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Liposomes-*in*-Chitosan Hydrogels. Challenges and Opportunities for Biomedical Applications

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1. Introduction

Chemical hydrogels are three-dimensional (3D) networks that are usually produced by chain cross-linking reactions between polymers and/or co-polymers. These materials are able to retain large amounts of water or biological fluids by exhibiting a thermodynamic compatibility in the equilibrium-swollen state. Such property along with their viscoelastic nature and remarkable

similarity to native extracellular matrices, make hydrogels compatible with numerous biological tissues where they can be implanted (1). As a consequence, hydrogels have become one of the most used soft scaffolds in plentiful biomedical applications during the last decades (2).

Hydrogel materials can be classified according to different criteria. For instance, according to their chemical composition, they can be made from natural or synthetic polymers. The latter can be homo-polymers, co-polymers or interpenetrating polymer networks (IPN). Regarding their physical structure, hydrogels can also be classified as amorphous, semi-crystalline or crystalline networks. This is usually influenced by the nature of the cross-linking (*e.g.* chemical bonds, non-covalent interactions or combination of both). Additional features like stimuli-responsive properties, physical form (*e.g.* matrix, film, microsphere), fate in living organisms (*e.g.* degradable, non-degradable) and charge state (*e.g.* neutral, zwitterionic, ionic) can be also used to classify such polymeric networks (3).

Since Wichterle and Lím proposed in 1960 the use of synthetic hydrogels based on poly(2-hydroxyethyl methacrylate) (pHEMA) for soft contact lenses (4), the enormous interest generated in synthesizing and engineering new hydrogels has exponentially grown. This revitalization in the field of biomaterials science has also allowed to obtain a wide number of “smart” hydrogels with improved mechanical and tailored properties. This interest has contributed to expand their biological response to certain stimuli (*e.g*. light, pH, temperature, ionic strength and redox potential) (5, 6). Such chemical and physical stimuli usually have induced some conformational changes in the polymeric network in

physiological conditions which have been exploited in different areas such as tissue engineering (7), controlled drug release (8) or bionanotechnology (9).

While a large number of synthetic hydrogels have been described as appropriate and effective materials in various biomedical applications (*e.g.* pHEMA, poly(ethylene glycol) (PEG), poly(vinyl alcohol) (PVA), block copolymers of PEG, polyacrylamide (PAAm) or poly(*N*-isopropylacrylamide) (PNIPAAm)) (10, 11, 12); natural and supramolecular hydrogels have also played a pivotal role in biomedicine (13, 14).

There is a good number of natural occurring hydrogels derived from polysaccharides (*e.g.* alginate, chitosan, cellulose, chitin, carrageenan), proteins (*e.g.* collagen, gelatin) and peptides, among others. The growing interest aroused by the use of these biopolymers in drug delivery and other biological applications has been due largely to their large abundance, sustainable nature, water solubility, biocompatibility, low toxicity as well as biodegradability properties. Furthermore, the presence of multiple functional groups (*e.g.* hydroxyl and amino groups) has made polysaccharide-based hydrogels ideal and versatile materials for encapsulation and controlled released of therapeutic drugs. This has worked in favor of using either covalent strategies or physical entrapment together with introducing stimuli-responsive units mainly against temperature and pH (15).

1.1 Chitosan

Chitosan (CS) is the second most abundant polymer in nature after cellulose and one of the most well-studied natural polymers in many applications. The scope of these uses is therefore very broad including wound dressings (16),

drug delivery (17) and tissue engineering (*e.g.* bone (18), cartilage (18, 19) or neural (20) tissue regenerations). From the structural point of view, CS is a linear polysaccharide made of randomly distributed mixtures of deacetylated residues (β-(1→4)-linked-D-glucosamine) and acetylated residues (*N*-acetyl-D-glucosamine). This polysaccharide is commercially produced by deacetylation of chitin (Figure 1A). The percentage of deacetylated and acetylated residues (so-called degree of deacetylation, DA) in CS determines its physicochemical properties as well as solubility (21), biodegradability and biological activity (22).

The protonation of the amino groups on the CS backbone under acidic conditions permits the biopolymer solubility before reaching pH values that can range from 6.2 and 6.5. As a consequence, the amino groups of the D-galactosamine units are predominantly positively charged at pH values below 6.2 (23, 24). The development of physical entanglements in CS hydrogels is governed by reversible non-covalent forces such as hydrogen, ionic and/or hydrophobic intermolecular interactions that depend on the pH, temperature, chemical composition and polymer chain length (24, 25, 26).

Importantly, the robustness of CS-based hydrogels permits the incorporation of small molecules during the gelation process under mild conditions. In this regard, β-glycerophosphate (GP) has been described as an efficient catalyst to trigger the CS *sol*-to-*gel* transition at physiological pH. This process has led to transparent physically cross-linked hydrogels with potential use as injectable biomaterials (24) (Figure 1B). Other small anionic molecules have also been described as effective ionic cross-linking agents including a) citrate and sulphates, b) transition metal ions like Pt (II) (27) or Mo (IV) (28), and c) the use

of alkali under controlled and well-defined conditions without adding additional components (29).

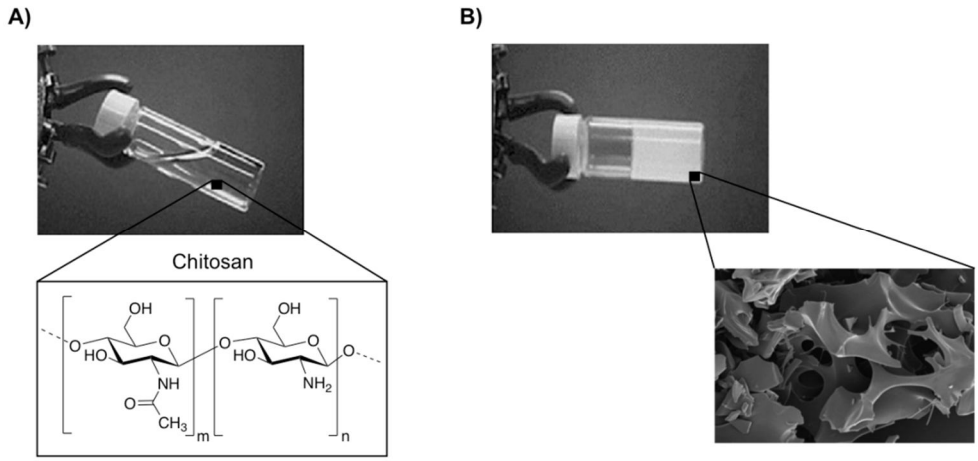

Figure 1. A. *Sol*-to-*gel* transition of CS polymers in combination with β glycerophosphate. A. Chemical structure of CS polymer. B. SEM micrographs of CS/GP xerogels obtained from the corresponding hydrogels. A. Adapted with permission from reference (24). Copyright 2015 MDPI. B. Adapted with permission from reference (127). Copyright 2015 Elsevier B.V.

The presence of free amino and hydroxyl groups in CS polymer network has opened up new possibilities for covalently engineering novel cross-linked materials. This has resulted in the fabrication of tailored hydrogels with improved stabilities, a greater control of pore size and better degradation profiles. A good number of chemical cross-linking strategies have been described and recently reviewed (30, 31). Within this context, small cross-linking molecules have been used to obtain covalent entanglements within CS hydrogels via Schiff's base formation between aldehydes and amines together with other linkage chemistries (*e.g*. Passerini and Ugi multicomponent condensations) (32). Thus, molecules such as glutaraldehyde (33), diisocyanate, (34), poly(ethylene glycol) (35), among others (36), have been

incorporated into CS networks affording materials with excellent mechanical properties. However, it is worth mentioning that most of these cross-linkers have displayed certain toxicity *in vitro* and have considered detrimental for their use in several biomedical applications (37). To circumvent this limitation, other bifunctional agents such as oxalic acid (38), diethyl squarate (39) and genipin (40) have been proved to be efficient cross-linked CS hydrogels by enhancing their biocompatibility. In particular, genipin has been considered a promising natural alternative to dialdehydes (41).

Polymer-polymer cross-linking is another interesting approach to obtain superior and biocompatible CS hydrogels without using bifunctional cross-linking agents. This strategy has enabled the combination of CS with other important biopolymers such as hyaluronic acid (42), alginate (43) or cellulose (44) by introducing reactive functional groups that facilitate polymer-polymer bonding through Michael addition reactions, Schiff's base and disulfide formation (45). Similarly, irradiation with UV light upon the introduction of photo-sensitive functional groups or the use of enzyme-catalyzed reactions have also become an alternative to classical chemical cross-linking approaches for obtaining *in situ* modified CS scaffolds (46, 47, 48).

Hydrogel networks provide a broad range of possibilities due to their physical and mechanical properties as well as their high degree of biocompatibility. These features have allowed hydrogel composites to be attractive for promoting controlled release of small molecules (8). In order to evaluate the efficiency of hydrogels as vehicles for drug release, the following factors must be taken into consideration: hydrogel components, network conformation, hydrogel porosity, gel swelling and drug encapsulation efficiency (17). In the case of hydrophilic

drugs, the encapsulation process can take place by either mixing the small molecule drug with the polymer previous to polymerization or swelling the hydrogel within an aqueous solution containing the drug. Once drug-loaded hydrogels are formed and come in contact with biological medium, erosion of the hydrogel surfaces takes place and sustainable drug release is usually triggered through a diffusion mechanism according to Fick's law (2, 49). Some examples using CS hydrogels have been described in previous reviews (50). In the case of entrapping hydrophobic drugs within hydrophilic materials, the encapsulation process may diminish in efficiency and thereby restrict their applicability in some drug delivery strategies. This effect might result in large aggregates and produce a great accumulation of the drug and thus causing toxicity. To overcome these drawbacks, several approaches have been successfully described for CS hydrogels such as modifying the CS network with hydrophobic residues (51), combining hydrophobic drugs with amphiphilic derivatives (52) and using vesicular carriers (53).

Herein, we summarize the impact and solutions that CS-based hydrogels containing liposomes have provided in biomedicine, with special emphasis on their advances in several applications such as ocular delivery, cancer and wound dressing therapies.

2. Liposomes

Liposomes are artificial vesicles that are able to self-assemble in aqueous media giving rise to one or more lipid bilayers. They are made of either natural or synthetic phospholipids (*e.g*. phosphatidylcholine (PC) and

phosphatidylethanolamine (PE) as the most representative ones). Additional components such as cholesterol (Chol) can also form part of the liposomes and depending on its concentration can affect both drug loading properties and vesicle size. Since G. Gregoriadis proposed the use of liposomes as reservoirs to encapsulate therapeutic drugs in the 70's (54, 55, 56), these spherical vesicles have become important tools for a good number of biomedical applications including drug delivery and diagnostic imaging. Multiple of these examples have been widely revised in the past few years (57, 58, 59, 60).

Liposomes can be classified into two categories: a) Multilamellar vesicles (MLVs) and b) unilamellar vesicles, which can be further classified into large unilamellar (LUVs) and small unilamellar vesicles (SUVs) (59, 61). Liposome diameters can vary from 30 nm to several micrometers and their size will depend on the manufacture process and lipid composition. Liposomes are capable of entrapping both hydrophilic molecules, which are localized in the core, and hydrophobic drugs, that are usually found throughout the lipid bilayer membrane (phospholipid membrane).

