Combined Gelatin−Chondroitin Sulfate Hydrogels for Controlled Release of Cationic Antibacterial Proteins

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Received October 22, 1999; Revised Manuscript Received February 18, 2000

ABSTRACT: Chemically cross-linked gelatin−chondroitin sulfate (ChS) hydrogels were prepared for the controlled release of small cationic proteins. The amount of chondroitin sulfate in the gelatin gels varied between 0 and 20 wt %. The chemical cross-link density, the degree of swelling, and the rheological behavior were determined to characterize the cross-linked hydrogels. Chemically cross-linked gelatin−ChS hydrogels were loaded with lysozyme, and the release was measured using phosphate-buffered saline. The lysozyme loading capacity of the hydrogels significantly increased with increasing chondroitin sulfate content of the gels. Compared to plain gelatin gels, the release rate of lysozyme slowed for the hydrogels containing 5 and 10 wt % of chondroitin sulfate, while the release was faster for hydrogels containing 20 wt % of chondroitin sulfate. The permeation of lysozyme through gelatin−ChS gels was measured using a two-compartment diffusion cell, and the effective diffusion coefficient was calculated. The effective diffusion of lysozyme in the gels was also qualitatively studied using fluorescence recovery after photobleaching. The Langmuir isotherms of lysozyme adsorption to gelatin−ChS gels and the lysozyme diffusion in the gels in the absence of electrostatic interactions were determined to evaluate the contributions of unspecific interaction between lysozyme and chondroitin sulfate and diffusion to the release. Both the interaction and the diffusion increase with increasing chondroitin sulfate content of the hydrogels, which resulted in a minimum value of the effective release rate for gels containing 5 wt % chondroitin sulfate.

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Introduction

Prosthetic valve endocarditis is an infrequent but serious complication of cardiac valve replacement.[1−3] The adherence of bacteria to the valve is considered to be the first step in the development of the infection. Lately, several antibacterial proteins have been isolated from saliva, blood platelets, and neutrophils, which are believed to play a role in the human defense system.[4,5] All these proteins are small, cationic, and stable. The application of such antibacterial proteins in a controlled release system in the Dacron sewing ring of a prosthetic heart valve may be a promising approach to reduce the incidence of infective endocarditis.[6]

A delivery system for antibacterial proteins was developed based on cross-linked gelatin, showing a good biocompatibility, a relatively fast in vivo degradation, and an in vivo lysozyme release time of 30 h while lysozyme could be detected in the surrounding tissue for a 50 h period.[7,8] The total amount of antibacterial protein released during this period was about 100 μg. Although the biocompatibility, the degradation rate, and the release properties of these gelatin gels are good, a higher maximum protein payload and a prolongation of the release time were considered to be advantageous to increase the efficacy of the antibacterial release system.

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The payload of the gels can be improved by increasing the number of binding sites for antibacterial protein, while the release time can be extended by increasing the interaction between the antibacterial protein and the gel. Antibacterial proteins are cationic, so that the introduction of negative charges into the gelatin gel may improve the release characteristics. In general, two methods can be applied to introduce anionic residues into the gelatin gels: direct derivatization of gelatin,[9,10] or incorporation of anionic (macro)molecules into the gelatin gels.[11] Many polysaccharides are anionic, and glucosaminoglycans were considered most suitable, because of their occurrence in mammalian tissue and their potential healing characteristics.[12−14]

Chondroitin sulfate (ChS) was chosen because it has a molecular mass of 20−60 kD, which may contribute to network formation, and it contains a substantial amount of sulfate and carboxylate residues, necessary for interaction and cross-linking.[13,15] Furthermore, chondroitin sulfate is the only glycosaminoglycan which is predominantly found in cartilage and skin, and it is associated with collagen in connective tissue and tendon in vivo.[16,17] This is probably advantageous for its compatibility with gelatin.

The present study describes the preparation and characterization of gelatin−chondroitin sulfate gels. As gelatin dissolves at temperatures higher than 35 °C, due to the breaking of the physical cross-links, the gelatin−ChS gels were chemically cross-linked with *N*,*N*-(3-dimethylaminopropyl)-*N*′-ethyl carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS). Although chondroitin sulfate is associated with collagen

10.1021/ma9917702 CCC: $19.00 © 2000 American Chemical Society
Published on Web 04/29/2000

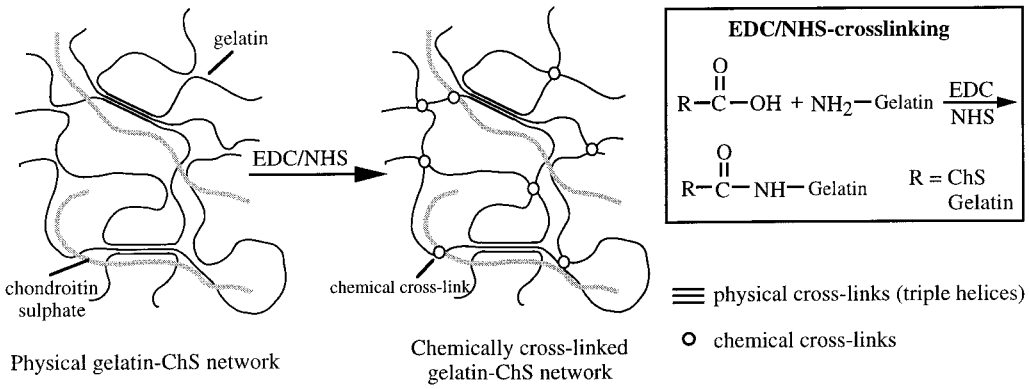

Figure 1. Schematic representation of a physical and a chemical gelatin−ChS network. A physical gelatin network contains triple helix junctions (≡) By chemical cross-linking of this physical network with EDC and NHS, chemical junctions are introduced (○).

in vivo, in vitro studies have shown that interactions between collagen and chondroitin sulfate were maximal at pH 3−4 and minimal at pH 7.[16,18] The network structure of a chemically cross-linked gelatin−ChS hydrogel is schematically represented in Figure 1.

The chemically cross-linked gelatin−ChS hydrogels were characterized by the free amine group content, the degree of swelling, and the mechanical properties. The lysozyme uptake and release from these chemically cross-linked gelatin−chondroitin sulfate hydrogels in phosphate buffered saline were studied as a function of chondroitin sulfate content. The permeation of lysozyme in the gels was measured with a two-compartment diffusion cell, while the mobility of lysozyme was evaluated by fluorescence recovery after photobleaching. As the effective diffusion coefficient is dependent on contributions from interaction as well as diffusion, both the lysozyme interaction with the gelatin−ChS network and the free diffusion of lysozyme in the gelatin−ChS network were studied.

