cross-linking density and molecular chain flexibility [159, 162].

Finally, the rheological data was also used to determine the fractal dimension (d_f), indicative of the homogeneity of the network, according to the model of Muthukumar [163] and the equation: $n = \frac{d(d+2-2d_f)}{2(d+2-d_f)}$, where d represents the space dimension (d=3).

While the rheological methods reveal important information concerning the rheological behavior of soft materials and their macroscopic properties, it is often desirable to acquire information related with the underlying microstructures [155, 164]. This can be achieved by combining standard rheometers to optical systems, *e.g.* via fluorescence microscopy or magnetic resonance imaging, or via scattering techniques such as SALS [155, 164]. In SALS, a laser beam is applied to the sample, 2D scattering patterns are acquired and the angular distribution (and intensity) of the scattered light is measured[164]. Specific orientations and deformations of a material can for instance be identified by a stretched scattering pattern [164], while the occurrence of aggregates can result in an augmented intensity and/or radius of the 2D pattern [165].

A picture of the combined Rheo-SALS instrument used in this work is shown in Figure 5. Simultaneously with the application of an oscillatory shear, a primary laser beam was focused on the sample. The scattered light was collected by a lens system and the 2D scattering patterns were transferred onto the CCD chip of a camera.

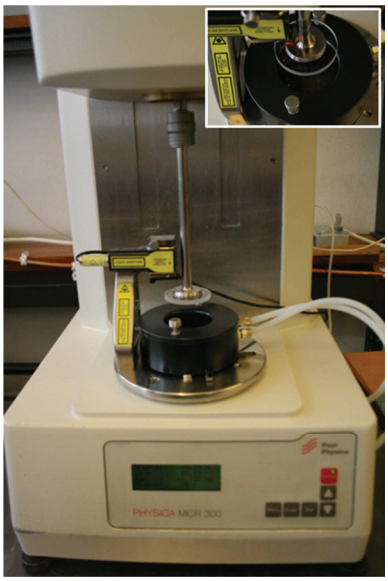


Figure 5. Rheo-SALS instrument used in this study (plate-plate geometry), showing the rotating upper plate and the laser beam (inset).

By analyzing the intensity of the scattered light, one can also determine the cloud points, taken as the temperature at which a very sharp intensity increase is observed.

3.2.2 Cell viability studies

In papers II and III, cell viability studies were carried out in order to investigate the cytotoxicity of the amino acid-based surfactants and the corresponding surfactant-EHEC gelling systems.

All cell viability studies were carried out in human HeLa cells (epithelial cervical carcinoma cell line).

Cells were incubated with the surfactants at different surfactant concentrations or with the gelling systems for a period of 24 h at 37 °C. After this time, cell viability was estimated by the modified Alamar Blue or resazurin assay. In this method, the dark blue resazurin (Sigma-Aldrich, USA) is reduced to the pink and highly fluorescent resorufin in the

presence of living cells [166] (Figure 6). By colorimetry, it is therefore possible to investigate the oxido-reductive capacity of the cells, which reflects the cell viability and proliferation. One important advantage of Alamar Blue in relation to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), the most conventionally used compound in toxicity assessment, is the fact that Alamar Blue is nontoxic, not causing cell death during the measurements, and allowing cell reuse [166]. Positive (untreated cells) and negative (absence of cells) controls were prepared in all experiments.

The cell viability was calculated according to the equation:

$$\text{Cell viability } (\%) = \frac{(A_{570} - A_{600}) \text{ of treated cells}}{(A_{570} - A_{600}) \text{ of control cells}} \times 100$$

In Paper V, similar viability studies were carried out in parallel with the gene delivery experiments. In this case, however, cell viability was measured 44 h after the initial transfection period (4 h), in agreement with the transfection protocol used.

The complete procedure concerning the viability studies can be found in Papers II, III and V.

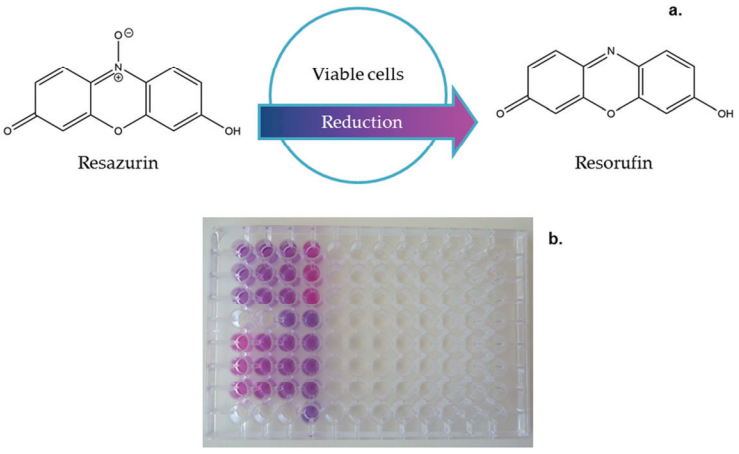


Figure 6. a. Reduction reaction of Alamar Blue (resazurin) in viable cells due to the presence of reducing metabolites; **b.** color modification observed in 98-well plates such as those used in this project (pink showing the presence of resorufin and indicating the presence of viable cells, and dark blue showing cells with low reducing capacity, interpreted as low viability wells).

3.2.3 Production and characterization of microparticles for drug delivery

In Paper IV, polymer-based microparticles were formed as carriers for a model drug (naltrexone). The microparticles were produced by an emulsification-solvent evaporation method. In the single emulsion strategy, the polymer and drug are firstly dissolved in an appropriate solvent and dispersed in a continuous immiscible phase by stirring in the presence of an emulsifier. Droplets are thereby formed and transformed into hard spheres as a consequence of the evaporation of the solvent [131, 133]. Finally, the microparticles can be recuperated, washed and dried or freeze-dried [133].

In the case of water-soluble drugs, it is sometimes advantageous to prepare a double emulsion, *i.e.* by dispersing the aqueous polymer + drug solution in an organic solvent (water-in-oil emulsion) and adding this primary emulsion to an aqueous phase containing an emulsifier, hence forming a water-in-oil-in-water (w/o/w) emulsion [132]. In this work, however, the model drug (naltrexone) was not significantly water-soluble and the production of a double emulsion was considered unnecessary.

The emulsification-solvent evaporation method is one of the most widely used methods to prepare microparticles, and it has important advantages such as the simplicity and inexpensiveness of the procedure and instruments, and the fact that it allows the control of process parameters [133].

Taking advantage of the possibility to control these parameters, an experimental design was developed, in order to investigate the influence of factors such as polymer concentration, needle diameter (used to add the polymer solution to the continuous phase), stirring speed and temperature (in the emulsification stage), on the microparticles' size properties (in the absence of drug). The procedure according to the emulsification-solvent evaporation method is outlined in Figure 7.

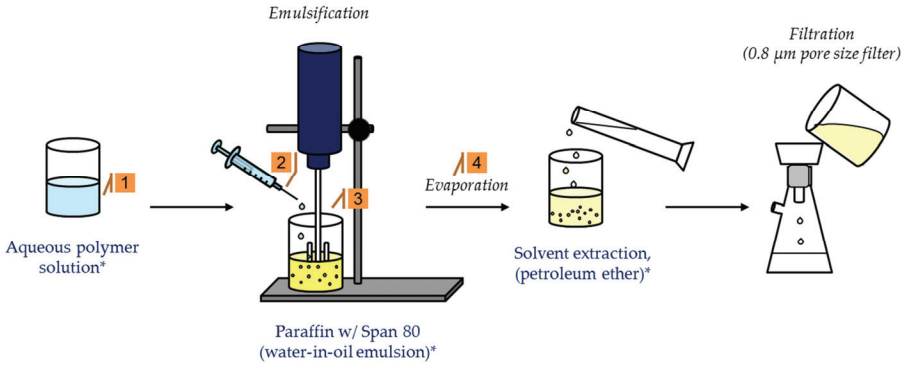


Figure 7. Emulsification-solvent evaporation method used for the production of chitosan and HM-chitosan microparticles. The numbers in the orange boxes represent the factors investigated in the experimental design, *i.e.* polymer concentration (1), needle diameter (2), stirring speed (3) and temperature (4).
* In the case of PLGA microparticles, the polymer was dissolved in dichloromethane, the emulsification of the polymer solution was carried out in water containing polyvinyl alcohol as the emulsifier, and water was added in the solvent extraction step.

In general, statistically designing the experiments and making several factors vary simultaneously has several advantages over the strategy of varying one factor at a time. A designed experiment requires fewer resources (time, material, etc.) and offers the possibility to estimate individual effects and the interaction between factors, and the response curvature in the experimental region (response deviation from linearity). In addition, the experiments are planned in advance and the developed model can give a good prediction of the response over the entire factor space [167, 168].

In this work, we planned to analyze the effect of 4 factors (indicated by the numbers in the orange boxes, Figure 7) on the size and size distribution of the polymeric microparticles. A full factor experimental design with two levels (high and low) would imply $2^4 = 16$ experiments plus 3 center points (analysis of the experimental curvature and analytical variability). Considering that 3 polymers (chitosan, HM-chitosan and PLGA) were used as the polymeric matrix, a full experimental design would involve a minimum of 48 experiments in addition to the center points. The large number of experiments was therefore the reason for designing a fractional factorial design, corresponding to $2^{4-1} = 8$ experiments (plus 3 center points) for each polymer (total of 33 experiments). By doing so, the main effects and interactions can still be determined, but this implies that the two-factor interactions cannot be distinguished from each other (confounding effect) [168].

After the determination of the effects of the process parameters, specific conditions were used to prepare naltrexone (NTX)-loaded microparticles based on the three polymers. Details of the emulsification conditions can be found in Paper IV. The microparticles were further analyzed in terms of production yield, drug content, morphological features and controlled release capacity, as described in the paper.

3.2.4 Production and characterization of polyplexes, and gene delivery studies

In Paper V, the thermoresponsive PNIPAAM-*b*-PAMPTMA(+) block copolymers were evaluated as gene carriers in HeLa cells.

Polyplexes were formed by incubating the polymer solutions with plasmid DNA coding for green fluorescent protein (GFP) for 20 min, at pre-defined polymer nitrogen-to-DNA phosphate (N/P, +/-) charge ratios. The cells were incubated with the polyplexes at 37 °C for 4h, the medium containing the polyplexes was then replaced with fresh medium and the cells were incubated for another 48h to allow gene expression. In the end, cells were detached from the plate wells and the fluorescence was measured by flow cytometry. In this instrument, physical and chemical features of cells are evaluated as the cells are made to flow through the instrument in a fluid stream. A laser light is focused onto the flow cell by a focusing lens. As the laser light passes through the cells, the light is scattered, filtered and directed to the photodetectors that convert the light into electric signals. Forward scatter detectors placed in line with the light beam provide a suitable detection of particles that are larger (such as masses consisting in two or more cells) or smaller (such as cell debris) than the normal cell size, while side scatter detectors, placed at approximately 90 degrees from the laser path, measure the side-scattered light that is proportional to cell granularity or cell internal complexity. If the cells contain fluorochromes such as GFP, these will be excited and emit fluorescence, which is then collected by fluorescence detectors. The scattered data is 'gated' *i.e.* a subset of measurements is created, so as to include the intended particle characteristics only in further analysis (dead cells, other cell types and debris can be excluded in this way) (information provided by the manufacturer, BD Biosciences, USA). The transfection efficiency (based on GFP fluorescence) was calculated with basis on the scattered data of the sample in relation to the untreated (control) cells.

The properties of the polyplexes prepared at specific N/P ratios were analyzed in terms of DNA protection ability, zeta potential, size and turbidity (in aqueous buffer). The detailed procedures concerning the transfection experiments and the characterization of the polyplexes are given in Paper V.

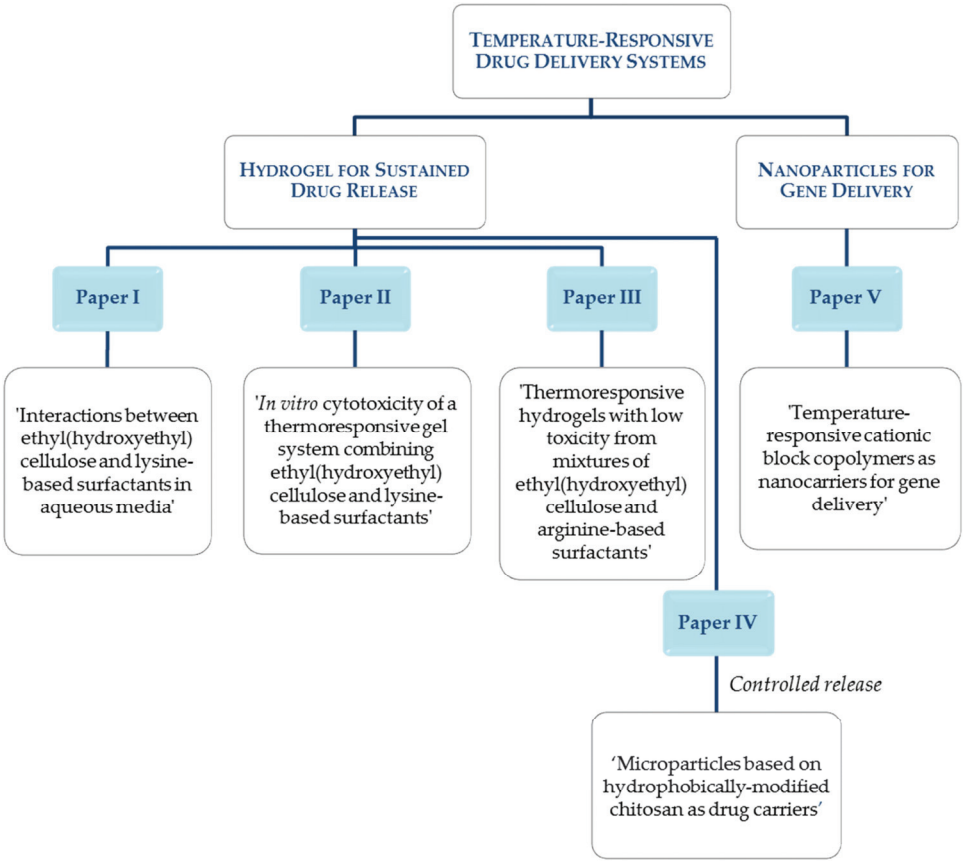
3.2.5 Other experimental techniques

In addition to the methods described above, a number of other techniques were used to characterize the systems under investigation. Specifically, in paper IV, the size and size distribution of the microparticles was evaluated by using a particle sizing instrument that measures particle size by detection of the magnitude of the obscured light, in dilute suspensions (detecting one particle at a time). The controlled release properties of the microparticles were investigated by an *in vitro* method. The released drug was quantified by measuring its absorbance at a specific wavelength, in a spectrophotometer, and with resource to a calibration curve.