Generally, the therapeutic effectiveness of liposomes as drug delivery systems mainly depend on the amount of the drug molecule encapsulated within the vesicular carrier system as well as other parameters such as ease of preparation, scale-up, liposomes charge, stability and relationship between drug and liposome (N/P ratio) (62). One of the bottlenecks displayed by the first-generation of liposomes is their tendency to aggregate in the presence of serum proteins and their clearance into the liver and spleen. To overcome these issues, second-generation liposomes have been prepared by introducing additional functional groups and targeted ligands. These strategies have helped

to increase the circulation longevity of the particles (stealth liposomes) (63) and the specificity in the drug delivery process into the tumor (64). These improvements have allowed liposomes to improve both pharmacokinetic and pharmacodynamic characteristics for a good number of encapsulated drugs.

2.1 Liposome preparation methods

Several methods have been described for the preparation of liposomes with defined sizes and dispersity degrees (58). Furthermore, each preparation can have influence on liposome properties such as lamellarity, size or EE. The thin-lipid film hydration is one of the most used methods for preparing liposomes however low encapsulation efficiencies are obtained (65). This procedure is based on forming a thin film of lipids by removing the organic solvents *in vacuo*. The corresponding liposomes are formed after hydration with aqueous buffer. MLVs, SUVs and giant unilamellar vesicles (GUVs) can also be obtained following this conventional method. In order to get homogeneity in liposomes with smaller sizes, it is convenient to place them in contact either using a water bath sonicator or employing multiple liposome extrusions through a polycarbonate membrane. In this latter case, the size reduction will depend on the number of extrusion cycles generated.

Alternatively, the reverse-phase evaporation method is also used as a conventional method in the preparation of liposomes (66). Briefly, lipid films formed after evaporation are dissolved with an organic solvent (*e.g*. diethyl ether, isopropyl ether) and the aqueous phase containing the drug of interest is added. This leads to the formation of a two-phase system, which forms a

homogeneous dispersion containing liposomes after sonication and organic solvent evaporation. This approach shows better drug encapsulation efficiencies than the thin-lipid method described above.

The third standard method is called solvent injection/vaporization technique (67). Phospholipids or other alternative lipids are dissolved in an organic solvent (*e.g.* ethanol or ether) and injected into an aqueous solvent that contains the drug of interest. This process leads to the formation of heterogeneous species of liposomes (LUVs).

While the preparation of coventional liposomes can be easily prepared in laboratories, novel methodologies have helped to optimize scaling-up processes for the industry with good yields and homogeneities. Some of these technologies are microfluidic (68), the supercritical reverse-phase evaporation by using supercritical CO_2 (69); freeze and spray drying techniques (70, 71) and the crossflow injection technique (72). To give the reader a clear and complete view of liposome preparation methods, excellent reviews have been reported which describe in depth the conventional methods and new techniques employed as well as their advantages and disadvantages of each process (58, 73).

The efficiency and success displayed by liposomes in the transport of small molecules have generated growing interest. Thus, this potential has allowed the use of liposomes to cover a good number of clinical applications (*e.g*. fungal diseases, viral vaccines, photodynamic therapy, analgesics and cancer therapy). Furthermore, this success has enabled liposomes to be launched to several phases of clinical trials (74). Within this context, efficient administration routes and development of liposomal formulations are required for their use in

clinical applications. Thus, depending on the loaded drug and therapeutic strategy, liposome properties can vary according to the administration routes. In addition to intravenous administration (parenteral administration), liposome surfaces have been coated with PEG in order to prepare more stable particles for being orally administered in gastric ulcer healing treatments (75) and oral vaccines (76). Liposomes have been also administered for lung delivery by developing aerosolized liposomal formulations containing paclitaxel and cyclosporine A with good *in vivo* results (77). Other examples involving liposomes for lung delivery have been recently reviewed (78).

Topical drug delivery is another application in which liposomes have been involved. Several experiments have confirmed the potential of liposomes to transport hydrophilic drugs, increasing the skin penetration and therefore reducing the risk of systemic side effects. Thus, extensive effort has been carried out in order to improve this application by obtaining a good number of vesicular carriers in addition to liposomes (*e.g*. transferosomes, ethosomes and niosomes) (79). The treatment of ophthalmic diseases involving liposomes, for instance, is often closely linked to topical administration (80). Liposomes have exhibited an extraordinary potential in ocular delivery by decorating their surface with targeting groups like sugar moieties (81). This strategy has allowed liposomes to cross the different biological barriers (*e.g*. cornea, conjunctiva epithelia and tear film) that protect the eye from the entrance of external substances.

3. Liposomes-*in*-Hydrogels

In addition to the advantages of using liposomes as drug delivery vehicles to improve their therapeutic action *in vivo* by using different administration routes, some additional issues need to be taken into account regarding liposome stability. For instance, conventional formulations of larger liposomes are easily removed from circulation by the effect of Kupffer cells and macrophages located in liver and spleen, respectively. Similarly, these drawbacks have been observed for positively charged liposomes. Although these particles have been mainly used in gene therapy, specifically for DNA/RNA delivery, they are prone to interact with serum proteins which result in aggregation, reduction of their half-life circulation time and therefore increasing their clearance rate by the reticuloendothelial system (RES). These rapid clearance of liposomes may be overcome either by obtaining stealth liposomes or varying other liposome properties including size, zeta potential and lipid composition; parameters that may affect the stability, kinetics and biodistribution of liposomes (82, 83, 84). However, the long-term therapeutic effect might be detrimental in certain applications.

In order to improve the pharmacokinetic profile and efficacy of liposomes, it would be desirable to achieve a sustainable drug release at the site of injection. The entrapment of liposomes within polymers, which are able to self-assemble and turn into hydrogels after injection, could represent a useful strategy for promoting sustained delivery of liposomal therapeutic drugs in a variety of therapeutic applications. For example, the simple combination of both systems allowed Weiner et al. to obtain for the first time liposomes embedded in collagen matrices (85). In this experiment, the authors were able to encapsulate two peptide hormones (insulin and growth hormone) forming liposomes within the

collagen matrix, obtaining sustainable cumulative releases for insulin (~ 5 days) and for growth hormone (~ 14 days). Since then, a good number of natural and synthetic hydrogels have been used as a depot to incorporate all kinds of vesicular carriers giving rise to important hybrid materials capable of fulfilling a broad range of biomedical applications (86, 87).

3.1 Liposomes-*in*-Chitosan hydrogels

Liposomes entrapped in CS-based hydrogels have become an attractive strategy for the biomedical field due to the possibility of obtaining smart injectable drug delivery systems *in situ* (86, 88). Consequently, this approach has helped to increase the biological effectiveness of encapsulated drugs (hydrophobic small drugs and macromolecules) when compared to traditional strategies.

Physical gelation of CS-based hydrogels can be obtained in aqueous solution either in combination with a cross-linking agent (*e.g*. glutaraldehyde, genipin, divalent and polyvalent anions) (17) or without adding any other additive (29). The use of β-glycerophosphate (GP) as a polyol counterionic dibase salt in combination with CS has resulted in one of the most used methods to form CS-based hydrogels (24) because of the response of this bifunctional system at temperature changes (89). The gelation process of these two systems has been thoughtfully studied by rheology (90). It can depend on multiple interactions based on CS polymer, deacetylation degree and concentration of GP and CS. While this system is maintained in solution at room temperature, the *sol*-to-*gel* phase transition takes place when temperature increases. Interestingly, the gel

formation can also be achieved at the body temperature, retaining its physical properties during a long period of time. This property has allowed this system to be injectable and has been also successfully used in areas like cancer treatment, ocular delivery, burn therapy as well as tissue engineering applications (86, 88).

Liposomes can be entrapped into CS hydrogels taking advantage of both hydrophobic and electrostatic interactions involving the positively charged of CS chains and the phospholipid polar head groups on the liposomal bilayer surface. However, it is well-known that other intermolecular interactions like van der Walls and hydrogen bonding may also play an important role in the formation of CS hydrogels. The dual system based on CS polymer and liposomes can be characterized either by FITR spectroscopy or TEM microscopy. In addition, liposomes-*in*-chitosan hydrogels can be prepared following two alternative strategies. The first one involves the addition of an acidic CS solution over a stirred liposomal suspension whereas in the second approach, liposomes can be added dropwise over the aforementioned CS solution. These two strategies would lead to the expected CS-coated liposomes after stirring and incubating at 4^{o}C. Morphology, surface charge, EE, sonication time, temperature, stirring speed and particle size are some of the parameters that may affect in the CS coating process (91, 92, 93).

The release of liposomes through CS networks is another parameter to take into account that may depend on membrane permeability, membrane stability and carrier fluidity. The optimization of such factors including CS concentration and chemical modifications of CS polymers may lead to better release of liposomal formulations by delaying and controlling drug release and reducing

the initial level of undesirable burst release of a particular cargo as well. Therefore, these physicochemical properties may be of crucial importance when using these hybrid systems as materials for therapeutic purposes.

Overall, liposomes-*in*-chitosan hydrogels may also provide a plethora of biological properties like high stability, an increase of the mucoadhesive capacity as well as an enhancement of both permeability and bioavailability. In addition, CS hydrogels can promote the protection of entrapped liposomes against RES and thereby increasing their lifetime, circulation time in the bloodstream and reducing the level of aggregation, fusion and drug leakage of liposomes (Figure 2).

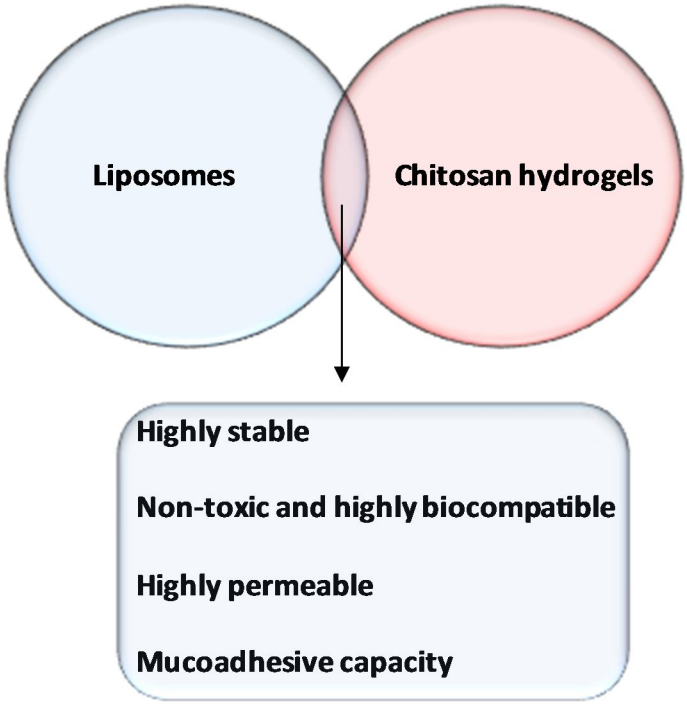

Figure 2. Physicochemical and biological properties of liposome-*in*-chitosan hydrogels

In this sense, the presence of liposomes within CS polymeric networks may provide a good number of biological properties and assure certain feasibility and

potential of this technology in therapy. In spite of the greater potential displayed by these hybrid materials made up of CS and liposomes, other alternative strategies including CS have been also carried out in various biomedical applications with promising therapeutic results as well.

For example, the preparation of modified CS nanoparticles (CSNPs) has been suggested with the aim at interacting with specific ligands and therefore triggering ligand-receptor interactions. This strategy may be useful to promote cellular internalization in targeted tumor cells (94). Other alternatives to liposomes-*in*-chitosan hydrogels have been also reported by entrapping liposomes (DPPC/Chol) and plasmid DNA into CSNPs. This strategy has enabled to protect CS from being deprotonated at physiological pH and thus overcoming important drawbacks associated to cytotoxicity and *in vivo* gene delivery (95, 96).

