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Interplay of thermal and covalent gelation of silanized hydroxypropyl methyl cellulose gels

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ABSTRACT

Silanized hydroxypropyl methyl cellulose (Si-HPMC) is a biocompatible polysaccharide that forms a covalently crosslinked hydrogel at all temperatures due to silanol condensation. Unmodified HPMC forms reversible turbid physical gels when heated above 55 °C. The interaction between thermal gelation and covalent crosslinking of Si-HPMC was investigated with rheology, turbidity and microscopy. Thermal gelation of the HPMC backbone was found to reinforce Si-HPMC gels at room temperature. However, simultaneous thermal and covalent crosslinking at higher temperatures led to weaker turbid gels at room temperature. The effect of the pH and the addition of orthophosphate on the elastic modulus and the gelation kinetics was investigated.

1. Introduction

The advancements in tissue engineering have motivated the use of polymer based hydrogels for regenerative medicine (Drury & Mooney, 2003; Van Vlierberghe, Dubruel, & Schacht, 2011; Vinatier, Guicheux, Daculsi, Layrolle, & Weiss, 2006). In order to develop less invasive surgical techniques notably for bone and cartilage tissue engineering, there is an increasing demand for aqueous biocompatible polymer solutions that are injectable and crosslink in situ under physiological conditions (Ashley & Lakshmi, 2012; Fiejdasz, Szczubiałka, Lewandowska-Łańcucka, Osyczka, & Nowakowska, 2013; Overstreet, Dutta, Stabenfeldt, & Vernon, 2012). Once implanted these injectable matrices must acquire the desired form, allow cell diffusion and growth and present mechanical properties in relation to the tissue to be repaired. One widely used biocompatible polymer is hydroxypropyl methyl cellulose (HPMC).

Aqueous HPMC solutions form gels with an elastic modulus (G_{el}) that increases sharply with increasing temperature above a critical temperature (T_c) where a phenomenon of localized phase separation occurs rendering the gels turbid (Allabash, Nicolai, Benyahia, Tassin, & Chassenieux, 2014; Bodvik et al., 2010; Fairclough et al., 2012; Haque & Morris, 1993; Haque, Richardson, Morris, Gidley, & Caswell, 1993; Hussain, Keary, & Craig, 2002; Sarkar, 1979; Silva et al., 2008; Yoo & Um, 2013). In pure water T_c is higher than 50 °C and depends on the degree of substitution of the cellulose chains. The critical temperature T_c can be reduced by addition of salt and in particular orthophosphate (Joshi, 2011). Thermal gelation of HPMC is reversed when the temperature is lowered but the gel melts at a temperature lower than T_c.

The HPMC chains can be covalently crosslinked at physiological conditions by grafting silanes to the HPMC chains (Bourges, Weiss, Daculsi, & Legeay, 2002; Vinatier et al., 2005; Weiss et al., 2008). The silanized hydroxypropyl methyl cellulose (Si-HPMC) is soluble at alkaline condition and transforms into a crosslinked gel after the pH is decreased below 11 due to silanol (SiOH) condensation. (Fatimi, Axelos, Tassin, & Weiss, 2008a; Fatimi, Tassin, Quillard, Axelos, & Weiss, 2008b; Fatimi, Tassin, Turczyn, Axelos, & Weiss, 2009). The rate of gelation increases with increasing temperature and increasing pH between 6 and 10. However the Si-HPMC gels formed in this way have a significantly lower modulus than cartilage.

The objective of the present investigation was to study the effect of reversible thermal gelation of the HPMC backbone on the covalent crosslinking of Si-HPMC. We will compare the case where the covalent gel is made at $T < T_c$ and subsequently heated, with that where the system is heated above T_c so that covalent crosslinking and thermal gelation occur simultaneously. Before discussing the effect of thermal gelation on covalent gelation of Si-HPMC, we show that T_c can be reduced to below 37 °C by addition of orthophosphate rendering this investigation potentially relevant for in vivo

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applications. We have also investigated the effect of varying the pH, because addition of orthophosphate influences the pH.

