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Research Paper

The effect of network charge on the immobilization and release of proteins from chemically crosslinked dextran hydrogels

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ABSTRACT

Size is the main protein characteristic that determines its release from non-degrading neutral hydrogels. The effect of network charge on the release of proteins has not been studied systematically so far. Therefore, we investigated the release of proteins from charged hydrogels that were obtained by co-polymerization of methacrylated dextran (Dex-MA) with either methacrylic acid (MA) or 2-*N*,*N*-dimethylaminoethyl methacrylate (DMAEMA). These hydrogels are stable under physiological conditions. The effect of incorporation of the charged monomers on hydrogel charge, equilibrium swelling, and release of model proteins was assessed at both low (10 mM HEPES) and physiological ionic strength (HEPES buffered saline, HBS). Model proteins were chosen on the basis of their charge at physiological pH; bovine serum albumin (BSA, negatively charged), myoglobin (neutral), and cytochrome C (positively charged). Interestingly, as opposed to myoglobin, both charged proteins were fully immobilized in the networks with opposite charge by electrostatic interaction at low ionic strength. On the other hand, at physiological ionic strength, the percentage of immobilized protein depended on the charge density of the hydrogel. For all proteins, the diffusion coefficient of the mobile fractions was not affected by opposite network charge. However, the release rate of BSA from similarly (negatively) charged networks significantly increased when a relatively high amount of charged monomers was incorporated. We conclude that incorporation of charge in a hydrogel network is suited as a tool for the immobilization of proteins and triggered release by increasing ionic strength.

1. Introduction

An increasing part of the pharmaceutical compounds introduced to the market consists of peptides and proteins [1]. At present, the therapeutic value of these compounds is compromised by the challenges related to their macromolecular complexity, such as immunogenicity [2–7], bioequivalency [8–11], and formulation [1,12,13]. Currently, most proteins are administered parenterally. In order to reduce the administration frequency and burden, research is dedicated to the development of sustained release formulations. Because of their tissue compatibility and possibilities to manipulate protein release, hydrogels are considered as attractive dosage forms for therapeutic proteins. The effect of parameters such as network density, solid content, and polymer degradation kinetics on protein release kinetics are well documented and commonly used to tailor the release of different proteins [14–21]. Provided that the protein is smaller than the mesh size of the network, protein diffusion in hydrogels is described by the free volume theory. According to this theory, diffusion of solutes occurs in the free space between solvent molecules. Inside the hydrogel, the presence of polymer chains decreases the amount of free space. Therefore, the diffusion rate is determined by hydration of the hydrogel and the size of the solute [22,23]. Size is therefore the most important protein characteristic determining its release from a non-degrading hydrogel with pores bigger than the diameter of the loaded protein. Other protein characteristics such as isoelectric point (*pI*) or hydrophobicity have hardly been exploited to tailor release. It can be expected that modulation of charge interaction between the hydrogel network and protein molecules has an effect on the protein release. In fact, it has been reported that the release rate of myoglobin from polyaspartic acid hydrogels at pH 5 increased with increasing ionic strength [24]. Furthermore, the release of VEGF from ionically crosslinked alginate beads can be modified by the use of different cations [25,26]. However, to our knowledge, the possibility to tailor the release rate of proteins by adaptation of the absolute charge and the charge density of the hydrogel network at physiological pH has never been explored. Therefore, in this paper, we studied the effect of network charge sign and density on the release of proteins from hydrogels.

Proteins can either carry a net positive or negative charge, or be neutral at a certain pH, depending on *pI*. Model proteins were

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Abbreviations

Dex-MA	methacrylated dextran	BSA	bovine serum albumin
MA	methacrylic acid	*pI*	isoelectric point
DMAEMA	2-*N*,*N*-dimethylaminoethyl methacrylate	TEMED	*N*,*N*,*N*′,*N*′-tetramethylethylenediamine
HEMA	hydroxyethyl methacrylate	APS	ammonium peroxodisulfate
HBS	HEPES buffered saline	DS	degree of substitution

chosen on the basis of their charge at physiological pH; bovine serum albumin (BSA, negatively charged), myoglobin (neutral), and cytochrome *C* (positively charged). To ensure that protein release from the hydrogels is governed by diffusion only, methacrylated dextran (Dex-MA) hydrogels were used as model gels. Dex-MA hydrogels are non-degradable at physiological conditions [21], which implies that hydrogel degradation does not play a role in protein release. Charge was introduced to the hydrogels by copolymerization of Dex-MA with various amounts of either methacrylic acid (MA) or 2-*N*,*N*-dimethylaminoethyl methacrylate (DMAEMA). To ensure equal network density, the total amount of additional monomer was kept constant by supplementing with neutral 2-hydroxyethyl methacrylate (HEMA).

