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Release of model proteins and basic fibroblast growth factor from in situ forming degradable dextran hydrogels

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Abstract

Our previous studies showed that degradable dextran hydrogels are rapidly formed in situ upon mixing aqueous solutions of dextran vinyl sulfone (dex-VS) conjugates and tetrafunctional mercapto poly(ethylene glycol) (PEG-4-SH) by Michael addition. The hydrogel degradation time and storage modulus could be controlled by the degree of vinyl sulfone substitution (DS) and dextran molecular weight. The degradation time could further be adjusted by the spacer between the thioether and the ester bond of the dex-VS conjugates (ethyl vs. propyl, denoted as dex-Et-VS and dex-Pr-VS, respectively). In this paper, the release of three model proteins, i.e. immunoglobulin G (d_h=10.7 nm, IgG), bovine serum albumin (BSA, d_h=7.2 nm) and lysozyme (d_h=4.1 nm), as well as basic fibroblast growth factor (bFGF) from these in situ forming dextran hydrogels is studied. Proteins could be easily loaded into the hydrogels by mixing protein containing solutions of dex-VS and PEG-4-SH. The release of IgG from dex-Et-VS hydrogels followed biphasic release kinetics, with a slow, close to first order release for the first 9 days followed by an accelerated release and over 80% of IgG was released in 12 to 25 days. Interestingly, the release of IgG from dex-Pr-VS hydrogels followed close to zero order kinetics, wherein approximately 95% was released in 21 days. The release of BSA from dex-Pr-VS hydrogels followed biphasic kinetics, with almost first order release followed by close to zero order release. Approximately 75% of the entrapped BSA could be released from dex-Pr-VS hydrogels in 16 days. Dex-Pr-VS hydrogels released 40% of lysozyme in 14 days, with full preservation of the enzymatic activity of the released lysozyme, as determined by bacteria lysis experiments. The release of basic fibroblast growth factor (bFGF) from dex-Pr-VS hydrogels showed first order kinetics, with quantitative release in 28 days. These results show that the in situ forming degradable dextran hydrogels can be used for the controlled release of proteins.

Keywords: Hydrogel; In situ forming; Michael addition; Protein delivery; Growth factor

1. Introduction

Nowadays, many pharmaceutically active proteins can be produced on a large scale by biotechnology. Unfortunately, parental administration of proteins is hampered by rapid clearance, whereas oral administration is generally not successful due to degradation in the gastro-intestinal tract. Also, the intestinal epithelium forms a major barrier towards protein absorption. Moreover, since the delivery is not localized, relatively high doses are needed to have a therapeutic effect. The administration of proteins may be greatly improved by the use of controlled delivery systems that allow for sustained and localized release, thereby decreasing the number of administrations, and enhancing the therapeutic efficacy. It is important that delivery systems allow modulation of the release of entrapped proteins and that the structural integrity of the proteins is preserved after being released.

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Hydrogels have been widely applied for controlled drug delivery, in particular for protein delivery. Many hydrogels have been shown to be compatible with proteins and living tissue. Hydrogels may be formed in situ upon mixing aqueous polymer solutions, thus allowing for the preparation of complex shapes and minimally invasive surgery. Moreover, bioactive compounds can be easily dissolved or suspended in the polymer solutions prior to gelation. Hydrogels are formed by either physical or chemical crosslinking of hydrophilic polymers [1]. Physical crosslinking generally occurs under mild conditions, thus allowing for the entrapment of labile compounds, such as proteins. However, physically crosslinked hydrogels are generally mechanically weak and may be disrupted by changes in the environment (e.g. pH, temperature and ionic strength). Chemically crosslinked hydrogels are generally stronger and more stable. Chemically crosslinked hydrogels have been prepared in situ by several methods. Photopolymerization of poly(ethylene glycol) (PEG) (meth)acrylates has been mostly used [2–4]. More recently, in situ forming chemically crosslinked hydrogels have been prepared by reaction of aldehyde-modified dextran with adipic acid dihydrazide compounds [5], reaction of amine groups of gelatin with aldehyde-modified alginate in the presence of small amounts of sodium tetraborate [6] and reaction of poly(*N*-isopropylacrylamide (PNIPAAm) derivatives modified with activated ester groups and amine terminated poly (amino acid)s [7]. Chemically crosslinked hydrogels have also been prepared in situ by Michael type addition of vinyl sulfones or acrylates with thiols [8–18]. Michael type addition is selective towards thiols under physiological conditions, thus preventing reaction with e.g. lysine residues of proteins present in the body and does not produce any side products. Hubbell et al. prepared hydrogels by Michael addition between multifunctional PEG acrylate and PEG dithiol or dithioerythritol (DTT). These hydrogels released albumin *in vitro* with zero order kinetics over a period of 4 days [19].