2. Materials and methods

2.1. Sample preparation

The hydroxypropyl methyl cellulose (Methocel® E4M Premium procured from Dow Chemical) was silanized by grafting 3-glycidoxypropyltrimethoxysilane GPTMS as described elsewhere (Bourges et al., 2002). The desired amount of Si-HPMC powder was dissolved in sodium hydroxide solution (NaOH = 0.2 M) by stirring at room temperature for 24 h, after which the solution was refrigerated for 24 h to ensure complete dissolution of the polymer. Si-HPMC solution was dialyzed (Spectra-por 6000–8000) against 3.8 l of NaOH solution (0.09 M) for 16 h and 4 l of NaOH solution (0.09 M) for 2 h. The final pH value of the basic Si-HPMC solution is 12.7. Pure aqueous HPMC solutions were prepared by dissolving the HPMC powder in water at the desired pH in the same manner.

The acidic buffer solution used to neutralize the basic Si-HPMC solution was prepared using 0.1 M hydrochloric acid and HEPES (4-2-hydroxyethyl piperazine-1-ethanesulfonic acid). The buffer solution was prepared by dissolving 6.2 g of HEPES and 1.8 g of NaCl in 60 ml HCl and the final volume was adjusted to 100 ml. The pH value of the final acidic buffer solution was 3.6. Si-HPMC hydrogels were prepared by rapidly mixing the polymer and buffer (HEPES) in the ratio of 2:1. This led to a reduction of the pH to 7.2 and initiated the silanol condensation. In some cases, the pH was varied by varying the amount of HCl in the buffer. The pH increased slightly during the reaction.

2.2. Methods

The rheological properties of the hydrogels were studied by carrying out oscillatory shear experiments on a stress controlled rheometer (AR G2, TA Instrument) using a cone and plate geometry (40 mm, 2°). The oscillatory stress was set sufficiently low to remain well within the linear response regime. Gelation was monitored by measuring the shear modulus at 1 rad/s. In order to avoid drying, a small amount of low viscosity mineral oil was added at the periphery of the cone.

The turbidity was determined as a function of the temperature using a UV-Visible spectrometer Varian Cary-50 Bio (Les Ulis, France). The path length through the cell was 10 mm and the temperature was controlled using a thermostatic bath. An optical microscope (Leica DMLP) coupled to a temperature controller was used to obtain the images of the gel.

3. Results and discussion

3.1. Thermal gelation of HPMC

As was mentioned in Section 1, thermal gelation of HPMC has been studied in detail in the past. Our aim here is to show that the critical temperature above which the elastic modulus of HPMC increases rapidly can be decreased by addition of sodium orthophosphate. The storage modulus at 1 rad/s for a solution of 2 wt% HPMC during a heating ramp is shown in Fig. 1a. Initially, G' decreased progressively with increasing temperature until it reached a minimum and started to increase with increasing temperature. At a critical temperature (T_c) G' dropped sharply before it increased again. As was discussed in detail by Allabash et al. (2014), this characteristic behavior of HPMC can be explained by a combination of three distinct phenomena. Disordered HPMC chains form a transient network with a terminal relaxation time that

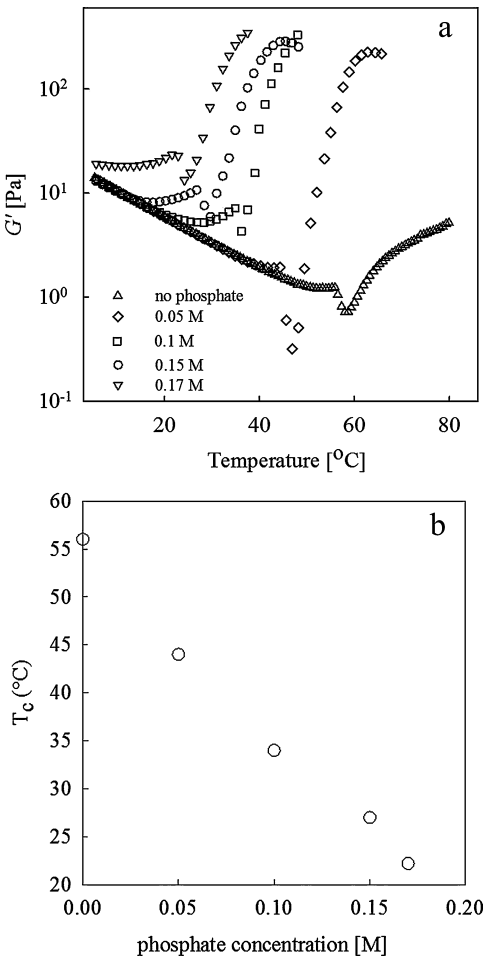

Fig. 1. (a) Evolution of G' at 1 rad/s during a heating ramp at a rate of 5 °C/min for 2 wt% HPMC in the presence of varying amounts of Na_3PO_4. (b) Dependence of the critical temperature on the Na_3PO_4 concentration.