2. Materials and methods

2.1. Materials

Dextran T40 (from *Leuconostoc spp.*), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), ammonium peroxodisulfate (APS), and 2-hydroxethyl methacrylate (HEMA) were obtained from Fluka (Buchs, Switzerland). Methacrylic acid (MA), 2-*N*,*N*-dimethylaminoethyl methacrylate (DMAEMA), bovine serum albumin (BSA, purity > 96%), cytochrome *C* from bovine heart (purity > 95%), and myoglobin from horse heart (purity > 90%) were purchased from Sigma–Aldrich (Zwijndrecht, the Netherlands). Methacrylated dextran (Dex-MA) with various degrees of substitution (DS) were synthesized according to van Dijk-Wolthuis et al. [27,28]. The DS was determined using ^{1}H NMR.

2.2. Preparation of Dex-MA hydrogels

Solutions of MA, DMAEMA, HEMA, and TEMED, respectively, were prepared in 10 mM HEPES or 10 mM HEPES buffered saline (HBS, 10 mM HEPES, 145 mM NaCl), and the pH was adjusted to 7.4 using HCl or NaOH. Dextran hydrogels with an initial Dex-MA content of 15% (w/w) were prepared by free radical polymerization. Dex-MA (0.15 g/g total weight), varying amounts of additional monomers (MA, DMAEMA, HEMA, total 350 μmol/g gel), and TEMED (67 μmol/g gel) were mixed in the appropriate buffer (HEPES/HBS). For protein loaded hydrogels, 20 μL of a protein stock solution (100 mg/mL) was added (8 mg protein per gram gel). APS in buffer (17 μmol/g gel) was added, and the mixture (final weight 250 mg) was transferred to a 1-mL syringe (radius 0.23 cm, height of the gel 1.5 cm). Polymerization was carried out under nitrogen atmosphere at room temperature for at least 2 h.

2.3. Equilibrium swelling

Hydrogels were removed from the syringe, weighed and transferred to vials containing HEPES or HBS. The weight of the gels was measured in time at 37 °C until a constant weight was reached. The swelling ratio is defined as the weight of the gel at equilibrium divided by the initial weight of the gel.

2.4. ζ-potential

Gels were washed with water and subsequently fragmented in 2.5 mL 5 mM HEPES pH 7.4 using an ultra-turrax tube drive (IKA® Werke GmbH & Co. KG, Staufen, Germany) for 10 min. The samples were incubated at room temperature for 2 h in order to let the larger particles settle. The ζ-potential of the particles in the supernatant was determined using a Malvern Zetasizer Nano-Z (Malvern Instruments, Malvern, UK) with universal ZEN 1002 'dip' cell and DTS (Nano) software (version 4.20). The system was calibrated with DTS 1050 latex beads (Zeta Potential Transfer Standard, Malvern Instruments, Malvern, UK).

2.5. Protein release

Protein loaded hydrogels were placed in vials containing 2 mL buffer (10 mM HEPES or HBS, pH 7.4). The vials were gently rotated at 37 °C. Samples (20 μL) were taken periodically and replaced with buffer. The protein concentration in samples was determined by means of a fluorometric assay using fluorescamine as described by Böhlen et al. [29], adapted to a 96-well plate format. Subtraction of the background signal measured in the release medium containing blank gels (without protein) was used to correct for possible interference of the gel components with the quantification.

2.6. Statistical analysis

First, cumulative release was compared using repeated-measures ANOVA with a post hoc test for linear trend. Subsequently, the total amount released was compared between gel formulations using a one-way ANOVA followed by post hoc analysis with a Bonferroni's multiple comparison test.

The slope of the best fit of the fractional release curves were compared using an *F*-test. Subsequently, the slopes were compared between gel formulations using a one-way ANOVA followed by post hoc analysis with a Bonferroni's multiple comparison test. A p-value < 0.05 was considered significant.