Materials and Methods

Materials. Gelatin B (lot no. 39238) was a kind gift of Vascutek (Sanofi, Paris, France). Chicken egg white lysozyme, gelatin B used in the rheological experiments (bovine skin, 225 bloom, lot no. 56H0658), radiolabeled chicken egg white lysozyme (^{14}C-methylated, 5 μCi), chondroitin sulfate C (ChS; 66 kD), and Amberlite cationic exchange material were purchased from Sigma Chemical Inc., St. Louis, MO. 2,4,6-Trinitrobenzenesulfonic acid solution (TNBS) (1 M) and *N*-hydroxysuccinimide (NHS) were purchased from Fluka, Buchs, Switzerland. 5-(Aminoacetamido) fluorescein was purchased from Molecular Probes, Leiden, The Netherlands. Coomassie Plus Protein Assay Reagent was obtained from Pierce, Rockford, IL. LumaSolve was purchased from LUMAC*LSC bv, Olen, Belgium, and OptiPhase "HiSafe" from Wallac, Milton Keynes, U.K. Phosphate-buffered saline (PBS) (pH 7.4, [NaCl] = 0.140 M) was purchased from NPBI, Emmer Compascuum, The Netherlands. Deionized water was obtained from a Milli-Q plus apparatus from Millipore (Molsheim, France). The phosphate buffer used for lysozyme loading was an aqueous solution of sodium dihydrogen phosphate and disodium hydrogen phosphate (pH 7.1, 66 mM phosphate). All other reagents were obtained from Merck, Darmstadt, Germany.

Conductometric Titration of Chondroitin Sulfate.[19] Chondroitin sulfate (100 mg) was dissolved in deionized water (2 mL) and perfused over a strong cation exchange column (Amberlite, IR-120). Fractions (5 mL) with a conductivity of 30 μS/cm and more were collected and diluted with deionized water to a final weight of 100 g. Solutions of chondroitin sulfate in water (20 g) were titrated with sodium hydroxide (0.1 M) at a constant addition rate, while the conductivity of the solution was recorded in time. The conductivity as a function of added hydroxide ions showed two inflection points. From these inflection points in the titration curves, the number of strong acid residues (first inflection point; sulfate) and weak acid residues (second inflection point; carboxylic acid) in chondroitin sulfate was determined. The experiments were performed in triplicate.

Preparation of Gelatin−Chondroitin Sulfate Gels. Gelatin B was dissolved in deionized water and chondroitin sulfate was added to a percentage of 0, 5, 10, or 20 wt % (total polymer concentration: 20 g in 180 mL) at 50 °C. For the rheological experiments, gels were made using a total polymer concentration of 60 g in 540 mL water. After 1 h of stirring the solution was sonicated to remove air bubbles. The solution was poured onto a silanated glass plate (28 × 38 cm) and allowed to dry on a flat surface overnight at room temperature. Smaller gels (6.5 × 8.5 cm) were cut from these gels.

Silanation of the glass plate was carried out with 50 mL of a mixture of a saturated dimethyl aminopyridine solution in toluene and chlorotrimethylsilane (7:3 v/v). After incubation of the glass plate under nitrogen for 5 h with this mixture, it was washed with ethanol (100 mL), petroleum ether 40−60 (100 mL), and acetone (100 mL) and dried under nitrogen.

Chemical Cross-Linking of Gelatin−Chondroitin Sulfate Gels. Before chemical cross-linking, the gels were dried in vacuo for at least 1 day. Cross-linking of gelatin−ChS sulfate gels with EDC and NHS was carried out in 2-morpholinoethane sulfonic acid (MES) buffer (pH 5.3, 0.05 M) at 4 °C during 16 h. In all experiments the amount of gel in buffer was 1 g in 50 mL of solution. The molar ratio of EDC to carboxylic acid groups of gelatin and chondroitin sulfate ($EDC/COOH_{gelatin+ChS}$) was 0.8 for all the gels. The cross-linking reaction was quenched by replacing the solution with a solution containing 0.1 M disodium hydrogen phosphate and 2 M sodium chloride for 2 h (pH 8.5, 100 mL), and the gels were subsequently washed four times for 1 h with deionized water (100 mL) to remove salt. All washings were performed at 4 °C. The cross-linked gelatin−ChS gels were dried in air. All experiments were performed on gels from the same batch, except the rheological measurements.

Swelling Measurements. Gelatin−ChS gels were dried at a reduced pressure for at least 1 day, and weighed (W_0). The gels were swollen in PBS for 2 h at 22 °C (after which equilibrium swelling was reached), blotted with a tissue, and weighed again (W). Experiments were carried out in triplicate. The swelling was calculated according to

$$S = \frac{W - W_0}{W_0} \tag{1}$$

Determination of Free Amine Groups in Gelatin−ChS Gels. Dried gelatin−ChS gels (2−4 mg) were incubated in 2 mL of a solution of TNBS (0.01 M) in sodium hydrogen carbonate (pH 8.2, 2 w/v%) during 2 h at 40 °C. Then

hydrochloric acid (6 M, 3 mL) was added to the solution to hydrolyze the gels in 1.5 h at 60 °C. After the reaction had cooled to room temperature, deionized water (5 mL) was added to the solution, and the absorbance at λ 345 nm was measured against a TNBS solution without gelatin−ChS, which had been treated in exactly the same way as the cross-linked gelatin samples. Using the absorption coefficient of 2,4,6-trinitrophenyl derivatized (hydroxy)lysine residues (ϵ = 14 600 L/mol·cm),[20] the fractional decrease in free amine group content was calculated from the number of free amine groups in the non-cross-linked gel and the corresponding cross-linked gel.

Rheological Characterization of Physical and Chemical Gelatin Gels. Rheological measurements were performed on an AR1000−N controlled stress rheometer from TA Instruments, Ghent, Belgium. The plate/plate geometry of the rheometer was adapted for measurements on hydrogels by sticking sandpaper on the plates to avoid slippage of the gels between the plates. All measurements were performed with an acrylic top plate (diameter 2 cm), in oscillation mode at 1 Hz in the linear viscoelastic range, by applying a constant strain of 5×10^{-4} rad. Before the measurement, the gelatin−ChS gels were swollen in PBS outside the rheometer. The swollen gels were applied between the plates of the rheometer. G_e, the equilibrium shear modulus, was measured following the methodology for elasticity measurements on gel slabs as recently developed by Meyvis et al.[21] By gradually decreasing the gap between the rheometer plates (in steps of 25 μm) and measuring G' at each position, this method allows one to find to which extent the hydrogel slab has to be compressed between the plates of the geometry in order to perform reliable G_e measurements. At optimal compression of the hydrogels G' is independent of the applied frequency (G' equals G_e), indicating the existence of a real rubbery network.

Lysozyme Release in PBS Solution. Gelatin−ChS gels were loaded with a solution of ^{14}C-labeled lysozyme in phosphate buffer (5 mg/mL, 2 mL) during 3 days at room temperature. The gels were blotted with a tissue to remove adherent solution and placed in PBS. The buffer solution was regularly replaced by fresh solution, and the lysozyme content of the supernatant was measured after the addition of OptiPhase "HiSafe" (18 mL), using a scintillation counter. The amount of lysozyme released was calculated using the specific activity of the radiolabeled lysozyme (3.2×10^{-3} μCi/mg).

