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Self-gelling hydrogels based on oppositely charged dextran microspheres

Sophie R. Van Tomme[a], Mies J. van Steenbergen[a], Stefaan C. De Smedt[b],
Cornelus F. van Nostrum[a], Wim E. Hennink[a],*

[a]*Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences (UIPS), University Utrecht, Sorbonnelaan 16, P.O. Box 800082, 3508 TB, Utrecht, The Netherlands*
[b]*Laboratory of General Biochemistry and Physical Pharmacy, Department of Pharmaceutics, Ghent University, Harelbekestraat 72, 9000, Ghent, Belgium*

Abstract

This paper presents a novel self-gelling hydrogel potentially suitable for controlled drug delivery and tissue engineering. The macroscopic gels are obtained by mixing dispersions of oppositely charged crosslinked dextran microspheres. These microspheres in turn were prepared by crosslinking of dextran derivatized with hydroxyethyl methacrylate emulsified in an aqueous poly(ethylene glycol) solution. Negatively or positively charged microspheres were obtained by addition of methacrylic acid (MAA) or dimethylaminoethyl methacrylate (DMAEMA) to the polymerization mixture. Rheological analysis showed that instantaneous gelation occurred when equal volumes of oppositely charged microspheres, dispersed in buffer solutions of pH 7, were mixed. The shear modulus of the networks could be tailored from 30 to 6500 Pa by varying the water content of the system. Moreover, controlled strain and creep experiments showed that the formed networks were mainly elastic. Importantly for application of these systems, e.g. as controlled matrix of pharmaceutically active proteins, it was demonstrated that the hydrogel system has a reversible yield point, meaning that above a certain applied stress, the system starts to flow, whereas when the stress is removed, gel formation occurred. Further it was shown that the network structure could be broken by either a low pH or a high ionic strength of the medium. This demonstrates that the networks, formed at pH 7 and at low ionic strength, are held together by ionic interactions between the oppositely charged dextran microspheres. This system holds promise as injectable gels that are suitable for drug delivery and tissue engineering applications.
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1. Introduction

Hydrogels are an important class of materials that have been studied extensively in the last decades for the controlled release of pharmaceutical proteins, and for tissue engineering applications [1–5]. Formation of hydrogels can be achieved by both chemical and physical crosslinking [6]. By chemical crosslinking covalent bonds between the different polymer chains are introduced. Chemical crosslinking results in a network with a relatively high mechanical strength and, depending on the nature of the chemical bonds in the building blocks and the crosslinks, in relatively long degradation times. However, chemical crosslinking can possibly damage the entrapped bioactive substance, leading to a loss of activity. Moreover, the crosslinking agents are mostly toxic and removal needs to be ensured before in vivo application. In recent years there is a growing interest in physically crosslinked hydrogels. In such systems non-permanent bonds, based on physical

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*Corresponding author. Tel: +31-30-253-6964; fax: +31-30-251-7839.

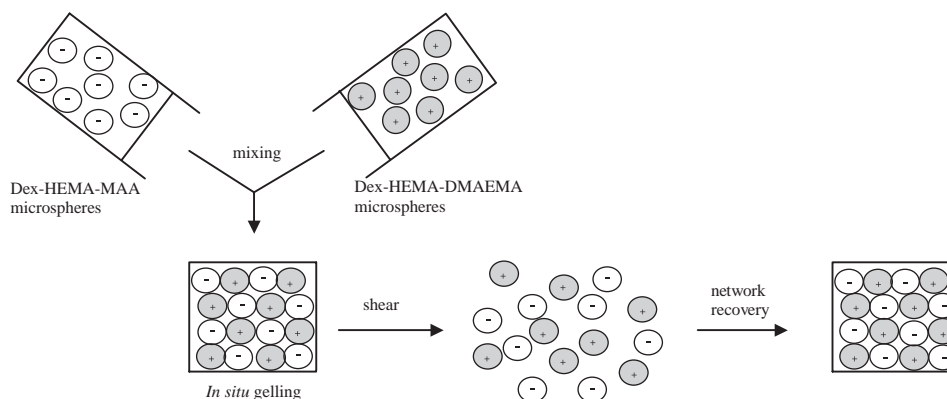*E-mail address:* w.e.hennink@pharm.uu.nl (W.E. Hennink).