3. Results and discussion

3.1. Hydrogel synthesis and characterization

Positively and negatively charged dextran networks were obtained by free radical polymerization of Dex-MA with negatively charged MA or positively charged DMAEMA, respectively. To assess whether the charged monomers were incorporated in the polymer network, the electrophoretic mobility of particles obtained by fragmentation of the hydrogels was determined as a function of charged monomer feed (Fig. 1). Dex-MA gel particles prepared without charged monomers had a slightly negative ζ-potential, which is in agreement with earlier reports [30], and is likely due to absorption of anions onto the hydrogel particle surface. Input of charged monomers caused a shift of the ζ-potential of hydrogel particles, negative for MA and positive in the case of DMAEMA. Fig. 1 also shows that an increase in charged monomer feed led

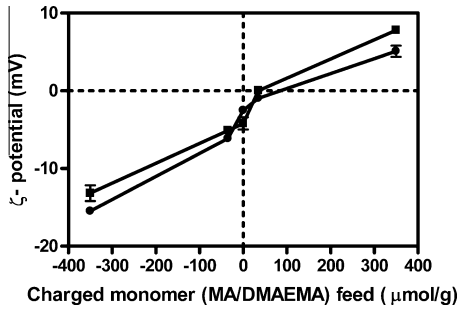

Fig. 1. ζ-Potential of hydrogel particles. Dex-MA hydrogels (squares = DS 4 and circles = DS 7) with varying concentrations of charged monomers were crushed, and the ζ-potential was measured at 25 °C in 5 mM HEPES pH 7.4. Values represent average and range of 2 separately prepared hydrogels.

to increased shift of ζ-potential. These findings indicate that the charged monomers copolymerized with Dex-MA to form charged Dex-MA hydrogels and that an increased charged monomer feed led to increased charge density.

3.2. Hydrogel swelling behavior

Once incubated in buffer, hydrogels reached an equilibrium swelling after 4 h. Fig. 2 shows the effect of charged monomers on the equilibrium swelling ratio of Dex-MA hydrogels. Without charged monomers, Dex-MA hydrogels with low DS (4) swell approximately 25% when incubated in buffer, while hydrogels with intermediate DS (7) are dimensionally stable, which is in agreement with earlier findings [22,27,31]. In 10 mM HEPES buffer, Dex-MA/DMAEMA or MA hydrogels (for both DS = 4 and 7) showed an increase in swelling when compared to gels without charged monomers. The degree of swelling increased with increasing charged monomer input. The incorporated charged groups caused a rise of the internal osmotic pressure due to accumulation of counterions (HEPES) in the network [32]. In HBS, a lower equilibrium swelling was observed for all charged Dex-MA hydrogels. Because of the high concentration of ions in this buffer the difference in osmotic pressure between the charged hydrogels and the surrounding buffer is small [32], and therefore the swelling was the same as that of the neutral gels. Similar findings were presented in earlier reports on hydrogels prepared by co-polymerization of Dex-MA and acrylic acid [33,34].

3.3. Protein release

The release of three model proteins varying in size and *pI* from neutral, positively, and negatively charged dextran networks (obtained from Dex-MA with DS 7) was studied in buffer at low and

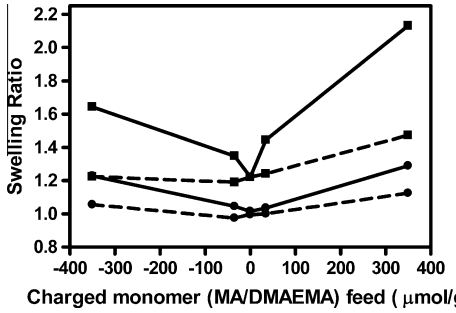

Fig. 2. Equilibrium swelling ratio of Dex-MA hydrogels (squares = DS 4 and circles = DS 7) with varying concentrations of charged monomers in HEPES (solid lines) and HBS (dashed lines). Values represent average and range of 2 separately prepared hydrogels.

Table 1
Molecular weight, D_0, *pI*, and charge at physiological pH of the selected proteins.

	Mw (kD)	D_0 (cm^2/s)	*pI*	Overall charge at pH 7.4
BSA	66	0.59×10^{-6} [41]	4.8	–
Myoglobin	17	1.06×10^{-6} [37]	7.2	±Neutral
Cytochrome *C*	12	1.30×10^{-6} [39]	10.2	+

physiological ionic strength. Table 1 lists the size, the diffusion coefficient in H_2O (D_0), *pI*, and overall charge at physiological pH of these model proteins.