In paper V, dynamic light scattering was used to determine the hydrodynamic radius (R_h) of the polyplexes. Zeta potential measurements were employed to characterize the electrostatic potential near the surface of the surfactant micelles (paper I) or the polyplexes (paper V). Turbidity measurements were carried out by measuring the transmittance of aqueous suspensions of the polyplexes (paper V), in order to investigate their structural arrangements, *e.g.* compactness, in suspension. Details of the procedure are given in the relevant papers.

4 Summary of papers

The papers included in this thesis reflect the steps taken towards the development of new thermoresponsive gel systems for the delivery of conventional drugs, and with potential in gene delivery applications, as summarized in the following chart.



Scheme 1. Outline of this thesis based on the investigated subjects and published papers.

4.1 Paper I

EHEC is a well-known amphiphilic polymer, and at sufficiently high concentrations (semidilute regime) it is able to form temperature-dependent hydrogels in the presence of ionic surfactants. In the first paper, the interactions between EHEC and three ionic surfactants were explored. The ionic surfactants investigated here had a gemini-like structure and lysine as the polar head group. The surfactants were distinct in terms of the length of the alkyl chains, comprising 6, 8 or 10 carbon atoms (respectively designated 6Lys6, 8Lys8 and 10Lys10). It was therefore hypothesized that these surfactants could replace the conventional and more toxic surfactants such as SDS and CTAB in the induction of sol-gel transitions of EHEC solutions. Rheological measurements were carried out by Rheo-Small Angle Light Scattering. The solutions showed a low viscosity in the presence of all surfactants at low temperatures. In addition, the viscosity tended to increase with temperature, in manner that greatly depended on the surfactant type and its concentration. Sol-gel transitions were observed at specific surfactant concentrations (lower for the longer chain-length surfactants) as the temperature was increased. At this point, the associations between the hydrophobic groups of EHEC are balanced by the swelling caused by charge repulsion, inducing gelation. Due to the higher association tendency of the longer chain-length surfactants, these were able to form gels at lower concentrations, but in this case the gel point was found to shift slightly to higher values. In the presence of all surfactants, soft, homogeneous gels were produced, as determined by the estimation of the gel strength parameter and fractal dimension values. Finally, the 2D SALS patterns allowed the estimation of the cloud points and provided further evidence of the association tendencies of the systems.

4.2 Paper II

The premise of the previous paper was that the amino acid-based surfactants could be more biocompatible than the conventional surfactants, which would open a window towards their potential use (and of the hydrogels produced by their combination with EHEC) in pharmaceutical and biomedical applications.

In Paper II, the toxicological properties of the lysine-based surfactants and of the EHEC-surfactant hydrogels were investigated, by using *in vitro* experiments on a human cell line

(HeLa cells). The EC_{50} of the surfactants, *i.e.* the concentration that induces a response halfway between the baseline and the maximum response, was found to decrease with the increasing chain length, suggesting a higher toxicity for the most hydrophobic surfactant. Importantly, the most hydrophilic surfactant (lower chain-length) was less toxic than the commercial SDS and CTAB. Having in mind the aforementioned potential applications of the surfactants, it was deemed important to evaluate the toxicity with respect to the 'effective concentration', *i.e.* the concentration previously shown to induce gelation of semidilute solutions of EHEC. In this context, the longer chain-length surfactant was found potentially interesting, since its higher toxicity is partly compensated by its high efficacy (implying the use of very low concentrations for the intended result).

In the final part of this paper, the cytotoxicity of the EHEC-surfactant hydrogels was evaluated. The results showed that the polymer contributes to cell stabilization and to a decrease of the toxicity caused by the presence of the ionic surfactant. The conjecture was that the presence of EHEC causes the surfactant molecules to preferentially interact with the polymer's hydrophobic groups, leaving the surfactant molecules less available to adsorb and disrupt the cell membranes.

Considering the applications for human use, this work showed that hydrogels comprising lower chain-length surfactants are less likely to cause cytotoxic effects, while the longest chain-length counterparts can also constitute promising alternatives, considering their high efficiency at low concentrations.

4.3 Paper III

This paper described the studies on an alternative system comprising EHEC and arginine-derived surfactants. One monomeric surfactant, N^{α}-lauroyl-L-arginine methyl ester (LAM) and two gemini surfactants with distinct spacer length (6 and 9 carbon atoms, respectively designated $C_6(LA)_2$ and $C_9(LA)_2$) were analyzed and compared in terms of the rheological properties in the presence of semidilute EHEC solutions. The complex viscosity was generally low at low temperatures and progressively increased as the temperature was raised and as the associations within and between polymer chains increase. It was noticeable that the concentrations at which sol-gel transitions were observed were significantly higher for LAM than for the more efficient gemini surfactants. The gel point, fractal dimension and gel

strength were all found dependent on the type and concentration of surfactant. Rheo-SALS also revealed that in the presence of very high concentrations of the gemini surfactants (*ca.* 1000 times the critical micelle concentration), a second viscosification peak is observed, which is probably related with the self-assembling properties of the surfactants in the bulk, rather than an effect of the interactions with the polymer.

The scattered intensities of the incident light on the samples (measured by SALS) were used to determine the cloud point values. A pronounced effect of surfactant concentration was observed when LAM was used. Conversely, changing the concentration of the gemini surfactants only had a minor effect on the cloud point.

This paper also describes the results of *in vitro* cytotoxicity experiments carried out on a human epithelial cervical carcinoma cell line. The monomeric surfactant was shown less toxic than the gemini counterparts, in consistency with its lower hydrophobicity created by the presence of a single alkyl chain. An interesting finding was also that $C_6(LA)_2$ was shown to be more toxic than $C_9(LA)_2$, in spite of the longer spacer present in the latter. This observation was related to the increased flexibility of longer spacers, a feature that can change the conformation of the molecules in the aqueous environment and affect their interaction with the cells. In the presence of the hydrogels, *i.e.* at the surfactant concentrations previously shown to induce sol-gel transitions of semidilute EHEC solutions, cell viability was found significantly higher in the presence of the gemini surfactants than in the presence of LAM. This was related to the higher gel-forming efficiency of the gemini surfactants compared to LAM, for which very high concentrations are necessary in order to induce gelation of EHEC at high temperatures.

4.4 Paper IV

Paper IV describes the study of polymer-based microparticulate systems for the delivery of a low water solubility drug – naltrexone (NTX).

Having in mind the use of the prepared hydrogels (Paper I, II and III) as sustained drug release systems, it was envisioned that the drug release rate could be improved by combining the hydrogels with drug-loaded microparticles. In other words, the two-component system would involve:

i. drug-loaded microparticles
ii. a temperature-responsive polymer solution, serving as a suspension medium for the microparticles.

The injection of this system should allow the *in vivo* gelation of the solution (due to the temperature increase), forming a depot system that, while restricting the movement of the microparticles, could allow the slow diffusion of the drug, and result in a long-standing therapeutic effect. A scheme describing this principle is shown in Figure 13.

In Paper IV, the first step into the development of this depot system is given, by exploring the production of NTX-loaded microparticles in the absence of the hydrogel and by investigating their controlled release properties.

A hydrophobically-modified (HM) chitosan (containing C_{12} groups) was used to prepare the microparticles, since it was expected that the chemical modification would improve the long-term stability of the polymer. For comparison, microparticles were also prepared from an unmodified chitosan and from the hydrophobic poly lactic-*co*-glycolic acid (PLGA). All samples were prepared using an emulsification-solvent evaporation method.

HM-chitosan microparticles showed the highest yield and drug encapsulation efficiency (EE), which was related with the high viscosity of the HM-chitosan polymer solutions and with the strong interactions between the polymer and NTX. Furthermore, this study showed that high HM-chitosan concentrations and rapid solvent evaporation rates contributed to increase the drug loading, while this also caused the particles to become larger. Excluding the cases where high concentrations of HM-chitosan were used, most microparticles were smaller than 50 μm. Drug encapsulation within chitosan and PLGA microparticles was generally less efficient, possibly due to the lower viscosity of the dispersed phase and due to weaker polymer-polymer interactions and fewer polymer-drug associations.

In this article, the *in vitro* release rate of NTX was also evaluated, and showed that, in spite of a moderate initial burst release, HM-chitosan microparticles were more able to control drug release than PLGA or chitosan microparticles, in the long-term. A slow drug release rate was observed for at least 50 days. It was assumed that the hydrophobic groups functioned as physical cross-linkers of the matrix, due to the establishment of hydrophobic inter-chain associations. It is also likely that NTX, being a low water solubility drug, has a high affinity to this polymer, which explains the high EE and the low drug release rates.

This study revealed the potential of hydrophobically-modified polymers in long-standing drug delivery applications.

4.5 Paper V

In paper V, the physicochemical characterization of PNIPAAM-based temperature-responsive polymers is described, when these were combined with DNA for gene delivery purposes.

The results shown in this paper derived from the cooperation with the Center for Neuroscience and Cell Biology (Molecular Biotechnology and Health – Vectors and Gene Therapy, University of Coimbra, Portugal), specialized in gene delivery studies. In this work, four temperature-responsive polymers, synthesized in our group, were evaluated in terms of their potential to form complexes (polyplexes) with plasmid DNA (pDNA, coding for green fluorescent protein or GFP) and deliver it efficiently to the nucleus of HeLa cells. The polymers consisted of two blocks, one of which comprising the temperature-responsive PNIPAAM and the other a positively charged PAMPTMA. The four block copolymers were additionally distinct in terms of the length of each block.

When incubated with the cells, the polyplexes were shown to efficiently deliver the pDNA into the nucleus, where the expression of GFP was monitored by a flow cytometer. The extent of gene expression and associated toxicity varied expressively among the four polymers, and these results were also found dependent on the charge ratio between polymer and DNA (N/P or +/-). All polymers were shown to protect DNA in an effective manner. Polymers containing a long PNIPAAM block and having a short charged block were found to be more efficient gene carriers. Higher concentrations also contributed to higher transfection efficiency, but caused greater toxic effects.

The differences observed between the systems were further investigated by studying the physicochemical properties of the polyplexes, namely size, zeta potential and turbidity, and at the temperatures used in the transfection experiment. The determination of these properties provided some insight into the relationship between polymer composition and the resulting polyplex structures. Ultimately, this study also revealed some important information regarding the influence of the structures of the temperature-responsive

polyplexes over their gene delivery efficiency. The key point was that the use of positively charged polymers as gene carriers allows a strong interaction with the oppositely charged DNA, while the thermoresponsive behavior contributes to encapsulate the payload at physiological temperature.

5 Results and Discussion

5.1 Thermoresponsive hydrogels as DDSs

In this thesis, the first objectives were related with the development of a thermoresponsive gel system demonstrating low viscosity at low temperatures and gelation at higher temperatures. This system was intended as a drug delivery formulation that can be easily injected due to the low viscosity at low temperatures, forming a gel *in situ* as a response to body temperature.

In view of these objectives, the EHEC-ionic surfactant system was investigated, using the lysine- and arginine- based surfactants indicated in Table 2 (page 36). The rheological properties of the systems were evaluated (Papers I and III), while their *in vitro* toxicity was also investigated on a human cell line (Papers II and III), due to the envisioned pharmaceutical use.

By using ionic amino acid-derived surfactants, it was anticipated that the toxicity of the system would be decreased as compared to the use of conventional surfactants. This principle has actually been claimed previously [169-172].

In this work, a total of 6 surfactants were investigated as components of semidilute aqueous EHEC solutions (polymer concentration= 2 wt%). The use of semidilute polymer solutions was deemed very important, since in this regime the polymer chains overlap, thus ensuring the connectivity of the network under the appropriate conditions. Of all surfactants, three were anionic and synthesized from lysine (Papers I and II), and contained two C_6, C_8 or C_{10} alkyl chains, hence being designated 6Lys6, 8Lys8 and 10Lys10. These double-chained surfactants contained two polar heads covalently linked to each other by a short alkyl chain of constant length (spacer). By analyzing the three lysine-based surfactants, it was therefore possible to evaluate the effect of the alkyl chain length on the rheological properties of EHEC semidilute solutions.

In contrast with the former, the arginine-based surfactants were cationic. Two of these also had a gemini-like structure, but in this case they differed in the spacer length, *i.e.* $C_6(LA)_2$

containing a C_6 spacer, and $C_9(LA)_2$ containing a C_9 spacer. The importance of the spacer length could consequently be probed in terms of the gel-forming properties of EHEC solutions. In addition, these gemini-like surfactants were compared with the corresponding monomeric LAM.

Figure 8 shows the effect of temperature and surfactant concentration on the viscosity of EHEC solutions. All systems demonstrated low viscosities at room temperature, and higher viscosities at 37 °C, depending on surfactant concentration.