Permeation Experiments. The permeation experiments were carried out using PBS and a solution of PBS, containing a total sodium chloride concentration of 0.5 M, respectively, to minimize the interaction between lysozyme and gelatin−ChS gels. Before the measurement, gelatin−ChS gels were swollen to equilibrium. The permeation of lysozyme through the gels was measured in time using a two-chamber diffusion cell (chamber volume 8.3 mL) at 37 °C. The membrane surface area was 2.27 cm^2. The initial lysozyme concentration in the donor chamber was 4 mg/mL. After an equilibration time, samples (300 μL) were taken from the acceptor chamber, and the protein concentration was measured with Coomassie Plus Protein Assay Reagent, using a calibration curve for lysozyme. The permeability of the gels was calculated from

$$\frac{\mathrm{d}(\ln((c_{\mathrm{source}} - c_{\mathrm{sink}})_t/(c_{\mathrm{source}} - c_{\mathrm{sink}})_0))}{\mathrm{d}t} = \frac{2AP}{Vh} \qquad (2)$$

In eq 2, A is the area of the membrane in the diffusion cell (cm^2), V is the volume of the compartments (cm^3), h is the thickness of the membrane (cm), and P is the permeability of the membrane (cm^2/s). The thickness of the gels was determined with a micrometer at the end of the experiment. From the permeability the diffusion coefficient was calculated by dividing P by the partition coefficient K_d which is the ratio between lysozyme uptake by a gel and the concentration in solution.

Determination of the Lysozyme Partition Coefficient (K_d). The partition coefficients were determined by incubating dry gelatin−ChS gels (ϕ = 8 mm, W_0 = weight) in a solution of 5 mg/mL ^{14}C-labeled lysozyme in PBS or in PBS containing 0.5 M sodium chloride, respectively. The gels were incubated during 3 days at 37 °C, which was sufficient to reach equilibrium. Subsequently, the gels were gently blotted with a tissue to remove adherent solution, hydrolyzed in LumaSolve (2 mL) at 50 °C for 5 h, and cooled to room temperature. OptiPhase "HiSafe" (18 mL) was added to the hydrolyzed gel and to the loading solution remaining after uptake, and the radioactivity was measured with a scintillation counter (Winspectral 1414, Wallac Turku, Finland). The partition coefficients were calculated from the ratio of the lysozyme concentration in the gel and in solution, which were determined based from the degree of swelling of the gel and the specific activity of radiolabeled lysozyme (6×10^{-4} μCi/mg). The experiments were performed in triplicate.

Fluorescent Labeling of Lysozyme. Lysozyme was fluorescently labeled by partial modification of the carboxylic acid residues with 5-(aminoacetamide) fluorescein. Lysozyme (22 mg) was dissolved in cold sodium hydrogen phosphate buffer (0.02 M; pH 4.5; 3 mL). EDC and NHS were added in 10× excess with respect to the number of carboxylic residues to be derivatized (2.2 and 0.5 mg, respectively) to this solution to preactivate the carboxylic acid groups in lysozyme. After 5 min, this solution was added dropwise to a solution of 5-(aminoacetamide) fluorescein in 25 mL borate buffer (sodium borohydrate; 0.2 M; pH 8.6) and the reaction mixture stirred for 2 h. Then sodium chloride solution (2 M; 25 mL) was added to the solution to remove urea derivatives, which may be complexated with lysozyme. The mixture was first concentrated to a volume of 15 mL, using an ultrafiltration cell (Amicon; low protein binding membrane: YM-10, molecular weight cutoff 10 kD), and then dialyzed against PBS until no significant fluorescence was detected in the dialysate (measured with a Perkin-Elmer LS-3 fluorescence spectrometer). The solution of fluorescent lysozyme (FITC−lysozyme) in PBS was frozen in liquid nitrogen and stored at −30 °C.

Fluorescence Recovery after Photobleaching (FRAP) Measurements. To screen the mobility of fluorescently labeled lysozyme in the gelatin−ChS hydrogels, FRAP measurements were performed on a confocal scanning laser microscope (Bio-Rad MRC1024; UK) using a 40-fold oil immersion lens.[22−24] After incubation in a solution of FITC−lysozyme in PBS (400 μL; 0.7 mg/mL lysozyme) for 24 h at 4 °C, the gelatin−ChS gels were placed in a small reservoir made on a microscopic slide. A cover slide was put on top, and the reservoir was sealed with blank nail polish to avoid dehydration of the gels by evaporation.

The FRAP experiments were performed according to the following procedure. First, the fluorescence in the hydrogels was measured by scanning a part of the $x-y$ plane at 50 μm below the surface of the hydrogels using an attenuated laser beam. Second, a 19 μm line segment from the $x-y$ plane was selected to be bleached. Photobleaching of this segment occurred at the time the laser beam scanned over this segment accompanied by a temporarily strong increase in the intensity of the laser beam. To measure the fluorescence recovery in this bleached stripe, a strongly attenuated laser beam scanned along this line segment. Because of the slow diffusion of lysozyme in the gels, intermittent scanning was used. Small time intervals (0.1 s) were considered just after bleaching while larger time intervals (20 s) were used at the end of the fluorescence recovery. At each scan session the line segment where bleaching occurred was scanned 200 times. The 200 data points per scan session were averaged and used as single time points in the fluorescence recovery curve. The normalized fluorescence recovery curves were obtained by dividing the fluorescence intensities during fluorescence recovery by the fluorescence intensity before photobleaching of the $x-y$ plane in which the bleached line segment was located.

Langmuir Adsorption of Lysozyme to Gelatin−ChS Gels. The adsorption measurements were carried out according to the procedure used in the determination of the partition coefficient. Dry gelatin−ChS gels were incubated in solutions containing ^{14}C-labeled lysozyme in PBS (concentrations of 0.5, 1.0, 2.0, 5.0, and 10 mg/mL) at 37 °C for 3 days. The lysozyme concentration in the loading solution and the amount of

Table 1. Characteristics of Chemically Cross-Linked Gelatin−Chondroitin Sulfate Hydrogels

sample	wt % chondroitin sulfate	cross-link density[a]	S
ChS 0	0	0.35 ± 0.03	2.37 ± 0.05
ChS 5	5	0.37 ± 0.04	2.63 ± 0.06
ChS 10	10	0.42 ± 0.03	3.03 ± 0.10
ChS 20	20	0.44 ± 0.02	4.25 ± 0.05

[a] The cross-link density is defined as the ratio between the number of free amine groups in EDC/NHS cross-linked gels and the number of free amine groups in the corresponding gels before chemical cross-linking.

lysozyme taken up by the gels were calculated using the specific activity of each of the radiolabeled lysozyme solutions. The experiments were performed in triplicate. At each lysozyme concentration, the amount of bound lysozyme was determined by correcting for the free lysozyme in the aqueous phase of the gels.