Fig. 3A and B show the release profiles of myoglobin from charged and neutral Dex-MA hydrogels in HEPES and HBS, respectively. Both at low and at high ionic strength, myoglobin was released gradually up to 70–90% of the initial load. This indicates that a fraction (10–30%) of myoglobin was trapped inside the hydrogel network during polymerization. This fraction was lower for the hydrogels containing 350 μmol/g charged monomer (in HEPES 9.2% ± 3.4 and 10.3% ± 4.0, and in HBS 13.4% ± 0.5 and 13.2% ± 1.4 for MA and DMAEMA, respectively), when compared to neutral hydrogels and hydrogels containing 35 μmol/g charged monomers (in HEPES 24.3% ± 0.6 and 25.8% ± 2.4, and 17.9% ± 3.7 and in HBS 29.1% ± 3.1 and 28.9% ± 0.8 and 28.4% ± 3.2 for 35 μmol MA/g, neutral and 35 μmol DMAEMA/g respectively). This could be due to the higher equilibrium swelling for the hydrogels containing 350 μmol/g charged monomers, resulting in a bigger hydrogel mesh size. With none of the hydrogel compositions, complete release of protein was obtained. This could be due to chemical crosslinking of the protein to the network [35], which leads to permanent entrapment.

To assess the effect of the hydrogel charge on the diffusion kinetics of myoglobin, the fractional release was plotted as a function of the square root of time (Fig. 3C). According to the early-time approximation equation of Fick's second law, diffusion controlled release is linear with the square root of time [36]:

$$\frac{M_t}{M_\infty} = 4\sqrt{\frac{D_m t}{\pi r^2}} \quad (1)$$

where M_t/M_∞ represents the fractional release of the entrapped protein, D_m is the diffusion coefficient of the solute in the matrix, t is the release time, and r is the radius of the hydrogel cylinder. It should be noted that the dimensions of the cylinder should ideally be such that release through radial diffusion is negligible. For the gels used in this study, the top and bottom plane together make up 15% of the total surface area. Although the D_m calculated using Eq. (1) is therefore an overestimation, this does not pose a problem since it is used only to compare the diffusion in hydrogels with the same dimensions. Fig. 3C shows that the release versus the square root of time of myoglobin was linear up to a fractional release of ±0.6 for all hydrogel formulations and that the slope was independent of the composition of the hydrogel and the ionic strength of the release medium ($p = 0.52$ and 0.62 for HEPES and HBS, respectively). From the slope of the release curves, the diffusion coefficient of myoglobin in the hydrogel network D_m was calculated using Eq. (1) (Table 2).

The reported D_0 of myoglobin is 1.06×10^{-6} cm^2/s [37]. The results show that the hydrogel matrix slows down the diffusion of myoglobin by approximately 1 order of magnitude. It becomes clear from Table 2 that the diffusion of myoglobin was not affected by the network charge. Since myoglobin is almost neutral at physiological pH, an effect of hydrogel charge on the release of myoglobin was not expected. The higher equilibrium hydrogel swelling at higher network charge did also not affect protein release kinetics. On the one hand, swelling can lead to a higher release rate due