The *in vitro* release of human growth hormone (hGH, precipitated with Zn^{2+} to prevent reaction with the gel precursors) followed zero order kinetics, wherein hGH was quantitatively released for up to a few months with preservation of the protein integrity [11]. Cell-adhesive, enzyme degradable hydrogels with covalently incorporated VEGF were prepared by first performing a Michael addition between RGDC peptides and VEGF-cysteine derivatives and excess of tetrafunctional PEG vinyl sulfone and subsequent gel formation by Michael addition with a matrix metalloproteinase (MMP) degradable bis-cysteine peptide [10]. When implanted subcutaneously in rats, these hydrogels were completely remodeled into native, vascularized tissue. Prestwich et al. prepared hydrogels by Michael addition between thiol-modified hyaluronic acid or chondroitin sulfate containing a small amount of thiol modified heparin, and PEG diacrylate [20–22]. These hydrogels were degraded by the enzyme hyaluronase and were shown to quantitatively release basic fibroblast growth factor (bFGF) *in vitro* for 28 days, wherein bFGF retained 55% of its original biological activity [14]. Moreover, bFGF loaded hydrogels dramatically increased neovascularization, when they were implanted into subcutaneous pockets in Balb/c mice.

We have previously reported on rapidly in situ forming degradable hydrogels by Michael addition between dextran vinyl sulfones and multifunctional mercapto PEG. These hydrogels showed good mechanical properties and their degradation time (ranging from 3 to 21 days) could be well-controlled by the degree of substitution (DS), polymer concentration, dextran molecular weight and length of the spacer between the ester bonds and the thioether. In this paper, the release of model proteins with different sizes, immunoglobulin G (IgG), bovine serum albumin (BSA) and lysozyme, as well as the release of basic fibroblast growth factor (bFGF) from these hydrogels is studied.

2. Materials and methods

2.1. Materials

Tetrafunctional mercapto poly(ethylene glycol) with M_n=2,100 (denoted as PEG-4-SH) and dextran vinyl sulfone conjugates (denoted as dex-VS) with different degree of substitution (DS, defined as the number of vinyl sulfone groups per 100 anhydroglucosidic rings, AHG, of dextran) and dextran molecular weights of 14 K and 31 K (denoted as dex14K and dex31K, respectively) were synthesized as reported previously [17]. Lysozyme (from hen egg white, MW=14 kDa) and dextran sulfate sodium salt (from *Leuconostoc* ssp.) were purchased from Fluka. Bovine serum albumin (BSA, fraction V, MW=67 kDa), bovine immunoglobulin G (IgG, MW=150 kDa), ethylenediaminetetraacetic acid (EDTA), heparin sodium salt (from porcine intestinal mucosa) and human recombinant basic fibroblast growth factor (bFGF, expressed in E. Coli, MW=17.2 kDa) were obtained from Sigma.

2.2. Model protein release

For the release of the model proteins, IgG, BSA and lysozyme, hydrogels were prepared in HEPES buffered saline (pH 7, 100 mM, adjusted to 300 mOsm with NaCl) by mixing solutions of dex-VS (250 μl) and PEG-4-SH (250 μl) both containing 1 wt % of protein with a double barreled syringe to a final total polymer concentration of 15 w/v%. The protein containing polymer solutions were prepared by adding 20 μl of concentrated protein solution to 230 μl of both the dex-VS and PEG-4-SH solutions just before preparation of the hydrogel, to minimize possible reaction of the protein with the gel precursors. The molar ratio of vinyl sulfone to thiol groups was kept at 1.1, since thiol groups may form some disulfide bonds due to exposure to air, thus lowering the effective concentration of free thiol groups. The hydrogels were formed in cylindrically shaped vials with a flat bottom with a diameter of 8.5 mm and a height of 8.8 mm, only exposing the upper surface of the hydrogel (device described in Ref. [23]). Subsequently, 3 ml of HEPES buffer was applied on top of the gels and the gels were gently shaken at 37 °C. Each hydrogel formulation was prepared in duplicate or triplicate. Samples of 500 μl were taken at regular time intervals (the first day after 30 min, 1 h, 2 h, 4 h, 8 h and 24 h, and subsequently after one or three days) and replaced by an equal volume of fresh buffer.

Samples were analyzed using reversed phase high-performance liquid chromatography (RP-HPLC), as described below.