Turbidity Measurements. A mixture of 1 mL of a solution of lysozyme (10 mg/mL) and 1 mL of a solution of chondroitin sulfate (1 mg/mL), both in 20 mM phosphate buffer (pH 7), was titrated with 0.5 M NaCl in steps of 100 μL to study the effect of salt concentration on the complexation of lysozyme with chondroitin sulfate. After each addition, 100 μL was removed from the solution to measure the turbidity in a SLT 340 ATTC plate reader.

Results and Discussion

Preparation and Characterization of Gelatin−ChS Hydrogels. Gels were prepared of gelatin solutions containing 0−20 wt % of chondroitin sulfate. The presence of chondroitin sulfate in these gels did not oppose the gel formation. Chondroitin sulfate is a polysaccharide consisting of D-glucuronic acid and *N*-acetyl galactosamine, having between 0.2 and 2.3 sulfate residues per disaccharide.[12] Conductometric titration was used to determine the number of carboxylic and sulfate residues in the batch of chondroitin sulfate used in this study. It was shown that this type of chondroitin sulfate contained about one carboxylate residue and one sulfate residue per disaccharide unit, assuming that these disaccharides consist of D-glucuronic acid and *N*-acetyl galactosamine.

The gelatin−ChS gels were cross-linked with a water-soluble carbodiimide (EDC). For all gels the molar ratio of EDC to carboxylic acid residues was 0.8. As EDC cross-linking results in the formation of amide bonds between carboxylic acid and amine residues, chondroitin sulfate will become cross-linked into the gelatin gel. To make sure that no chondroitin sulfate had leaked out of the gels during cross-linking, the gels were analyzed before and after cross-linking using elemental analysis. This revealed that chondroitin sulfate was quantitatively present in the gels after cross-linking (results not shown).

The degree of swelling, the free amine group content, and the rheological behavior of the gels were studied after cross-linking. Compared to non chemically cross-linked gelatin−ChS gels, as expected, chemical cross-linking decreases the free amine group content of the gels (Table 1). Increasing the amount of chondroitin sulfate resulted in a less efficient chemical cross-linking, as shown by the lower reduction in free amine groups, and the increase in swelling (Table 1). It was expected that the higher number of carboxylic acid groups of gelatin−ChS hydrogels, and the proportional increase in EDC concentration, would result in a higher consumption of free amine residues in gelatin−ChS gels compared to carbodiimide cross-linked gelatin gels without ChS. The decrease in consumed amine groups with increasing chondroitin sulfate content may be explained by two reasons. First, the number of free amine groups is lower, upon increasing the chondroitin sulfate content of the gels as the gelatin content decreases. Second, gelatin−ChS hydrogels swell stronger in aqueous solution resulting in a larger distance between the network chains, which may prevent effective proceeding of the cross-linking reaction. Both these effects limit the availability of amine residues for the coupling reaction, which explains a decrease in cross-link density with increasing chondroitin sulfate content of the gels.

Table 2. Rheological Characterization of Physically Cross-Linked and Chemically Cross-Linked Gelatin−Chondroitin Sulfate Gels, Depending on the Composition of the Gelatin−ChS Gels

sample	G_e (kPa) physically cross-linked gel	cross-link density[a]	G_e (kPa) chemically cross-linked gel
ChS 0	17 ± 1	0.40 ± 0.05	180 ± 30
ChS 5	13 ± 1	0.41 ± 0.02	130 ± 20
ChS 10	11 ± 1	0.44 ± 0.02	190 ± 30
ChS 20	12 ± 1	0.39 ± 0.02	260 ± 40

[a] The cross-link density is defined as the ratio between the number of free amine groups in EDC/NHS cross-linked gels, and the number of free amine groups in the corresponding gels before cross-linking.

As shown in Table 1, the chemically cross-linked gelatin−ChS hydrogels show a higher degree of swelling with increasing chondroitin sulfate content. A polymer network swells or shrinks to obtain a condition of minimal internal energy. The total Gibbs free energy accompanying the swelling process (ΔG_{tot}) depends on contributions from the elastic free energy (ΔG_{el}), the free energy of mixing solvent molecules with network chains (ΔG_{mix}), and the free energy associated with ionic attraction and repulsion for the chains in a polyelectrolyte cross-linked gel (ΔG_{ion})

$$\Delta G_{tot} = \Delta G_{el} + \Delta G_{mix} + \Delta G_{ion} \qquad (3)$$

At equilibrium ΔG_{tot} is zero, which means that for swollen hydrogels, a decrease in free energy, due to mixing of ions and solvent molecules with the network chains, is balanced by an increase in free energy due to stretching of the network chains. It can be expected that, upon increasing the number of anionic residues in the hydrogel, the driving force for swelling increases.

Table 2 shows the outcome of the rheological measurements on the hydrogels. For non chemically cross-linked gelatin−ChS gels, G_e decreases with incorporation of chondroitin sulfate. This is related to the lower gelatin content, which results in a lower number of physical cross-links in the hydrogels. Upon chemical cross-linking, as expected, the elastic modulus clearly increases. However, for the chemically cross-linked gelatin−ChS gels the influence of the amount of chondroitin sulfate on G_e remains unclear. While G_e of gels containing 0, 5, and 10 wt % chondroitin sulfate does not differ significantly, G_e of the gels with 20 wt % ChS shows a marked increase. This is hard to explain by an increase in the cross-link density as, compared to the other gels, the 20 wt % gels do not show a higher consumption of free amine groups, which does not suggest a higher number of cross-links in these gels.

A hypothesis which may explain the trends in G_e values of the chemically cross-linked gelatin−ChS gels

Table 3. Lysozyme Uptake (μg/mg Dry Sample) by Cross-Linked Gelatin−Chondroitin Sulfate Gels after Loading from a Solution of 5 mg/mL Lysozyme in 66 mM Phosphate Buffer Solution during 3 Days at 20 °C (mean ± sd, n = 3)

sample	lysozyme uptake (μg/mg)
ChS 0	80 ± 2
ChS 5	165 ± 5
ChS 10	268 ± 8
ChS 20	680 ± 47

deals with the deformation behavior of the polymer network. In an affine network, it is assumed that the junctions of the network do not fluctuate and that they transform affinely (linearly) with the macroscopic deformation.[25,26] For an affine network it is assumed that only the network chains contribute to the decrease of entropy of the network (which gives rise to the elastic force) upon deformation. For a phantom network, it is assumed that the junctions do fluctuate over time.[27] For the affine network model, the equilibrium shear modulus of the network (G_e) is given by[25]

$$G_e = \nu RT \tag{4}$$

In this equation, ν is the number of moles of elastic chains per unit volume. For the phantom network, due to fluctuations of the junctions, G_e is lower than that of the corresponding affine network:[27]

$$G_e = (\nu - \mu)RT \tag{5}$$

In eq 5, μ is the number of moles of elastic cross-links per unit volume of the network. Real networks are expected to show characteristics which lie somewhere between the properties of both affine and phantom models. This phenomenon was treated by Flory and Erman in the constrained junction model which allows for this intermediate behavior:[26]

$$G_e = (\nu - h\mu)RT \tag{6}$$

In eq 6, h ranges between 0 (for an affine network) and 1 (for a phantom network).