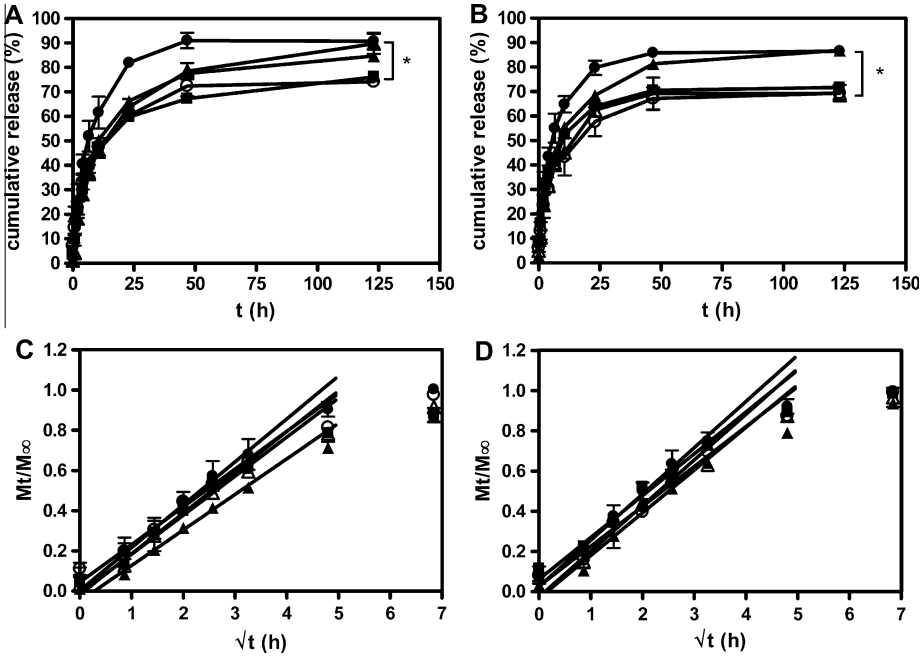

Fig. 3. Cumulative release of myoglobin from Dex-MA hydrogels (DS 7) in HEPES (A + C) and HBS (B + D). Values represent the average (n = 3, ±s.d.) released from neutral (squares), negatively charged (open circles = 35 μmol, closed circles = 350 μmol MA/g), and positively charged (open triangles = 35 μmol, closed triangles = 350 μmol DMAEMA/g) hydrogels. The symbol * indicates p-values < 0.05.

Table 2
The diffusion coefficient of myoglobin in Dex-MA hydrogels (DS 7) with varying charge density in HEPES and HBS calculated using the early-time approximation of Fick's second law (n = 3). Values between brackets represent the 95% confidence interval.

Charge density (μmol charged monomer/g hydrogel)	D_m in HEPES × 10^7 (cm^2/s)	D_m in HBS × 10^7 (cm^2/s)
350 MA	1.3 (0.8–1.9)	1.5 (1.1–2.0)
35 MA	1.0 (0.8–1.3)	1.5 (1.0–2.1)
0	1.2 (0.6–1.9)	1.2 (0.7–1.9)
35 DMAEMA	1.1 (0.5–1.9)	1.1 (0.7–1.7)
350 DMAEMA	0.9 (0.8–1.1)	1.3 (0.9–1.8)

to the increased hydration of the network; on the other hand, the increased diffusional pathlength resulting from the increase in hydrogel volume leads to slower release kinetics. Overall, for these hydrogels, the moderate increase in hydrogel swelling did not affect the release rate of myoglobin.

The release of cytochrome *C* from neutral, positively, and negatively charged dextran networks is shown in Fig. 4. In 10 mM HEPES, the release was substantially affected by the charge of the hydrogel network (Fig. 4A). The total amount of released cytochrome *C* was approx. 70% for neutral and positively charged gels (69.2% ± 1.0, 69.7% ± 2.0 and 69.4% ± 1.4 for neutral and 35 and 350 μmol DMAEMA/g gel, respectively). However, no release of this protein from negatively charged dextran networks was observed. At 35 μmol MA/g gel, the hydrogel contained an excess of MA when compared to the number of positively charged amino acid residues in cytochrome *C* (assuming full co-polymerization of MA; 50 mol MA/mol protein = 2.4 MA/positively charged amino acid residues), which explains that cytochrome *C* was immobilized quantitatively. Fig. 4B shows the release of cytochrome *C* from neutral, positively, and negatively charged gels in HBS. The amount of released cytochrome *C* from both neutral and positively charged gels in HBS (77.7% ± 0.9, 77.1% ± 3.2 and 77.8% ± 1.5 for neutral and 35 and 350 μmol DMAEMA/g gel, respectively) was similar to the release in HEPES, with similar release kinetics. However, as opposed to what was found in HEPES, cytochrome *C* was not quantitatively immobilized in negatively charged gels in HBS. The amount of released cytochrome *C* depended on the charge of the hydrogel, varying from 54% ± 3.2 for 350 μmol MA/g to 70.2% ± 0.39 for 35 μmol MA/g. These results demonstrate that the immobilization in HEPES was reversible and indeed due to electrostatic interactions between the protein and the dextran network. A similar effect of charge and ionic strength was described by Hirota et al. who found that the partitioning of myoglobin in negatively charged λ-carrageenan hydrogels at low ionic strength strongly depended on the pH of the medium but was almost the same for all pH values at 0.5 M KCl [38].