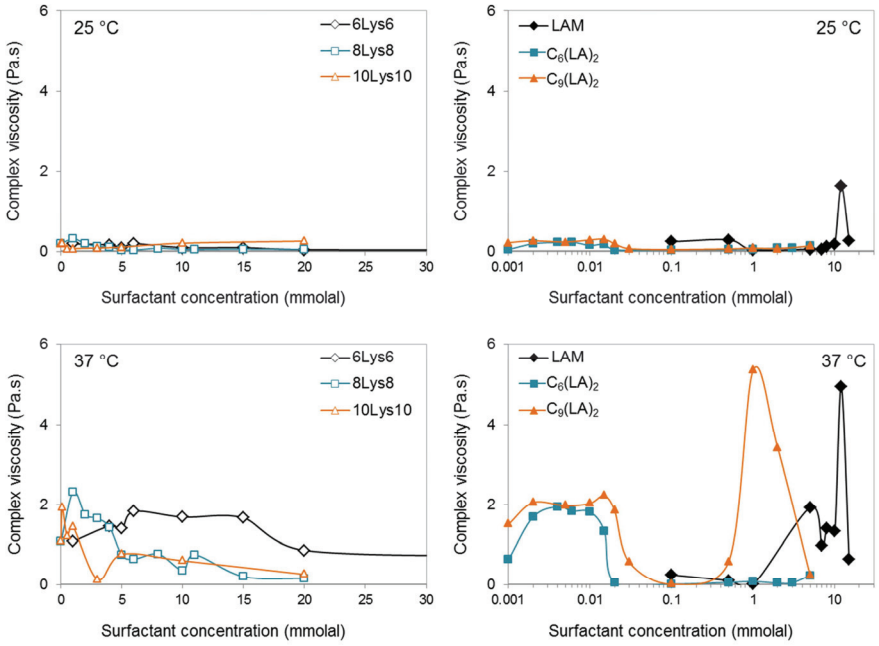


Figure 8. Complex viscosity of EHEC-surfactant systems at 25 and 37 °C, at different surfactant concentrations. EHEC concentration = 2 wt% in all systems.

When small amounts of the amphiphilic surfactant molecules are present, they assemble in the vicinity of the hydrophobic groups (Figure 9 b.). In addition, one of the most striking properties of mixed polymer-ionic surfactant systems is the fact that the formation of mixed micelles at moderately high surfactant concentrations causes a viscosification of the solution, as shown in Figure 8 for all systems at 37 °C. The underlying reasons are twofold, since the mixed micelles act as cross-linking points (connecting two or more polymer

chains) and cause the matrix to swell due to charge repulsion between the ionic surfactant molecules. It is the balance between these effects (Figure 9 c.) that causes the viscosification of the polymer solution, and allows the sol-gel transition at adequate surfactant concentrations. This behavior between amphiphilic polymers and ionic surfactants has been extensively discussed before [87, 88, 90, 94].

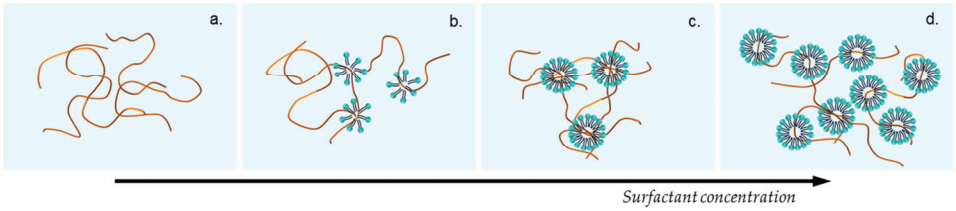


Figure 9. Effect of surfactant concentration on the network structures. **a.** Absence of surfactant; **b.** In the presence of small amounts of surfactant, the surfactant molecules arrange around the hydrophobic microdomains of the polymer; **c.** At sufficiently high surfactant concentrations, the widespread formation of mixed polymer-surfactant micelles increases the connectivity of the network (the connectivity is balanced by the charge repulsion effect of the ionic surfactant); **d.** At high surfactant concentrations, the connectivity of the system will be disrupted due the combinations of an excessive swelling effect and the solubilization of the hydrophobic microdomains by the surfactant molecules.

In general, the results in Figure 8 show that the surfactant concentration at which the increase in viscosity is observed follows the *cmc* trend, *i.e.* occurring at lower concentrations for the surfactants with lower *cmc* (values shown in Table 2, page 36). In the case of the Lys-based surfactants, the increase in alkyl chain length causes an increase in the hydrophobicity of the surfactant that tends to associate at lower concentrations. In the presence of the amphiphilic EHEC, this implies a decrease in the critical aggregation concentration (*cac*) and a decrease in the surfactant concentration that leads to the viscosity increase at elevated temperatures. Accordingly, the remarkably low *cmc* of the gemini arginine-based surfactants (2×10^{-3} mM for $C_6(LA)_2$ and 3×10^{-3} mM for $C_9(LA)_2$, Table 2) causes them to associate at very low concentrations. This has major implications from a prospective industrial use, since very low amounts are needed in order to cause viscosification of the EHEC solution (surfactant concentration in the range 1×10^{-3} to 1×10^{-2} mM). In addition, the need for lower surfactant concentrations is expected to contribute to a lower toxicity, as will be discussed later. In contrast, the lower efficiency and higher

cmc of the monomeric surfactant LAM causes it to associate (and increase the viscosity) at significantly higher concentrations, as shown in Figure 8.

In all cases, the viscosity tends to decrease as the surfactant concentration is further increased. At this stage, the connectivity of the system will be disrupted due the combinations of an excessive swelling effect (due to charge repulsion) and the solubilization of the hydrophobic domains by the excess of surfactant molecules [86, 90]. This scenario is illustrated in Figure 9 d.

In Paper III, it was also discussed that $C_9(LA)_2$ probably forms self-associating structures at very high concentrations (*ca.* 1000 times higher than the *cmc*), as a consequence of strong hydrogen bonding within and between the surfactant molecules and the surrounding water. This behavior was suggested as the reason for the observed second viscosity maximum.

The sol-gel transitions were determined as described before in the section 'Experimental considerations'. Pictures of some gels (no flow observed as the temperature is increased and the vial is inverted) are shown in Figure 10. The most significant gel points and gel parameters are also indicated in Table 3.

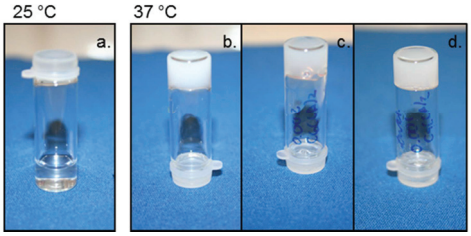


Figure 10. Examples of 2 wt% EHEC in the presence of the surfactants at 25 °C (**a.**) and at 37 °C (**b.-d.**). **a.** and **b.** – 5 mmolal 6Lys6; **c.** – 2 x 10^{-3} mmolal $C_6(LA)_2$; **d.** – 1 x 10^{-3} mmolal $C_9(LA)_2$.

The surfactant concentration inducing a sol-gel transition in semidilute EHEC solutions was found to vary among the different surfactants, essentially following the previously discussed relationship between *cmc* and concentration at which the viscosity increase is observed. The most effective surfactants were the arginine-based surfactants with a gemini-like structure. These surfactants aggregate at low concentrations (low *cac*) due to the presence of the long alkyl chains and spacer (structures shown in Table 2). In contrast, the highest surfactant concentrations inducing gelation of EHEC were found in the case of LAM, the only

monomeric surfactant analyzed, which clearly demonstrates the lower surface activity and efficiency of the monomeric surfactants with respect to the dimeric or gemini counterparts.

The gel points were found between 36 and 44 °C. The fact that in most cases the fractal dimension (d_f) was 2.4 suggests the formation of relatively homogeneous, closed networks [87, 93]. However, the fact that the gel strength was not higher than 19 $Pa \cdot s^n$ indicates the presence of soft gels which are weaker than those reported before in the presence of CTAB (S = 25 $Pa \cdot s^n$) and SDS (S = 45 $Pa \cdot s^n$) [93]. Nonetheless, it is likely that stronger gels are produced using higher polymer concentrations, as shown previously for the EHEC/SDS and EHEC/CTAB systems [93].

When gels were formed at different concentrations of the same surfactant (*i.e.* for $C_6(LA)_2$ and $C_9(LA)_2$), an increase in surfactant concentration generally caused the gel point to increase and the gel strength parameter to decrease. This is caused by the excess of surfactant which leads to a higher solubilization of the polymer's hydrophobic microdomains. As a result, a higher temperature is necessary to create the connectivity necessary to cause the sol-gel transition, and the resulting gels still demonstrate a weakened network (lower *S* values).

Table 3. Gel properties of the thermoresponsive EHEC-surfactant systems investigated in this work.

SURFACTANT	SURFACTANT CONCENTRATION (mmolal)	GEL POINT (°C)	GEL STRENGTH PARAMETER (S, $Pa \cdot s^n$)	FRACTAL DIMENSION (d_f)
6Lys6	5.0	37.0	14.2	2.4
8Lys8	3.0	39.0	17.2	2.4
10Lys10	0.1	40.0	17.5	2.4
$C_6(LA)_2$	2.0×10^{-3}	36.5	18.3	2.4
	1.0×10^{-2}	40.0	17.1	2.4
	1.5×10^{-2}	44.0	14.3	2.4
$C_9(LA)_2$	2.0×10^{-3}	39.0	18.9	2.5
	1.0×10^{-2}	43.0	16.4	2.4
	3.0×10^{-2}	39.0	5.6	2.4
LAM	12.0	36.0	14.2	2.1

In papers I and III, the polymer-surfactant systems are also characterized in terms of the cloud point profile at different surfactant concentrations (Figure 11). The CP is defined by the temperature at which the polymer solution undergoes a microscopic phase separation and this is shown by a turbidity increase as this temperature is reached [173, 174].

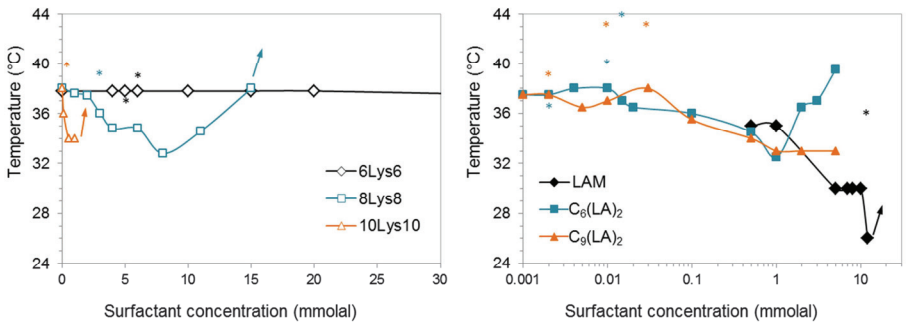


Figure 11. Effect of surfactant type and concentration on the cloud point values of the EHEC-surfactant system. The arrows in the figure indicate an increase in the cloud point to values higher than the instrument's upper temperature limit (44 °C). The gel points at specific surfactant concentrations are also indicated by the star symbols in the figure.

For 8Lys8 and 10Lys10, the cloud point was shown to decrease with increasing surfactant concentration until a minimum value was reached. At this concentration, the cloud point increased with increasing surfactant concentration. Similar observations were made for LAM, in Paper III. The presence of a minimum CP has also been reported before for the EHEC/SDS system [86, 90], and it indicates a higher tendency for aggregation as the concentration is increased, possibly due to the formation of mixed aggregation sites and due to the high hydrophobicity of the long-chain surfactants. Only at sufficiently high surfactant concentrations, the charge repulsion effect becomes significant, inducing the repulsion between the polymer chains and causing the CP to increase. However, the cloud point of 6Lys6 solutions remained unchanged at the measured range of concentration and temperature, which was related with the high hydrophilicity and low association tendency of this surfactant. Interestingly, none of the gemini arginine-based surfactants showed a significant decrease of CP at the gel-forming concentration range (1 x 10^{-3} to 1x10^{-2} mmolal, see star symbols in Figure 11 for GP), in spite of the presence of the long alkyl chains and hydrophobic spacer. This may be explained by the presence of two charged polar

head groups (instead of one, as in the Lys-based surfactants, Table 2, page 36). This causes a prominent charge repulsion effect, which balances the hydrophobic phenomena that are caused by the surfactant tails and by the association of EHEC at these temperatures.

The CP was generally closely related with the estimated GP, due to the high connectivity of the system as the temperature is increased. In the vicinity of the gel point, small clusters of hydrophobic groups form, trigging an increase in the turbidity of the solution. In addition, the CP was always higher than the GP, suggesting the formation of turbid gels, with the exception of 6Lys6 (GP= 37.0 °C; CP=37.8 °C) and $C_6(LA)_2$ (GP= 36.5 °C; CP=37.5 °C) at the lowest concentration (Table 3).

It is also worth discussing the relationship between structure and properties observed for the surfactants. As mentioned before, the different length of the alkyl chains in the Lys-based surfactants was shown to have a prominent effect on the viscosity profiles, GPs and CPs. For these surfactants, an increase in the alkyl chain length generally caused the viscosification, the sol-gel transition and the CP upturn to occur at lower concentrations. In contrast, this tendency was not very clear for the gemini Arg-based surfactants, in which the increase in spacer length did not reveal a very pronounced effect over the measured rheological properties, since similar profiles were observed. However, the lower efficiency of the monomeric surfactant was clearly demonstrated, as pointed out previously.

Surfactants, and, particularly, those bearing a positive or negative charge, are commonly observed to be environmentally toxic and poorly biocompatible and biodegradable [95]. This is a major issue that compromises their use in biomedical and pharmaceutical applications. In this work, the investigation of the toxicological implications of the surfactants was therefore considered very important, having in mind a potential pharmaceutical use. The results for the *in vitro* experiments on a human epithelial cervical carcinoma cell line (HeLa) were presented and discussed in papers II and III. In Figure 12, a summary of the estimated EC_{50} values (concentration that induces a response halfway between the baseline and the maximum response) and the minimum effective surfactant concentration (MESC) that induces the thermoresponsive gelation of EHEC is presented.

In Paper II, where the Lys-based surfactants were investigated, the EC_{50} values followed the tendency: 6Lys6 > 8Lys8 > 10Lys10, indicating that the toxicity increases with the alkyl

chain length, due to the increased hydrophobicity of the molecule and higher capacity to destabilize the biological membranes.

In Paper III, it was not surprisingly shown that among the Arg-based surfactants, the monomeric LAM caused the lowest toxic effects (higher EC_{50}), due to the presence of a single alkyl chain and only one charged group. This implies that the surfactant is less prone to disturb the cell membranes. In opposition, the higher hydrophobicity and charge density of the gemini counterparts causes them to be more cytotoxic.