2.3. bFGF release

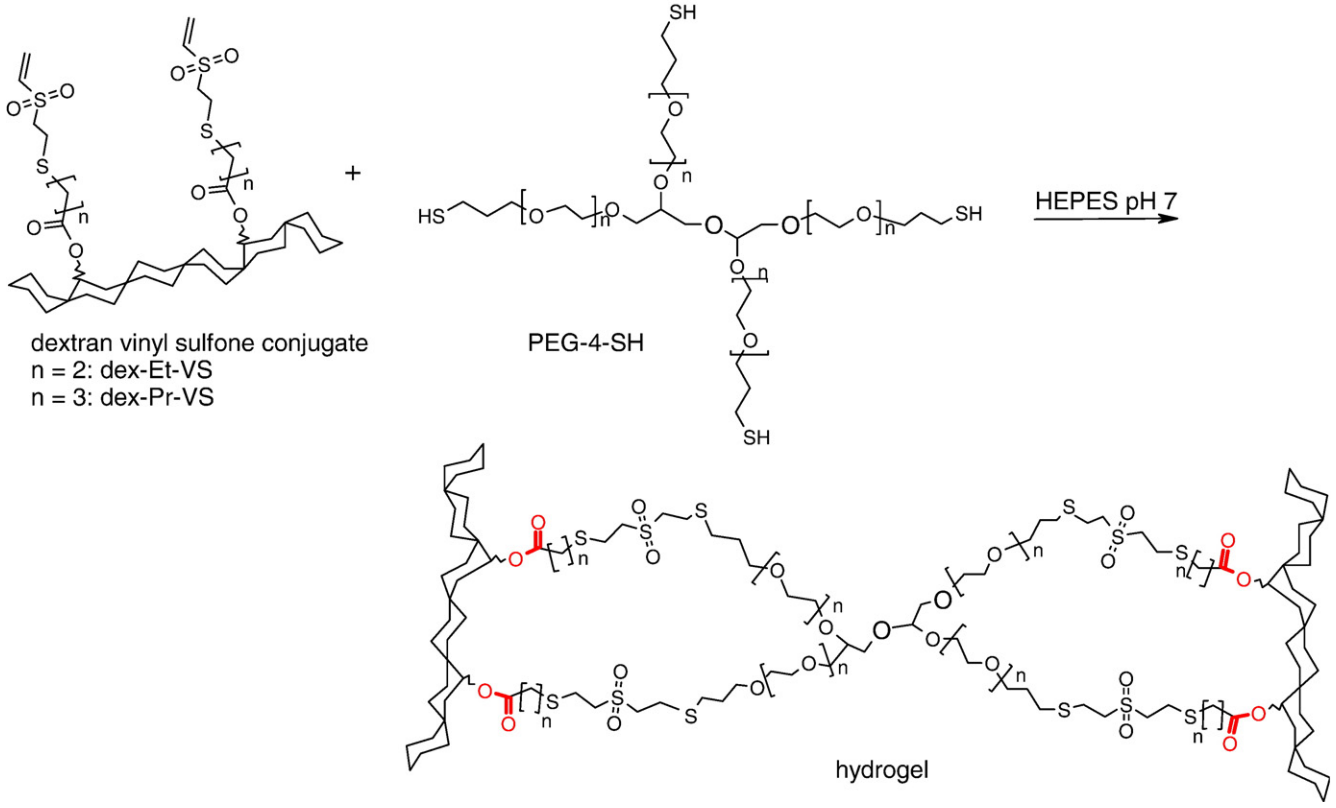
For the release of bFGF, hydrogels were prepared in PBS (pH 7.4, 10.5 mM, 300 mOsm) containing 0.1 wt.% of BSA, 5 wt.% of sucrose, 0.01 wt.% of EDTA and 0.15 w/v% of dextran sulfate ($M_r \approx 500{,}000$) and as release buffer PBS supplemented with 10 μg/ml heparin, 1 wt.% of BSA and 1 mM of EDTA was used to retain bFGF activity and to prevent surface adsorption [14]. Hydrogels with a total polymer concentration of 15 w/v% were prepared by mixing solutions of dex-VS (125 μl) and PEG-4-SH (125 μl) with a double barreled syringe. Each gel contained 250 pg of bFGF. Release medium (3 ml of PBS release buffer) was added to the gels and they were gently shaken at 37 °C. The release experiment was performed in quadruplicate. Samples of 500 μl were taken at 12 and 36 h, and at 3, 6, 10, 14, 21 and 28 days, and replaced by an equal volume of fresh buffer. The samples were stored at −30 °C until measurement. Samples were analyzed using a bFGF ELISA kit as described below.

2.4. Analysis of lysozyme release samples by RP-HPLC

A 600E Multisolvent Delivery System with a 717plus Autosampler, two concentration detectors: a 2487 Dual Wavelength Absorbance and a 2475 Multi λ Fluorescence Detector, where used (Waters Associates Inc.). An analytical column (Prosphere, 5 μm C18 300 A) was used for separation. Standard protein solutions (concentration range 0.75–37.5 μg/ml) were prepared to generate calibration curves. All samples were centrifuged for 1 min (10,000 g) and 10 or 50 μl of the supernatant was injected onto the column. A linear gradient was run from 70% A (water/acetonitrile/TFAA 95/5/0.1 w/w) and 30% B (water/acetonitrile/TFAA 95/5/0.1 w/w) to 45% B in 15 min. The flow rate was set to 1.0 ml/min and the column oven was set at 4 °C. The fluorescent emission at 300 nm (excitation wavelength of 295 nm) was measured. Instruments where controlled by and peak areas were determined with Empower 2 Chromatography Data Software (Waters Associates Inc.).