In this sense, this review is entirely focused on strategies that have been described for entrapping liposomes in CS hydrogels. In particular, the use of these hybrid materials in ocular, vaccine delivery, cancer and wound therapy will be discussed in the following sections.

3.1.1 Liposomal release through CS hydrogels with model drugs

Liposomes were entrapped within CS/GP-based hydrogels for the first time in 2002 (97). Leroux et al. prepared both large unilamellar and multilamellar liposomes containing carboxyfluorescein (CF) as a model drug. Different concentration of egg phosphatidylcholine (EPC)-Chol liposomes were prepared (from 0 to 15 µmol/mL) and encapsulated within the CS/GP polymer network.

Rheological measurements of this system showed that gelation rate and gel strength parameters relied on the liposome concentration used. While storage modulus and gelation time of CS hydrogels increased when liposome concentrations were up to 15 µmol/mL, concentrations above this value gave rise to a decrease in the gel strength. *In vitro* release experiments confirmed that the presence of liposomes within the polymer network enabled to prolong the CF liberation in the receptor-phase. CF release rates depended on the composition and liposome size (72 % and 26 % after 2 weeks of CF released for EPC-Chol liposomes containing 100 and 280 nm, respectively). In contrast, the use of larger liposomes (589 nm) abolished the CF release. Interestingly, the presence of phospholipase A_2 in the receptor-phase, which is able to catalyze the hydrolysis of the glycerophospholipid *sn*-2 fatty acyl bonds (98), contributed to acceleration in the CF release.

An important key aspect to study with these CS/GP hydrogels is the exact role of liposomes in biodistribution processes. In this respect, Rouini et al. studied the influence of lipid composition as well as the biodistribution with labeled liposomes *in vitro* and *in vivo* (99, 100). The authors prepared by the thin film method a series of liposomes that differed in both alkyl chain lengths (DMPC, DPPC, DSPC) and charge (negative -DSPG- and positive -DOTAP-) at several molar ratios in phosphate-buffered saline at pH 7.4. The prepared liposomes were characterized in terms of size and zeta potential showing average sizes that ranged from 110 to 125 nm with *Đ* values less than 0.2 and surface charges of -22 (DSPC, DPPC and DMPC) and 15 mV (DSPG and DOTAP). Liposomes were conveniently labeled by incorporating ^{99m}Tc-HMPAO (101) and *in vitro* stability studies in the presence of human plasma were

assessed previous to their entrapment within the CS/GP polymeric matrix. The authors found different release profiles of ^{99m}Tc from the prepared liposomes. While changes in the length of the hydrophobic residues did not affect the release of the radiolabeled marker (~ 3% was released from DSPC, DPPC and DMPC at 30 min), positively charged liposomes were able to liberate the maker with good efficiencies (15 % released at 24 h incubation). In the case of DSPG liposomes, the corresponding marker rapidly diffused (38 % released at only 30 min). Labeled liposomes did not alter the gelation properties of the CS/GP hydrogels according to their gelation times that were above 32 ^{o}C for all formulations (100). After characterizing the *sol*-to-*gel* transition and confirming CS hydrogel and liposome interactions by FITR spectra, the authors carried out a detailed study involving the biodistribution of the released liposomes *in vivo* by intraperitoneal injection. This study took both lipid composition and charge of liposomes into consideration. Regarding the first effect, it was observed that ^{99m}Tc-HMPAO in solution form cleared faster than liposomal formulations and did not remain in the peritoneum because of its low molecular weight. However, in the case of all liposomal CS hydrogels significantly increased such retention according to AUC values, which showed similar radioactivity levels, regardless of lipid composition and lipid type. Additionally, the release of the radiolabeled marker was mainly located in the liver and spleen for all liposomal formulations (suspension and hydrogel), especially for DSPC liposomes, which displayed the highest AUC values. Moreover, interesting results were obtained for negatively (DSPG) and positively (DOTAP) charged liposomes. While DOTAP showed better peritoneal retention levels, when were entrapped into the CS hydrogels; in the case of entrapped DSPG liposomes, the effect observed was the

opposite which increased up to 8 times the AUC values for the abdominal cavity. The distribution of liposomal hydrogels in other organs revealed that DOTAP had a greater tendency to be accumulated in the spleen more than 2.3 times than DSGP liposomal formulation and also were detected in lungs. This systematic study performed by Rouini et al. demonstrated the ability of releasing liposomal formulations through CS networks and promoting their accumulation in the peritoneal area (over two weeks). These properties might be of interest for future cancer treatments such as gastrointestinal, colorectal and ovarian cancers.

While in most cases small molecules like GP has been combined with CS solutions to activate the *sol*-to-*gel* transition with the aim to obtain temperature-sensitive CS hydrogels, Ladavière et al. also prepared physical CS hydrogels from aqueous solutions without using GP. In particular, this group was able to entrap liposomes containing model drugs (*e.g.* carboxyfluorescein) (102) within such physical CS hydrogels. MLVs were prepared by hydrating the lipid film with a CF solution in a carbonate buffer at pH 8.6. The CS polymer amino groups were first protonated with acetic acid and the resulting solution was combined with CF forming liposomes. To confirm the presence of liposomes, the authors added NBD-PC (1 % molar) to the final DPPC formulation (99 % molar), analyzing the final material by fluorescence microscopy (Figure 3A). Finally, the use of an ammonia solution, which neutralized the system and favored the hydrophobic effect among CS chains, enabled the formation of the expected CS-based hydrogel containing MLVs. Importantly, these extreme conditions (acid and basic pH) did not affect the total integrity of the liposomes as well as CF, which maintained constant its fluorescence emission peak (518

nm) at both conditions. The authors also showed that liposomes entrapped within physical CS-based hydrogels did not affect the gelation properties of the hydrogel (*G' > G"*) according to rheology measurements. After characterizing the hybrid system by fluorescence and cryo-SEM microscopy, the release of the liposomal formulation from CS hydrogels was also evaluated. *In vitro* release experiments were carried out in basic conditions, taking advantage that CF was soluble in aqueous buffer at pH ranging from 6 to 12. The authors observed that the presence of liposomes in such CS hydrogels were able to delay the release of CF when compared to the release behavior of free CF at the same experimental conditions (Figure 3B). These preliminary results involving physical CS hydrogels have contributed to further explore new factors that may play an important role in the drug release like CS weight-average, molecular-weight, CS acetylation degree as well as liposome concentration and size.

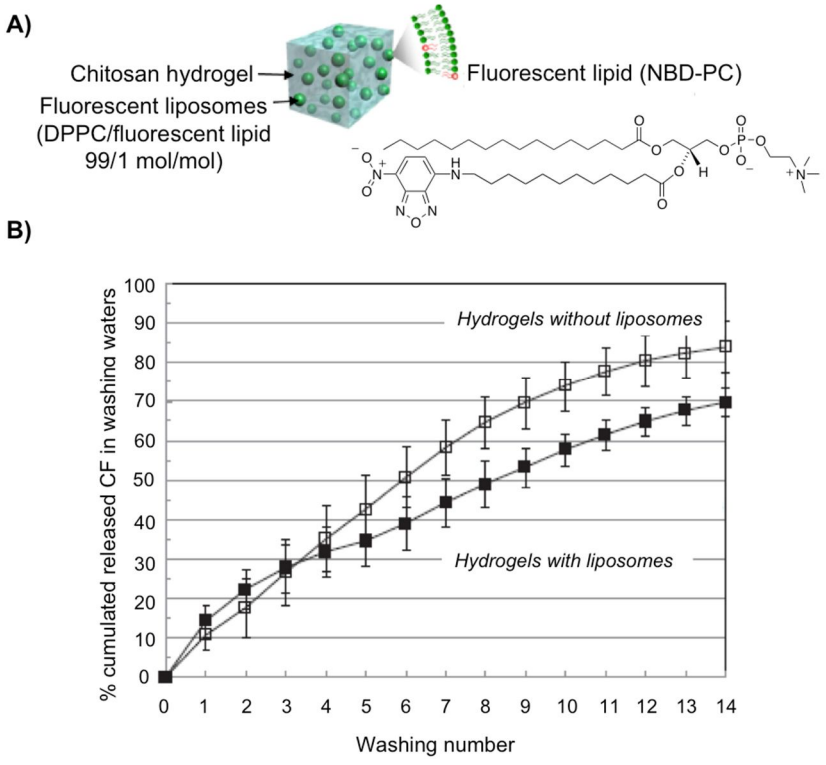

Figure 3 A. CS/GP-based hydrogels containing fluorescently labeled liposomes. B. Cumulative release of carboxyfluorescein (CF) from CS/GP hydrogels showed a slow release when CF was formulated into liposomes. Adapted with permission from reference (102). Copyright 1992 Elsevier B.V.

3.1.2 Ocular delivery

Classical treatments in ocular delivery in form of ophthalmic solutions have resulted in several drawbacks due to a considerable lack of effectiveness of free drugs as they are not able to efficiently reach intraocular tissues (103, 104). A variety of strategies have been proposed to overcome this limitation (*e.g.* liposomes (105), solid lipid nanoparticles (106), *in-situ* hydrogels (107) and microemulsions (108), among others). This restriction has been observed in the case of ofloxacin (OFX), a fluoroquinolone with a potent bactericidal activity against Gram-positive and negative pathogens (109). When administered as ophthalmic solution, OFX shows a number of deficiencies that reduces its therapeutic effect. In particular, it needs to be usually administered in a continuous manner, depending on the infection degree. Furthermore, OFX stability can also be detrimental as unionized OFX fractions increase and are in contact with the corneal fluid.

To overcome these drawbacks, Hosny proposed the use of liposomal hydrogels based on CS/GP polymeric networks (110). This author prepared the formation of MLVs containing liposomes according to the lipid film hydration and reverse-phase evaporation techniques (REV) at pH 7.4. Several lipid compositions and molar ratios were used and systematically studied. The

results showed that lipid film hydration approaches achieved better encapsulation efficiencies than REV technique. In particular, higher encapsulation efficiencies of OFX (65.5 %) were found when dipalmitoyl-L-α-phosphatidyl-choline (DPPC), Chol and stearylamine (SA) in a ratio of 4:3:1 was used. However, the average liposomal size of MLVs was slightly larger than in the case of the particles prepared by the REV technique. Interestingly, the entrapment of MLVs containing OFX within CS/GP polymeric network resulted in a one-degree decrease in the gelation time compared to the native CS/GP hydrogel. This was confirmed by rheological measurements in which the gelation lag time was reduced from 5 min to 1 min. Several *in vitro* transcorneal permeation experiments involving OFX in solution, liposomal suspension and liposomal hydrogel were designed. As might be expected, the penetration profile of the drug varied according to the strategy used. Thus, the use of liposomal hydrogel afforded a permeabilization degree seven times higher than in the case of free OFX in solution after 10 h of release (47.5 % *vs* 6.8 %). In the case of using plain liposomes, they produced five times higher permeation than the aqueous solution (33.8 % *vs* 6.8 %) (Figure 4). These results may indicate the applicability of CS/GP liposomal gels for ocular treatments due to its prolonged release and bioavailability and therefore minimizing the ocular side effects of free OFX.