In short, surfactants with longer alkyl chains and with a dimeric structure were the most cytotoxic. However, these are also the most efficient and only very low amounts are needed in order to induce the temperature-responsive gelation of EHEC. In this context, it is imperative that the results are analyzed in terms of the relationship between the toxicity and the MESC. If one analyzes Figure 12, it is clear that 10Lys10, $C_6(LA)_2$ and $C_9(LA)_2$ were the only surfactants where the MESC was lower than the EC_{50}. This suggests that the higher toxicity of the surfactants is considerably compensated by their enhanced efficiency at lower concentrations. Importantly, the same condition was not observed for the conventionally used surfactants SDS and CTAB (Figure 12).

In addition, EHEC was not shown cytotoxic under the conditions of the in vitro experiment, and its presence was found to reduce the toxic effects of the surfactants. For instance, at the MESC of 6Lys6, cell viability increased from an average 49.9% to 90.8%, in the presence of the polymer (data presented in paper II). It is anticipated that in the presence of EHEC, the surfactant molecules preferentially interact with the polymer's hydrophobic groups, forming mixed micelles, as discussed before for the gelation of EHEC in the presence of ionic surfactants. The lower availability of the surfactant in the medium makes it less available to interact with the cells, thereby contributing to a reduced toxicity.

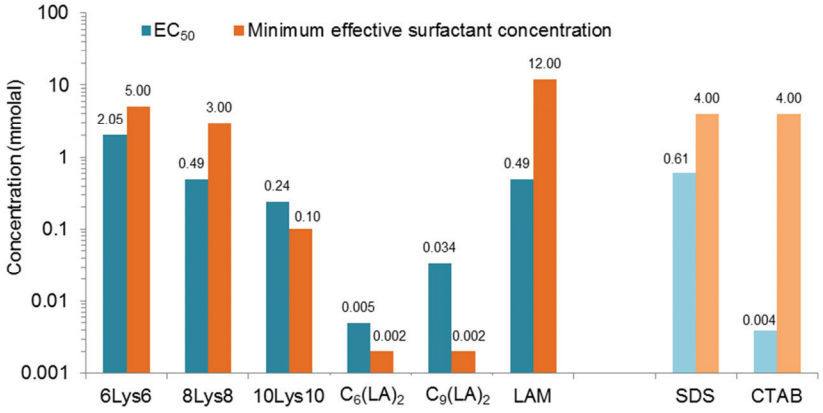


Figure 12. EC_{50} values estimated for the amino acid-based surfactants on HeLa cells, and minimum effective surfactant concentration inducing the temperature-responsive sol-gel transition on 2 wt% EHEC solutions. EC_{50} and minimum effective concentration of SDS and CTAB from refs. [175] and [93], respectively.

From the point of view of the prospective pharmaceutical application of the EHEC-surfactant systems, the following considerations can be made from the rheological and toxicological results:

i. All systems demonstrated low viscosities at room temperature and formed gels at 37 °C or close to body temperature. This means that the systems can constitute promising DDSs once injected, forming a depot *in situ* from which the drug can slowly be released. In case of sol-gel transitions at temperatures higher than body temperature, it is anticipated that the gel point can be tuned to lower values by increasing polymer concentration or the formulation composition;

ii. The gels were in general soft, as revealed by the gel strength (*S*) values, which is expected to increase patient's comfort at the injection site. In addition, the fractal dimension (d_f) values suggest that a relatively homogeneous network is formed at the gel point, indicating that the drug would be evenly distributed within the network. The drug retention properties should nonetheless be contemplated in future studies;

iii. The cloud point determinations suggest that most gels are turbid at the GP, which is reminiscent of the formation of small hydrophobic clusters in the network. In the context of a DDS, the hydrophobic microdomains can be interesting for the retention of hydrophobic drugs with low water solubility. However, because the cloud point is also indicative of the onset of phase separation [174], the long-term stability of the systems should be probed;

iv. Surfactants with gemini-like structure were shown to be more effective, inducing gelation at lower concentrations than the monomeric LAM. Remarkably low surfactant concentrations are necessary for $C_6(LA)_2$ and $C_9(LA)_2$, which makes them particularly appealing from both the economical and the toxicological points of view;

v. Among all EHEC-surfactant systems, formulations with $C_6(LA)_2$ showed the most promising results, since the GP is lower than body temperature (36.5 °C), and very low concentrations are needed to induce the sol-gel transition. In addition, the low turbidity suggests that gels with higher stability may be formed under these conditions. These gels also exhibited some of the highest values of *S*;

vi. The higher efficiency of the long-chain and double-chained surfactants contributed to a lower toxicity *in vitro*, since the gelation takes place at very low concentrations. This also confirms that these surfactants are the most promising for pharmaceutical applications. *In vitro* studies on other cell lines and *in vivo* experiments should be performed so as to confirm the biocompatibility of the hydrogels.

vii. Finally, it cannot be disregarded that the rheology data were acquired in water, and that different observations can be made when the medium is replaced with the isotonic injectable solution. Further experiments should be carried out to accommodate for this issue.

5.2 Microparticles as controlled drug release systems

Bearing in mind the development of a DDS that allows for a long-standing effect, it was hypothesized that suspending polymeric drug-loaded microspheres in a temperature-responsive hydrogel could significantly contribute to control the drug release. In other words, the microspheres would be the first barrier, from which the drug could be released by diffusion and matrix erosion phenomena, while the gel should be able to confine the microparticles and further reduce the drug release rate. This combined system is illustrated in Figure 13.

Preliminary experiments concerning the production and development of polymeric microparticles are described in Paper IV.

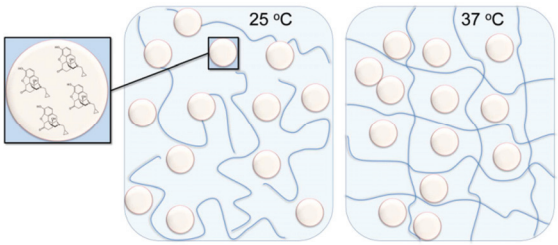


Figure 13. Two-component system involving drug-loaded microparticles and a suspension medium that forms a gel after injection, due to the higher temperature *in situ*.

In order to fulfill these objectives, three different polymers were tested as polymeric matrices for microparticulate systems (Table 1, page 34), with particular emphasis being given to a hydrophobically-modified chitosan derivative (HM-chitosan). Naltrexone (NTX), an opioid antagonist, was used as a model drug.

Chitosan is a cationic polysaccharide that has been widely used in DDSs such as nano and microparticles [176-179]. Chitosan is highly biocompatible and biodegradable and its degradation products are nontoxic [180, 181]. Its bioadhesive character, immunostimulating activity and antimicrobial properties are also well-known [181]. In drug delivery, chitosan demonstrates a high ability to control the release of the active agents. In addition, the drug

encapsulation typically does not require the use of harsh solvents, since the polymer is soluble in aqueous acidic solution. Importantly, the polymer contains a number of free amine groups that can be cross-linked for an improved stability of the DDS [181]. These free amine groups allow the chemical cross-linking of the matrix, thereby improving the stability and strength of the matrix [181, 182]. However, chemical cross-linkers (*e.g.* glutaraldehyde) are usually toxic [182].

In contrast, the hydrophobic modification of chitosans has been suggested to improve the stability of the polymer, while retaining its biocompatibility and biodegradability [183, 184]. In this thesis, this principle motivated the use of a hydrophobically modified chitosan derivative, prepared by chemical modification of the amine groups on the polymer chains with a C_{12} aldehyde (Table 1). By doing so, it was expected that the drug-loaded microparticles showed enhanced stability and control release properties.

For the matter of comparison, PLGA microparticles were produced in a similar way, due to the broad use of this polymer in DDSs such as micro and nanoparticles, pellets, films, implants and *in situ*-formed devices [128, 185]. PLGA is approved by the Food and Drug Administration, due to its biodegradability and biocompatibility, stability, mechanical properties and ease of processing [185].

Spherical and polydisperse particles were produced in most cases and the size measurements revealed that they were generally smaller than 50 µm. However, the multivariate analysis demonstrated that larger sizes were obtained if high HM-chitosan concentrations were used (up to 350 µm), due to the high viscosity of these solutions, making the droplets in the emulsion difficult to divide by the shear forces. The formation of large particles was also shown to be favored by the evaporation at high temperatures, which causes the particles to solidify rapidly. Other factors evaluated in the experimental design, namely needle diameter and stirring speed during the emulsification were not found significant in the planned experimental domain.

The encapsulation efficiency (EE) of naltrexone was higher when HM-chitosan was used as a polymeric matrix (86.5% in the best case), than when chitosan and PLGA were employed. This high EE is probably related with the amphiphilic nature of HM-chitosan. Particularly, it is anticipated that the C_{12} groups are involved in hydrophobic associations with the drug, thereby contributing to a superior retention of NTX within the matrix.

Importantly, HM-chitosan demonstrated the highest drug retention properties in release experiments performed *in vitro*. After a small burst release during the first 6 hours, NTX was very slowly and continuously released for at least 50 days (Figure 14). This slower release rate is also probably related with the presence of the C_{12} groups that act as cross-linkers due to the hydrophobic associations, and due to polymer-drug associations, as discussed previously. Furthermore, it is likely that the higher hydrophobicity of this polymer in relation to chitosan, contributes to reduce the water penetration. The drug release rate from HM-chitosan microparticles was further reduced when the particles were prepared at high temperatures, which suggests that compact structures of low porosity are formed under these conditions.

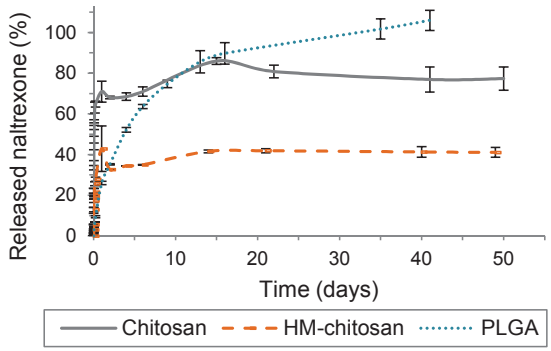


Figure 14. *In vitro* NTX release from chitosan, HM-chitosan and PLGA microparticles (particles prepared at 1.5 wt% and at 60 °C for chitosan and HM-chitosan, and 25 °C for PLGA).

In a context of the development of an injectable formulation allowing for a long-standing effect, stability and *in vitro* release studies should be carried out, namely by combining the temperature-responsive hydrogel with the drug-loaded microparticulate system. The results described in Paper IV showed that the conditions used in the preparation of the microparticles should be carefully considered, since they can significantly affect the EE and drug release properties of the system.

5.3 PNIPAAM-*b*-PAMPTMA(+) copolymers as gene carriers

In this thesis, a selection of cationic polymers, synthesized or modified by the Polymer Chemistry Group, University of Oslo, were screened with respect to their potential as carriers for pDNA. These polymers included cationic hydroxyethyl cellulose (HEC^+) (MW=400,000), hydrophobically-modified HEC^+ (MW=400,000), poly-β-cyclodextrin (poly-β–CD) (MW=280,000) and poly(*N*- isopropylacrylamide)$_n$-*block*-poly((3-acrylamidopropyl)trimethylammonium chloride)$_m$ (PNIPAAM$_n$-*b*-PAMPTMA(+)$_m$) block copolymers (Table 1). The preparation conditions of the polyplexes and the procedure details for the transfection of HeLa cells are described in 'Experimental considerations' and in Paper V.

The screening experiments revealed that both HEC^+ and poly-β–CD were mostly unsuccessful gene carriers, since 6% and 11% were the maximum transfection efficiencies estimated for the former and the latter, respectively. No gene delivery activity was detected for HM-HEC^+. The assumption is that the high molecular weight of these polymers compromised the formation of small, compact structures and the transport across the plasma membrane. It is also possible that these polymers form very strong polyplexes with DNA, delaying its release, or that the polyplexes aggregate, as observed for high molecular weight chitosans [186].

In contrast, the temperature-responsive PNIPAAM-*b*-PAMPTMA(+) block copolymers were shown to successfully deliver the pDNA into the nucleus of HeLa cells, as assessed by the detection of GFP by flow cytometry. These block copolymers combined the thermoresponsive properties of PNIPAAM with the polycationic character of PAMPTMA. The four block copolymers were distinct in terms of the length of the two blocks, and so these differences were assessed with regard to the resulting polyplex structures and transfection potential. The increase in the length of the charged PAMPTMA further resulted in the increase of the LCST of the polymer, as shown in Table 1 (page 34).

It was hypothesized that the presence of the positively charged block could allow the interaction with the oppositely charged DNA through electrostatic interactions, in a process that is facilitated by incubation at room temperature (< LCST), due to the expansion of the polymer chains at this temperature. Furthermore, the temperature-responsive properties of

PNIPAAM at 37 °C were theorized to contribute to form compact particles in contact with the cells, while the resulting positively charged surface is known to facilitate the adherence to cell surface and the endocytic process [146]. The principles supporting the complex formation and nanoparticle uptake by HeLa cells are illustrated in Figure 15.

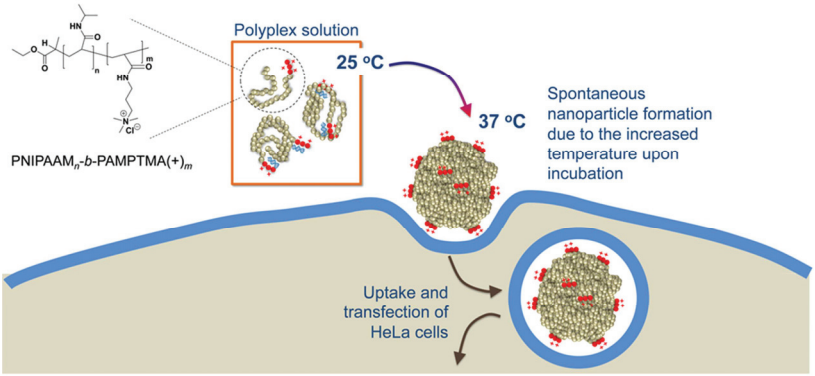


Figure 15. Suggested model leading to complex formation, cell interaction and uptake, using PNIPAAM-*b*-PAMPTMA(+) block copolymers.