2.5. Analysis of BSA and IgG release samples by RP-HPLC

A 600E Multisolvent Delivery System with a 717plus Autosampler, two concentration detectors: a 2487 Dual Wavelength Absorbance and a 2475 Multi λ Fluorescence Detector, where used (Waters Associates Inc.). An analytical column (Tosoh Biosciences TSKgelG3000SWXL, 7.6x300 mm, 5 μm) was used for separation. Standard protein solutions (concentration range 0.1–50 μg/ml) were prepared to generate calibration curves. All samples were centrifuged for 1 min (10,000 g) and 50 μl of the supernatant was injected onto the column. PBS (pH 7.4, 10.5 mM, 300 mOsm) was used as the mobile phase. The flow rate was set to 1.0 ml/min and the column oven was set at 4 °C. The fluorescent emission at 300 nm (excitation wavelength of 295 nm) was measured. Instruments were controlled by and peak areas were determined with Empower 2 Chromatography Data Software (Waters Associates Inc.).



Scheme 1. In situ hydrogel formation by Michael addition of dextran vinyl sulfone conjugates (dex-Et-VS or dex-Pr-VS) with tetrafunctional mercapto PEG (PEG-4-SH) [17].

Table 1
Characteristics of dex-VS conjugates used in this study

Dex-VS conjugates	MW dextran [a]	DS [b]	Type of spacer [c]	Degradation time (days)[d]
dex14K-Et-VS DS 13	14 K	13	Ethyl	9
dex14K-Et-VS DS 22		22	Ethyl	21
dex14K-Pr-VS DS 10		10	Propyl	17
dex31K-Et-VS DS 9	31 K	9	Ethyl	14
dex31K-Et-VS DS 13		13	ethyl	16
dex31K-Pr-VS DS 8		8	propyl	21

[a] Determined by GPC.
[b] Degree of substitution (DS), defined as the number of vinyl sulfone groups per 100 anhydroglucosidic rings, AHG, of dextran, determined by ^{1}H NMR.
[c] Spacer between the thioether and the ester bond. The degradation time of hydrogels with 15 w/v% total polymer concentration was determined by swelling tests [17]. The degradation time is defined as the time required to completely dissolve at least one of the three hydrogels used for testing one type of hydrogel.

2.6. Determination of the enzymatic activity of lysozyme

The enzymatic activity of released lysozyme was determined for a few samples. The assay is based on the lysis of the outer cell membrane of *Micrococcus lysodeikticus*, resulting in solubilization of the affected bacteria and consequent decrease of light scattering [24]. The release samples were diluted to a concentration of 50–100 μg/ml and 10 μl of the sample was added to 1.3 ml of the bacteria suspension (0.2 mg/ml, HEPES buffered saline, pH 7.0). The decrease in turbidity was measured at 450 nm and the percent enzymatic activity was determined by comparing the activity of the sample with that of a freshly prepared reference lysozyme solution (0.1 mg/ml).

2.7. Analysis of bFGF release samples

bFGF release samples were analyzed using a bFGF ELISA kit. 100 μl of sample was added to each well of a 96-wells plate coated with human bFGF specific antibody. After incubation for 2.5 h at room temperature the solutions were discarded and the wells were washed 4 times. Subsequently, 100 μl of biotinylated antibody solution was added to each well and incubated for 1 h at room temperature. The solutions were discarded after incubation and the wells were washed 4 times. Next, to each well 100 μl of horseradish peroxidase–streptavidin solution was added and incubated for 45 min at room temperature. Subsequently, the solutions were discarded and each well was washed 5 times. In the next step 100 μl of 3,3′-5,5′-tetramethylbenzidine (TMB) solution was added to each well and after 30 min incubation at room temperature in the dark, 50 μl of 2 M sulfuric acid was added. The absorbance was read at 450 nm with a plate reader (SLT 340 ATC).

3. Results and discussion

Our previous studies showed that hydrogels are rapidly formed by mixing aqueous solutions of dextran vinyl sulfone conjugates (dex-VS) and tetrafunctional mercapto poly(ethylene glycol) (PEG-4-SH) [17]. The crosslinks are formed by Michael addition between the vinyl sulfone and thiol groups (Scheme 1). The dex-VS conjugates used in this study are listed in Table 1. Different degrees of substitution (DS, defined as the number of vinyl sulfone groups per 100 anhydroglucosidic rings, AHG, of dextran) ranging from 8 to 22, and dextran molecular weights of 14 K or 31 K (denoted as dex14K and dex31K, respectively) were used. The hydrogel degradation time increases with increasing DS and dextran molecular weight, as was determined previously by swelling tests (Fig. 1) [17]. The degradation time is defined as the time required to completely dissolve at least one of the three hydrogels used for testing one type of hydrogel. Furthermore, two types of dex-VS, having either an ethyl or a propyl spacer between the thioether and the ester bond (denoted as dex-Et-VS and dex-Pr-VS, respectively, Scheme 1), were used. Dex-Pr-VS hydrogels have prolonged degradation times but otherwise similar properties compared to the corresponding dex-Et-VS hydrogels [17].