Fig. 1. The concept of the physically crosslinked hydrogel system. The hydrogel is obtained after mixing aqueous dispersions of negatively charged dex-HEMA-MAA and positively charged dex-HEMA-DMAEMA microspheres. When shear is applied, the interactions between the microspheres are broken and the sample flows. Upon removal of the shear stress, the network rebuilds itself.

interactions between the polymer chains, are created. Different methods have been investigated to prepare physically crosslinked hydrogels. An attractive class of physically crosslinked gels is those where gel formation is not instantaneous, but occurs a certain time after mixing the hydrogel components (e.g. stereocomplex gels [7–10]) or after a certain trigger (e.g. temperature [3,11–15]). Such systems can be administered by injection as liquid formulation and gellify in situ. Gel formation through chemical crosslinking can also occur using UV light as a trigger [16,17].

In our Department, both chemically and physically crosslinked dextran hydrogels have been developed in recent years. An organic solvent free approach to obtain crosslinked microspheres has been described where preparation occurs in an all-aqueous environment [18,19]. The in vivo biocompatibility of dextran-based hydrogels and microspheres has been demonstrated as well as the relation between in vitro and in vivo degradation behavior [20–22].

In this paper a novel injectable hydrogel system, as schematically outlined in Fig. 1, is investigated. The macroscopic hydrogels are designed by combining the injectability of microspheres with physical crosslinking through ionic interactions. Anionically or cationically charged microspheres were prepared and gels were obtained by mixing aqueous dispersions of the oppositely charged microspheres. Gel formation was studied by rheological experiments and special attention was given to the reversibility of the system.

2. Materials and methods

2.1. Materials

Dextran T40 (from *Leuconostoc* ssp.), *N,N,N′,N′*-tetramethylethylenediamine (TEMED) and 2-hydroxyethyl methacrylate (HEMA) were obtained from

Fluka (Buchs, Switzerland). Poly(ethylene glycol) (PEG) 10000 and potassium peroxodisulfate (KPS) were provided by Merck (Darmstadt, Germany). *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid (Hepes) was purchased from Acros Chimica (Geel, Belgium). Methacrylic acid (MAA) and dimethylaminoethyl methacrylate (DMAEMA) were provided by Sigma-Aldrich (Zwijndrecht, The Netherlands).

2.2. Hydroxyethyl methacrylate-derivatized dextran (dex-HEMA)

Dextran was derivatized with hydroxyethyl methacrylate (dex-HEMA) (Fig. 2A) as described previously [23]. The degree of substitution (DS, i.e. the number of HEMA groups per 100 glucopyranose units) used in this study was 6.