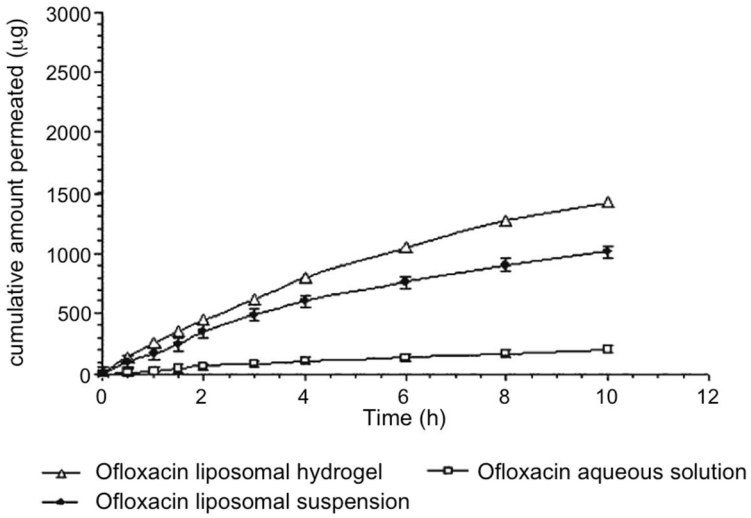

Figure 4. Transcorneal permeation profile of ofloxacin from liposomal hydrogel and liposomal suspension in comparison to its solution (mean ± SD, *n* = 3). Adapted with permission from reference (110). Copyright 2009 Springer.

3.1.3 Vaccine delivery

Vaccine delivery is other important therapeutic strategy in which a good number of drug delivery systems (*e.g*. liposomes, emulsions, polymeric carriers) have been used to promote controlled release of subunit antigens as well as introducing immune adjuvants into vaccine formulations (111, 112). CS/GP-based hydrogels have been used as a depot to release ovalbumin (OVA) protein as a model of subunit antigen *in vitro* and *in vivo* (113). Gordon et al. found a different behavior in the release of OVA when CS polymer was used either as a nanoparticle or hydrogel. While the use of CS nanoparticles did not show any significant immunogenicity, the slow release of OVA observed for CS hydrogels was attributed to the strong interactions between the protein model and CS polymer chains.

Inspired by these results, the same authors proposed to formulate OVA protein into liposomes, which also contained an immunopotentiator, Quil-A (QA). an adjuvant usually used for the liposomal delivery of antigens (114). This liposomal formulation was, in turn, entrapped within CS/GP-based hydrogels (115) (Figure 5) in order to promote a sustainable release of OVA and therefore maximize immunogenicity without putting tolerability and safety at risk. Two vesicular carriers were used: liposomes, which were made of a mixture of PC, SA and Chol in a mixture of 63:7:30 % (molar ratio), respectively, and cubosomes (116), which were prepared according to the lipid-film hydration method by mixing phytantriol, propylene glycol and poloxamer 407. Both liposomal and cubosomal formulations were characterized in terms of size, dispersity (*Đ*), zeta potential and EE. Cubosomes showed lower Z-average sizes (190 nm) with low *Đ* values (0.221) and greater entrapment efficiencies (85 %) than liposomal formulations. Both vesicular carriers were able to induce cluster of differentiation (CD)8^+ and $CD4^+$ T-cell proliferation as well as production of interferon (IFN-Y), which afforded the production of an OVA-specific antibody. Unfortunately, liposomes entrapped in CS/GP hydrogels resulted in certain instability *in vivo* and showed similar responses in the case of OVA and QA when were directly entrapped within the same polymer network. To overcome the problem of liposome stability, the authors prepared cubosome formulations containing OVA and QA. The results showed that there was no significant difference in the immune response *in vivo* when these complexes were entrapped either within the polymeric matrix or used in solution. The authors argued that the highly negative surface charge (-35.3) of the particles could be determinant to promote unspecific electrostatic interactions between

OVA-containing cubosomes and the CS polymer network, which might annul the expected OVA release.

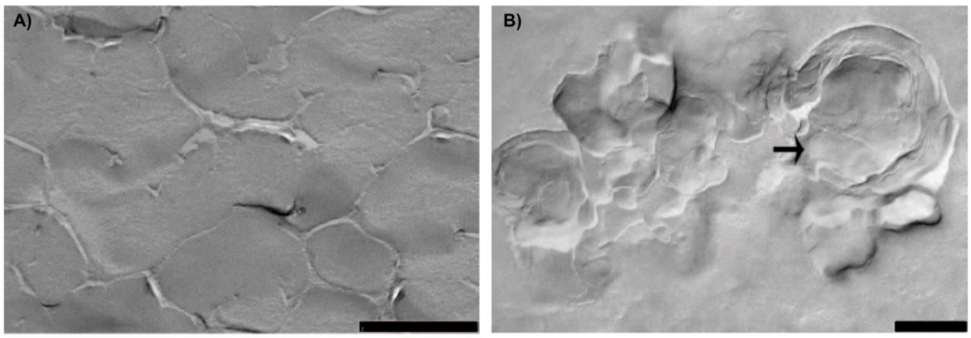

Figure 5. Freeze-fracture TEM images of an unloaded chitosan hydrogel (A) and chitosan hydrogel containing liposomes (B). An area of possible liposomal membrane pore formation is indicated in (B) by the arrow. Scale bars shown represent 500 (A) and 200 nm (B). Adapted with permission from reference (115). Copyright 2012 Informa Healthcare.

3.1.4 Drug delivery in cancer therapy

The growing interest of nanomedicine has allowed the design of novel therapeutic strategies to obtain more efficient drug delivery systems for cancer therapy. The activity and properties of traditional chemotherapeutic molecules can be enhanced either by increasing their solubility and bioavailability in aqueous media or adding superior protection to the therapeutic agent when is in contact with harsh environments. These approaches might lead to enhancing the cellular uptake processes of small molecule drugs and an increase of their half-life in plasma, respectively (117).

Although the majority of chemotherapeutic drugs used in clinic are usually hydrophobic drugs (*e.g*. paclitaxel), some hydrophilic drugs such as

biomacromolecules and small molecules have also been employed in the treatment of some cancers (118, 119). For instance, cytarabine (ara-C) is a small chemotherapeutic drug that has shown effectiveness in most leukemia and certain lymphoma when administered in the form of liposomal formulations. Murthy et al. developed a therapeutic strategy in which CS/GP-based hydrogels were used to entrap a liposomal formulation based on ara-C (120). The authors achieved an optimized formulation based on 60:40 EPC:Chol (% ratio) and 1:20 drug:lipid components (molar ratio), which afforded greater encapsulation efficiencies of the drug (up to 85 %) and low *Đ* values (0.213). Several concentrations of CS (1.5, 1.8 and 2.1 %; *w/v*) and GP (4.7, 5.7 and 6.7 %; *w/v*) were chose in order to obtain the corresponding hydrogels just below the body temperature. This optimization allowed the formation of CS-based hydrogels in less than 5 min at 36.6 ºC when 1.8 % of CS and 5.7 % of GP were used. The *in vitro* release of ara-C was investigated in three different ways: a) the chemotherapeutic drug was directly encapsulated in CS hydrogels; b) the drug was formulated in liposomes, and c) liposomes based on ara-C were entrapped in CS hydrogels. In the case of free ara-C, 90 % of the drug was released in the receptor phase in the first 12 h of the experiment, whereas liposomal ara-C was able to afford a sustainable release for 48 h. Additionally, an undesirable burst effect was produced at the first min of the experiment probably due to the non-encapsulated ara-C. Finally, the system based on CS/GP-liposomes was able to liberate the liposomal formulation in a controlled release manner for more than 60 h. After this time, Triton-X100 was added in order to confirm the presence of intact liposomes within the hydrogel. The authors also designed *in vivo* experiments in albino rats involving the optimized formulation as well as the

corresponding controls. The three formulations were intramuscularly administered at a dose of 5.4 mg drug/Kg body weight. After isolating ara-C from plasma at several time intervals, the authors found good correlations among *in vitro* and *in vivo* control release experiments, which may open the door to the development of novel drug delivery systems for cancer treatments.

Doxorubicin (DOX) is another chemotherapeutic agent used for the treatment of numerous cancers including, among others, breast cancer, bladder cancer and lymphoma. DOX belongs to the anthracycline family of medicaments and because of its planar configuration, DOX is able to intercalate between neighboring DNA base pairs in the DNA minor groove (121). DOX is a hydrophobic small drug and this leads to various limitations during its administration such as low water solubility, systemic toxicity and rapid renal clearance despite of its a great efficacy as a chemotherapeutic agent. DOX is usually distributed in a non-specific manner to the tumor site when is administered systemically and it may be deposited at high concentrations in others organs reducing its therapeutic potential. To increase DOX accumulation at the tumor site, the development of safer and implantable delivery systems may represent an ideal strategy to overcome these limitations (122, 123).

Recently, Chen and Li et al. carried out the encapsulation of DOX liposomes into CS/GP-based hydrogels in order to study both the antitumor efficacy and the systemic toxicity of liposomal DOX *in vitro* and *in vivo* (124). Previously, liposomes were prepared in a mixture of PC and Chol. The resultant lipid film was hydrated with ammonium sulfate. This strategy gave the authors high encapsulation efficiencies (up to 95 %) and particle sizes of 94.2 nm. In addition to entrapping DOX liposomes into CS/GP hydrogels, free DOX was also

incorporated in the same polymeric network with the aim of studying the *in vitro* DOX release profiles at several pH values. In this case, the authors found an undesirable burst effect during the first 4 h of the experiment over a wide range of pH (Figure 6A). Interestingly, DOX liposomes were able to impart a sustainable release during 21 days when released from CS/GP hydrogels at pH of 6.8 and remarkably reduced the burst effect (Figure 6B). *In vitro* cytotoxicity analysis of CS/GP-DOX and CS/GP-DOX-liposomes confirmed the cellular toxicity effect of DOX in both systems after eight days of incubation. However, much more efficiency was found in the case of embedded DOX liposomes than free DOX at the same concentration (95.4 % *vs* 80 % of dead cells, respectively).

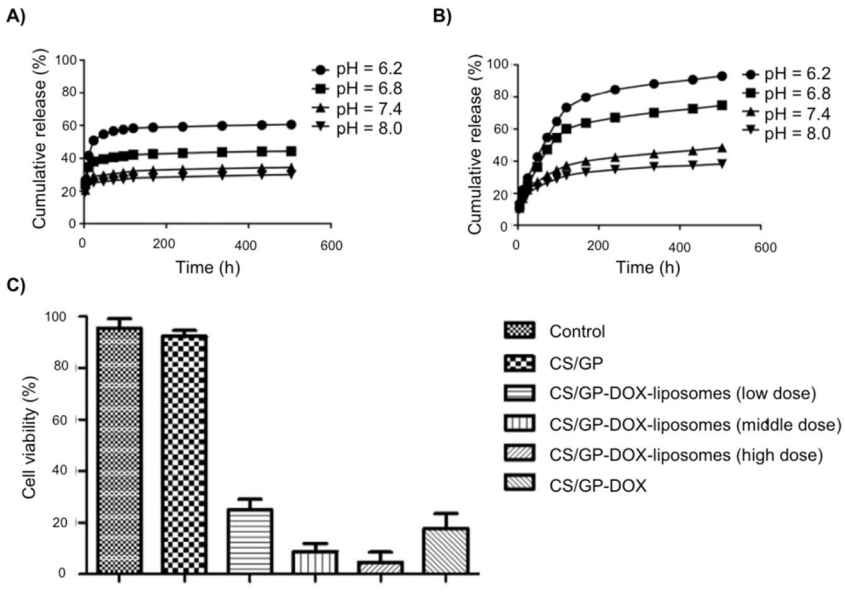

Figure 6. A. Cumulative release of DOX showing an intense burst effect at different pH values when the drug was directly entrapped into CS/GP-based hydrogels. B. Cumulative release of liposomal DOX through CS/GP-based hydrogels at several pH values. Adapted with permission from reference (124). Copyright 2004 Wiley-VCH.