Fig. 4C and D shows that the release of cytochrome *C* from all hydrogels investigated was linear with the square root of time. The intercept of the linear fit of the release curves (0.1–0.2) indicates there was a burst release of cytochrome *C*. Most likely, this is a result of inhibition of the polymerization by oxygen at outer layer of the hydrogels. The slope of the curves and therefore the diffusion coefficient (Table 3) is comparable to that observed for myoglobin, which is expected considering the similar size. D_0 for cytochrome *C* is 1.30×10^{-6} cm^2/s [39], meaning that also in this case diffusion of the protein through the polymer matrix is 1 order of magnitude slower that the diffusion in H_2O. Fig. 4C shows that at high charge density, the similarly charged DMAEMA had a minor effect on the release rate of cytochrome C in HEPES. The slope of the fractional release curve was slightly lower for the release from gels containing 350 μmol DMAEMA/g compared to neutral and 35 μmol DMAEMA/g ($p < 0.001$ for each comparison). This indicates that the diffusion of cytochrome *C* in the hydrogel was hindered by the repulsive forces at high network charge density.

Strikingly, despite the overall charge on cytochrome *C*, there were no differences for the release rates from the different hydrogels in HBS ($p = 0.19$). Clearly, the ions in HBS effectively screen the polymer and protein charges, thereby breaking the electrostatic interaction between the hydrogel network and cytochrome *C*. This is in line with the assumption of Zhang and Amsden that at physiological strength the diffusivity of globular proteins is dominated

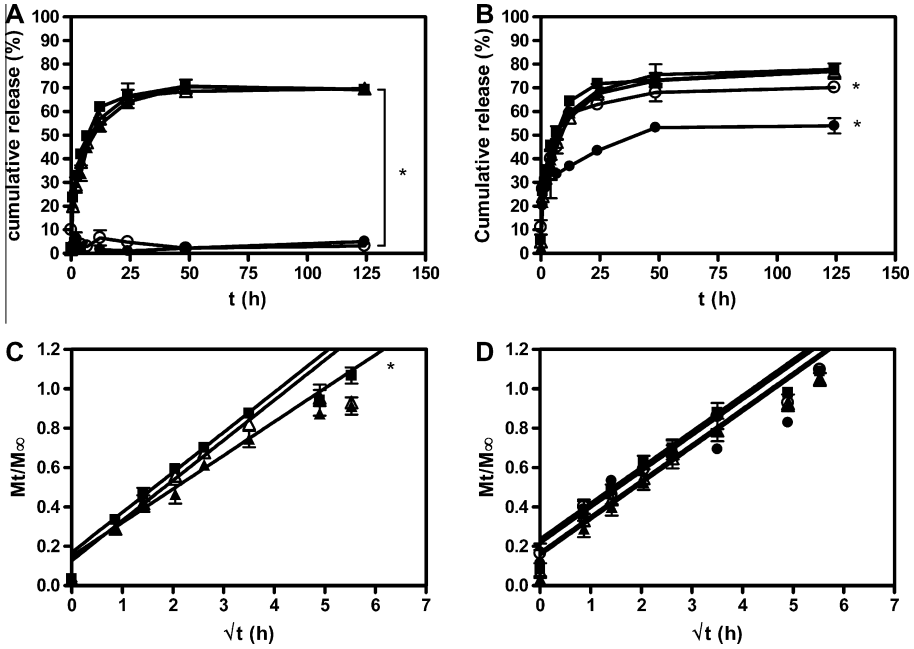

Fig. 4. Cumulative release of cytochrome C from Dex-MA hydrogels (DS 7) in HEPES (A + B) and HBS (C + D). Values represent the average (n = 3, ±s.d.) released from neutral (squares), negatively charged (open circles = 35 μmol, closed circles = 350 μmol MA/g), and positively charged (open triangles = 35 μmol, closed triangles = 350 μmol DMAEMA/g) hydrogels. The symbol * indicates p-values < 0.05.

Table 3
The diffusion coefficient of cytochrome C in Dex-MA hydrogels (DS 7) with varying charge density in HEPES and HBS calculated using the early-time approximation of Fick's second law (n = 3) Values between brackets represent the 95% confidence interval.

Charge density (μmol charged monomer/g hydrogel)	D_m in HEPES × 10^7 (cm^2/s)	D_m in HBS × 10^7 (cm^2/s)
350 MA	–	1.3 (0.8–2.0)
35 MA	–	1.0 (0.8–1.3)
0	1.2 (1.0–1.4)	1.4 (1.2–1.7)
35 DMAEMA	1.2 (1.0–1.4)	1.1 (1.0–1.3)
350 DMAEMA	0.8 (0.7–1.1)	1.3 (1.1–1.6)

by steric hindrance and that electrostatic interactions can be neglected [40].