decreases with increasing temperature. With increasing temperature an increasing fraction of the HPMC chains associates into long rigid fibrils that form a permanent network. At lower temperature this permanent network is very weak and G' is dominated by the transient network so that it decreases with increasing temperature. But with increasing temperature the permanent network becomes increasingly stronger and causes an increase of G'. At T_c the HPMC chains are no longer soluble and phase separation sets in, which leads to a collapse of the transient network and causes a sharp drop of G' at T_c. Macroscopic phase separation is inhibited by the permanent network but microphase separation causes the system to become turbid at T_c. The increase in the shear modulus of the permanent network becomes strong for $T > T_c$.

T_c depends on the content of propylene and methylene groups, but for commercially available HPMC it is higher than 50 °C. It can however be decreased by addition of large amounts of salt that reduce hydration of HPMC. Orthophosphate was reported to be particularly effective in reducing T_c (Joshi, 2011). The effect of addition of different amount of Na_3PO_4 is shown in Fig. 1a. Clearly addition of more phosphate induced both gelation and phase separation at lower temperatures. The critical temperature decreased approximately linearly with increasing phosphate concentration, see Fig. 1b. Notice that addition of phosphate changed the pH, but we checked that varying the pH in itself did not modify the thermal gelation of HPMC contrary to the covalent gelation of Si-HPMC that will be discussed in the following section.

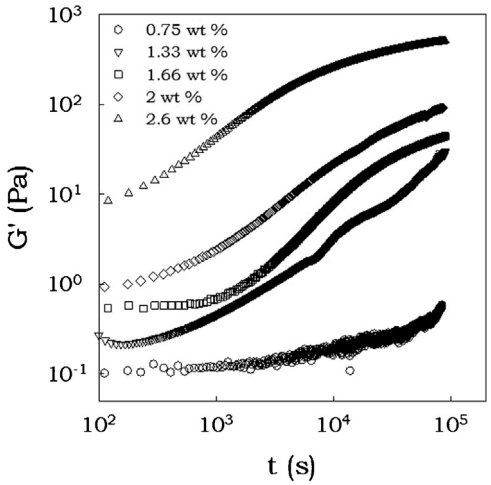

Fig. 2. Evolution of G' at 1 rad/s with time at 37 °C of Si-HPMC solutions at different concentrations after setting the pH to 7.2.

3.2. Covalent gelation of Si-HPMC

Fig. 2 shows the evolution of the storage modulus at 1 rad/s after mixing the Si-HPMC and the buffer at different polymer concentration close to physiological conditions (pH 7.2 and 37 °C). Mixing and loading onto the rheometer took approximately 1 min. Silanol condensation created crosslinks between the HPMC chains and led to the formation of hydrogels with an elastic modulus that increased with time. Gelation was faster at higher polymer concentrations and a weak gel was already formed during mixing at the highest concentration. However, at all concentrations steady state was not reached even after 24 h for the given temperature and pH.

Frequency sweeps between 0.01 and 10 Hz showed that G' was almost independent of the frequency and much larger than G'' confirming that a gel was formed (data not shown) and G' at 1 rad/s is a good approximation of the elastic modulus. The range of polymer concentrations that could be explored was limited, because at lower concentrations no significant increase of G' was found and higher polymer concentrations were too viscous to permit rapid homogeneous mixing with the buffer.

It was shown earlier that the pH has a strong effect on the gelation rate of Si-HPMC (Fatimi et al., 2009), but the effect of the pH on the elastic modulus at steady state has not yet been investigated. In order to determine the pH dependence of the elastic modulus we monitored G' as a function of time at 37 °C for Si-HPMC solutions at different pH. In two cases the pH was set by addition of phosphate. Fig. 3a shows that the storage modulus increased more rapidly at higher pH. A master curve could be obtained by horizontally shifting the results at different pH onto those at pH 7.2 as shown in Fig. 3b, implying that the effect of the pH is only on the kinetics of the reaction and not on the gel stiffness at steady state.