It is generally considered that stretching of chains in polymer networks by swelling favors an affine-like behavior of the junctions as they will be less able to fluctuate.[28,29] From these observations and on the basis of eq 6, it may be hypothesized that, although the number of cross-links in the 20 wt % gelatin−ChS gels is lower compared to the other gels, the stronger swelling of these gels, which favors a more affine-like behavior, may increase G_e.

Lysozyme Uptake and Release from Gelatin−ChS Hydrogels. Before release, the chemically cross-linked gelatin−chondroitin hydrogels were loaded with lysozyme by incubation in a solution of 5 mg/mL lysozyme in 66 mM phosphate buffer (Table 3). A buffer with low ionic strength was used as, at low ionic strength, the interaction between lysozyme and the negatively charged hydrogel may be favored. The lysozyme loading capacity of the combined hydrogels increased more than 8-fold by increasing the chondroitin sulfate content of the gels from 0 to 20 wt %. For the gel containing 20 wt % of chondroitin sulfate, about 600 μg lysozyme is bound to 200 μg of chondroitin sulfate. Considering that lysozyme has a net positive charge of +8 and a total molecular weight of 14.4 kD and chondroitin sulfate contains about 1 sulfate residue per 500 g/mol, equimolar amounts of cationic and sulfate residues were present in the gels.

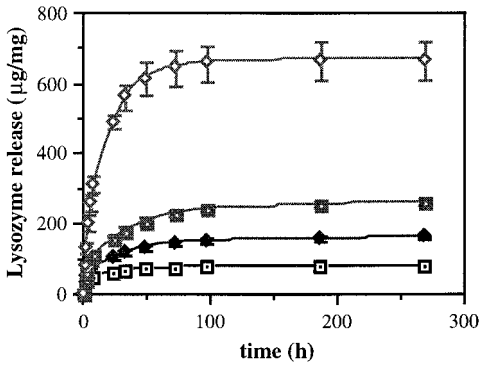

Figure 2. Lysozyme release (μg/mg) profiles in PBS at 37 °C for gelatin−ChS hydrogels, containing different amounts of chondroitin sulfate, cross-linked with an $EDC/COOH_{gelatin+ChS}$ molar ratio of 0.8 in time (h): 0 wt % ChS (solid box in solid box); 5 wt % ChS (◆); 10 wt % ChS (◘), 20 wt % ChS (tilted-square solid with circle) (mean ± sd, n = 3).

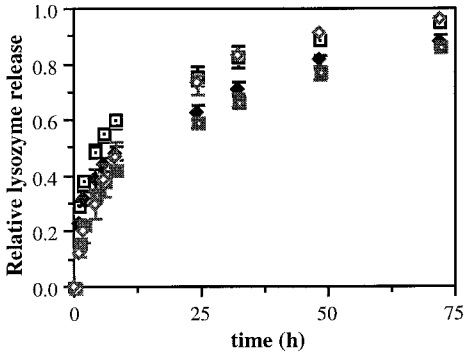

Figure 3. Relative lysozyme release profiles in PBS at 37 °C for gelatin−ChS hydrogels, containing different amounts of chondroitin sulfate, cross-linked with an $EDC/COOH_{gelatin+ChS}$ molar ratio of 0.8 in time (h): 0 wt % ChS (solid box in solid box); 5 wt % ChS (◆); 10 wt % ChS (◘), 20 wt % ChS (tilted-square solid with circle) (mean ± sd, n = 3).

Subsequently, the release of lysozyme in PBS (13 mM phosphate buffer, 140 mM sodium chloride) at 37 °C was measured, with a regular refreshment of the medium. PBS was selected as release medium, because a good agreement was found between in vitro lysozyme release from cross-linked gelatin gels using PBS and in vivo lysozyme release from these gels in subcutaneous tissue.[7]

Figure 2 shows that after 1 week, the lysozyme release from all the gels was complete, while no lysozyme remained in the gels. To consider the release rate, the amount of lysozyme released, relative to the initial amount of lysozyme which was initially present in the gels, was calculated (Figure 3). Compared to plain gelatin (ChS 0), the release is obviously prolonged in the gels containing 5 and 10 wt % chondroitin sulfate, while for the gel containing 20 wt % chondroitin sulfate the lysozyme release is only slowed during the initial release.

From Table 3 and Figure 3, it was concluded that the lysozyme uptake increases significantly with increasing chondroitin sulfate content, while the release was only substantially retarded for ChS 5 and ChS 10. The following paragraphs study the mechanisms governing these release profiles.

Lysozyme Diffusion in Cross-Linked Gelatin−ChS Hydrogels. The permeation of lysozyme in PBS

Table 4. Calculation of the Permeability, and the Effective Diffusion Coefficient of Lysozyme for Cross-Linked Gelatin−ChS Gels (PBS; 37 °C), from the Permeation Experiments (eq 3), the Thickness of the Gels (*h*), and the Partition Coefficient (K_d), Where the Initial Lysozyme Concentration in the Donor Chamber Was 4 mg/mL

sample	slope (h^{-1})[a]	*h* (mm)	*P* (cm^2/s)[b]	K_d	D_{eff} (cm^2/s)[c]
ChS 0	5.561×10^{-4}	0.546 ± 0.001	1.54×10^{-8}	7.2	2.13×10^{-9}
ChS 5	8.140×10^{-4}	0.636 ± 0.008	2.63×10^{-8}	12.5	2.10×10^{-9}
ChS 10	2.115×10^{-3}	0.700 ± 0.010	7.52×10^{-8}	19.7	3.82×10^{-9}
ChS 20	1.565×10^{-2}	0.669 ± 0.009	54.22×10^{-8}	35.6	15.23×10^{-9}

[a] These values are the slopes of the curves in Figure 4. [b] *P* is calculated from the slope of the curves in Figure 4, a surface area of the gels (*A*) of 2.27 cm^2, and a compartment volume (*V*) of 8.3 mL. [c] D_{eff} is calculated from *P*, dividing *P* by K_d.