The results were shown to depend on the N/P charge ratio and on the type of polymer (Figure 16). In general, low N/P ratios resulted in very low transfection efficiency, due to the absence of a positively charged surface (confirmed by zeta potential measurements). In contrast, at higher N/P ratios, the DNA is coated and protected from the action of nucleases and the overall charge density increases, facilitating the adsorption and uptake of the polyplexes by the cells.

Polyplexes containing the longest PNIPAAM (PNIPAAM$_{65}$-*b*-PAMPTMA(+)$_{20}$) were the most effective gene carriers. When the PNIPAAM length was kept constant (PNIPAAM$_{48}$), the transfection efficiency essentially followed the sequence PAMPTMA(+)$_6$ > PAMPTMA(+)$_{10}$ > PAMPTMA(+)$_{20}$, showing that the process is favored by the presence of a shorter charged block.

However, in the cases where higher transfection efficiency was observed, the cell viability was found to decrease. At an N/P ratio of 20/1, the transfection efficiency was 64.4 ± 2.8%

for PNIPAAM$_{65}$-*b*-PAMPTMA(+)$_{20}$ and 61.2 ± 7.6% for PNIPAAM$_{48}$-*b*-PAMPTMA(+)$_{6}$, with a cell viability of 41.2 ± 3.5% and 56.2 ± 3.6%, respectively. At the highest N/P ratio (30/1), the 65:20 block copolymer transfected 80.8 ± 4.5% of the cells, and the cell viability was 30.5 ± 1.7%.

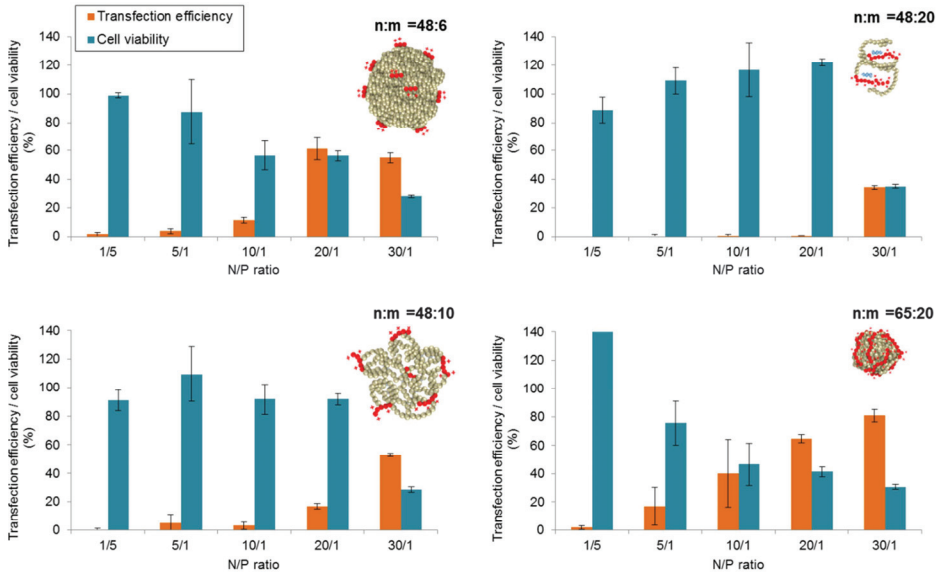


Figure 16. Transfection efficiency and viability of HeLa cells after treatment with the polyplexes. The postulated structures formed by the polyplexes at 37 °C are shown in the inset.

This work demonstrated that the composition and molecular weight of the polymers has an important effect on the gene delivery efficiency and associated toxicity. Since temperature-dependent structural modifications were expected at the incubation temperature, physicochemical data concerning the thermosensitive behavior of the polyplexes in solution were acquired (zeta potential, size and turbidity), so as to build polymer-DNA structural models. These models are shown in the insets in Figure 16.

The most efficient carrier, *i.e.* PNIPAAM$_{65}$-*b*-PAMPTMA(+)$_{20}$ was characterized by forming small (100-300 nm), closely packed polyplexes, and by the presence of a highly charged corona around the condensed PNIPAAM core. In this case, the formation of the

closely packed core was associated with the very long PNIPAAM and with a high degree of hydrophobic associations taking place at 37 °C (CP estimated as 34 °C, Table 1, page 34).

Among the polymers with a similar PNIPAAM length, the polymer containing the smallest PAMPTMA(+) (*n:m* = 48:6) led to the highest transfection efficiency, as mentioned previously. Here, the presence of a very short charged block was insufficient to cause charge repulsion and larger structures were formed (approx. 400 nm). Nonetheless, the high association tendency of the polymer led to the formation of compact polyplexes, as assessed by turbidity measurements. As in the former case, it is likely that the compact core was decorated with positive charges (zeta potential at 37 °C of +20 to +25 mV depending on N/P ratio). The combination of these features is expected to have contributed to a higher adsorption to the cell surface and to the cell uptake and endosomal escape.

Compact systems such as those made from the two former polymers are further expected to cause the inclusion of the DNA, which enhances the protection effect against the damaging action of nucleases. Experiments with Picogreen dye (method for quantifying double-stranded DNA in solution) revealed that the polymers effectively protected the DNA from the external environment, even when the lower polymer concentrations were used (methods and data shown in Paper V).

In contrast, when the length of PNIPAAM was kept constant, polymers with longer PAMPTMA(+) blocks (PNIPAAM$_{48}$-*b*-PAMPTMA(+)$_{10}$ and PNIPAAM$_{48}$-*b*-PAMPTMA(+)$_{20}$) were the poorest gene carriers (Figure 16). This is because the long PAMPTMA(+) blocks cause some electrostatic repulsion between polymer chains, inhibiting to some extent the hydrophobic association of the chains into a compact structure. As a consequence, the CP of the polymers increases (Table 1, page 34). In the extreme case of PNIPAAM$_{48}$-*b*-PAMPTMA(+)$_{20}$, the compactness and hydrophobicity of the core is the lowest, making the transfection a very difficult process. For this reason, no levels of transfection are detected, except when very high polymer concentrations are used with respect to the DNA payload (N/P = 30/1).

Overall, the results showed that the polymers effectively protected the DNA from the external environment, and that the payload can be delivered into the nucleus, where the gene that codes for GFP was expressed. However, the production of compact particles that condense the DNA seems to be a prerequisite for an efficient transfection.

In view of a prospective use of the PNIPAAM-*b*-PAMPTMA(+) block copolymers in gene therapy, this work also demonstrated that the molecular weight and the hydrophilic-hydrophobic balance of the copolymer should be carefully planned, so as to ensure that compact particles are formed at physiological temperature. Furthermore, an adequate polymer/DNA ratio can contribute to increase the transfection efficiency with low cytotoxicity levels. One very appealing advantage of the use of PNIPAAM-*b*-PAMPTMA(+) copolymers is that the formation of nanoparticles and entrapment of DNA can be achieved by simply increasing the temperature above the LCST of the polymer.

Kurisawa et al. demonstrated that the gene delivery efficiency of temperature-responsive polymers can be improved by momentarily lowering the incubation temperature below the LCST during transfection. In this article, the authors related the increased transfection efficiency with the dissociation of the complexes at lower temperatures [187]. Takeda et al. similarly showed that the DNA could be released from temperature-responsive polymers by decreasing temperature [188]. These authors further addressed the potential of these polymers as gene delivery carriers to the epidermal region (by employing a skin cooling procedure), or to the internal organs, if a cooling catheter is used. In a similar fashion, it is expected that higher transfection efficiencies are achieved if a cooling step is included in the transfection protocol involving the PNIPAAM-*b*-PAMPTMA(+) block copolymers. Ultimately, in an optimized system, an increase of the transfection efficiency can imply a decrease in the necessary amount of polymer, which contributes to decrease the cytotoxicity levels. Future work should therefore accommodate for these hypotheses.

In addition, it is known that the presence of anionic serum proteins can affect the stability of the polymer/DNA complexes and result in reduced endocytosis [189, 190]. Due to the importance of this effect *in vivo*, the sensitivity of the polyplexes to the presence of serum should be investigated in future studies.

Concluding Remarks

First of all, this work demonstrated that thermoresponsive hydrogels can be developed by combining EHEC with lysine- or arginine-based surfactants. The polymer solutions generally show a low viscosity at room temperature and a viscosification at higher temperatures. At specific surfactant concentrations, a sol-gel transition is observed, demonstrating the potential of the solutions as *in situ* gelling systems. Among all the tested surfactants, those with longer alkyl chains and with a dimeric structure are the most efficient, modulating the temperature-responsive gelation of EHEC at very low concentrations. The produced gels are mostly soft but relatively homogeneous.

Furthermore, *in vitro* toxicity studies demonstrated that the most effective surfactants are also the most cytotoxic, as evaluated by the estimated EC_{50} values. However, the toxicity of these surfactants in the presence of EHEC is significantly reduced. In addition, the fact that remarkably low amounts of the long chain or gemini-like surfactants are needed in order to produce the gels significantly contributes to decrease the overall cytotoxicity of the system.

In the context of the development of a DDS allowing for a long-term drug delivery, it is proposed that the thermoresponsive hydrogel can be combined with drug-loaded microparticles. Spherical particles, smaller than 50 µm, are in general formed by the emulsification-solvent evaporation method. Among the three polymers evaluated, chitosan and HM-chitosan demonstrate higher production yield, while the latter also shows superior efficiency in encapsulating naltrexone. When HM-chitosan is employed, larger particle size can be achieved if high concentrations are used, and if the solvent is evaporated at high temperatures. Ultimately, particles formed by this polymer also show the highest control of drug release.

In this thesis, it was also shown that DNA-polymer complexes prepared from PNIPAAM-*b*-PAMPTMA(+) block copolymers effectively carry the nucleic acids to the cell nucleus, suggesting a potential use of these thermoresponsive polymers in gene delivery applications. The transfection efficiency is however highly dependent on the molecular weight and hydrophilic-hydrophobic balance of the copolymer, and on the polymer/DNA ratio.

Polymers containing the longest PNIPAAM block or the shorter PAMPTMA(+) are the most effective gene carriers, due to the strong PNIPAAM aggregation at 37 °C. These structures have a hydrophobic PNIPAAM core and a positively charged corona, and were demonstrated to efficiently protect the DNA from the external environment.

In cases where a high transfection is observed, high cytotoxicity levels are also observed. This emphasizes the importance of controlling the structure of the polymers and the polymer/DNA ratio, so as to achieve an optimal result.

Future Studies

In this thesis, the first steps towards the development of new drug and gene delivery systems are described. To further explore these systems, additional experiments are suggested.

With regard to the thermoresponsive hydrogel, rheological studies should be carried out in the isotonic injectable solution intended for pharmaceutical use. It is also suggested that the polymer concentration is modified in order to produce gels with a phase transition temperature appropriate for human use. Once the mechanical properties of the gel are optimized, its stability should be evaluated in a buffer environment. Ultimately, a binary system combining drug-loaded microparticles and the thermoresponsive hydrogel should be evaluated *in vitro* in terms of its pharmacokinetic profile and potential for long-term applications. In this respect, HM-chitosan microparticles may be preferably studied, due to the high drug retention capacity shown in this work.

Taking into consideration a prospective use of the PNIPAAM-*b*-PAMPTMA(+) block copolymers in gene delivery, further studies should be carried out in other cell lines. Studies on polymers with different molecular weight and block length may allow for the optimization of the system in terms of the balance between high transfection efficiencies and low cytotoxicity. In one alternative approach to the protocol used in this thesis, a cooling step can be introduced in the transfection method, so that complex dissociation is promoted, thereby increasing the transfection efficiency. Furthermore, transfection studies should be carried out in the presence of serum, with the purpose of more closely mimicking the *in vivo* milieu conditions.

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Papers I-V

I

II

III

IV

Microparticles based on hydrophobically-modified chitosan as drug carriers

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ABSTRACT: In this work, a hydrophobically-modified (HM) chitosan derivative was prepared by covalent linkage of C_{12} groups to the chitosan backbone. HM-chitosan microparticles were prepared according to an emulsification-solvent evaporation method and naltrexone (NTX) was used as a model drug. For comparison, unmodified chitosan and poly lactic-*co*-glycolic acid (PLGA) microparticles were also tested as carriers for NTX.

HM-chitosan formed viscous semi-dilute solutions, suggesting a high level of chain entanglements and hydrophobic associations. HM-chitosan microparticles generally showed higher production yield and encapsulation efficiency, as compared to chitosan and PLGA. At pH 7.4, the burst release shown by chitosan microparticles was significantly reduced when using the HM-chitosan derivative. Reduced release rate from the HM-chitosan microparticles was further favored when these were prepared at a higher temperature. An enhanced control of drug release was observed over at least 50 days. PLGA particles demonstrated inferior controlled release properties as compared to HM-chitosan subsequent to the initial release stage.

These results revealed the potential of hydrophobic modification of chitosan as a means to improve the stability and sustained delivery properties of the polymer. HM-chitosan could thus be a promising carrier for drugs such as NTX.