2.3. Preparation of charged microspheres

The dextran microspheres with a water content of 70% were obtained through radical polymerization of dex-HEMA, emulsified in an aqueous PEG solution [18,24]. In short: aqueous solutions of PEG (40% (w/w)) and dex-HEMA (20% (w/w)) were prepared in Hepes buffer (100 mM pH 7.0). PEG, dex-HEMA and buffer solution, 197.6, 18.3 and 284.1 g respectively (total weight 500 g) were transferred into a 500 mL glass cylinder. Subsequently, either 12.5 mmol of MAA (Fig. 2B), or 12.5 mmol of DMAEMA (Fig. 2C) was added to the two-phase system (molar ratio HEMA/MAA or DMAEMA = 0.53). The two-phase system was flushed with nitrogen and intensively mixed (30 min, 11000 rpm, IKA Ultra-Turrax® T 25 basic, IKA-®WERKE GMBH & CO.KG, Staufen, Germany). In this way, a water-in-water emulsion was created that was allowed to stabilize for 15 min. Next, a TEMED solution (10 mL, 20% v/v, adjusted to pH 7 with 4 M HCl) and a KPS solution (18 mL, 50 mg/mL), both freshly prepared, were
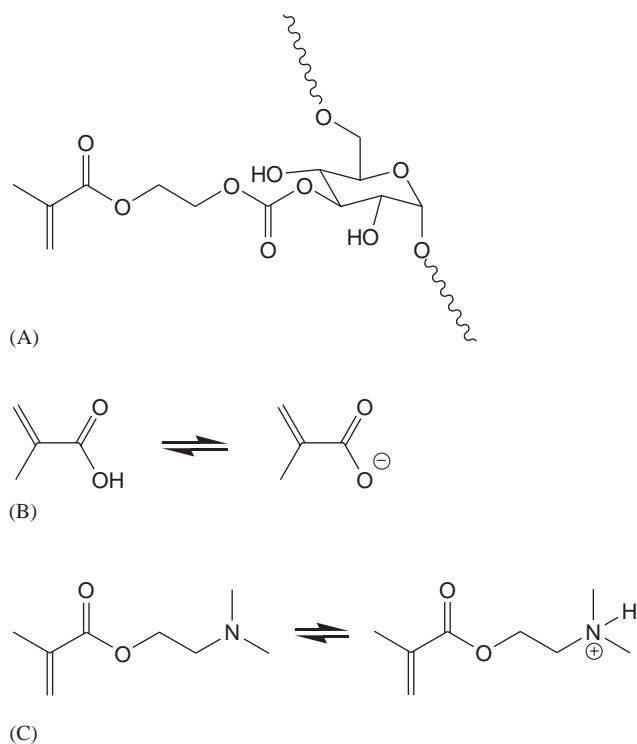
Fig. 2. Chemical structures of dex-HEMA (A), methacrylic acid (B) and dimethylaminoethyl methacrylate (C).

added to the mixture. The emulsified droplets were allowed to polymerize for 30 min at ambient temperature. Under these conditions the HEMA conversion is >90% [25]. Two types of microspheres were prepared, containing either MAA (dex-HEMA-MAA) or DMAE-MA (dex-HEMA-DMAEMA). The crosslinked particles were collected and purified by multiple washing and centrifugation steps (thrice with reversed osmosis water, 15 min, 3000 rpm). Ultimately the microspheres were lyophilized.

The particle size distribution of the microspheres was determined using a Coulter Counter Multisizer® 3 (Beckman Coulter Nederland B.V., The Netherlands) with a 100-μm orifice.

2.4. Formation of macroscopic gels with charged microspheres

Lyophilized microspheres (dex-HEMA-MAA or dex-HEMA-DMAEMA) were dispersed in Hepes buffer (100 mM pH 7; solid content between 10% and 25%). The dispersions were stored at 4 °C for 2 h to allow full hydration of the microspheres. The equilibrium water content of the rehydrated microspheres was determined using the blue dextran exclusion assay [26]. To study possible gel formation, equal volumes (200 μL) of the two different microsphere dispersions were mixed.

2.5. Rheological experiments

The rheological measurements were performed using a controlled stress rheometer (AR1000-N, TA Instruments, Etten-Leur, The Netherlands), equipped with an acrylic flat plate geometry (20 mm diameter) and a gap of 500 μm. Immediately after mixing equal volumes of both dispersions (see Section 2.4), the sample was placed between the plates. A solvent trap was used to prevent evaporation of the solvent. The viscoelastic properties of the sample were determined by measuring the G' (shear storage modulus) and G'' (loss modulus) at 20 °C with a constant strain of 1% and constant frequency of 1 Hz. Also frequency sweep and strain sweep experiments were performed. Creep experiments were performed to evaluate the extent of recovery of the material after deformation. In the creep experiment a shear stress of 1 Pa was applied while the strain was monitored. After 1 min the stress was removed and the recovery of the sample was monitored by measuring the strain during 2 min. As a control, the same rheological experiments were performed on dispersions containing only dex-HEMA-MAA or dex-HEMA-DMAEMA microspheres.