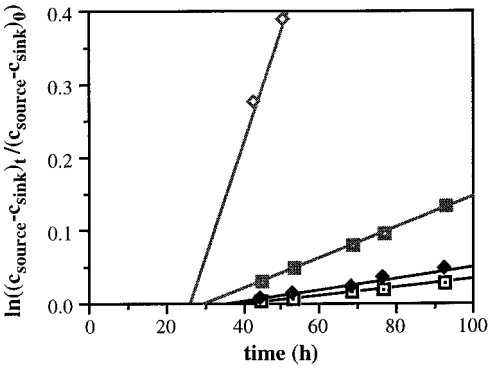

Figure 4. Permeation of lysozyme in PBS at 37 °C through gelatin−ChS hydrogels, containing different amounts of chondroitin sulfate, cross-linked with an $EDC/COOH_{gelatin+ChS}$ molar ratio of 0.8 in time (h): 0 wt % ChS (solid box in solid box); 5 wt % ChS (◆); 10 wt % ChS (◘), 20 wt % ChS (tilted-square solid with circle).

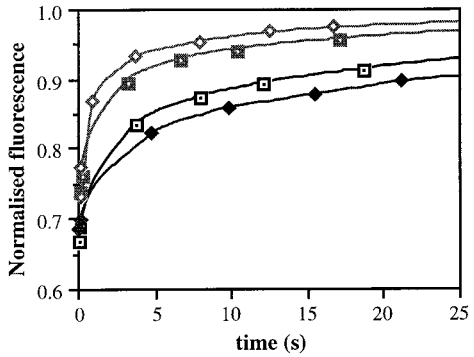

Figure 5. Normalized fluorescence after photobleaching as a function of time for fluorescently labeled lysozyme (PBS; 20 °C) within gelatin−ChS hydrogels, containing different amounts of chondroitin sulfate, cross-linked with an $EDC/COOH_{gelatin+ChS}$ molar ratio of 0.8; 0 wt % ChS (solid box in solid box); 5 wt % ChS (◆); 10 wt % ChS (◘), 20 wt % ChS (tilted-square solid with circle).

through the cross-linked gelatin−chondroitin sulfate hydrogels was measured using a two-compartment diffusion cell at 37 °C. These experimental conditions were equal to the conditions used for the release experiments. Figure 4 shows the results of the permeation experiments. The lysozyme permeation shows a lag time. With increasing chondroitin sulfate content of the hydrogels, the lag time shortens while the permeation through the membranes occurs faster.

On the basis of the permeation experiments, the permeability of the gelatin−chondroitin sulfate hydrogels for lysozyme in PBS was calculated (Table 4). With increasing chondroitin sulfate content, the permeability of the hydrogels increases. Table 4 also shows the experimental values for K_d. It clearly shows that, upon increasing the chondroitin sulfate content, K_d of lysozyme with the gelatin−ChS gels increases. As the following equation holds for *P*, the increase in K_d partially explains the increased lysozyme permeability of the gelatin−ChS gels.

$$P = K_d D_{eff} \tag{7}$$

Table 4 shows the effective diffusion coefficient D_{eff} as calculated from eq 7. For the gels containing 10 and 20 wt % ChS, the diffusion coefficient seems to be significantly higher than the diffusion coefficient in the gels containing 0 and 5 wt % ChS. This may be attributed to the higher degree of swelling with increasing chondroitin sulfate content.

Fluorescence recovery after photobleaching (FRAP) was used to confirm that the lysozyme diffusion occurs faster in gelatin−ChS gels with a high amount of chondroitin sulfate. Figure 5 shows the normalized fluorescence recovery profiles. All the normalized fluorescence profiles showed a total fluorescence recovery after several minutes (data not shown), which indicated that the interactions between lysozyme and the gelatin−ChS gels were reversible. Figure 5 further shows that the recovery of the fluorescence in the bleached areas, which is due to diffusion of fluorescently labeled lysozyme, indeed occurred significantly faster in the 10 and 20 wt % gelatin−ChS gels than in the 0 and 5 wt % gelatin−ChS gels. Both the increase in K_d and the increase in *D* explain the increase in permeability of the gels with increasing amount of chondroitin sulfate.

Mathematical Evaluation of the Release Profiles. The results of the permeation measurements were used to evaluate the experimental release profiles (Figures 3 and 4) using a mathematical release model. Equation 8 shows Fick's second law of diffusion:

$$\partial C_A/\partial t = D\partial^2 C_A/\partial x^2 \tag{8}$$

In this equation C_A is the free drug concentration (mg/mL), *x* is the spatial position in the matrix, *t* is time, and *D* is the drug diffusivity (cm^2/s). This equation was solved assuming two-sided release from a slab, using a time interval of 100 h, and integrating over the thickness, *h*, of the gels.

Figure 6 shows the experimental release profiles and the release profiles, calculated using the effective diffusion coefficients as derived from the permeation experiments (Table 4). The calculated release profiles for the gelatin−ChS gels containing 0 and 5 wt % chondroitin sulfate show an underestimation of the experimental release. This underestimation is explained by the fact that the actual release surface is 14−17% larger than was assumed in these model calculations, as the surface of the rim of the gels was not accounted for. The mathematical release profile for the gel containing 20 wt % of chondroitin sulfate predicts a faster release than was experimentally observed. This difference is caused by the fact that the diffusion experiments

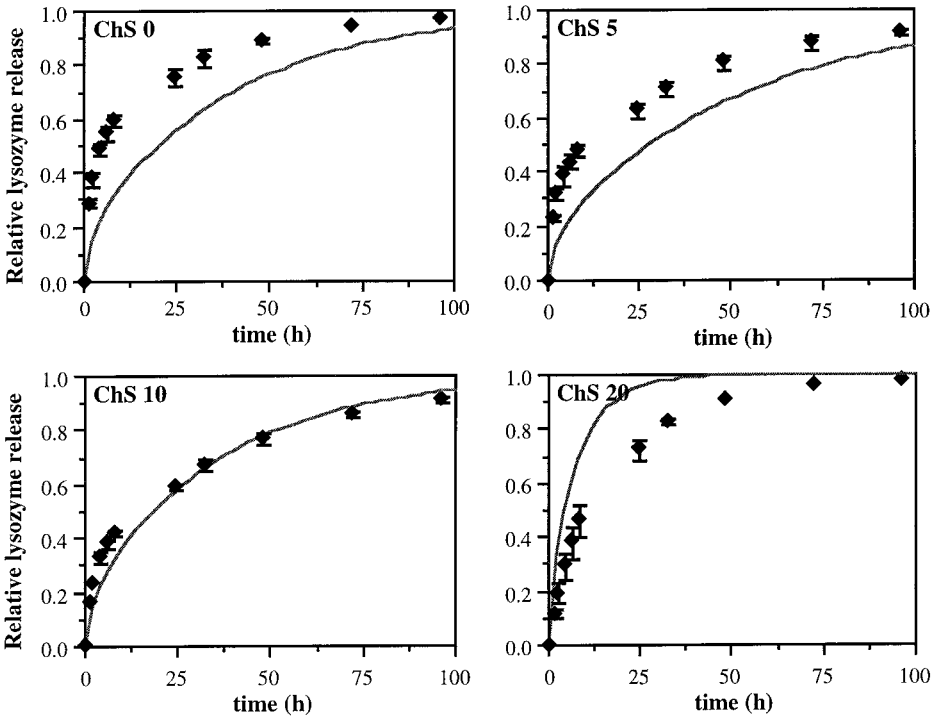

Figure 6. Comparison between the calculated (■), and experimental (◆) lysozyme release profiles for gelatin−ChS hydrogels, containing 0, 5, 10, and 20 wt % of chondroitin sulfate, and cross-linked with an EDC/$COOH_{gelatin+ChS}$ molar ratio of 0.8.