3.1. Release of model proteins in vitro

The release of three model proteins with different hydrodynamic diameters (d_h), i.e. immunoglobulin G (IgG, d_h=10.7 nm [25]), bovine serum albumin (BSA, d_h=7.2 nm [26]) and lysozyme (d_h=4.1 nm [26]) from dex-VS hydrogels was studied, using a polymer concentration of 15 w/v% (defined as the total dry weight of both PEG and dextran per volume of buffer) in HEPES buffered saline (pH 7.0, 100 mM, 300 mOsm) at 37 °C.

Fig. 2 shows the cumulative release of IgG. All dex-Et-VS hydrogels showed a biphasic release profile of IgG (Fig. 2a), with slow release for the first 9 days, which was close to first order kinetics (as the release scaled almost linearly with the square root of time, insert Fig. 2a), followed by an accelerated release. Although the initial first order release suggests a typical diffusion controlled release mechanism [27], the actual situation is more complex, since the hydrogels swell in time due to degradation of the hydrogel network. The acceleration in the release is attributed to progressive degradation of the hydrogel network and indicates that the initial hydrogel mesh size is at

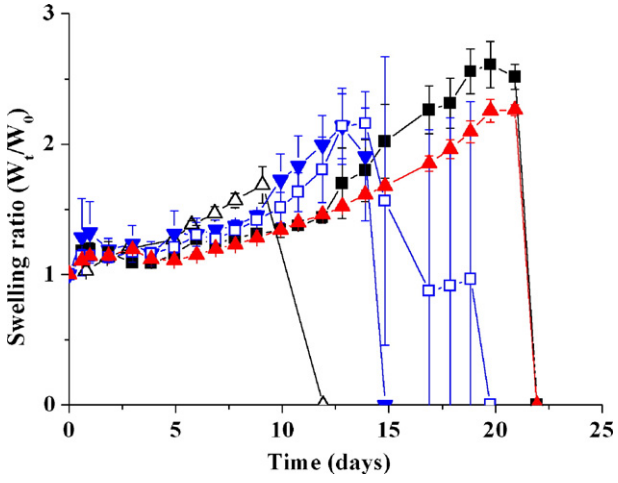


Fig. 1. Swelling ratio (W_t/W_0) profiles of dex-VS hydrogels in HEPES buffered saline at pH 7.0 and 37 °C (average±S.D., n=3) [17]. (a) Dex14K-Et-VS DS 13 (△), dex14K-Et DS 22 (■), dex31K-Et-VS DS 9 (▼), dex31K-Et-VS DS 13 (□) and dex31K-Pr-VS DS 8 (▲). The data have been published previously in Ref. [17].

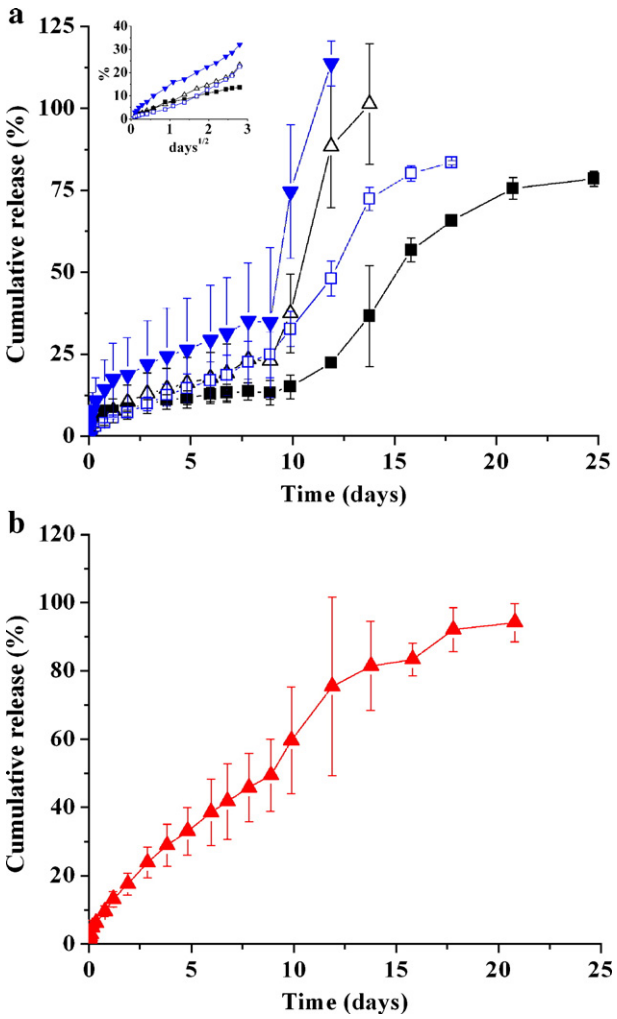


Fig. 2. Cumulative release profiles of IgG from dex-VS hydrogels in HEPES buffered saline (pH 7.0) at 37 °C (average±S.D.). (a) Dex14K-Et-VS DS 13 (△, $n=3$), dex14K-Et DS 22 (■;, $n=2$), dex31K-Et-VS DS 9 (▼, $n=2$) and dex31K-Et-VS DS 13 (□, $n=2$); insert shows the cumulative release as a function of the square root of time, for the sake of clarity error bars are not shown. (b) Dex31K-Pr-VS DS 8 ($n=3$).

least equal to or smaller than the hydrodynamic diameter of IgG. After sufficient degradation, the hydrogel mesh size becomes large enough to allow easy diffusion of IgG from the hydrogels. Importantly, no burst-release was observed.