To determine the yield point of the system, stress sweep experiments were performed at 20 °C. During these experiments the G' and G'' were monitored while the stress was increased. The frequency was kept constant to 1 Hz. The experiment was performed 4 times in a row using the same sample. After each experiment the sample was allowed to recover for 1 h.

Most experiments were performed on hydrogels containing 15% (w/w) of freeze-dried microspheres. Controlled strain and creep experiments were also performed on hydrogels with different percentages (10%–25% w/w) solid content. The influence of pH and ionic strength on the systems was studied by using different buffers (phosphate buffer (100 mM, pH 3) or Hepes buffer (100 mM, pH 7) with variable ionic strengths (NaCl, from 17–1000 mM)).

3. Results and discussion

3.1. Preparation of charged dex-HEMA microspheres

Charged dextan particles were obtained by radical copolymerization of dex-HEMA with either MAA or DMAEMA. At physiological pH both MAA and DMAEMA are mainly ionized (pKa MAA = 4.7; pKa DMAEMA = 8.4; [27]), resulting in charged microspheres at this pH. The mean volume diameters of the dex-HEMA-MAA and the dex-HEMA-DMAEMA microspheres were comparable (8.3 and 7.5 μm respectively; 90% < 12.5 μm (Fig. 3)). The equilibrium water
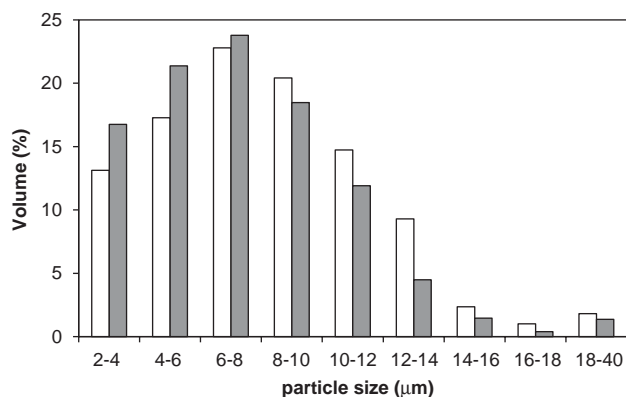
Fig. 3. Volume diameter distribution of dex-HEMA-MAA (light) and dex-HEMA-DMAEMA (dark) microspheres.

content of the rehydrated microspheres was 70%, as determined with the blue dextran exclusion assay [26].

3.2. Gel formation through ionic interactions between microspheres

Addition of buffer to freeze-dried microspheres created a homogenous opalescent dispersion. It should be noted that the lyophilized microspheres absorbed water resulting in a dispersion of hydrated microspheres in a continuous aqueous phase. The percentage of free water depends on the amount of dried particles dispersed in the aqueous phase and amounts 50% when the solid content of the dispersion was 15%. When the solid content was 10% or less, the dispersions were freely flowing. Increasing the solid content, and so decreasing the amount of free water, yielded more viscous dispersions. With a solid content above 25% (free water is <16%), dispersions with a very high viscosity were obtained.

When equal volumes of dex-HEMA-MAA and dex-HEMA-DMAEMA microsphere dispersions were mixed, gelation clearly occurred instantly. However, the obtained gel could be easily handled by a positive displacement pipette. This implicates that the network can be easily broken and rebuilt when exposed to stress and deformation, as expected for physical crosslinking. This aspect is studied in more detail is Sections 3.3 and 3.5.

3.3. Rheological characterization of the system

The viscoelastic properties of dex-HEMA-MAA/dex-HEMA-DMAEMA microsphere dispersions (15% solid content) were investigated with controlled strain experiments.