The master curve shows more clearly that after an initial steep increase the elastic modulus continues to increase slowly for a long time after the gel has been formed. The effect of the pH on the gel time was derived from the shift factors that were required to obtain the master curve in Fig. 3b, see Fig. 4. Here we define the gel time as the time when G' is 10 Pa, i.e. at the start of the steep increase. This definition is of course rather arbitrary and a more precise definition can be obtained from studying the frequency dependence (Fatimi et al., 2008a). However the dependence of the gel time on the pH does not depend on its exact definition. It appears that the gel time decreases exponentially with increasing pH in the range covered in the experiment, from pH 5 to pH 9.2. At higher pH gelation is very fast and starts during mixing and loading on the rheometer,

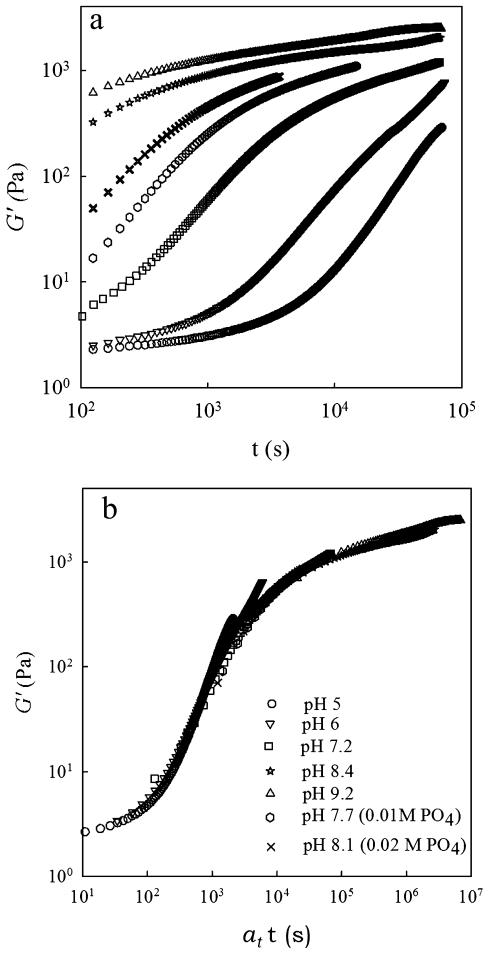

Fig. 3. (a) Evolution of G' at 1 rad/s with time at 37 °C for Si-HPMC at $C = 2.6$ wt% and various values of the pH at steady state. In two cases the pH was set by addition of 0.01 M or 0.02 M Na_3PO_4. (b). Master curve obtained by superposition of the data shown in Fig. 3a using shift factors (a_T) with respect to the reference at pH 7.2.

which explains why G' was already quite large at the start of the experiment. The results obtained in the presence of Na_3PO_4 are in line with the other results which means that gelation of Si-HPMC is not influenced by addition of Na_3PO_4.

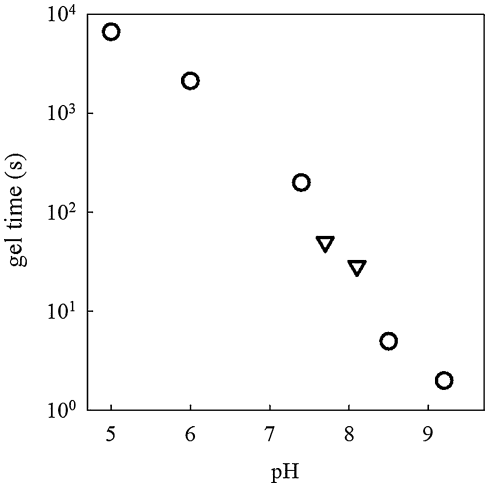

Fig. 4. pH dependence of the gel time for Si-HPMC at 37 °C and $C = 2.6$ wt%. The triangles indicate gels formed in the presence of Na_3PO_4.