Dex14K-Et-VS DS 13 and dex31K-Et-VS DS 9 hydrogels quantitatively released IgG in 12 to 14 days, while dex14K-Et-VS DS 22 and dex31K-Et-VS DS 13 released up to approximately 80% of IgG in 18 and 25 days, respectively. Previous studies showed that the hydrogel degradation time as well as the storage modulus increase with increasing DS and dextran molecular weight [17]. A higher storage modulus indicates a higher crosslinking density and thus a smaller hydrogel mesh size. It should be noted that the release of IgG continued after the degradation time as determined by swelling tests. This is most likely due to some damaging of the hydrogel during the swelling tests when removing the buffer prior to weighing, thereby underestimating the degradation time. The faster release of IgG from dex14K-Et-VS DS 13 hydrogels as compared to dex14K-Et-VS DS 22 and dex31K-Et-VS DS 13 hydrogels, having either a higher DS or a higher dextran molecular weight, respectively, may be due to a faster degradation as well as a larger initial hydrogel mesh size.

The incomplete retrieval of IgG for dex14K-Et-VS DS 22 and dex31K-Et-VS DS 13 hydrogels is due to partial precipitation of the protein, as the release media contained a small amount of precipitate at the end of the release experiment (after 30 days). HPLC chromatograms showed an extra peak at shorter retention time, which corresponds to a compound with a higher molecular weight than IgG, indicating the presence of water-soluble IgG aggregates. Possibly, denaturation, aggregation and subsequent precipitation may have occurred in time, due to reaction of the reactive groups of IgG (ε-amines of the lysine amino acids or the terminal α-amines or disulfide bonds) with the reactive groups of the gel precursors (vinyl sulfone and thiol groups). Hubbell et al. found that 80% of the added VEGF was covalently linked to the hydrogel matrices, which were prepared by first mixing aqueous solutions of VEGF and a large stoichiometric excess of tetrafunctional PEG vinyl sulfone for 60 min and subsequent addition of an aqueous solution of bis-cysteine MMP peptide to induce gelation at pH 8.0 and 37 °C [10]. They suggested that the incorporation of VEGF was due to reaction of ε-amines or the α-amine of VEGF with vinyl sulfone groups of the tetrafunctional PEG vinyl sulfone. Kim et al. showed that all amine groups of polyethylenimine (PEI) reacted with bifunctional vinyl sulfone-PEG-(*N*-hydroxysuccinimidyl) (VS-PEG-NHS) within 2 h at pH 7.0 and room temperature [28]. The difference in percentage of retrieved IgG from the different dex-VS hydrogels may be due to the differences in hydrogel degradation time, as most IgG is retrieved from the most rapidly degrading hydrogels (dex14K-Et-VS DS 13 and dex31K-Et-VS DS 8).

Release of IgG from dex31K-Pr-VS DS 8 hydrogels followed almost zero order release kinetics, wherein approximately 95%

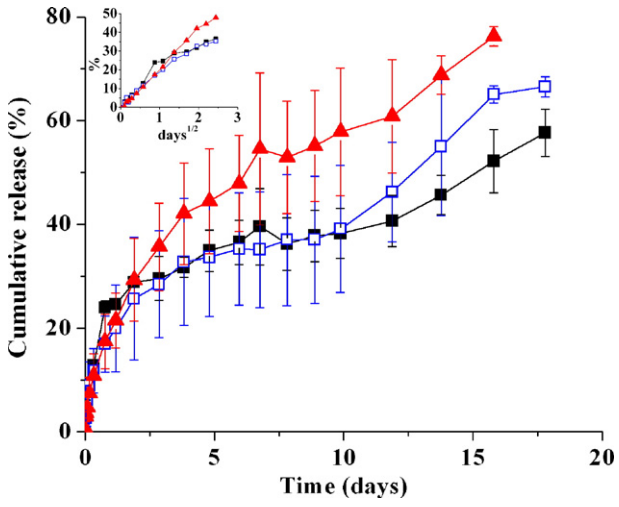


Fig. 3. Cumulative release profiles of BSA from dex-VS hydrogels in HEPES buffered saline (pH 7.0) at 37 °C (average±S.D., $n=3$). Dex14K-Et-VS DS 22 (■), dex31K-Et-VS DS 13 (□) and dex31K-Pr-VS DS 8 (▲). Insert shows the cumulative release as a function of the square root of time, for the sake of clarity error bars are not shown.