Fig. 4 shows that when a strain of 1% is applied the sample is still in the linear viscoelastic deformation range. As Fig. 5a shows, after mixing the anionic and cationic dex-HEMA microspheres, the storage modulus
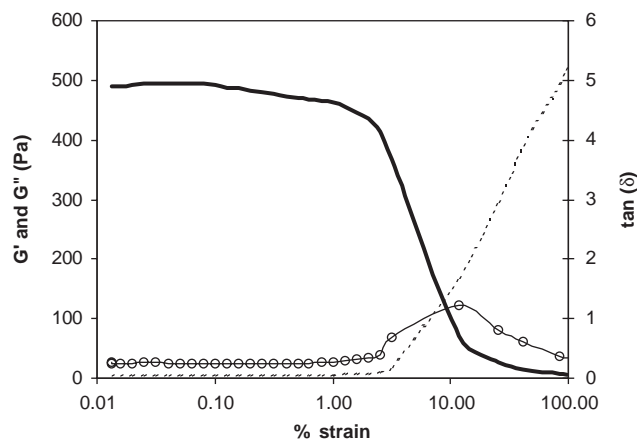

Fig. 4. Storage modulus G' (—), loss modulus G'' ($\circ\circ\circ\circ$) and tan(δ) (- - -) of a dex-HEMA-MAA/dex-HEMA-DMAEMA microsphere dispersion (solid content 15% (w/w)) at 20 °C as a function of the % strain.
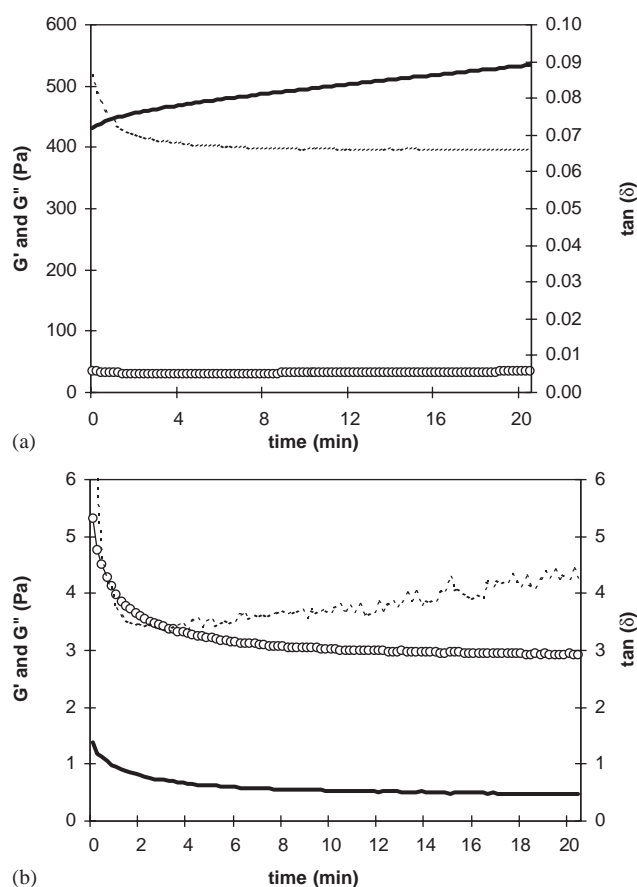

Fig. 5. (a) Storage modulus G' (—), loss modulus G'' ($\circ\circ\circ\circ$) and tan(δ) (- - -) of a dex-HEMA-MAA/dex-HEMA-DMAEMA microsphere dispersion (solid content 15% (w/w); 20 °C). G', G'' and tan(δ) were followed in time after mixing the anionic and cationic dex-HEMA microspheres. (b) Storage modulus G' (—), loss modulus G'' ($\circ\circ\circ\circ$) and tan(δ) (- - -) of a dex-HEMA-MAA microsphere dispersion (solid content 15% (w/w)) at 20 °C as a function of the time.