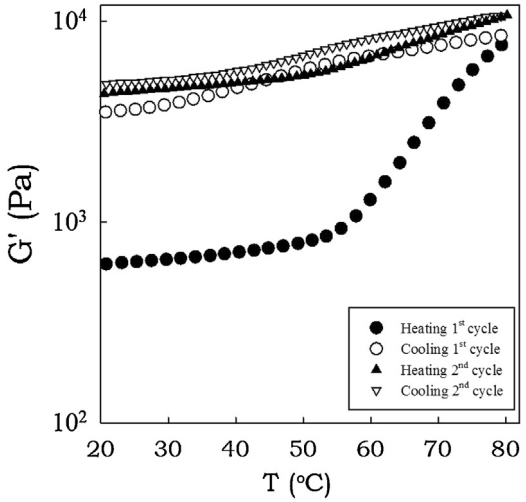

Fig. 5. Evolution of G' during heating (closed) and cooling (open) ramps at a rate of 5 °C/min for 2.6 wt% Si-HPMC. The temperature ramps were carried out after crosslinking at 37° and aging for 1.5 h.

3.3. Covalent and thermal gelation of Si-HPMC

We studied the effect of covalent and thermal gelation of Si-HPMC in two ways. In the first experiment we aged the system at 37 °C which caused covalent gelation without significant thermal gelation. Subsequently we heated the system to 80 °C in order to induce thermal gelation. In a second experiment we heated the solution directly to 80 °C so that covalent and thermal gelation occurred simultaneously.

Fig. 5 shows the effect of thermal gelation of the HPMC backbone on the covalently crosslinked Si-HPMC gel at $C = 2.6$ wt% that was formed below T_c. The Si-HPMC was crosslinked at 37 °C and pH 7.2 during 1.5 h after which the gel was heated from 20 °C to 80 °C at a rate of 5 °C/min. Increasing the temperature did not significantly increase the shear modulus until 55 °C beyond which it increased sharply. The increase started close to T_c of HPMC solutions suggesting that it is caused by thermal gelation of the HPMC backbone of Si-HPMC. During subsequent cooling to 20 °C at the same rate the shear modulus decreased again at a somewhat lower temperature as is also observed for HPMC solutions. However, contrary to the HPMC the modulus of the Si-HPMC gel decreased only slightly upon cooling. When the temperature was raised a second time the modulus increased again above T_c to a slightly higher value than during the first heating ramp and after subsequent cooling to 20 °C, the gel remained somewhat stronger than after the first heating and cooling cycle. A third heating and cooling cycle no longer significantly modified the system indicating that the system had reached steady state.

During aging at 37 °C (below T_c) the gels remained transparent. The effect of heating on the turbidity of gels aged at 37 °C is compared with that of a HPMC solution in Fig. 6. In both cases the turbidity increased sharply at T_c due to microphase separation, which was apparently not inhibited by the covalent crosslinks. The turbidity of the Si-HPMC gel decreased steeply during cooling but contrary to the HPMC solution the gel did not become fully transparent. The fact that the turbidity induced by heating to 80 °C was not completely reversed for the gels aged at 37 °C after cooling to 20 °C indicates that some additional covalent crosslinks were formed at high temperatures. This also explains the observation of higher modulus of this system at 20 °C after the heating cycle.

In a second experiment we heated the Si-HPMC solution immediately to 80 °C and let the system age at this temperature. The rate of silanol condensation increases with increasing temperature and