In vivo antitumor activity was also assessed in tumor-bearing mice by an intratumoral injection of the two DOX formulations (CS/GP-DOX and CS/GP-DOX-liposomes, respectively) at the same dose of 20 mg/Kg. Interestingly, the authors found statistically differences in the tumor volume on the sixth day when treated with CS/GP-DOX and CS/GP-DOX-liposomes in comparison with controls. Furthermore, this difference was even more apparent between both DOX formulations for longer daily treatment times, in particular at 18^{th} days after injection. Furthermore, CS/GP-DOX-liposomes showed better antineoplastic effect and less toxicity than free DOX. These encouraging results may open the door to future clinical applications involving this kind of formulations.

In addition to DOX, topotecan hydrochloride (TPTC) is a topoisomerase I inhibitor with potential antitumor activity as well. This synthetic water-soluble analogue of camptothecin has been used to treat a good number of cancers, especially ovarian and lung cancer as an injectable solution (125). Despite this effectiveness, free TPTC is sensitive at physiological and alkali pH which results in reducing its efficacy due to the presence of an inactive carboxylate moiety in neutral or basic solutions generated from undesirable lactone hydrolysis of TPTC (126). To overcome these drawbacks, TPTC has been efficiently formulated in liposome-based drug delivery systems in order to preserve the lactone ring stability (127). However, some concerns need to be taken into account when PEGylated liposomes containing TPTC are used. It has been demonstrated that repeated injections of these liposomes have produced at certain time intervals an “accelerated blood clearance” from the circulation (128). Although the mechanism is not yet clear, it is believed that there is a

production of immunoglobulin M (IgM) after the first liposome injection. As a consequence, IgM is able to bind selectively to another PEGylated liposome which leads to rapid elimination by promoting hepatic uptake (129, 130).

Xing et al. proposed to obtain liposomal injectable hydrogels made of CS/GP in order to preserve the stability of this active lactone and therefore trigger a sustainable release of the liposome formulation avoiding undesirable secondary effects (130). Firstly, TPTC was formulated in liposomes with a soybean phospholipid:Chol relationship of 4:1. Liposomes were hydrated with ammonium sulfate solution in order to ensure high drug loading and were subsequently entrapped within CS/GP-based hydrogels. The authors carried out an *in vitro* release study by varying both the pH of the receptor-phase (5.0, 6.8 and 7.4) and the ionic concentration with the aim to evaluate how these parameters could affect to the liposomal release. The results indicated that acid solutions produced an acceleration in the hydrogel erosion and consequently in the release profile (80 % of drug release in 50 h), whereas increasing pH (up to 7.4) slightly reduced the drug cumulative release (60 % of drug release) (Figure 7). Additionally, rheological measurements showed that TPTC liposomes produced an increase both in *sol*-to-*gel* phase transition and *sol*-to-*gel* transition temperature (40.2 ^{o}C).

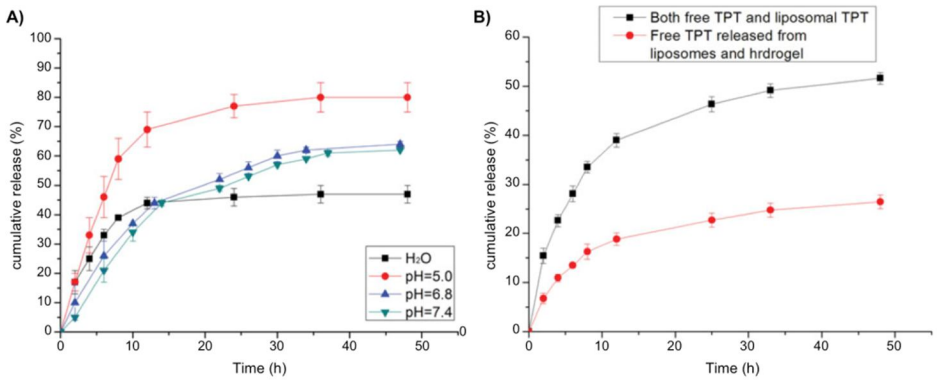

Figure 7. A) The release profiles of TPT loaded CS/β-GP hydrogel in different pH (PBS, pH 5.0; PBS, pH 6.8; PBS, pH 7.4) and ionic concentration (deionized water; PBS, 0.05 M, pH 7.4) solutions at 37 ± 0.5 ºC (mean ± SD, *n* = 6). B) *In vitro* cumulative release of TPT, free or both free and liposomal, released from the TPT liposomes-loaded CS/β-GP hydrogel in PBS (pH 7.4, 0.05 M) at 37 ± 0.5 ºC (mean ± SD, *n* = 6). Adapted with permission from reference (130). Copyright 2014 Taylor & Francis.

In vivo experiments with Kunming mice bearing H22-tumor were carried out by intratumoral injection employing CS/GP-TPTC-liposomes with several control experiments (*i.e.* plain CS/GP-liposomes, free TPTC and CS/GP-TPTC). The authors observed that tumor size was significantly reduced as well as the survival rate without observing any side effects when TPTC liposomes loaded in the CS/GP-based hydrogel was used. These results demonstrated the therapeutic potential of such injectable formulations for the protection of camptothecin analogues and their ability to release the active chemotherapeutic agent in a sustained manner into the tumor.

In addition to obtaining hydrogel materials that are able to respond to temperature stimuli and trigger the release of a chemotherapeutic drug, other important therapeutic strategies have been also developed to favor controlled drug release. In this respect, hyperthermia is one of the strategies that have been utilized in cancer treatments since 1970 (131). In this technique, malignant cells are heat-treated at 43 ^{o}C, around six degrees above the body temperature which leads to cellular death. Generally, hyperthermia has been successfully

combined with both chemotherapy and radiation therapies however some secondary effects have been found in healthy tissues.

In addition to using and modifying magnetic nanoparticles for treatments involving hyperthermia (132), temperature-sensitive liposomes (TSLs) have been used due to their capacity of releasing drugs at 42 ^{o}C when reaching at the melting-phase transition temperature of the lipid bilayer (133, 134). Ruiz-Hernández et al. proposed to use *in situ* injectable CS/GP-based hydrogels containing DOX-loaded TSLs, which were entrapped by the pH gradient method (135) (Figure 8A and 8B). Thus, the combination between CS/GP polymers and TSLs in a homogeneous solution afforded the expected hybrid CS hydrogel after a local injection. Curiously, the gelation temperature of the hybrid system took place at 33 ^{o}C. Two *in vitro* release experiments were carried out in order to characterize the release behavior of DOX-loaded TSLs through the hydrogel. The authors detected a dual mechanism when control release experiments were performed. Firstly, they initially observed a small burst release effect (~ 10 % of free DOX) followed by a sustainable liposomal release during the next 8 days at 37 ^{o}C. Interestingly, when liposomes were externally activated by hyperthermia treatments involving radiofrequency microwaves (a pulse of 1 h at 42 ^{o}C), the liberation of DOX increased at day 3 and provided a therapeutic effect that lasted for three additional days (Figure 8C). This result confirmed the activation of the TSLs (approximately seven-fold increase when compared to non-pulsed treatments) by hyperthermia.

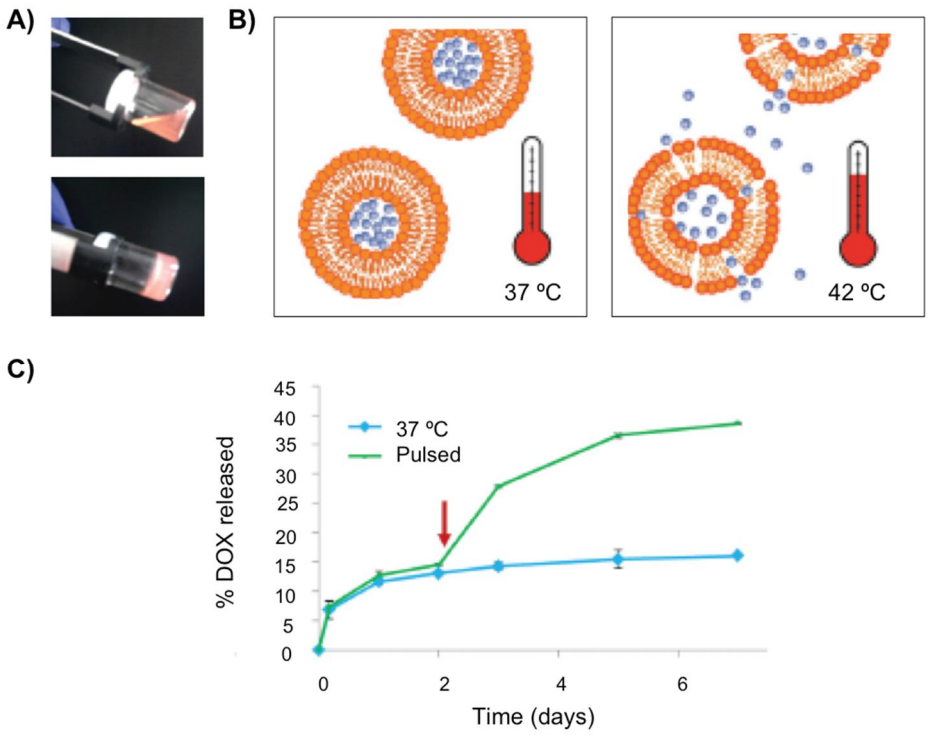

Figure 8. A. *Sol*-to-*gel* transition of CS/GP-based hydrogels containing liposomal DOX formulations. B. The use of thermosensitive liposomes (TSLs) for hyperthermia treatments liberated DOX at 42 ^{o}C. C. Cumulative release of liposomal DOX at 37 ^{o}C at several days. The use of hyperthermia helped to increase the release of the therapeutic drug. Adapted with permission from reference (135). Copyright 2012 Elsevier B.V.

The activity of the CS/GP-based containing thermosensitive DOX liposomes was confirmed in human A2780 ovarian carcinoma cells. The authors designed two types of experiments: firstly, tumor cells were incubated in the presence of hydrogels. After 48 h incubation, a decrease in dsDNA levels was observed which demonstrated the diffusion mechanism produced by DOX. The second experiment involved the incubation of non-treated cells with the CS hydrogel previously used. Interestingly, the authors observed an extraordinary reduction of dsDNA levels at day 4 when the hybrid system was subjected to a 1 h heat

pulse at 42 ^{o}C. Furthermore, this pulse did not significantly affect the cellular viability which demonstrated the efficacy of this treatment.