Keywords: Chitosan, biomaterials, hydrophobic modification, microparticles, factorial design, controlled release

INTRODUCTION

Chitosan is a natural cationic polysaccharide derived from the deacetylation of the naturally occurring chitin. It is a linear copolymer composed of β-(1,4)-linked 2-acetamido-2-deoxy-β-D-glucopyranose and 2-amino-2-deoxy-β-D-glycopyranose.[1] This homo-polymer is soluble in water at low pH, due to the protonation of the free amine groups.[2] In addition, the excellent biocompatibility and biodegradability properties of chitosan make this a particularly appealing material for pharmaceutical and biomedical applications. Its degradation products, amino sugars, are also non-toxic and completely absorbed by the human body.[1,2]

Chitosan has been extensively used in drug delivery systems, such as nanoparticles and microparticles,[3-6] where the controlled delivery of encapsulated or entrapped active agents has been demonstrated. In addition, chitosan is recognized for its mucoadhesiveness to epithelial tissues.[1]

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The presence of free amine groups additionally allows the chemical cross-linking of the matrix with agents such as glutaraldehyde,[2,5,7,8] which contributes to increase the stability and strength of the network. However, the use of such cross-linkers has been questioned due to toxicity concerns.[5] Another approach has been to physically cross-link the polymer with ionic molecules such as the anionic tripolyphosphate (TPP).[9,10] In spite of the lower toxicity of the ionic cross-linkers, the cross-linking density of the network can change significantly *in vivo* in response to pH changes. Physically cross-linked chitosan networks may also lack mechanical stability and dissolve rapidly, which seriously compromises their use in long-term applications.[10]

Hydrophobically modified chitosans can show excellent biocompatibility and biodegradability *in vivo*, and form high stability systems.[11,12] The presence of hydrophobic moieties makes these polymers very appealing as drug delivery systems for poorly water-soluble drugs.[11-13] It has been shown that hydrophobically modified chitosans can self-assemble into nanoparticles with a hydrophobic core, improving the encapsulation efficiency and release profile of hydrophobic drugs.[11-14]

Tien et al.[15] also reported that N-acetylated chitosans with long chain side chains (C_8-C_{16}) showed improved mechanical properties and drug release features when used in tablets, which was attributed to the establishment of hydrophobic interactions between side chains. Furthermore, the authors suggested that as the length of the side chains increased, the swelling degree of the matrices decreased, causing the drug release to depend mostly on diffusion.[15]
Water-soluble hydrophobically modified chitosans are therefore an innovative field with great potential in drug delivery. In this work, a hydrophobically modified chitosan (HM-chitosan) was produced by chemical modification of the amine groups on the polymer chains with a C_{12} aldehyde (Figure 1 a.). The potential of this modified polymer in controlled drug delivery was investigated by formulating microparticles containing the model drug naltrexone (NTX). Throughout the study, HM-chitosan particles were compared with counterparts based on an unmodified chitosan and on poly lactic-*co*-glycolic acid (PLGA), allowing estimation of the effect of increasing the hydrophobicity of chitosan, and comparison with an established hydrophobic polymer.
The work described here is part of a broader project in which the ultimate aim is to develop a two-component formulation involving drug-loaded microparticles and a thermoresponsive suspension medium that forms a gel *in situ*, once injected. Through this combination, it is anticipated that the control of drug release is highly improved, as shown previously for analogous formulations.[16] Even though the work presented in this article is strictly related with the development of the microparticulate system, studies on low toxicity thermosensitive gel systems have also been carried out by our group.[17-19]

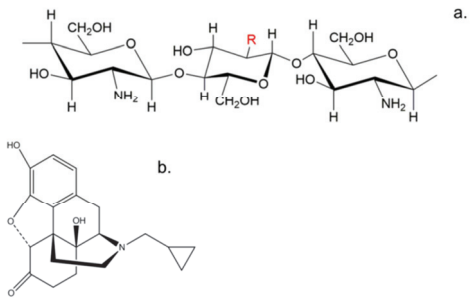

Figure 1. a. Molecular structure of chitosan and HM-chitosan. Chitosan: R= NH_2 or R= NH-CO-CH_3; HM-chitosan: R=NH_2 or R=NH-CO-CH_3 or R= N=CH-$C_{11}H_{23}$. **b.** Structure of naltrexone (NTX).

MATERIALS AND METHODS

Materials

The hydrophobically-modified chitosan (HM-chitosan) was prepared by modification of a chitosan sample (Pronova Biopolymers, Norway) by reaction of the amine groups on the polymer chains with a C_{12} aldehyde (Figure 1 a.).
The unmodified chitosan had a degree of N-deacetylation of 84% and a molecular weight of 400 kDa. The degree of C_{12}-aldehyde substitution in HM-chitosan was 5 % with respect to free amine groups. Details on the modification can be found elsewhere.[20]
The unmodified chitosan used in this study had a degree of deacetylation of 75-85% and a molecular weight of 300-500 kDa (information provided by the manufacturer, Sigma-Aldrich, USA). PLGA (RESOMER® RG 502), ester terminated, (lactide:glycolide (LA/GA) 50:50), Mw 70-17,000 was obtained from Aldrich (USA). Naltrexone, USP Reference Standard (Rockville, MD) was used as a model drug (Figure 1 b.).

Rheology measurements

Viscosity measurements were performed in a Paar-Physica MCR 301 rheometer (Physica Messtechnic GmbH, Germany) using a cone-and-plate geometry, with a cone angle of 1° and a diameter of 75 mm. Temperature control was ensured by a temperature unit (Peltier plate). Chitosan and HM-chitosan samples were dissolved in 1% (v/v) acetic acid at a concentration of 0.5, 1.0 or 1.5 wt%. The samples were introduced onto the plate and a thin layer of low viscosity silicone was added to the free surface of the sample to prevent evaporation during the measurements.

Preparation of blank microspheres

Chitosan, HM-chitosan and PLGA microspheres were prepared by an emulsification-solvent evaporation method, as briefly described below.
Chitosan and HM-chitosan were dissolved in 3.3-10 ml of 1% (v/v) acetic acid, depending on concentration (indicated in Table 1). The polymer solution was added dropwise to the continuous phase comprising of 50 g liquid paraffin and 0.7 wt% sorbitan monooleate as emulsifier. Addition of the polymer solution was performed through a needle of specific diameter, while the continuous phase was kept under constant stirring (Eurostar power control-visc stirrer, IKA-WERKE, Germany). The stirrer was connected to a Neslab RTE-101 thermostat (Neslab Instruments, USA), allowing temperature control of the process. Stirring continued for a minimum of 5 hours in order to ensure

complete evaporation of the aqueous solvent. At the end, the microspheres were extensively washed with petroleum ether and recovered by vacuum filtration through a 0.8 µm pore size membrane filter (MF-Millipore Membrane, mixed cellulose esters, Millipore, USA).
When producing PLGA microparticles, the polymer was dissolved in dichloromethane. The polymer solution was added as described before to the continuous phase, which in this case consisted of an aqueous polyvinyl alcohol (PVA) solution (0.5 wt%). In the final step, the PLGA particles were thoroughly washed with water and vacuum filtered as described previously.
According to a planned experimental design, four experimental parameters were varied during the emulsification process: i) polymer concentration, ii) needle diameter, iii) stirring speed and iv) temperature. The details are described in the following section.

Experimental design and multivariate analysis

The effects of the four formulation factors at two levels were investigated in a reduced factorial 2^{4-1} design with three center points. Subsequent to preliminary multivariate evaluation, the design was extended. The factors and levels of the experimental design are shown in Table 1.
Once all batches were prepared, the size and size distribution of the microspheres was analyzed (see section 'Size and morphological features of naltrexone-loaded microspheres' for procedure details).
Multiple linear regression (MLR) was used (Modde 5.0, Umetrics AB) to evaluate the effects of the variables on the particle size. The initial fitting was performed on a full model with maximum available main factors and interactions, followed by stepwise backward elimination of the least significant regression coefficients until only significant coefficients remained ($p<0.05$). The statistical significance of the model and individual regression coefficients were evaluated by Analysis of Variance (ANOVA).

Table 1. Experimental design for the preparation of the blank-microspheres.

Factors	Low Level	High level
Polymer concentration (wt %)	0.5	1.5
Needle diameter (mm)	0.5	1.1
Stirring speed (rpm)	700	900
Temperature (°C)[a]	30	60

[a] In the case of preparation of PLGA microspheres, the low and high level were 5 and 25 °C, respectively.

Preparation of naltrexone-loaded microspheres

Suitable conditions indicated by the preparation of blank microspheres (selected after multivariate analysis – Table 1) were used to prepare the naltrexone-loaded samples. In this case, stirring speed was kept at 750 rpm and all polymer solutions were added dropwise to the continuous phase through a 0.5 mm diameter needle. The drug was dissolved together with the polymer (50% drug:polymer mass ratio).
The preparation conditions of the different naltrexone-loaded samples are shown in Table 3.
From hereafter and in order to clarify the text for the reader, each sample will be shortly named according to the polymer used (C for chitosan, HM for HM-chitosan and P for PLGA), polymer concentration (L for low=0.5 wt% and H for high=1.5 wt%) and preparation temperature (indicated by the last number, *i.e.* 5, 25, 30 or 60 °C). The subsequent preparation steps of the NTX-loaded microparticles essentially followed the procedure described above for the blank microparticles.

The yield of production of NTX-loaded microparticles was estimated as follows:

$$Yield\ \% = \frac{weight\ of\ naltrexone\ and\ polymer\ initially\ used}{weight\ of\ produced\ particles\ after\ drying} x100$$

Size and morphological features of naltrexone-loaded microspheres

Size and size distribution were evaluated by suspending a small amount of microspheres in ethanol, so that particle concentration was lower than the instrument's threshold for individual detection of the particles. Samples were stirred for a few minutes in order to ensure homogenization and dispersion of aggregates. The analysis was performed using an Accusizer 780 (Optical Particle Sizer, PSS NICOMP, Santa Barbara, California, USA). Size parameters, *i.e.* mean, standard deviation (SD), mode and median were determined. For particles larger than 2.5 µm, the median volume was also calculated.
The morphological features of the microspheres were evaluated by scanning electron microscopy (SEM). Samples were sprinkled on carbon taps mounted on aluminum stubs followed by sputtercoating with 5 nm platinum in a Cressington 308 UHR Coating System. Samples were imaged in a Hitachi S-4800 FE SEM at 1.0 kV and a working distance of 2.5 mm.

Drug content: encapsulation efficiency and loading capacity

In order to determine the drug content present in chitosan and HM-chitosan microspheres, an accurately weighed

amount of microspheres (3-5 mg) was firstly suspended in ethanol, in order to facilitate the dispersion of aggregates. The microspheres were subsequently dissolved in 0.1 N hydrochloric acid (pH 1) by vigorously stirring the samples at room temperature for 4 days. Samples were filtered through a 0.22 µm syringe filter (Millex, Millipore, Ireland). Naltrexone was released from PLGA microspheres by dissolving the polymer in dichloromethane. The amount of drug released was quantified by measuring the absorbance of the solution at 282 nm, using a Spectronic Helios Gamma spectrophotometer (Thermo Fisher, UK).
The encapsulation efficiency (EE) and loading capacity (LC) of the microspheres were calculated as follows:

$$EE(\%) = \frac{measured\ amount\ of\ NTX\ in\ microspheres}{total\ amount\ of\ NTX\ added\ during\ manufacturing} x\ 100$$

$$LC(\%) = \frac{measured\ amount\ of\ NTX\ in\ microspheres}{weight\ of\ microspheres} x\ 100$$

In vitro release experiments

The *in vitro* release experiments were carried out in phosphate buffered saline (PBS), pH 7.4, prepared according to the European Pharmacopoeia[21] and adding 0.02% sodium azide, to prevent bacterial growth during the experiment. NTX-loaded microspheres were accurately weighed (10 mg) and transferred to a dialysis tube (Spectra/Por MWCO 50,000, Spectrum Laboratories, USA). 400 µl of PBS were carefully added to suspend the particles. The dialysis tube was placed in a stoppered vial containing 25 ml of PBS solution, ensuring sink conditions. The vials were further positioned inside a water bath connected to a thermostat, keeping the temperature at 37 °C. Samples were stirred throughout the experiment by a 15-position magnetic stir plate (RO15 power, IKA, Germany), placed beneath the water bath (position 2, ~100 rpm).
At specific time intervals, the absorbance of the buffer solution was measured at 282 nm, using a UV Ultrospec II spectrophotometer (LKB Biochrom, UK).

RESULTS AND DISCUSSION

Viscosity measurements of chitosan and HM-chitosan solutions

In the emulsion-solvent evaporation method, one of the critical factors that define the size of the droplets in the emulsion is the viscosity of the dispersed phase. The general tendency is that high viscosity solutions are more difficult to disrupt into small droplets, thereby yielding larger particles.[22] In this work, the viscosities of chitosan and HM-chitosan solutions were determined at different concentrations and temperatures, as shown in Figure 2.
First of all, it is obvious that chitosan solutions are less viscous than the corresponding ones prepared using HM-chitosan. This effect is very prominent for the highest polymer concentration (1.5 wt%). The viscosity of semi-dilute solutions of unmodified- and HM-chitosan samples has previously been attributed to both entanglement effects and hydrophobic associations.[20,23] The viscosity has also been shown to increase as the hydrophobicity of the polymer increases, due to the high tendency of the hydrophobic groups to associate.[20,23] The association of the hydrophobic moieties results in aggregates that act as cross-links between the polymer chains, causing the formation of an interconnected network.[24,25]
Figure 2 also shows that, in most cases, the viscosity decreased with increasing temperature. This is explained by the fact that the intermolecular associations are broken to some extent at higher temperatures, due to the enhanced mobility of the chains.[23] However, the viscosity of the HM-chitosan sample prepared at a concentration of 1.5 wt% is less affected by the temperature increase, because of strong entanglements and association effects.

Experimental design and multivariate analysis

Blank polymeric microspheres were prepared by an emulsification-solvent evaporation method, using different conditions, as summarized in Table 1.
Chitosan, HM-chitosan and PLGA samples prepared according to the experimental design were compared in terms of the size parameters. The control of particle size was deemed very important, since this parameter can have a crucial effect over the particles' degradation profile,[26] loading and encapsulation efficiency[27] and drug release rate.[28]
The investigated factors were hence chosen based on their possible effect on particle size. It has been shown, for instance, that smaller particle sizes may be obtained by using low polymer concentrations. Low viscosity of the solutions causes them to be easily separated into small droplets by shear forces[29] leading to reduction of the final particle size. Temperature may consequently also be an important parameter, due to its effect on the viscosity of the polymer solution, while the stirring speed can be determinant of the size of the droplets.[29,30] Apart from polymer concentration, temperature and stirring speed, we also investigated the effect of needle diameter on particle size, since it was hypothesized that the initial droplet size of the emulsion could affect the final particle size.
As the size distributions of blank microparticles were generally observed to be bimodal, the size parameters were estimated separately for two regions, above and below 2.5 µm, where nearly all the curves exhibited a minimum.