(G') increased gradually in time while the loss modulus (G'') remained low. The G''/G' ratio or $\tan(\delta)$ was lower than 0.1, which indicates that the obtained material is mainly elastic. Fig. 5b shows the rheological characteristics of a dex-HEMA-MAA microsphere dispersion with the same solid content. Compared with the mixture of oppositely charged microspheres Fig. 5b shows that the $\tan(\delta)$ was substantially higher (>4). It indicates that, as expected, an elastic network does not exist in a dispersion of negatively charged dex-HEMA spheres. Positively charged dex-HEMA microspheres showed comparable results (data not shown).

Fig. 6a shows the results of a creep experiment on a mixture of dex-HEMA-MAA and dex-HEMA-DMAEMA microspheres. Upon applying the shear stress (1 Pa), the system deformed, evolving to 0.15% strain. When the stress was removed, the sample recovered almost completely, confirming the almost fully elastic properties of the material, and indicating the presence of a network. In contrast, the dex-HEMA-MAA microsphere dispersion showed mainly viscous behavior

(Fig. 6b). The deformation in the retardation phase was more than a 10000 fold stronger than the one in Fig. 6a. Also, after removal of the stress the sample did not recover, indicating that the dispersion is not elastic. These results are in full agreement with the high value of $\tan(\delta)$ for this dispersion (Fig. 5b).

3.4. Influence of solid content, pH and ionic strength on gel properties

Fig. 7 shows the G' and $\tan(\delta)$ of dex-HEMA-MAA/dex-HEMA-DMAEMA microsphere dispersions as a function of the solid content of the dispersions. When the solid content of the mixture was 10%, the microspheres did not establish a network structure as evidenced from the high $\tan(\delta)$ ($\sim$1.6). Obviously, due to the high water content, anionic and cationic dex-HEMA microspheres are too far separated from each other to form a network. From 12.5% solid content (58% free water) on, the microspheres do interact and create a network, clearly illustrated by the increase in G' and the low $\tan(\delta)$. For dispersions with a solid content of 25%, G' equaled 6500 Pa while $\tan(\delta)$ was 0.09. For a 25% dispersion of dex-HEMA-MAA microspheres, G' equaled 700 Pa while $\tan(\delta)$ was 0.3. The high G' of the dex-HEMA-MAA dispersion can be explained by the low free water content (16.5%), which forces the microspheres to be closely packed despite their negative charge. The higher $\tan(\delta)$ indicates that there is less elasticity in these dispersions when compared to the dex-HEMA-MAA/dex-HEMA-DMAEMA system.

Table 1 shows the rheological properties of a dex-HEMA-MAA/dex-HEMA-DMAEMA dispersion (solid content 15%), prepared at respectively pH 3 and pH 7. Interestingly and in contrast to pH 7-dispersions, at pH 3 the system shows mainly viscous behavior
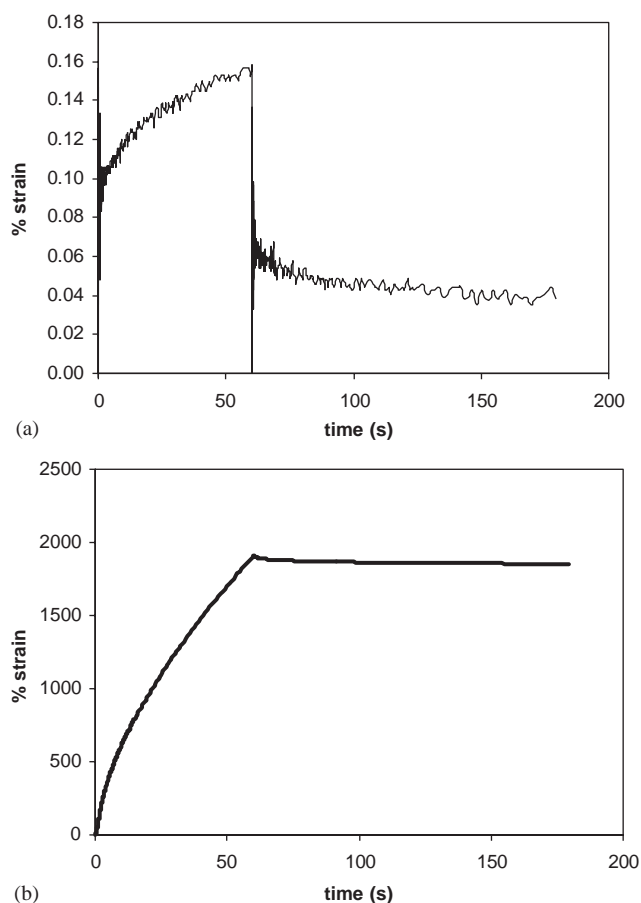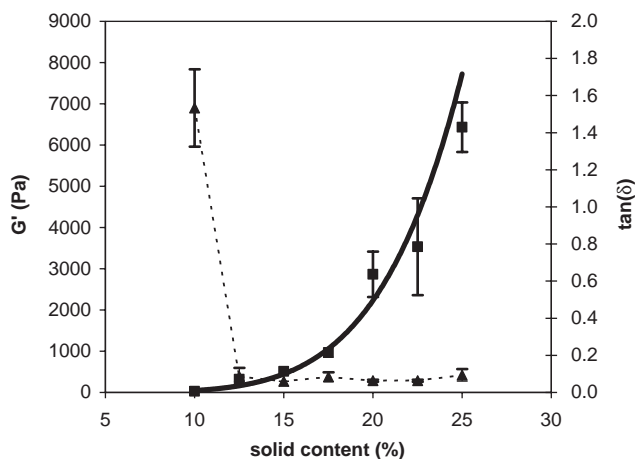