Recently, the same authors were able to tailor the release kinetics of drugs by affording a passive diffusion of one therapeutic molecule through the same polymeric hydrogel as described before and activating the release of a second drug when loaded into TSLs after hyperthermia treatments (136). In particular, it has been described that a dual combination between hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) have resulted in better angiogenic responses *in vivo* (137). This dual effect aimed the authors at designing “smart materials” based on CS/GP-based hydrogels to administer this therapeutic combination. This strategy was based on developing a proangiogenic therapeutic platform by promoting the passive release of HGF followed by deferoxamine (DFO) release, a pro-angiogenic drug, using hyperthermia in mesenchymal stem cells (MSCs). TSLs containing DFO were composed of DPPC, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[amino(polyethyleneglycol)-2000] (DSPE-PEG) and monostearoylphospha-tidylcholine (MSPC) in a molar ratio of 85.3:9.7:5.0, respectively and were easily entrap within CS/GP hydrogels along with HGF by stirring both drug solutions. Initially, the authors characterized the diffusion of DFO and HGF separately through the hydrogel. There was a burst release when DFO was directly entrap within the hydrogel (40 % in the first 4 h) whereas the liposomal formulation of DFO resulted in reducing this undesirable burst effect and achieving a slow sustainable release for 14 days. This release rate was modulated by applying 1 h hyperthermic pulses at 42 ^{o}C (transient hyperthermia) at different days (2, 6 and 10), which increased the amount of DFO released (30, 24 and 15 % on

days 3, 7 and 11, respectively). Previous to the bioactivity effect in MSCs, the authors carried out an *in vitro* release experiment involving TSLs-loaded DFO and free HGF from CS/GP-based hydrogels, which allowed the authors to prove the potential of the hybrid system. As expected, there was an initial burst release of HGF for the first 24 h until reaching *a plateau*. While a hyperthermic pulse was not applied, the liposomal formulation of DFO was slowly released. However, when a thermal pulse was subjected to the hydrogel containing the dual system, an increase in the release of DFO was obtained (57 %). The applicability of hyperthermia in the CS hydrogel was then assessed with different thermal pulses in MSCs. Interestingly, it was shown the appearance of VEGF levels were significant when compared to untreated cells, demonstrating that DFO remained active up to 10 days (Figure 9). These results suggest the therapeutic efficacy of this system by modulating the depot with external stimuli and therefore modifying the release of therapeutic drugs.

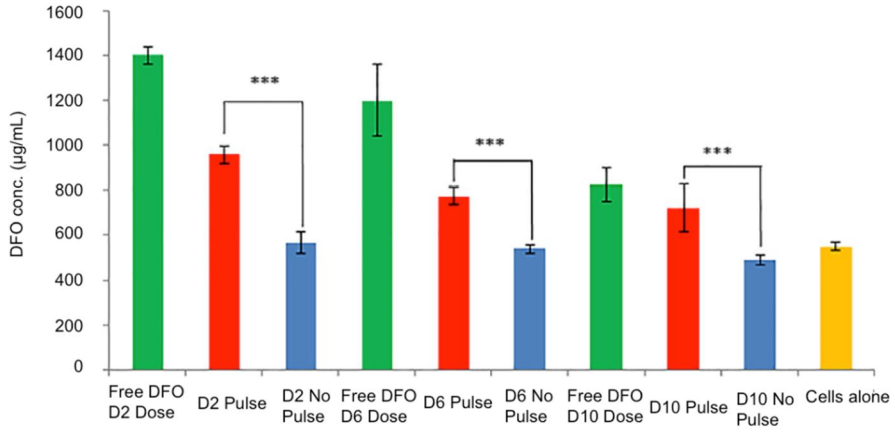

Figure 9. Cumulative release of liposomal DFO (VEGF expression) in mesenchymal stem cells through CS/GP-based hydrogels during several days. Free DFO showed an intense burst effect whereas the use of hyperthermia afforded an increase in the VEGF expression. The effect remained until 10

days. Adapted with permission from reference (136). Copyright 2014 Wiley-VCH.

3.1.5 Modified chitosan-based hydrogels in cancer therapy

The therapeutic potential shown by CS-based hydrogels in different applications has motivated the search of new synthetic strategies with the aim at providing improved CS polymers but without changing the functional properties of the natural CS. In 2013, Liang et al. reported the use of liposomal DOX encapsulated in CS/GP-based hydrogels for topical cancer therapy (138). This group used a soluble CS derivative, *N*-[(2-hydroxy-3-trimethylammonium)-propyl] chitosan chloride (HTCC) by introducing an additional quaternary ammonium unit, which remarkably improved the solubility of CS polymer in aqueous solutions (139). Liposomes containing DOX were prepared by hydrating the thin lipid film with 0.3 M ammonium sulfate yielding the expected MLVs. The excess of ammonium sulfate was removed by dialysis and subsequent ultrasonication generated the corresponding SUVs with average particle size of 108.1 nm and *Đ* of 0.382. Unfortunately, an increase in the liposomes size (10.3 μm) and *Đ* (2.53) was observed as a consequence of unexpected aggregation processes when liposomes were entrapped into HTCC/GP-based hydrogels. The *sol*-to-*gel* transition of HTCC/GP hydrogels containing DOX-loaded liposomes was investigated by rheology. The authors found that the presence of liposomes did not affect the gelation process of the mixture HTTC/GP. Additionally, both plain HTCC/GP and HTCC/GP containing liposomes showed similar properties at room temperature showing good fluidity.

However, when temperature increased to 37 ^{o}C, both solutions became more viscous and generated the expected hydrogels within 5 min. Finally, *in vitro* release behavior and *in vivo* antitumor activity were evaluated. In the first case, authors were able to prolong the release of liposomal DOX when the drug was entrapped into HTCC/GP-based hydrogels (around 22% of drug release in 9 days) (Figure 10). Furthermore, this system was also able to reduce the initial burst effect observed when DOX was formulated only in liposomes. *In vivo* experiments were carried out by evaluating the effect of the formulations (*i.e.* HTCC/GP-DOX, HTTC/GP-liposomes and liposomal DOX) in ten hepatoma-22 (H22)-tumor-bearing mice. After intraperitoneally injections, the results demonstrated that introducing liposomal HTCC/GP hydrogel significantly enhanced the antitumor activity (20 % of the mice survived more than 60 days after treatment) and also reduced the side effects observed when compared with other formulations used (HTCC/GP-DOX and liposomal DOX, respectively).

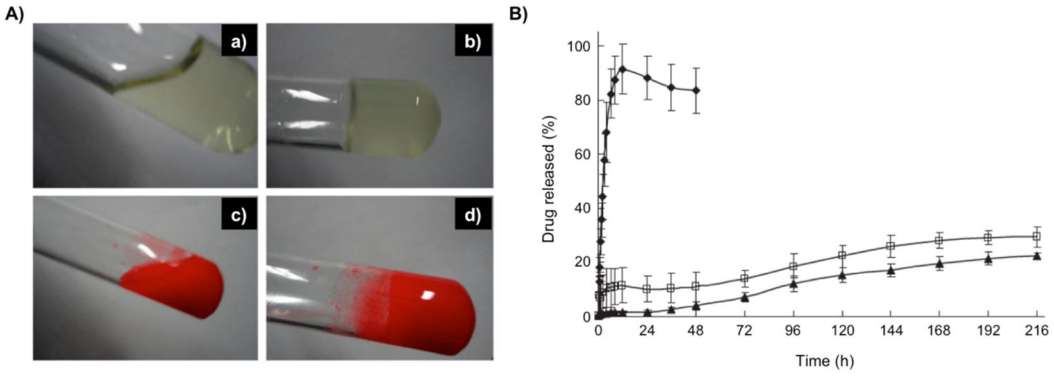

Figure 10. A) HTCC-GP (a) and DOX-LP/HTCC-GP (c) solutions at room temperature. HTCC-GP (b) and DOX-LP/HTCC-GP (d) formed hydrogels at 37 °C. B) *In vitro* drug release from doxorubicin solution mixed to HTCC (♦), DOX-LP suspension (□) and DOX-LP/HTCC-GP gel (▲) in natural saline at 37 °C performed using the dialysis membrane method. Each point represents the

mean value ± SD (*n* = 3). Adapted with permission from reference (138). Copyright 2013 Taylor & Francis.

CS polymers have also been modified by introducing hydrophobic residues. In particular, Raghavan et al. introduced covalently *n*-dodecyl hydrocarbonated alkyl chains to the CS backbone (140). In previous experiments, these authors combined hydrophobically modified CS (hmCS) with positively charged vesicles (a mixture of cetyl trimethylammonium tosylate, -CTAT- and sodium dodecyl benzene sulfonate, -SDBS- in a mixture of 70:30, respectively) (141), which resulted in the formation of the expected hybrid materials. HmCS hydrogels containing cationic vesicles were characterized by rheology and small-angle neutron scattering (SANS). These measurements confirmed the presence of intact vesicles within the polymeric network due to the hydrophobic interactions of hmCS and the vesicular bilayer. Curiously, the entrapment of these vesicles did not produce the expected hydrogel in unmodified CS polymers.

The applicability of hmCS-based hydrogels was carried out by Lee et al. (142) by entrapping SUVs that were obtained by hydrating lipid films with ammonium sulfate. These authors prepared hmCS hydrogels containing both DOX (hmCS-DOX) and DOX liposomes (Doxil) (hmCS-Doxil). While the first system resulted in a viscous solution, the second one showed the characteristics of elastic hydrogels according to their rheology measurements (elastic *G'* exceeded viscous *G"* moduli at low frequencies) (Figure 11A). Furthermore, the injectability capacity of hmCS-Doxil was also characterized by steady-shear rheology, displaying high viscosities at low shear-rates followed by strong shear-thinning. However, this viscosity was considerable less

pronounced at a shear-rate of 5 s^{-1}. Such low viscosity under high shear confirmed the suitability of hmCS-Doxil as an injectable depot-based delivery system. Moreover, the release of DOX liposomes through hmCS-based hydrogels was investigated *in vitro*. After removing free DOX (*i.e.* drug not encapsulated into liposomes) by using a size-exclusion column, the liberation of DOX through the hydrogel afforded a sustainable cumulative release of 20 % after 30 h. To confirm the presence of intact liposomes, the authors added Triton X100 (TX100) which caused the disruption of liposomes producing an uncontrolled release of the drug (Figure 11B). Finally, the potential of this material was confirmed by cell viability experiments using SK-BR-3 cells. HmCS-Doxil was placed into a transwell insert and the percentage of cells was measured after 11 days using trypan blue. The results confirmed the cytotoxic effect of the hybrid system, which showed high levels of dead cells (more than 80 %). This result confirmed the stability and sustainable property of the hybrid material over a week long-period. These promising results may display some advantages of this material against the well-known system CS/GP due to liposomes are able to take actively part of the polymer network by the presence of hydrophobic interactions between the polymer and colloid vesicles.

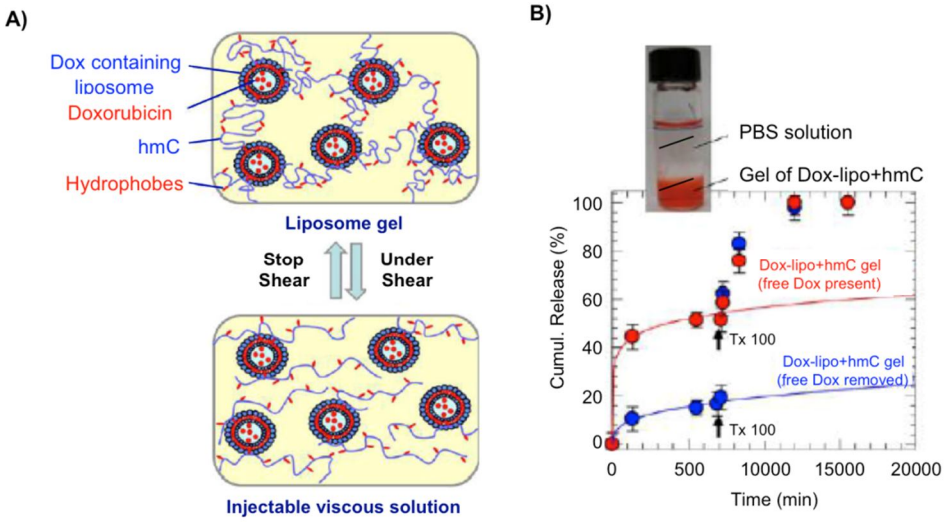

Figure 11. A. *Sol*-to-*gel* transition of hydrophobically modified CS (hmCs) containing liposomal DOX formulations. HmCS polymer chains interact with liposomes giving rise to the formation of the expected hydrogel. B. Cumulative release of liposomal DOX when released from hmCS hydrogels in the presence of additional free DOX (red) or free DOX removed (DOX is exclusively released from liposomes (red). Triton X100 was added to confirm the presence of liposomes within the hmCS-based hydrogel**.** Adapted with permission from reference (142). Copyright 2000 American Chemical Society.