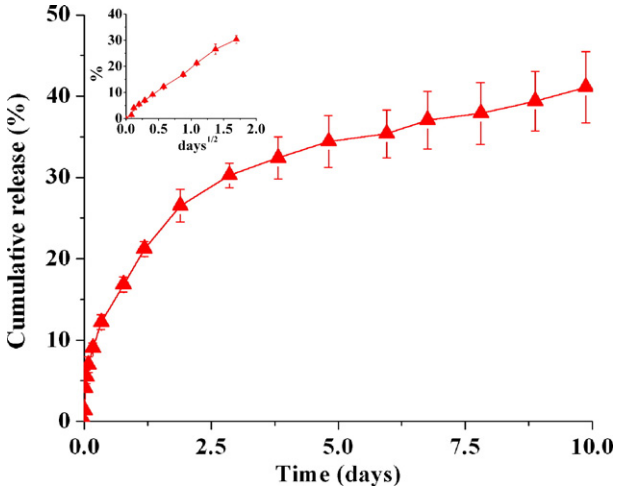


Fig. 4. Cumulative release profiles of lysozyme from dex31K-Pr-VS DS 8 hydrogels in HEPES buffered saline (pH 7.0) at 37 °C (average±S.D., $n=3$). Insert shows the cumulative release as a function of the square root of time.

of IgG was released in 21 days (Fig. 2b). The difference in release profiles of dex31K-Pr-VS DS 8 hydrogels and similar dex31K-Et-VS DS 9 hydrogels is attributed to slower hydrogel degradation (Table 1). The difference in release profile of dex31K-Pr-VS DS 8 hydrogels compared to dex14K-Et-VS DS 22 hydrogels with similar degradation time, is attributed to the larger initial pore size of the dex31K-Pr-VS DS 8 hydrogels due to the lower DS. Furthermore, swelling tests showed that the swelling ratio of dex31K-Pr-VS hydrogels increased smoothly in time compared to dex14K-Et-VS DS 22 hydrogels, which showed accelerated swelling after 12 days (Fig. 1). The smooth increase in swelling indicates a gradual degradation of the dex31K-Pr-VS hydrogels. Therefore, the close to zero order release of IgG from dex31K-Pr-VS DS 8 hydrogels is most likely due to a combination of degradation/swelling and diffusion.

The release of BSA from dex-Et-VS and dex-Pr-VS hydrogels was biphasic, with close to first order kinetics (insert Fig. 3), followed by a (slightly) accelerated release after 9 days (Fig. 3). The release did not show a burst-effect. BSA was released much faster compared to IgG from these hydrogels, due to its smaller size. For instance, approximately 10% of IgG vs. approximately 40% of BSA was released from dex14K-Et-VS DS 22 hydrogels after 10 days. This is most likely because the initial hydrogel mesh size is much smaller than the hydrodynamic diameter of IgG, but equal to or somewhat larger than the hydrodynamic diameter of BSA. While BSA may diffuse out of the hydrogel without significant hydrogel degradation, further degradation is needed to facilitate the diffusion of IgG. The observed acceleration in release rate for both proteins indicates that the hydrogel mesh size becomes larger than the size of the proteins after approximately 9 days. The acceleration is less pronounced for BSA compared to IgG, since the cumulative release of BSA was higher compared to IgG before the acceleration. Dex14K-Et-VS DS 22 and dex31K-Et-VS DS 13 hydrogels released approximately 55 and 65% of BSA in 18 days, respectively, while dex31K-Pr-VS DS 8 hydrogels released approximately 75% of BSA in 16 days. The incomplete retrieval of BSA is due to precipitation of the protein, as the release media contained small precipitates after the release experiment (after 30 days). The precipitation may be due to denaturation caused by reaction with the gel precursors, similar to IgG.

Dex31K-Pr-VS DS 8 hydrogels released approximately 40% of lysozyme in approximately 10 days, wherein the release followed first order kinetics for the first 3 days, followed by an almost constant release (Fig. 4). Dex14K-Et-VS DS 22 and dex31K-Et-VS DS 13 hydrogels released 10 and 20% of lysozyme in 10 days, respectively (results not shown). The cumulative release of lysozyme was low compared to IgG and BSA. This is due to precipitation of lysozyme, as the release media contained quite some precipitates after the release experiment (after 30 days). Similar to BSA en IgG the precipitation may be due to denaturation of lysozyme caused by reaction with the gel precursors. The underlying reasons for the increased precipitation of lysozyme as compared to IgG and BSA need to be studied further. Lysozyme released after 1 week from dex31K-Et-VS DS 13 and dex31K-Pr-VS DS 8 hydrogels did retain all of its enzymatic activity after 7 days, as was shown by bacteria lysis experiments (results not shown). Lysozyme released after 1 week from dex14K-Et-VS DS 22 retained 50% of its enzymatic activity. The lower activity of released lysozyme and the lower cumulative release for dex14K-Et-VS DS 22 hydrogels (having the highest concentration of reactive groups prior to gelation) compared to the dex31K-Et-VS DS 13 and dex31K-Pr-VS DS 8 hydrogels agrees well with the view that lysozyme may react with the gel precursors.