were performed at a relatively high, and constant donor concentration (4 mg/mL), while during release, the free lysozyme concentration in the gel continuously decreases. The influence of interaction with the gel on the effective diffusion coefficient will be more pronounced with decreasing lysozyme concentration. For the gels containing 10 wt % chondroitin sulfate, the prediction of the release profiles is rather good, as the deviation in surface area and the higher interaction at lower lysozyme concentrations have opposite effects on the effective diffusion coefficient.

Mechanism of Lysozyme Release: Diffusion vs Interaction. From the release experiments, it was observed that the release rate of lysozyme shows a minimal value for the gels containing 5 wt % of chondroitin sulfate. To gain more insight in the mechanisms governing the release profiles, both the contribution of the free diffusion of lysozyme in the gels and the interaction of lysozyme with the gelatin−ChS matrix were evaluated.

As turbidity measurements showed that at a sodium chloride concentration higher than 0.25 M no complex formation occurred between lysozyme and chondroitin sulfate (Figure 7), the permeation experiments were carried out in PBS containing 0.5 M sodium chloride. Compared to the degree of swelling of the gels in PBS, the swelling in 0.5 M salt increased at most 5%. Furthermore, the increase in salt concentration hardly affects the hydrodynamic radius of lysozyme.[30]

Figure 8 shows the results of the permeation experiments using lysozyme in PBS containing 0.5 M sodium chloride. Compared to the permeation experiments with PBS, these profiles show a shorter lag time, and a lower slope. The permeability of the gelatin−ChS gels was calculated from the slope of the permeation profiles (Table 5), and increased with increasing chondroitin sulfate content. Determination of the partition coefficient (K_d) of lysozyme in gelatin−ChS gels in PBS containing 0.5 M NaCl revealed that K_d equaled 2 for

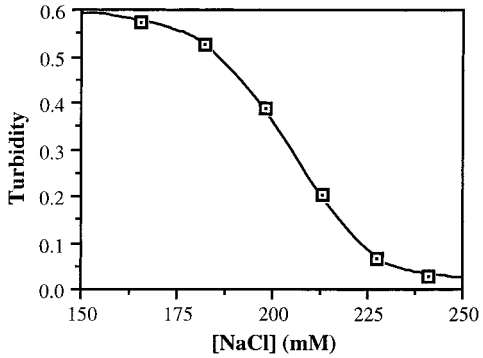

Figure 7. Turbidity of a solution containing lysozyme (5 mg/mL) and chondroitin sulfate (0.5 mg/mL) in 20 mM phosphate buffer (pH 7.0), during titration with sodium chloride.

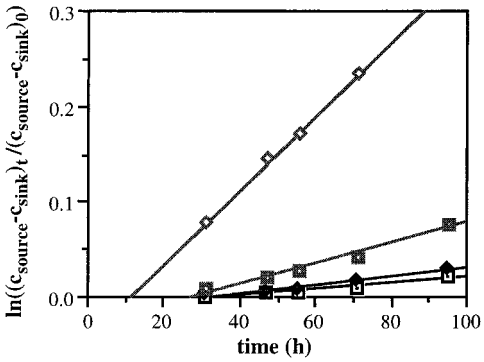

Figure 8. Permeation of lysozyme in PBS containing 0.5 M sodium chloride at 37 °C through gelatin−ChS hydrogels, containing different amounts of chondroitin sulfate, cross-linked with an EDC/$COOH_{gelatin+ChS}$ molar ratio of 0.8 in time (h): 0 wt % ChS (solid box in solid box); 5 wt % ChS (◆); 10 wt % ChS (◘), 20 wt % ChS (tilted-square solid with circle).

all the gels. Compared to the partition coefficient of lysozyme in PBS (Table 4), K_d is significantly reduced, but even in 0.5 M sodium chloride, there is some

Table 5. Calculation of the Permeability (*P*) and the Free Diffusion Coefficient (*D*) of Lysozyme for Cross-Linked Gelatin−ChS Gels in PBS Containing 0.5 M NaCl at 37 °C, from the Permeation Experiments (eq 2), the Thickness of the Gels (*h*), and the Partition Coefficient (K_d), Where the Initial Lysozyme Concentration in the Donor Chamber Was 4 mg/mL

sample	slope (h^{-1})[a]	*h* (mm)	*P* (cm^2/s)[b]	K_d	*D* (cm^2/s)
ChS 0	3.226×10^{-4}	0.547 ± 0.003	7.35×10^{-9}	2.1	3.50×10^{-9}
ChS 5	4.370×10^{-4}	0.641 ± 0.002	11.66×10^{-9}	1.9	6.14×10^{-9}
ChS 10	1.082×10^{-3}	0.699 ± 0.001	31.48×10^{-9}	2.1	14.99×10^{-9}
ChS 20	3.861×10^{-3}	0.670 ± 0.011	107.66×10^{-9}	2.3	46.81×10^{-9}

[a] These values are the slopes of the curves in Figure 8. [b] *P* is calculated from the slope of the curves in Figure 8, a surface area of the gels (*A*) of 2.27 cm^2, and a compartment volume (*V*) of 8.3 mL. [c] *D* is calculated from *P*, dividing *P* by K_d.

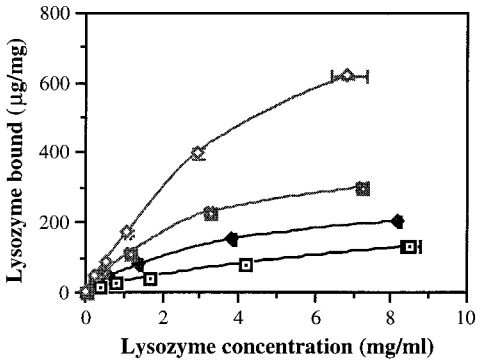

Figure 9. Lysozyme bound to gelatin−ChS hydrogels (μg/mg), containing different amounts of chondroitin sulfate, cross-linked with an $EDC/COOH_{gelatin+ChS}$ molar ratio of 0.8, as a function of lysozyme concentration in PBS (mg/mL) at 37 °C: 0 wt % ChS (solid box in solid box); 5 wt % ChS (◆); 10 wt % ChS (◘), 20 wt % ChS (tilted-square solid with circle) (mean ± sd, $n = 3$).