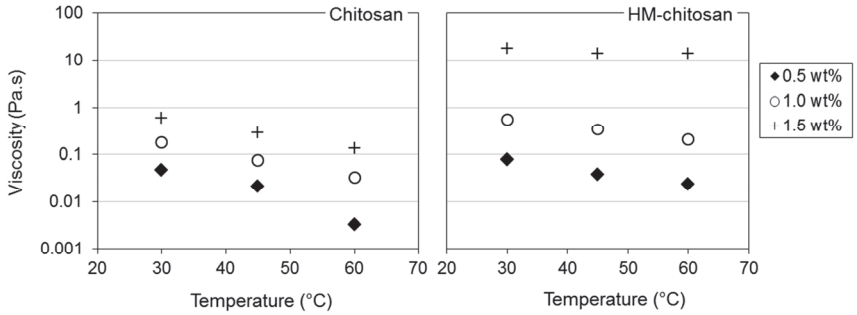

Figure 2. Temperature-dependencies of the shear viscosity of chitosan and HM-chitosan solutions prepared in 1% (v/v) acetic acid at the polymer concentrations of 0.5, 1.0 and 1.5 wt% (shear rate 0.4 s^{-1}).

It is clear that the type of polymer affected the size distribution of the particles. In this respect, chitosan microparticles were generally shown to have the largest proportion of particles larger than 2.5 µm, whereas the differences between HM-chitosan and PLGA microparticles were not found significant (analysis of variance followed by a post hoc Tukey's test).

Looking at the individual polymers and size parameters for the chitosan and PLGA particles, no significant effects of the formulation factors were observed. Even though this might indicate a robustness of the process, it is likely that different results might have been obtained if the experimental domain had been increased (Table 1). However, this was not the purpose of this study, but rather to screen for possible important process factors within reasonable limits and obtain an insight into the inter-batch variation.

In contrast to the other polymers, the production of HM-chitosan microparticles was found to be significantly affected by polymer concentration (Figure 3, Table 2) and to some extent temperature (Table 2).

An interesting observation is that the effect of HM-chitosan concentration showed an opposite trend for the lower and higher size region, *i.e.* increasing polymer concentration reduced the size of the small particles and increased the mean size of the large particles (Figure 3).

The effect on the large particles is as expected, since the concentration-induced viscosification promotes the formation of larger particles. The effect of the high concentration on the smaller particles may be explained by the high viscosity of the solution and lower solubility of the polymer in the dispersed phase, causing the polymer to precipitate faster on the droplets' surface, and limiting the coalescence between droplets.

For some of the models, Lack of Fit was observed, indicating possible additional significant terms. However, such terms would be interactions or squared terms that are not possible to estimate with the present screening design.

The initial screening of the blank microspheres focused on size but it is also well-known that the density and/or porosity of the microspheres can significantly affect the drug release rate.[31] Bearing in mind this principle and considering the factors significantly affecting the blank microspheres (concentration and temperature), formulation conditions were varied as shown in Table 3, while the remaining factors were kept constant.

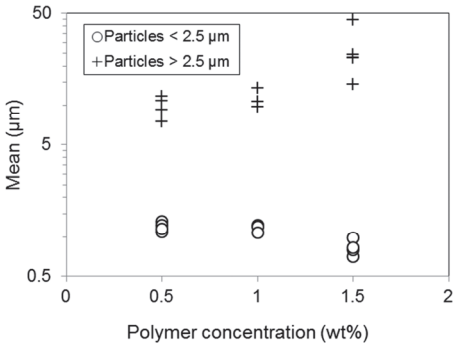

Figure 3. Illustration of the effect of polymer concentration on the estimated mean particle size of blank HM-chitosan microparticles. Each point in the figure indicates a sample that was prepared at the specified polymer concentration and at different conditions of temperature, stirring speed and needle diameter, according to the planned experimental design.

Table 2. Summary table of the size responses and significant factors.

		Significance	*Factor*	*Direction*
< 2.5 µm	Mean	S	Concentration	-
	SD	S/LOF	Concentration	-
	Mode	NS		
	Median	S	Concentration	-
> 2.5 µm	Mean	S/LOF	Concentration	+
	SD	S	Concentration	+
	Mode	S/LOF	Concentration	+
	Median	S/LOF	Concentration	+
	Volume-Median	S	Temperature	+

S=Significant; NS=Non-significant; LOF=Lack of Fit.
Directions +/- indicate whether the factor increases or decreases the response, respectively.

Production of naltrexone-loaded microspheres and drug encapsulation efficiency

The yield of the NTX-loaded particles was generally higher than 85% for chitosan- and HM-chitosan microspheres, but significantly lower for PLGA-based particles (51-66%). The reason for the discrepancy is the likely formation of a high number of PLGA particles smaller than 0.8 µm that are filtered out after the emulsification-solvent evaporation process. Other studies have similarly shown that low viscosity drug/polymer solutions (such as those produced here for the PLGA dispersed phase), usually result in the formation of small particles.[32]

In contrast, samples prepared from chitosan and HM-chitosan were shown to produce viscous phases. Droplets formed by these polymer solutions were therefore more difficult to disrupt during the emulsification process, forming larger droplets that ultimately produced larger particles. These particles were easily recovered after filtration, resulting in higher yields.

The presence of a high drug to polymer ratio typically results in low encapsulation efficiencies.[33,34] As a high drug to polymer ratio (50% w/w) was used in this work in order to produce particles with high drug content, this probably accounts for the low entrapment efficiencies observed for many formulations, even though higher values were found in some cases (Table 3).

When chitosan was used as the polymeric matrix, the estimated values of EE and LC (Table 3) do not seem to be significantly influenced by the emulsification conditions (EE 42-46% and LC 14-15%). Interestingly, the encapsulation of naltrexone was more efficient in the presence of HM-chitosan, with the values of EE varying between formulations but being as high as 87% in the best case (HM-H60). The overall higher encapsulation efficiency in the presence of HM-chitosan is probably related to the higher hydrophobicity of the polymer due to the presence of the C_{12} groups. This means that in addition to the formation of hydrogen bonds between the hydroxyl and amine groups of the polymer and the hydroxyl and ketone groups of the drug, it is also likely that the C_{12} groups of the polymer form hydrophobic interactions with the aryl ring and cycloalkyl rings of the drug (Figure 1). The high affinity between the molecules explains the enhanced presence of naltrexone within the HM-chitosan microparticles, as depicted in Figure 4. In addition, our experiments have shown that HM-chitosan solutions exhibit a significantly higher viscosity (Figure 2) than the unmodified counterpart, which can further reduce the diffusion of drug out of the droplets during emulsification.[35] Another important fact is that HM-chitosan is less soluble in water than the unmodified analogue. In fact, it has been shown that the lower solubility of the polymer in the dispersed phase causes it to precipitate faster, thereby contributing to increase the encapsulation efficiency.[36]

The highest drug loading was found for the HM-H60 formulation, where a high polymer concentration was used, and the solvent was evaporated at a high temperature. In this respect, it is generally accepted that high polymer concentrations also contribute to a higher encapsulation efficiency.[36]

It should also be emphasized that the high viscosity of 1.5 wt% HM-chitosan solutions is an indication of a high degree of chain entanglements and hydrophobic associations – acting as physical junction zones of the network –, which can further account for the retention of the drug.

Table 3. Formulation conditions of naltrexone-loaded microspheres and encapsulation properties (encapsulation efficiency (EE) and loading capacity (LC)). Results shown as average ± standard deviation, n=3.

	Polymer	Polymer concentration (wt%)	Temperature (°C)	EE (%)	LC (%)
C-L30	Chitosan	0.5	30	45.9 ± 1.5	15.3 ± 0.5
C-L60		0.5	60	44.1 ± 3.6	14.7 ± 1.2
C-H30		1.5	30	44.5 ± 2.3	14.8 ± 0.8
C-H60		1.5	60	42.1 ± 1.9	14.0 ± 0.6
HM-L30	HM-chitosan	0.5	30	48.7 ± 1.9	16.2 ± 0.7
HM-L60		0.5	60	64.6 ± 2.6	21.5 ± 0.9
HM-H30		1.5	30	54.4 ± 0.2	18.1 ± 0.1
HM-H60		1.5	60	86.5 ± 4.2	28.8 ± 1.4
P-L5	PLGA	0.5	5	49.1 ± 3.5	16.4 ± 1.2
P-L25		0.5	25	20.8 ± 2.0	6.9 ± 0.7
P-H5		1.5	5	51.0 ± 3.2	17.0 ± 1.1
P-H25		1.5	25	52.2 ± 6.5	15.4 ± 2.7

It is interesting to note that the HM-H60 sample was prepared at a high temperature (60 °C). As mentioned previously, low viscosity solutions are generally obtained with increasing temperature, which in theory could lead to an increased diffusion of the drug into the continuous phase and a corresponding decrease in EE. Since the rheological measurements showed that the viscosity of 1.5 wt% HM-chitosan solutions is only slightly affected by a temperature increase (Figure 2), this effect is not observed here. The differences between HM-H30 (prepared at 30 °C) and HM-H60 (prepared at 60 °C) are therefore probably due to a fast solvent evaporation for the latter, inducing rapid particle solidification and limiting the diffusion of the drug to the continuous phase. The fact that large microspheres were produced under these conditions may also explain the high EE, as observed before for other polymeric particles.[37] A detailed interpretation of the size distribution of the NTX-loaded particles will be given later.

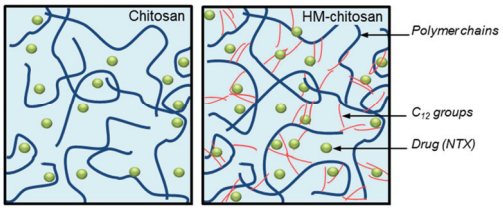

Figure 4. Schematic illustration of chitosan/HM-chitosan networks in the presence of NTX. Within the chitosan network, NTX is mostly physically entrapped due to chain entanglement. In the HM-chitosan network, the hydrophobic intra- and inter-molecular interactions are also very important, forming cross-linking points due to the presence of the C_{12} groups. In this case, both the entanglements and the hydrophobic interactions are responsible for NTX retention.

The EE of PLGA particles was mostly found in the range 21-52%. The exceptionally low EE and LC values found for P-L25 (21 and 7%, respectively) are consistent with the low polymer concentration (0.5 wt%, and thus a very low viscosity) of the dispersed phase in this formulation. In addition, it has been shown that a fast evaporation can induce pore formation in PLGA microspheres,[38] enabling the drug to diffuse out to the aqueous phase during the washing procedure, which further explains the low encapsulation of NTX.

Morphological characterization of NTX-loaded formulations

The microparticles produced in this work were generally spherical, as shown by SEM (Figure 5), even though different sizes and degrees of aggregation were obtained in different formulations. The micrographs showed that chitosan-based microparticles did not differ significantly in terms of size distribution and surface porosity. The detailed observation of samples at different magnifications showed the occasional presence of merged and/or irregularly-shaped particles in C-L30 and C-L60, suggesting that the low

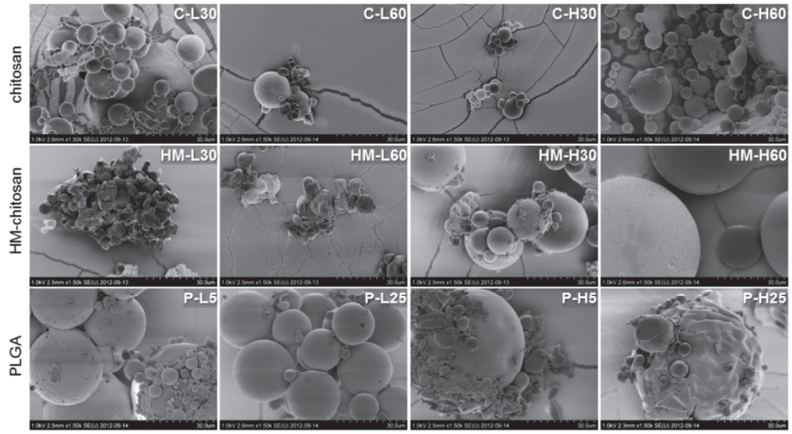

Figure 5. SEM micrographs of naltrexone-loaded microparticles prepared from chitosan, HM-chitosan and PLGA. The preparation conditions of all samples are described in Table 3. Scale bar = 30 µm.

viscosity of the polymer solution (0.5 wt%) facilitates the coalescence between droplets during the emulsification.

In contrast, C-H30 and C-H60 formulations, prepared at a higher polymer concentration (1.5 wt%), formed stable droplets that resulted in well-defined microspheres.

HM-chitosan produced very different samples depending on the formulation conditions. Samples prepared at the lowest polymer concentration (HM- L30 and HM-L60) also produced irregularly-shaped particles that occurred in aggregates. Conversely, particles prepared at a polymer concentration of 1.5 wt% (HM-H30 and HM-H60) were shown to be spherical and well-defined, but significantly larger when prepared at higher temperatures (HM-H60).

This observation is probably related with the fast solvent evaporation and particle solidification, as mentioned above.

PLGA microparticles were largely polydisperse, with a high presence of small particles (< 3 µm). Regardless of the process conditions, PLGA microparticles had a porous surface, as shown by micrographs taken at higher magnifications. P-L25, particularly, showed the occasional presence of very large pores (up to 1 µm). P-H5 and P-H25 samples also contained a number of rod-like particles, which may be related to the elongation or coalescence of droplets at high concentrations, in a similar way to the observations made by Li et al..[39]

Size and size distribution of naltrexone-loaded particles

Figure 6 shows the size distribution of two formulations for each polymer and illustrates the influence of the drug loading on the size and size distribution of the microspheres. It is noticeable that, in most cases, similar size distribution profiles are observed both in the presence and absence of NTX, even though the size is shifted to slightly higher values when the drug is present. Moreover, the amount of small particles tends to decrease, while the number of large particles increases. This relationship between drug loading and particle size has been reported before.[40] The increase in particle size in the presence of NTX may also be an indication of an increase in the viscosity of the dispersed phase, which in turn can suggest that polymer-drug interactions take place within the droplets. These interactions are probably favored by high polymer concentrations and temperature, as shown by the prominent size increase observed for C-H60 (chitosan) and HM-H60 (HM-chitosan) formulations, in the presence of NTX.