3.1.6 Cross-linking associations with other polymers

Covalent cross-linked-based CS hydrogels can be obtained from three different strategies: a) CS hydrogels can promote cross-linking associations with itself; b) the formation of hybrid polymer networks (HPN) and c) full-interpenetrating polymer networks (IPN) (43). The first approach usually takes place when different units of CS are associated to each other giving rise to a final hydrogel in which the entanglements of the CS polymeric network show restricted mobility. In the second case, the gelation process occurs when some functional groups from CS polymers are able to react with a second polymer. In the latter case, IPN hydrogels are made of non-reacting polymers that have been combined with CS polymers before cross-linking (143). This process allows the formation of cross-linked hydrogels in which the second polymer is efficiently entrapped. Although covalent bonds are preferentially formed in the three strategies, the contribution from secondary interactions should not be excluded.

Within this context, Peptu et al. prepared a series of covalently cross-linked CS/gelatin hydrogels with several concentrations of glutaraldehyde, sodium sulphate (NaS) or sodium tripolyphosphate (NaTPP), as ionic cross-linking agents (144). This double cross-linking strategy allowed these authors to prolong and achieve a greater sustainable release of a model drug. Furthermore, MLVs and SUVs were prepared from PC according to well-established protocols, generating the corresponding liposomes with diameter sizes of 1.27 μm and 112 nm for MLVs and SUVs, respectively. Both vesicular carriers, which contained calcein as a model hydrophilic drug, were efficiently entrapped into different ratios of cross-linked CS/gelatin hydrogels. As a control experiment, calcein was directly loaded into the hydrogels cross-linked with NaS and NaTPP, respectively. In both cases, they displayed an intense burst release (30 % of calcein was diffused) during the first hour and there was an increase in the concentration of the calcein in the first three days reaching at 50 - 80 % of efficiency at pH 7.4. Furthermore, the authors characterized the behavior of calcein when released from liposomes (MLVs and SUVs) and CS/gelatin-liposomes hybrid systems. They found that the liberation of calcein from liposomes was extremely fast (all content was released in less than 24 h). However, the combination of hydrogels and the two liposomes resulted in a marked decrease of the burst release effect. This result was due to the presence of two barriers to overcome: the lipid membrane and the cross-linked CS/gelatin hydrogel. In addition, this release could be effectively tuned after modifying the parameters of the hydrogel such as hydrogel composition, cross-linked agent and vesicle size. Thus, when MLVs containing calcein were entrapped in NaTPP cross-linked CS hydrogels, the drug remained unchanged

within the hybrid system at least three weeks. This effect was attributed to the density increase in the entanglements of the hydrogel, which reduced the mobility of the MLVs. In the case of SUVs, while a sustained and controlled release of calcein was achieved, the liberation displayed a different profile than the one found for MLVs. A burst release effect was observed for SUVs particles probably due to the smallest size of the particles that helped to reach easily the hydrogel surface by diffusion processes. The authors also studied the integrity of the MLV particles entrapped when released through different cross-linked CS hydrogels by adding TX-100 in the receptor phase (PBS). This provided a direct relationship between both the drug released and calcein liposomes (*latency*) and it gave information about the stability of MLVs in PBS. In the case of NaTPP cross-linkers, the *latency* values were lower than in CS/gelatin hydrogels prepared with NaS. This result allowed the authors to reinforce the argument of the compaction effect produced by ionically NaTPP cross-linkers in this kind of hydrogels.

In addition to perform the release of small molecules, the combination of two biodegradable hydrogels has allowed the preparation of interesting materials for the delivery of macromolecules. Díaz-Sales *et al*. prepared a polyelectrolyte complex made up of CS and xanthan gum (XG) containing liposomes (chitosomes) with the aim to prepare a drug delivery system for protein C-phycocyanin (C-PC) (145). This phycobiliprotein is a protein derived from eukariotic blue-green algae (*Spirula maxima*) and cyanobacteria. C-PC protein has shown interest in biomedicine due to their antioxidant and anti-inflammatory properties *in vivo* by acting as a scavenger of oxygen free radicals and H_2O_2

(146, 147). Furthermore, other applications have been reported not only in neurological disorders (148) but also in cancer (149).

Liposomes and other colloidal systems have been employed as main vehicles for the transport of C-PC in order to be topically administered *in vivo* experiments (150, 151). Alternative administration routes have been also investigated and CS has played a pivotal role in the search of developing new strategies. Thus, the combination of CS and XG polymers may afford hydrophilic matrices in which the corresponding microparticles are formed by electrostatic attraction (152). These systems are highly soluble in aqueous solutions and have been used to improve the oral bioavailability and therefore protect the activity of the desired drug in the gastrointestinal tract (153).

Díaz-Sales *et al*. were able to form MLVs containing C-PC protein from thin film hydration methods with a liposome mean size of 416 nm. Subsequently, the corresponding liposomes were entrapped in a mixture based on CS (0.5 %, w/w) and several ratios of XG polymer that ranged from 2 % to 10 %, respectively. The formation of the microparticles was achieved by freeze-dried (F-D) and spray-dried (S-D) methods. Finally, the authors fully characterized this dual system by rheology studying the contribution of each CS-XG complexes. Extensive rheological studies at different concentrations of CS-XG complexes showed the storage modulus (*G'*) greater than the loss modulus (*G"*) and it was proportional to the XG concentration. Interestingly, the relationship between the two polymers was different depending on the methodology used for the preparation of the microparticles. Thus, in the case of F-D approach, optimal concentrations of CS-XG ranged from 0.5/2.0 to 0.5/10 whereas S-D methodology the optimal ratio was found from 0.5/6.0 to 0.5/10, respectively.

The effect produced in liposomes when incorporated into the CS-XG microparticles was also studied. The authors showed that F-D methodology generated a system where entrapped liposomes displayed an irregular shape as well as aggregates whereas in the case S-D approach liposomes were remained practically intact. The *in vitro* release of liposomal C-PC involving CS-XG (0.5/2.0) and (0.5/8.0) prepared from F-D and S-D techniques were investigated in simulated gastric (pH 1.2) and intestinal fluids (pH 7.4), respectively (Figure 12). Both microparticles showed similar low diffusion profiles at acid pH whereas C-PC liposomes were efficiently released at neutral pH. In particular, systems prepared by the F-D approach generated faster cumulative release of liposomes probably due to porosity detected in this system (49 % and 68 % after 4 h in simulated and intestinal fluids, respectively). However, in the case of S-D methodology, it afforded more compact microparticles that were able to maintain a better control in the release of the liposomes in both fluids. Additionally, this release data confirmed a Fickian diffusion mechanism and was fitted according to the Korsmeyer-Peppas equation model. Finally, the authors carried out *ex vivo* test for mucoadhesive properties with both CS-XG systems. They demonstrated that CS-XG system (0.5/8.0) prepared from the S-D technique displayed better interaction with the colon mucosa and prolonged the contact time. As a consequence, an achievement in the mucoadhesive strength between CS-XG polymers and the mucus layer. These results confirmed this promising strategy and the suitability of the S-D technique to prepare chitosomes for colon drug delivery strategies.

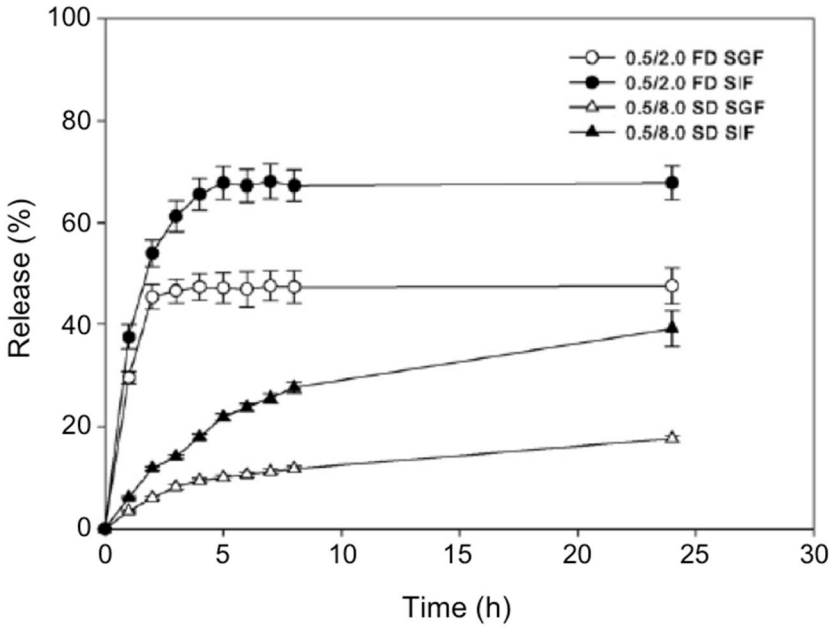

Figure 12. *In vitro* phycocyanin release from tablets prepared using spray-dried (SD CH/XG 0.5/8.0) and freeze-dried (FD CH/XG 0.5/2.0) chitosomes, in different test medium (*n* = 3). Adapted with permission from reference (145). Copyright 2010 Elsevier B.V.

3.1.7 Wound therapy

Wound therapy is the other major application in which CS hydrogels have emerged due to their adhesiveness properties. Ineffective treatments could lead to undesirable bacterial infections and, therefore, the development of microbial resistance. This situation may be solved by implementing the use of topical rather than oral administrations and thus achieving longer sustainable releases of antimicrobial drugs, which might enhance the effectiveness and reduce the risk of burn infections.

Polysaccharides have been extensively studied as wound management aids because of their natural origin, bioadhesive properties and their ability to impart

wound healing (154). Furthermore, it is worth mentioning the antimicrobial property against several fungi and bacteria promoted by chitin and CS polymers as well as their level of permeability to oxygen make these biopolymers interesting candidates for delivering antimicrobial agents (155).

Škalko-Basnet et al. preformed a complete release study using rhodamine model drugs loaded in liposomes according to their liposomal composition, charge and size. This optimization allowed the authors to develop promising hydrogel-liposome hybrid systems for wound therapy (156). Neutral, negatively or positively charged liposome formulations were prepared containing two rhodamine dyes (MP-4 and MTJ-12): a) PC liposomes which contained a low positive charge; b) PC/PG liposomes that exhibited a negative charge and c) PC/SA liposomes which contained a highly positive charge. So-prepared liposomes were finally entrapped within CS hydrogels obtained from diluted acid (2.5 %, *w/w*) and glycerol (10 %, *w/w*), respectively. The *in vitro* release studies confirmed that lipid composition and charge affected remarkably the release behavior of the liposomal drugs. However, liposome size did not have any influence on the liposomal release. In particular, negatively charged liposomes (PC/PG) liberated faster both rhodamine dyes than positive liposomes (PC/SA) (Figure 13). This opposite behavior might be due to the result of electrostatic interactions between CS polymer chains and the corresponding liposomes. Thus, the rapid diffusion displayed by negatively charged PC/PG particles might be caused to an unexpected liposomal membrane destabilization. In the case of entrapped cationic liposomes within CS hydrogel, electrostatic repulsions might be produced generating a slow diffusion of the rhodamine formulations without altering the liposomes integrity (157).