The release of BSA from neutral, positively, and negatively charged dextran networks is shown in Fig. 5. The release of BSA from neutral gels was significantly slower than observed for myoglobin and cytochrome *C*, which is caused by the higher molecular weight of BSA compared to myoglobin and cytochrome *C*. The calculated diffusion coefficients of BSA are shown in Table 4. As can be seen from the diffusion coefficient in a neutral gel obtained from Dex-MA with DS 7, the hydrogel network decreases the diffusion coefficient by 2 orders of magnitude when compared to the diffusion in H_2O ($D_0 = 5.90 \times 10^{-7}$ cm^2/s [41]). As expected, decreasing

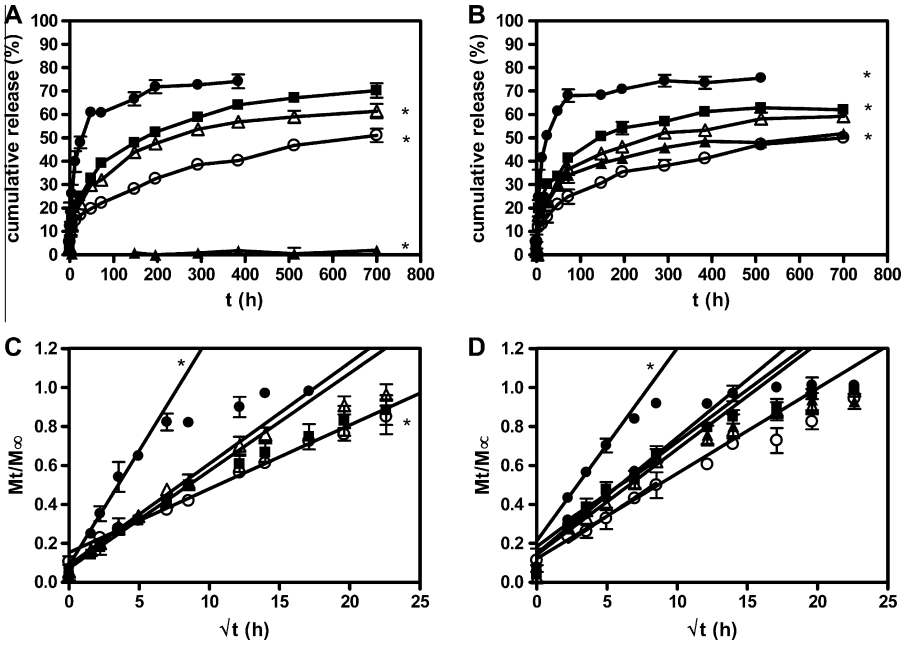

Fig. 5. Cumulative release of BSA from Dex-MA hydrogels (DS 7) in HEPES (A and C) and HBS (B and D). Values represent the average (n = 3, ±s.d.) released from neutral (squares), negatively charged (open circles = 35 μmol, closed circles = 350 μmol MA/g), and positively charged (open triangles = 35 μmol, closed triangles = 350 μmol DMAEMA/g) hydrogels. The symbol * indicates p-values < 0.05.

Table 4
The diffusion coefficient of BSA in Dex-MA hydrogels (DS 7 and 4) with varying charge density in HEPES and HBS calculated using the early-time approximation of Fick's second law ($n = 3$) values between brackets represent the 95% confidence interval.

Charge density (μmol charged monomer/g hydrogel)	DS 7		DS 4	
	D_m in HEPES × 10^9 (cm^2/s)	D_m in HBS × 10^9 (cm^2/s)	D_m in HEPES × 10^9 (cm^2/s)	D_m in HBS × 10^9 (cm^2/s)
350 MA	40 (23–61)	28 (14–46)	22 (19–25)	18 (17–20)
35 MA	3.1 (2.6–3.6)	5.5 (3.3–8.3)	10 (8.5–12)	11 (9.9–12)
0	7.2 (5.8–8.8)	8.3 (5.3–12)	16 (12–21)	15 (13–16)
35 DMAEMA	7.8 (6.2–9.6)	8.5 (6.9–10)	16 (15–17)	15 (14–16)
350 DMAEMA	–	10 (7.7–13)	–	13 (12–14)

the network density (using Dex-MA with a lower number of methacrylate units, i.e. DS 4) increased the diffusion coefficient (Table 4).