The size distribution of NTX-loaded microspheres prepared according to all formulations is depicted in Figure 7. First of all, it is noticeable that particles smaller than 50 µm were produced in most cases. Particles smaller than 2.5 µm were often observed in high amounts (inset plots in Figure 7) and it is likely that even smaller microspheres were produced during the emulsification, but were eliminated during the washing and filtration process.

In the case of chitosan and PLGA particles, the profile differed slightly between formulations, but in general all the preparation conditions led to the production of polydisperse samples, with a high presence of particles in the range 5-25 µm. The differences observed between formulations were not found to be statistically significant with respect to size and size distribution. In contrast, clear differences were observed between formulations prepared from HM-chitosan.

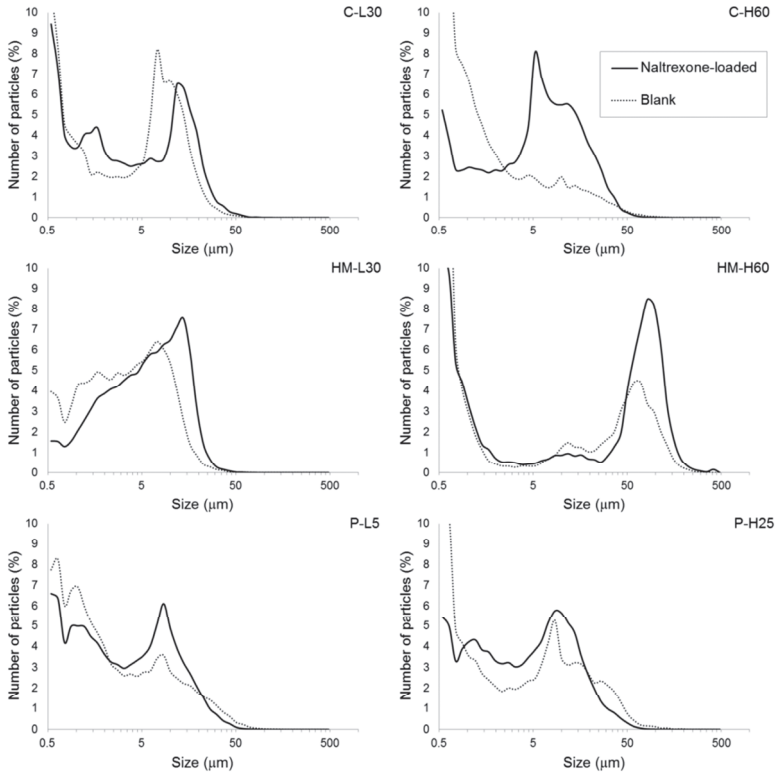

Figure 6. Effect of the presence of drug on the size distribution of chitosan, HM-chitosan and PLGA microspheres (the preparation conditions of each sample are described in Table 3). The solid and dashed lines represent the NTX-loaded and the blank microspheres, respectively.

The polymer concentration was observed to play an important role, in agreement with the results from the experimental design for the blank microparticles (Figure 3). In general, the conditions used in HM-L30 and HM-L60 formulations yielded smaller particles with profiles comparable to chitosan and PLGA. This is possibly related to the lower viscosity of the dispersed phase (Figure 2). However, the size distribution of these samples also shows the presence of larger structures (up to 50 μm in HM-L30 and 100 μm in HM-L60) that are possibly large aggregates, as shown by SEM (Figure 5).

The formulations that stand out compared to the others are HM-H30 and HM-H60, which produced microparticles with sizes up to 200 and 350 μm, respectively, in agreement with the high viscosity of 1.5 wt% HM-chitosan solutions. This implies that the droplets of the dispersed phase are difficult to divide by the shear forces. The largest particles were obtained at higher temperatures (HM-H60), which explains the higher loading capacity of the particles in this case (Table 3). As discussed above, the viscosity of 1.5wt% solutions of HM-chitosan is largely unaffected by temperature (Figure 2). For this reason, the large size and EE may be due to the fast solidification of the particles at the high temperature used during the emulsification step.

In vitro drug release studies

The *in vitro* release profile of NTX from the different types of polymeric microparticles is shown in Figure 8. Chitosan microparticles generally showed a pronounced burst release during the first 6 hours – 55 to 69% of the drug was released within this period –, followed by a second stage, where the drug was slowly released or remained virtually constant over time. The initial burst release is quite commonly observed in controlled drug delivery systems that are unable

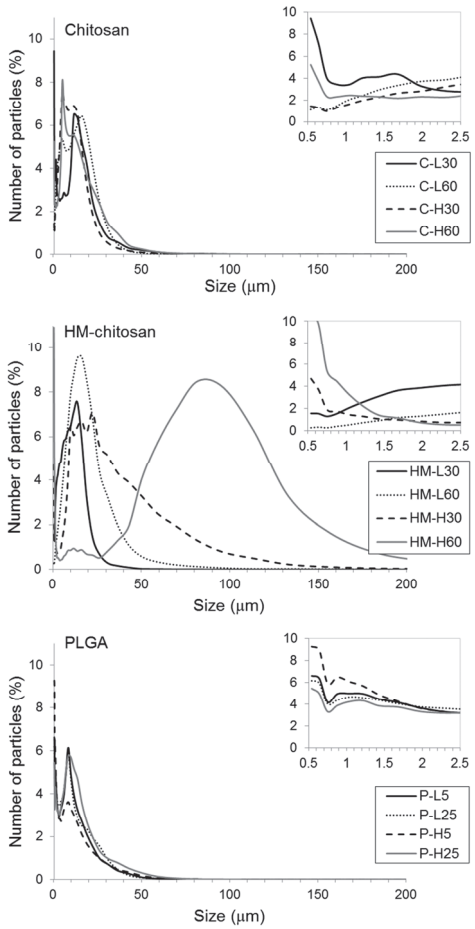

Figure 7. Size distribution of naltrexone-loaded microparticles prepared from chitosan, HM-chitosan and PLGA. The inset shows the number of particles smaller than 2.5 μm. Samples were prepared as described in Table 3.

to retain the drug located at the surface of the particles. The constant release stage that follows, reflects a slower release of the drug, which is usually caused by both diffusion through the matrix and degradation of the polymer.[31]

A substantial reduction in the initial burst release was observed for HM-chitosan microparticles. In this case, only 23 to 40% of NTX was released within the first 6 hours, depending on the formulation. After this initial release, NTX was very slowly and continuously released for more than 50 days. Only 13-26% of the total drug content was released during this time period, which suggests that HM-chitosan microparticles can constitute promising carriers in long-term applications. The slower release rate is probably related to the presence of the C_{12} groups. These hydrophobic groups can act as cross-linkers due to the hydrophobic associations. In addition, stronger polymer-drug interactions are expected, as discussed previously (Figure 4). The presence of the hydrophobic groups is probably also causing a lower water penetration.

Particles prepared according to HM-L60 and HM-H60 conditions showed a very similar release profile and the slowest *in vitro* release rate. Interestingly, these samples were very different in terms of size distribution (Figure 7) and morphology (Figure 5). However, both of them were prepared at a high temperature (60 °C), and it is likely that the fast solvent removal resulted in a rapid solidification of the droplets. It may further be hypothesized that at the same time that the solvent evaporated, the extent of hydrophobic associations increased, prompting the formation of a dense network with limited possibilities for drug diffusion. Conversely, the slow evaporation rate used to prepare HM-L30 and HM-H30 microparticles may have induced the formation of more porous structures. Similar findings have been reported for microspheres based on a hydrophobic dextran derivative.[41] In that study, particles prepared at a lower solvent evaporation rate were formed slowly, and demonstrated large pores and cracks at the surface. These microspheres were shown to release the drug faster than the microspheres prepared at higher temperatures.[41]

In the case of HM-chitosan microparticles, the formation of compact, low porosity particles seems to be a decisive factor when it comes to the drug release rate, while particle shape, aggregation status and size distribution seem to be less important. However, it should be stressed that the differences in morphology and size between the samples may cause them to have different fates *in vivo*.[26]

Finally, PLGA microspheres showed a very low burst release, followed by a continuous drug release that reached 72-94%, depending on the formulation, within 16 days. PLGA-based controlled delivery systems have been extensively investigated, due to the polymer's hydrophobicity, long-term stability, and its biocompatibility and biodegradability.[42,43] In this work, we used PLGA with a LA/GA composition of 50:50, since this is the most commonly used PLGA composition in drug delivery.[43] However, this copolymer also degrades relatively fast, and other compositions may be employed for improved polymer stability.[43,44] After the second day, drug release levels from PLGA microparticles were comparable with those observed for HM-chitosan, while the latter provided a slower release rate at longer times.

In spite of the different conditions used to produce the PLGA microparticles, similar release profiles were observed for three of the formulations, namely P-L5, P-H5 and P-H25. These results are explained by the similar EE and LC (Table 3) and similar size distribution (Figure 7) between these samples. The rate of NTX release was significantly

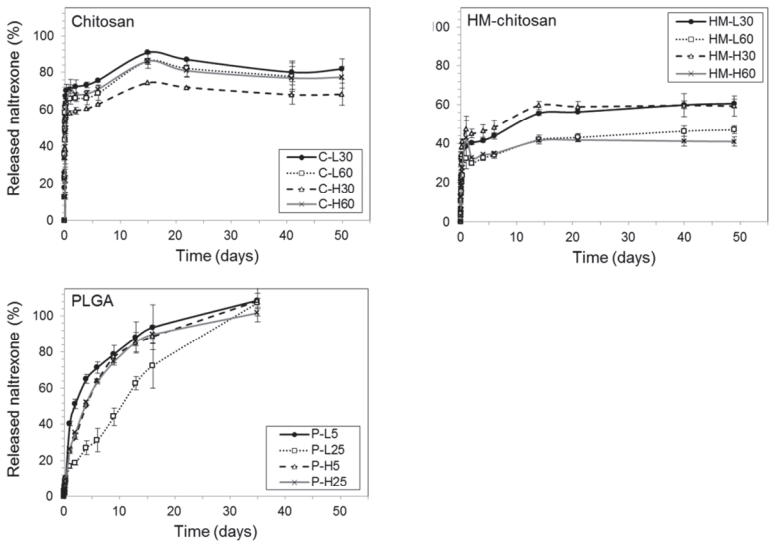

Figure 8. Influence of the microparticle preparation conditions on the *in vitro* release of naltrexone. Error bars= max/min values, n=2. The preparation conditions are described in Table 3.

lower for particles prepared according to P-L25. This is probably due to the low drug loading of these particles (Table 3). For poly(L-lactide) (PLA) microspheres containing NTX,[45] decreasing the drug content similarly resulted in a significant reduction in the drug release, due to the lower presence of NTX at the surface of the particles. In the case of P-L25, the processing conditions had an important influence on this outcome. The low polymer concentration and the fast evaporation rate formed a number of large pores at the surface of the particles, as discussed before. As a consequence, a high amount of NTX was probably eliminated from the surface during the particle washing step, which accounts for the low EE. The low presence of drug at the surface of the microparticles also explains the reduced burst release in this case.

In summary, this study revealed the potential of HM-chitosan for long-term drug delivery applications. HM-chitosan microparticles demonstrated improved controlled delivery properties in relation to the samples prepared with the unmodified counterpart. PLGA microparticles revealed the lowest burst effect, but subsequent drug release was significantly faster than for HM-chitosan. The faster release of NTX from PLGA particles is probably due to the hydrolytic cleavage of the polymer's ester bonds.[46] From HM-chitosan microparticles, the slower release rate suggests that the drug is mostly released by diffusion, to the external medium.

It is important to note that the drug release kinetics may differ *in vivo*. Chitosan is known to be hydrolytically degraded by enzymes present in human body fluids, such as lysozyme[47,48] and N-acetyl-β-D-glucosaminidase.[47] For injectable formulations, it has also been suggested that chitosan nano- and micro-structures may be taken up and degraded by macrophages.[48] The degradation rate is also known to depend strongly on the degree of acetylation of the polymer.[47,48] In a study on the oral delivery of insulin, chitosan particles were shown to be enzymatically degraded over time in simulated fluids containing pepsin, trypsin or chymotrypsin.[49] Interestingly, an HM-chitosan derivative containing succinyl and lauryl moieties revealed improved drug protection properties and drug release characteristics in relation to the native chitosan.[49]

With respect to our work, it is similarly expected that the presence of C_{12} groups in HM-chitosan provides the polymer with enhanced drug delivery properties *in vivo*, due to a higher mechanical strength and resistance to enzymatic activity and hydrolysis.

CONCLUSIONS

HM-chitosan and chitosan microparticles were manufactured in higher yields than PLGA microparticles. HM-chitosan particles showed a higher encapsulation efficiency of NTX. The encapsulation efficiency was further improved when high polymer concentrations were used, and when the solvent was evaporated at high temperatures. In addition, the drug loading caused the number of small particles to decrease and the amount of large particles to increase, in comparison with the blank microparticles. In most cases, particle size was smaller than 50 µm, with a large number of small particles produced when PLGA was used as the matrix.

HM-chitosan microparticles showed a significantly higher controlled delivery capacity than chitosan microparticles. It is hypothesized that this is due to the presence of the hydrophobic groups that limit the water diffusion through the pores, act as physical junction zones in the network, and promote strong interactions with the drug molecules. The release rate of HM-chitosan particles was even lower when the particles were prepared at higher temperatures, suggesting that compact structures of low porosity are formed under these conditions. PLGA particles showed a markedly lower burst release in the first hours. However, after a few days, these were shown to be less able to control drug release, as compared to HM-chitosan microparticles.

In conclusion, the results from this study indicate that HM-chitosan can be a promising material for a sustained delivery of drugs such as NTX.

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