Fig. 6. (a) Creep experiment on a dex-HEMA-MAA/dex-HEMA-DMAEMA microsphere dispersion (solid content 15% (w/w), applied stress 1 Pa, 20 °C). (b) Creep experiment on a dex-HEMA-MAA microsphere dispersion (solid content 15% (w/w), applied stress 1 Pa, 20 °C).

Fig. 7. Storage modulus G' (━) and $\tan(\delta)$ (- - -) as a function of the solid content of dex-HEMA-MAA/dex-HEMA-DMAEMA microsphere dispersions. The data are shown as sample mean $\pm$ the standard deviation ($n = 3$).

Table 1
Storage modulus G' loss modulus G'' and tan(δ) of dex-HEMA-MAA/dex-HEMA-DMAEMA microsphere dispersion as a function of the pH. The hydrogel solid content was 15% (w/w). All data are shown as sample mean $\pm$ the standard deviation ($n = 3$)

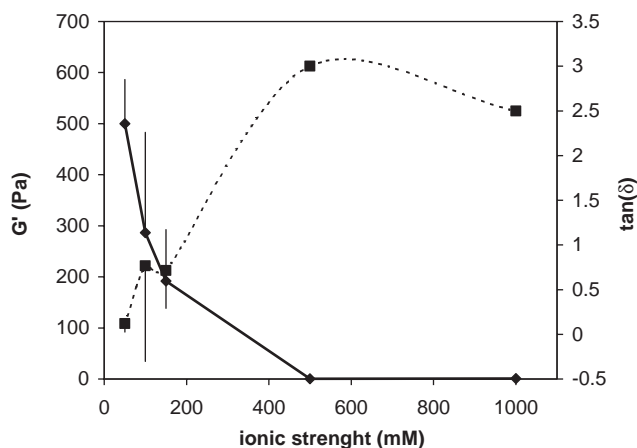| pH of the buffer	G' (Pa)	G'' (Pa)	tan(δ)
3	11 ± 1	29 ± 5	2.7 ± 0.2
7	509 ± 18	29 ± 2	0.06 ± 0.00

Fig. 8. Storage modulus G' (—) and tan(δ) (- - -) of a dex-HEMA-MAA/dex-HEMA-DMAEMA microsphere dispersion as a function of the ionic strength of the buffer. The hydrogel solid content was 15% (w/w). The data are shown as sample mean $\pm$ the standard deviation ($n = 3$).