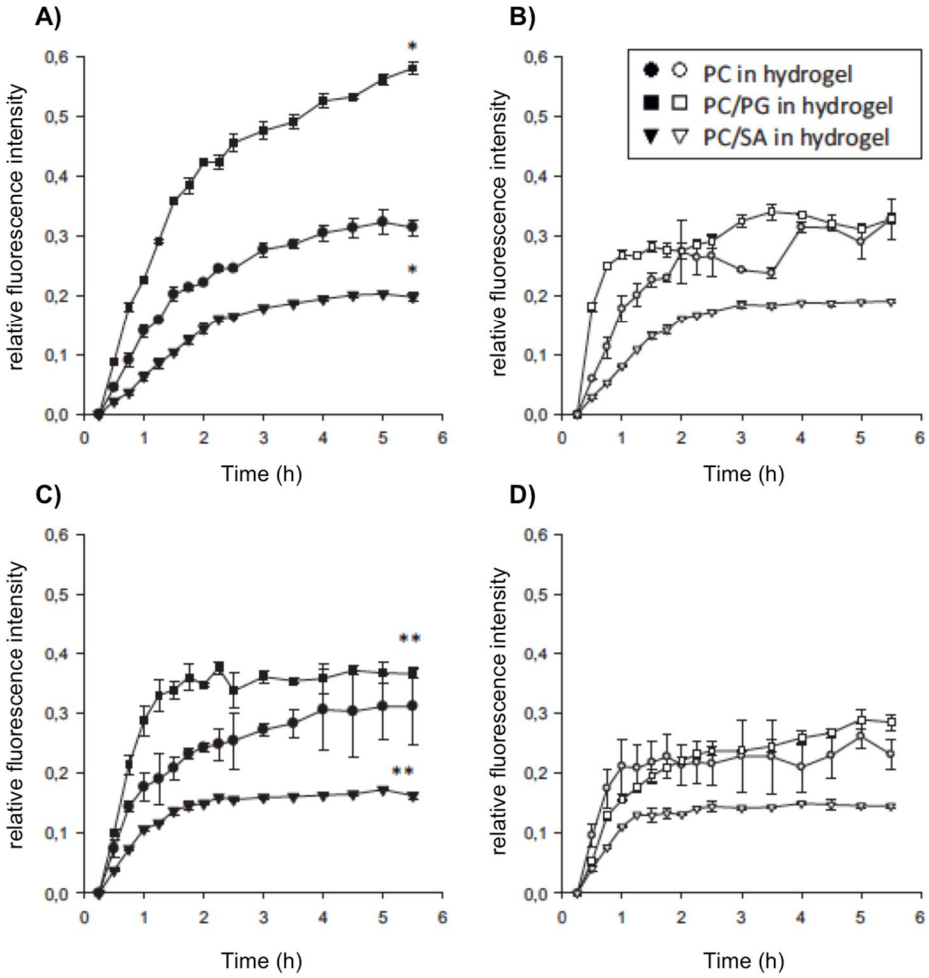

Figure 13. Release of MP-4 (A, B) and MTJ-12 (C, D) from phosphatidylcholine liposomes-in-chitosan hydrogel. Both non-sonicated liposomes (filled symbols) and sonicatedliposomes (open symbols) were tested. *significant vs. PC MP-4 ($p < 0.05$); ** significant vs. PC MTJ-12 ($p < 0.05$) ($n = 3$). Adapted with permission from reference (156). Copyright 2013 Elsevier B.V.

Additionally, the rheological properties of CS hydrogels containing PC liposomes were assessed. These studies confirmed that CS hydrogel was prone to modify its physical properties when additional components were part of the polymeric network. Several concentrations of CS polymer were studied at different molecular weights containing the same PC liposomes ratio (10 %, *w/v*):

a) Low molecular weight (LMW) CS (6.0 %); b) Medium molecular weight (MMW) and c) HMW CS (2.5 %) (158). The authors found that CS-based hydrogels (2.5 %) in combination with glycerol displayed improved texture properties when compared to carbopol-based hydrogels (159, 160). Furthermore, smaller sizes and surface charge of the liposomes, in particular PC/PG and PC/SA significantly increased hardness, adhesiveness and cohesiveness properties of CS-based hydrogels compared to hydrogel controls. In another experiment, the authors entrapped the simplest liposomal formulation based on PC containing mupirocin (MPC) within the same CS hydrogel described before in order to develop an efficient drug delivery model for burn therapy (159). MPC is an antibiotic that has proved to be effective *in vitro* and *in vivo* in the treatment of methicillin-resistant *Staphylococcus aureus* strains (MRSA), pathogens that are mainly found in burn wounds (161). The same group found that the presence of entrapped liposomes were able to prolong the cumulative release of MPC in 24 h (80 % of free MPC *vs* ~ 30 % of liposomal MPC) *in vitro* and *ex vivo* experiments when diffused through CS hydrogels. Interestingly, this delivery system also reported promising antimicrobial results and superior bioadhesiveness. These results were corroborated in cell culture and *in vivo* mice burn models (162). The authors nicely found that CS hydrogels containing MPC forming liposomes were also non-toxic in the presence of human skin cell lines (HaCaT) and showed antibiofilm activity against *S. aureus*. Preliminary *in vivo* results confirmed that the corresponding delivery system based on CS hydrogels enhanced wound healing and its efficiency was comparable to commercially available antibiotic drugs after 28 days of treatment.

The versatility of CS hydrogels has been also studied by incorporating other antimicrobial drugs in form of liposomes like chloramphenicol (CAM) in order to find effective treatments against drug-resistant pathogens (163). Liposomes were prepared from the dual centrifugation (DC) method, which involved the lipid film hydration (egg lecithin, 80 % PC) with a mixture of propylene glycol and distilled water (164) in a ratio of 2:1:2 (*w/v/v*), respectively. The authors obtained good entrapment efficiencies (~ 55 %) with liposome sizes of 120 nm and good *Đ* (< 0.2) having the possibility to scale-up this process, as well. The resultant liposomes were finally loaded into HMW CS hydrogels (2.5 %, *w/w)* containing glycerol (10 %, *w/w*). *Ex vivo* skin permeation of CAM was carried out in a vertical Franz diffusion cell setup using pig skin as a model and three different formulations: a) free CAM; b) Liposome-CAM; c) CS-CAM and d) CS-Liposome-CAM. Holsæter et al. confirmed that CAM was efficiently introduced into the skin when was formulated either into liposomes or in CS hydrogels. Curiously, the use of CS-Liposome-CAM resulted in a skin penetration enhancement as well as a dermal retention on the skin surface when compared to other formulations. After confirming an appropriate skin bioadhesion mediated by this CS-Liposome-CAM delivery system, the authors evaluated the antimicrobial activity from two species of bacteria (*S. aureus* strains and *S. epidermidis* isolates) at increasing concentrations of CAM (500 µg/mL and 750 µg/mL, respectively) after 24 h incubation. Interestingly, CS-based hydrogels containing CAM liposomes were able to demonstrate promising antimicrobial activities than free CAM, indicating the suitability of CS hydrogels as a universal platform for the delivery of antimicrobial drugs.

Conclusions and future prospects

The rapid expansion experienced in the field of hydrogels in recent years has helped to open up new possibilities and thus find novel therapeutic strategies for a good number of diseases. This great variety has allowed both synthetic and natural hydrogels to be promising depot-based delivery systems for a good number of molecules with therapeutic interest. Although there are many examples in which synthetic hydrogels have been used in different applications, natural hydrogels have become an alternative of use due to their natural origin and high compatibility degree.

The direct encapsulation of therapeutic molecules into hydrogels can lead to uncontrolled release of the drug (burst release) and thus produce certain toxicity at the site of action. To avoid this, the double encapsulation of the drug both in liposomes and hydrogels may increase its sustainable release and therefore afford a better therapeutic activity. An important feature of some hydrogels is their ability to promote the *sol*-to-*gel* transition with temperatures soaring to up to 37 ^{o}C. Importantly, the presence of liposomes within the polymeric CS matrix has not disturbed the CS gelation process in most cases. Therefore, this strategy of combining CS/GP with liposomes has been able to reduce minimally the invasive nature by injecting the CS/GP solutions and thus facilitating the integration of the hybrid materials into the target area.

The use of this approach has allowed the CS/GP depot-based delivery system to become an interesting and non-toxic alternative for ocular, cancer and wound therapies. Despite progress and benefits of these hybrid materials have showed both *in vitro* and *in vivo* experiments, any liposome-*in*-chitosan

hydrogel has been launched to clinical trials so far. This drawback may occur mainly as a result of expensive manufacturing processes, a committed physical stability and the need of these materials to be implanted due to their dimensions and elastic properties in most cases. These disadvantages might limit the clinical use and preparation in large-scale of such liposome-*in*-chitosan materials.

This chapter has described a good number of examples in which the CS/GP system has taken part in the release of various liposomal formulations with special emphasis on the biomedical applicability of these systems. Overall, the results suggest, *a priori*, the success of this kind of hydrogels in biomedicine due to their versatility of obtaining stimuli-sensitive-materials that are able to respond to certain stimuli such as pH or temperature and, therefore, increase the therapeutic activity in a specific target site.

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Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this article

Glossary of Abbreviations

Ara-C: cytarabine

CAM: chloramphenicol

CF: carboxyfluorescein

CHOL: cholesterol

C-PC: C-phycocyanin

CS: chitosan

CTAT: cetyl trimethylammonium tosylate

DA: degree of deacetylation

DC: dual centrifugation

DFO: deferoxamine

DMPC: dimyristoyl phosphatidylcholine

DOTAP: *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium chloride

DOX: doxorubicin

DPPC: dipalmitoyl-L-α-phosphatidylcholine

DSPC: 1,2-distearoyl-sn-glycero-3-phosphocholine

DSPE-PEG: 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[amino(poly-ethylene glycol)-2000]

EE: encapsulation efficiency

EPC: egg phophatidylcholine

F-D: freeze-dried

GP: β-glycerophosphate

GUVs: giant unilamellar vesicles

HGF: hepatocyte growth factor

HmCS: hydrophobically modified chitosan

HMW: high molecular weight

HPN: hybrid polymer network

HTTC: *N*-[(2-hydroxy-3-trimethylammonium)-propyl]-chitosan chloride

IGM: immunoglobulin M

IPN: Interpenetrating network

LMW: low molecular weight

LUVs: large unilamellar vesicles

MLVs: multilamellar vesicles

MMW: medium molecular weight

MPC: mupirocin

MSCs: mesenchymal stem cells

MSPC: monostearoylphosphatidylcholine

NBD-PC: 1-palmitoyl-2-(12-[7-nitro-2,1,3-benzoxadiazol-4yl)amino]dodecanoyl)-*sn*-glycero-3-phosphocholine

OFX: ofloxacin

OVA: ovalbumin

PAAm: polyacrylamide

PC: phosphatidyl choline

PE: phosphatidylethanolamine

PEG: polyethylene glycol

PG: propyleneglycol

PHEMA: poly(2-hydroxyethylmethacrylate)

PNIPAAm: poly(*N*-isopropylacrylamide)

PVA: poly(vinylalcohol)

RES: reticuloendothelial system

REV: reverse-phase technique

SA: stearylamine

SANS: small-angle neutron scattering

S-D: spray-dried

SDPS: sodium dodecylbenzene sulfonate

SUVs: small unilamellar vesicles

TPTC: topotecan hydrochloride

TSLs: temperature-sensitive liposomes

VEGF: vascular endothelial growth factor

XG: xanthan gum

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