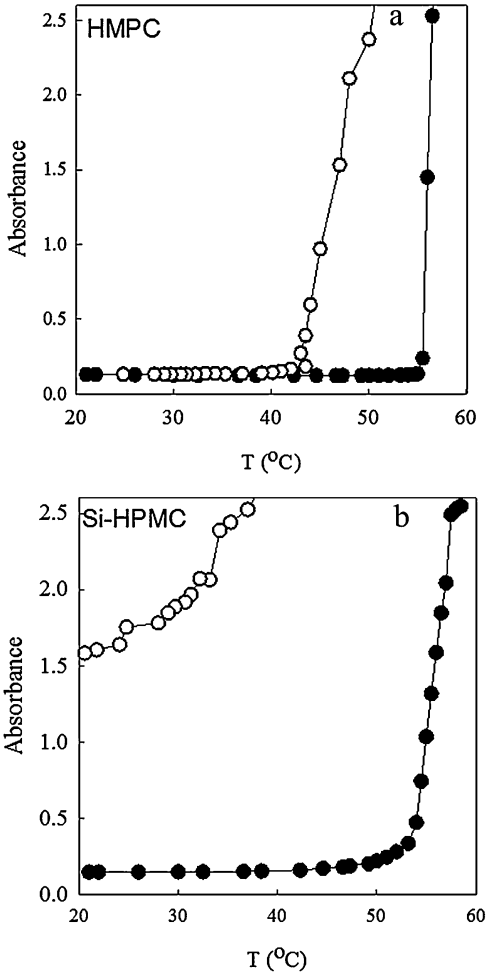

Fig. 6. Evolution of the turbidity during a heating (filled symbols) and cooling (open symbols) ramp for a HPMC solution (top) and a Si-HPMC gel aged at 37 °C (bottom).

it was observed that the elastic modulus reached rapidly a steady state value of about 800 Pa. For this system subsequent cooling and heating cycles gave the same results. In Fig. 7 the evolution of G' during heating and cooling ramps of the gel formed at 80 °C is compared with the one formed at 37 °C. For the latter the third heating and cooling cycle is shown, i.e. when the system had reached steady

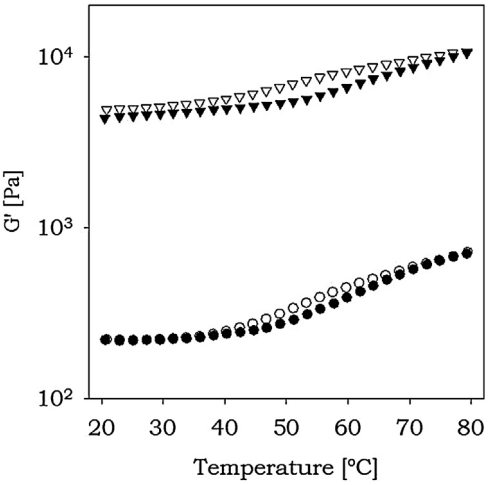

Fig. 7. Evolution of G' for 2.6% Si-HPMC after aging at 37 °C (triangles) or 80 °C (circles) during heating (closed symbols) and cooling (open symbols) cycles at 5 °C/min.

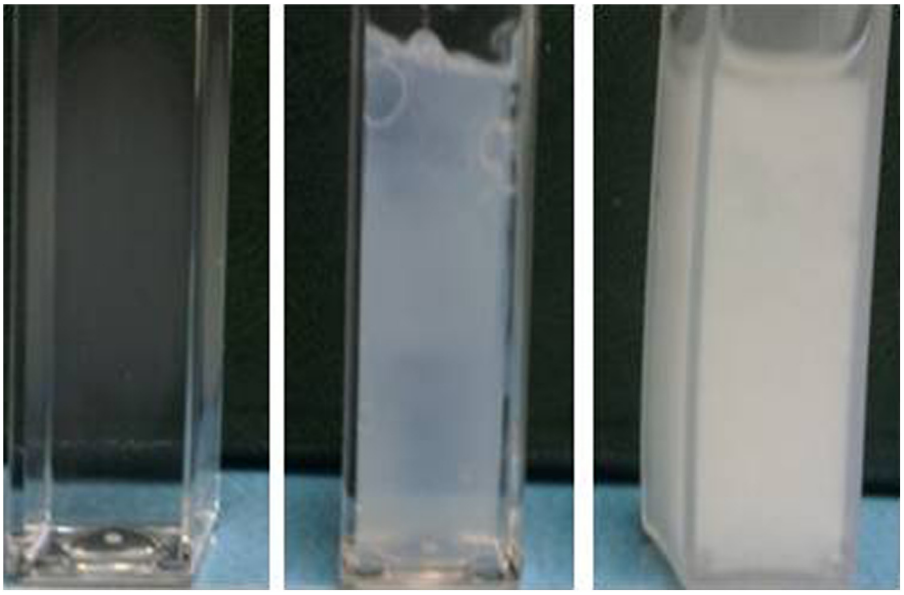

Fig. 8. Photographs taken at 20 °C of a solution of HPMC (left) and gels of Si-HPMC at $C = 2.6$ wt% that were aged at 37 °C (middle) or at 80 °C (right). In each case the samples were heated to 80 °C and subsequently cooled back to 20 °C.