Fig. 5A shows that release of BSA from neutral gels was 70.3% ± 3.1. Co-polymerization of Dex-MA with DMAEMA decreased the total amount of BSA released in HEPES, depending on the input of charged monomer (59.2% ± 2.3 and 1.9% ± 1.6 for 35 and 350 μmol/g, respectively). It becomes clear from Fig. 5B that the immobilization of BSA was counteracted in HBS (61.7% ± 1.5 for neutral and 59.2% ± 2.3 and 52% ± 1.7 for 35 and 350 μmol DMAEMA/g, respectively), as was observed with cytochrome *C*.

The effect of incorporation of MA on the release of negatively charged BSA was twofold. On the one hand, incorporation of 35 μmol MA/g gel surprisingly led to a decrease in the total amount of released BSA (51% ± 2.9, Fig. 5A), and a significant decrease in the release rate in HEPES ($p < 0.05$), especially at relatively high network density (Table 4). On the other hand, at higher charge density (350 μmol MA/g gel), the total amount of released BSA was unaffected, while the release rate substantially increased when compared to the neutral gel. This effect was also most pronounced at relatively high network density ($p < 0.001$). The same effect was observed in HBS (Fig. 5B and D, Table 4). It is certain that the increased release rate from these gels is not caused by increased equilibrium swelling, since in HBS the equilibrium swelling was not dependent on the network charge density (Fig. 2). The observed dualistic effect of the incorporation of MA can be explained by the quaternary structure of BSA and the relative amount of charged moieties in the hydrogel network. Despite the overall negative charge at pH 7.4, BSA has a positively charged surface domain [42]. Probably, during polymerization with a relatively low amount of MA (i.e. 35 μmol/g corresponds to 2.9 mol MA/mol BSA), preferred electrostatic interaction between MA and the positively charged domain of BSA occurred, leading to the formation of pockets in the hydrogel network complementary in charge and shape, a phenomenon often referred to as "molecular imprinting" [43–45]. Oriented binding of the asymmetric BSA to these pockets may account for the retention of BSA in the hydrogel at low charge density (35 μmol/g gel). At higher charge density, however (350 μmol/g gel), the excess of negative charges distributed throughout the network (29 mol MA/mol BSA) renders orientation impossible, resulting in repulsion of the overall negatively charged BSA. The repulsive forces restrict the accessible space for BSA in the hydrogel network, thereby favoring the release from the hydrogel, leading to a higher release rate. In networks with a lower crosslink density (i.e. with DS 4), this effect was also observed, although less pronounced, because in those gels the protein already had a higher release rate (as illustrated by the higher diffusion coefficients in the neutral gels).

As opposed to BSA, the release of cytochrome *C* from similarly (positively) charged dextran networks was independent of the network charge (Fig. 4). First, the surface charge distribution of cytochrome *C* is more homogenous than that of BSA, and no negatively charged domains are present in this protein [46], which explains the observation that cytochrome *C* is not retained in similarly charged gels with low charge density. Second, at higher similar charge density, no increase in release rate of cytochrome *C* when compared to neutral gels was observed. This second difference in behavior of cytochrome *C* and BSA can be explained by the fact that the diffusion of the considerably larger BSA is influenced by the hydrogel matrix to a much larger extent than the diffusion of cytochrome *C*. Therefore, it can be anticipated that charge of a relatively dense crosslinked network can have a larger effect on BSA then on cytochrome *C*. In other words, the effect of a similar network charge is not observed for cytochrome *C* because the diffusion of the small protein is not inhibited by density of the gel to a sufficiently large extent. This explanation is supported by the release of BSA from hydrogels with a lower crosslink density (i.e. using Dex-MA of DS 4), where the effect of the similar charge is only just noticeable.

Based on the above results, we propose that incorporation of charged monomers in a hydrogel can be used for the triggered release of proteins. First, electrostatic interaction between a protein and an oppositely charged network can fully immobilize proteins in the hydrogel, while the release is triggered by a change of the ionic strength of the surrounding medium. It is anticipated that a change in pH leading to a change in protonation state of either protein or network could terminate the electrostatic interactions as well.

4. Conclusion

The results presented in this paper show that charged proteins are immobilized in oppositely charged dextran hydrogels by electrostatic interaction at low ionic strength. At higher ionic strength, the amount of immobilized protein depends on the charge density of the hydrogel. The diffusion coefficient of the mobile fraction is determined by the network density and is unaffected by the opposite charge of the network. Therefore, we conclude that incorporation of charge in Dex-MA hydrogels can be used for immobilization and ionic strength triggered release of proteins.

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