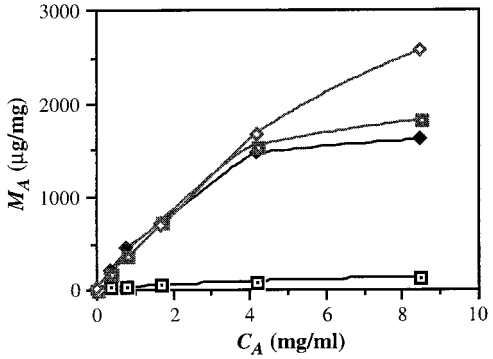

Figure 10. Lysozyme bound (μg/mg) to gelatin (solid box in solid box) and chondroitin sulfate, calculated from the binding curves of cross-linked gelatin−ChS hydrogels, containing 5 wt % (◆), 10 wt % (◘), and 20 wt % (tilted-square solid with circle) chondroitin sulfate, as a function of lysozyme concentration in PBS solution (mg/mL) at 37 °C.

interaction between lysozyme and gelatin−ChS gels, probably due to hydrophobic interactions. The free diffusion coefficient of lysozyme increases with increasing chondroitin sulfate content of the gels, due to the increased swelling with higher chondroitin sulfate content (Table 5).

To study the interaction between lysozyme and gelatin−ChS gels, Langmuir isotherms were determined at 37 °C in PBS. The Langmuir adsorption model is valid for reversible, monolayer adsorption of molecules onto a homogeneous surface. The experimental conditions were equal to the conditions used in the release measurements. The amount of lysozyme bound to the matrix was calculated and represented as a function of the lysozyme concentration in solution (Figure 9). An increase in chondroitin sulfate content of the gelatin matrix from 0 to 20 wt % resulted in an 5-fold increase in bound lysozyme, at equal lysozyme concentration in solution.

The contribution of gelatin and chondroitin sulfate, respectively, to the binding of lysozyme to the gelatin−ChS hydrogels was investigated. The relation between the lysozyme concentration in solution and the amount of lysozyme bound to either gelatin or chondroitin sulfate was described by a Langmuir isotherm:

$$M_A = \frac{K_g M_{Ag}{}^{max} C_A}{1 + K_g C_A} + \frac{K_{cs} M_{Acs}{}^{max} C_A}{1 + K_{cs} C_A} \qquad (9)$$

In this equation M_A is the total amount of lysozyme bound to the polymer matrix (mg/mg), C_A is the free protein concentration in the gel (mg/mL), $M_A{}^{max}$ is the maximum mass of lysozyme bound per mass of gelatin (g) or chondroitin sulfate (cs) and *K* ($(mg/mL)^{-1}$) is the binding constant (Langmuir adsorption constant).

Figure 10 shows the binding curves of lysozyme to either gelatin or chondroitin sulfate. The lysozyme adsorption to chondroitin sulfate was calculated by subtracting the binding curve for gelatin from the binding curves for the gelatin−ChS gels, containing 5, 10, and 20 wt % of chondroitin sulfate, respectively. The (three) calculated binding curves of lysozyme to chondroitin sulfate are equal at low lysozyme concentrations. At higher lysozyme concentrations, the curves deviate from each other: the maximum lysozyme loading capacity is higher when the chondroitin sulfate content of the gel increases. Thus, the presence of gelatin in the gel competes with the adsorption of lysozyme to chondroitin sulfate.

The values of $M_A{}^{max}$ for gelatin and chondroitin sulfate were derived by extrapolation of the curves (Figure 10), and *K* was subsequently calculated from the initial slope of the curves (at $KC_A \ll 1$, this slope is equal to $KM_A{}^{max}$). For gelatin K_g was 0.144 mL/mg and $M_{Ag}{}^{max}$ was 0.229 mg/mg, while for chondroitin sulfate K_{cs} was 0.149 mL/mg and $M_{Acs}{}^{max}$ was 5.17 mg/mg, calculated for ChS-20, as for this gel the highest maximum loading capacity was observed. The calculated values for *K* are in the same range as the strength of polylysine interaction with collagen, which had been derivatized with anionic residues.[31]

Upon incorporation of 20 wt % of chondroitin sulfate into gelatin, the number of carboxylic acid residues is somewhat increased (from about 100 per 10^5 g/mol for gelatin to 120 per 10^5 g/mol for ChS 20), as gelatin is replaced by chondroitin sulfate. The number of sulfate residues increases considerably (from zero to about 40 per 10^5 g/mol). It is remarkable that an increase in sulfate groups does significantly increase the maximum binding capacity but hardly affects the binding strength. Therefore, the lysozyme adsorption to gelatin should be considered carefully.

Conclusions

The incorporation of chondroitin sulfate in the gelatin gels did not inhibit the formation of physical cross-links. Furthermore, the gelatin−ChS gels could be characterized using similar methods as for plain gelatin gels. The combination of chondroitin sulfate with cross-linked gelatin gels led to a significant increase in the lysozyme loading capacity of the gel and a prolonged lysozyme release time.

Permeation measurements in two-compartment diffusion cells using PBS showed that the effective diffusion coefficient of lysozyme in the gelatin−ChS hydrogels had the lowest value for the gels containing 0 and 5 wt % chondroitin sulfate. Diffusion of lysozyme within the gelatin−ChS gels was also studied by fluorescence recovery after photobleaching. These results confirmed that the diffusion of lysozyme is significantly slower in the 0 and 5 wt % gelatin−ChS gels than in the 10 and 20 wt % gelatin−ChS gels.

To gain more insight in the mechanisms governing the release, the diffusion of lysozyme in the gels and the interaction of lysozyme with the gels were evaluated. The free diffusion of lysozyme in the gelatin−ChS gels was measured by performing the permeation measurements in a PBS solution containing 0.5 M sodium chloride to minimize interactions between lysozyme and the gels. The diffusion coefficients increased with increasing chondroitin sulfate content of the gels, which is related to the degree of swelling. The Langmuir isotherm of the lysozyme adsorption to gelatin−ChS gels was determined and showed that the interaction increased with increasing chondroitin sulfate content. Separation of the Langmuir isotherms for lysozyme binding to gelatin and to chondroitin sulfate revealed that the lysozyme binding capacity of chondroitin sulfate is significantly higher than that of gelatin, while no difference in binding strength is observed. The incorporation of chondroitin sulfate increases both the interaction and the free diffusion, which have net opposite effects on the effective diffusion.

It has been shown that, by incorporation of chondroitin sulfate into gelatin gels, the uptake of cationic proteins is significantly increased and their controlled release retarded, which is expected to improve their suitability for controlled release applications and to increase their range of possible other applications. Summarizing, the properties of natural polymers may make them favorable for biomedical applications but also contributes to the complexity of interactions, which makes it difficult to predict their properties in controlled release applications.

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MA9917702