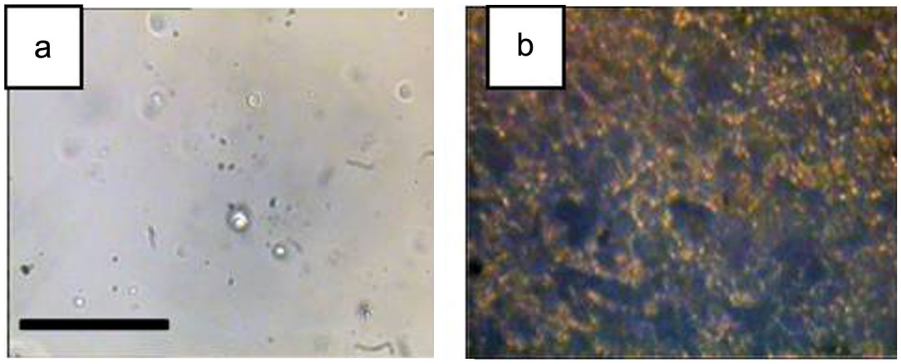

Fig. 9. Optical microscope images taken at 20 °C of a Si-HPMC gel aged at 37 °C before (a) and after (b) heating to 80 °C. The scale bar represents 75 μm.

state. For both systems the elastic modulus increased reversibly with increasing temperature, but it was much higher for the system that was aged at 37 °C. It is clear that covalent crosslinking of Si-HPMC is less effective at 80 °C when the HPMC backbones are microphase separated and have formed a network of rigid fibrils. Si-HPMC gels that were aged at 80 °C remained very turbid after cooling to 20 °C, see Fig. 8, indicating that microphase separation was 'frozen-in' by the covalent crosslinks (Fig. 9).

It was shown in Fig. 1 that T_c can be decreased by adding Na_3PO_4 as was already reported by Joshi (2011) for unmodified HMPC. Therefore the effect of thermal gelation and microphase separation on covalent gelation of Si-HPMC gels at 37 °C can be induced by adding Na_3PO_4. However, whereas Joshi (2011) found that the presence of phosphate rendered HMPC gels stiffer, no significant effect on the elastic modulus was observed for Si-HMPC gels at lower Na_3PO_4 concentrations for which $T_c > 37$ °C. At higher concentrations of Na_3PO_4 so that $T_c < 37$ °C, the elastic modulus of the gels was even lower. These observations corroborate the results shown above that microphase separation reduces the crosslink density of Si-HPMC gels. In order to take advantage of thermal gelation of the HPMC backbone one needs to form the covalent gel first and subsequently induce thermal gelation. This obviously limits the usefulness of this procedure for applications in regenerative medicine.

4. Summary

Thermal gelation of HPMC can be shifted to lower temperatures by adding orthophosphate. Also the critical temperature (T_c) at which HPMC solutions phase separate decreases after addition of orthophosphate from 56 °C in pure water down to 22 °C in 0.17 M Na_3PO_4. The rate of covalently crosslinking Si-HPMC increases exponentially with increasing pH between pH 5 and pH 9.2, but the elastic modulus is not influenced by the pH.

The onset of the strong increase of G' due to thermal gelation of the HPMC backbone takes place at the same temperature when the chains are covalently crosslinked in Si-HPMC gels. Contrary to HPMC gels the reinforcement of Si-HPMC gels is to a large extent maintained after cooling to 20 °C. Si-HPMC gels are transparent when formed below T_c, but become turbid at T_c indicating that microphase separation also takes place in the covalently crosslinked gels. They remain weakly turbid after cooling back to 20 °C, probably due to additional crosslinking at high temperatures that partially 'freezes-in' the microphase separation.

Covalent crosslinking of Si-HPMC is less efficient in thermally set HPMC gels, probably because at $T > T_c$ the HPMC chains are microphase separated. As a consequence, the gels have a significantly lower elastic modulus than those formed at $T < T_c$. When

Si-HPMC gels are crosslinked at $T > T_c$ they remain highly turbid when cooled to room temperature.

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