Proteins can be protected from reaction with the gel precursors by appropriate formulations. For instance, Hubbell et al. incorporated human growth hormone (hGH) by first precipitation of dissolved hGH with Zn^{2+} ions and subsequent hydrogel formation by Michael reaction upon addition of aqueous solutions of eight-arm PEG acrylate and dithiothreitol

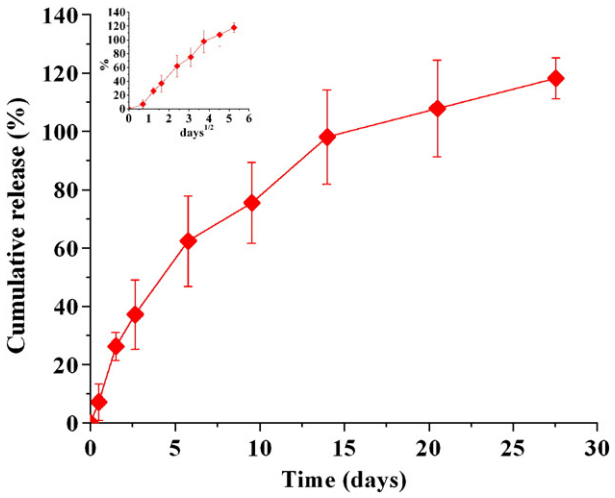


Fig. 5. Cumulative release profile of bFGF from dex14K-Pr-VS DS 10 hydrogels in PBS (pH 7.4) at 37 °C (average±S.D., $n=4$). Insert shows the cumulative release as a function of the square root of time.

(DTT) [11]. SDS-PAGE experiments showed that hGH retained its integrity after the Michael reaction.

3.2. Release of bFGF

The release of basic fibroblast growth factor (bFGF) from dex14K-Pr-VS DS 10 hydrogels was studied, using supplemented PBS release buffer (pH 7.4) at 37 °C (Fig. 5). In the hydrogels, 0.01 wt% EDTA was used to prevent trace metal-induced disulfide exchange between the hydrogels and bFGF, 5 wt% sucrose was added to maintain the bFGF conformation, 0.1 wt.% BSA was added to prevent bFGF adsorption to plastic surfaces and 0.15 w/v% dextran sulfate was added to maintain bFGF activity [14,28]. In the release medium 10 μg/ml heparin was added to maintain and sequester bFGF activity after it is released, 1 wt.% BSA was added to prevent adsorption and 1 mM EDTA was added as a chelator. The concentration of bFGF in the release samples was determined by a bFGF ELISA assay. The bFGF release scaled almost linearly with the square root of time, according to first order release (insert Fig. 5), wherein bFGF was quantitatively released in 28 days. Importantly, the release of bFGF from these hydrogels did not show a burst-effect. In general, bFGF was released much faster than BSA and IgG, due to a smaller hydrodynamic diameter of bFGF compared to BSA and IgG. The hydrodynamic diameter of bFGF is similar to that of lysozyme (d_h=4.1 nm), since both have similar molecular weights (17.2 and 14 kDa respectively). The potential of released bFGF to stimulate tissue regeneration will be subject of future study. Prestwich. et al showed that bFGF releasing hydrogels formed by Michael addition induced neovascularization when implanted subcutaneously in Balb/c mice [14]. Hubbell et al. showed that hydrogels containing covalently incorporated vascular endothelial growth factor (VEGF) formed by Michael type addition completely remodeled into native, vascularized tissue when implanted subcutaneously in rats [10].

4. Conclusions

Dex-VS hydrogels were rapidly formed in situ upon mixing aqueous solutions of dex-VS and tetrafunctional mercapto poly (ethylene glycol) (PEG-4-SH). Dex-VS conjugates with either an ethyl or a propyl spacer between the thioether and the ester bonds (dex-Et-VS and dex-Pr-VS, respectively) were used. Proteins could be easily loaded into the hydrogels by mixing protein containing aqueous polymer solutions. The release profile of the relatively large protein immunoglobulin G (IgG, d_h=10.7 nm) was dependent on the type of hydrogel. Biphasic kinetics were observed for dex-Et-VS hydrogels and almost zero order kinetics for the slower degrading dex-Pr-VS hydrogels, wherein dex-Pr-VS hydrogels released approximately 95% of IgG in 21 days. The release rate of IgG from dex-Et-VS hydrogels was dependent on the DS and dextran molecular weight and over 80% of IgG was released in 12 to 25 days. Lysozyme was released up to 40% from dex-Pr-VS hydrogels in 14 days, with full preservation of its enzymatic activity. Basic fibroblast growth factor (bFGF) was released quantitatively from dex-Pr-VS hydrogels with close to first order kinetics in 28 days. Importantly, the release of proteins from these dextran hydrogels did not show a burst-effect. In some cases, the proteins were not completely released and recovered, due to precipitation of the protein. Methods to prevent precipitation of proteins have been presented in literature. In conclusion, these rapidly in situ forming, degradable dex-VS hydrogels are very promising for the controlled release of proteins.

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