(tan(δ) > 2), comparable to the results of the dex-HEMA-MAA dispersion (Fig. 5b). These results can be explained by the fact that the dex-HEMA-MAA microspheres essentially loose their negative charge at pH 3.

Fig. 8 shows the influence of the ionic strength of the buffer on the rheological properties of dex-HEMA-MAA/dex-HEMA-DMAEMA microsphere dispersions. Increasing the concentration of NaCl significantly decreases G' and increases tan(δ). It indicates that a higher salt concentration inhibits network formation, which is explained by the shielding of the microsphere charges at high ionic strength.

Taken together the results presented in Table 1 and Figs. 7 and 8 demonstrate that the observed network formation is indeed due to electrostatic interactions between oppositely charged microspheres, creating a physically crosslinked hydrogel network.

3.5. Determination of the yield point

In view of the possible application as an injectable dispersion which gellifies in situ, it is important to know whether the material flows when shear forces are
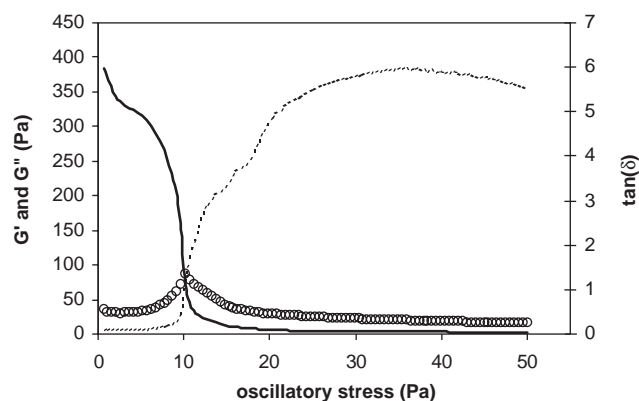

Fig. 9. Storage modulus G' (—), loss modulus G'' (ⵔⵔⵔⵔ) and tan(δ) (- - -) of a dex-HEMA-MAA/dex-HEMA-DMAEMA microsphere dispersion (solid content 15% (w/w)) at 20 °C as a function of the oscillatory stress.

applied. The dispersions should flow during injection, however the network structure should be established at the place of injection (Fig. 1). The shear at which flow starts is referred to as the yield point [28]. Because the obtained yield stress value is dependent on the applied technique, it is preferable to use the term 'apparent yield stress' [29,30].

To determine the apparent yield point, stress sweep experiments were performed on a hydrogel formed at pH 7 at 20 °C by mixing dex-HEMA-MAA and dex-HEMA-DMAEMA dispersions (Fig. 9). With increasing stress (from 0.1 Pa to 50 Pa), the G' gradually decreased and the tan(δ) increased simultaneously. However, when the applied stress exceeds 10 Pa the G' dramatically dropped from 300 Pa to 3 Pa whilst the tan(δ) increased from 0.08 to 5. Next, the stress was removed and the system was allowed to recover for 1 h. When an increasing stress was put on the gel, a similar rheogramme as shown in Fig. 9 was observed. Four consecutive stress sweep experiments were performed, each giving comparable values for G', G'' and tan(δ). The results of Fig. 9 show that the system of oppositely charged dextran microspheres is plastic in a rheological sense, meaning the ionic interactions between the microspheres can be broken by mechanical stress and that the network rebuilds itself when the stress is removed.

As expected, the yield stress was dependent on the gel composition and amounted 150 Pa for 25% systems.

4. Conclusion

This paper reports on a novel method to design macroscopic hydrogels, combining injectability of hydrogel microspheres with physical crosslinking through ionic interactions. The ionic interactions between the cationic and anionic microspheres and so creating a

physical network, can be broken when exposed to stress. The gel forms again when the stress is removed, indicating the reversible character of the system. A number of possible applications for this novel system can be foreseen among which controlled delivery of pharmaceutically active proteins and entrapment of living cells for tissue engineering. At present, we are studying the release of proteins from these systems.

Acknowledgement

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