

Gelation studies of a cellulose-based biohydrogel: the influence of pH, temperature and sterilization.

Ahmed Fatimi, Jean-François Tassin, Roman Turczyn, Monique Axelos,
Pierre Weiss

► **To cite this version:**

Ahmed Fatimi, Jean-François Tassin, Roman Turczyn, Monique Axelos, Pierre Weiss. Gelation studies of a cellulose-based biohydrogel: the influence of pH, temperature and sterilization.. Acta Biomaterialia, Elsevier, 2009, 5 (9), pp.3423-32. 10.1016/j.actbio.2009.05.030 . inserm-00507127

HAL Id: inserm-00507127

<https://www.hal.inserm.fr/inserm-00507127>

Submitted on 29 Jul 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Gelation studies of a cellulose-based biohydrogel: the influence of pH, temperature and sterilization

Ahmed Fatimi^a, Jean-François Tassin^b, Roman Turczyn^c, Monique A. V. Axelos^d, Pierre Weiss^{a,*}

^a*INSERM, U791, Laboratory for Osteo-Articular and Dental Tissue Engineering (LLOAD),
Faculty of Dental Surgery, University of Nantes, 1 place Alexis Ricordeau, 44042 Nantes
Cedex 01, France.*

^b*CNRS, Université du Maine, UMR 6120, Laboratoire Polymères, Colloïdes, Interfaces,
avenue Olivier Messiaen, 72085 Le Mans Cedex 09, France.*

^c*Department of Physical Chemistry and Technology of Polymers, Silesian University of
Technology, Strzody 9, 44-100 Gliwice, Poland.*

^d*UR1268, Unité Biopolymères Interactions Assemblages, INRA, rue de la Géraudière, 44316
Nantes Cedex 03, France.*

*Corresponding author:

Prof. Pierre Weiss
INSERM, U791, LLOAD
Laboratory for Osteoarticular and Dental Tissue Engineering
Faculty of Dental Surgery, University of Nantes
1 place Alexis Ricordeau, 44042 Nantes Cedex 01, France.
Tel.: +33 2 40 41 29 14; Fax: +33 2 40 08 37 12.
E-mail address: pierre.weiss@univ-nantes.fr

Abstract

The present paper investigates the rheological properties of silylated hydroxypropylmethylcellulose (Si-HPMC) biohydrogel used for biomaterials and tissue engineering. The general property of this modified cellulose ether is the occurrence of self-hardening due to silanol condensation subsequently to decreasing pH (from 12.4 to nearly 7.4). The behavior of unsterilized and sterilized Si-HPMC solutions in diluted and concentrated domains is firstly described and compared. In addition, the influence of physiological parameters such as pH and temperature on the rate of the gelation process is studied. In dilute solution, the intrinsic viscosity ($[\eta]$) of different pre-steam sterilization Si-HPMC solutions indicates that macromolecular chains occupy a larger hydrodynamic volume than the post-steam sterilization Si-HPMC. Although the unsterilized Si-HPMC solutions demonstrate no detectable influence of pH upon the rheological behavior, a decrease in the limiting viscosities (η_0) of solutions with increasing pH is observed following steam sterilization. This effect can be explained by the formation of intra and intermolecular associations during the sterilization stage originating from the temperature-induced phase separation. The formation of Si-HPMC hydrogels from injectable aqueous solution is studied after neutralization by different acid buffers leading to various final pH. Gelation time (t_g) decreases when pH increases (t_g varies from 872s to 11s at pH 7.7 and 11.8, respectively). The same effect is observed by increasing the temperature from 20 to 45°C. This is a consequence of the synergistic effect of the increased reaction rate and acid buffer diffusion. pH and temperature are important parameters in the gelation process and their influence is a key factor in controlling gelation time. By adapting the gel parameters one could propose hydrogels with cross-linking properties adapted to clinical applications by controlling the amount of pH of neutralization and temperature.

Keywords: Self-cross-linkable hydrogels, *in situ* gelling, hydroxypropylmethylcellulose; silanisation; rheology.

1. Introduction

In the past few years, several polymers of natural origin have been proposed as alternative materials for application within the tissue engineering field. Numerous hydrogels are already used for this application [1] but none entirely satisfy the applications requested. These polymers, that are capable of forming hydrogels, are of a natural origin (hyaluronic acid, alginate, chitosan, chondroitin sulfate, collagen, fibrin adhesive and cellulose ether) or synthetic (polylactide-co-glycolic, polyethylene glycol, polyvinyl alcohol, polypropylene fumarate, etc.). Reticulated three-dimensional (3D) structures which enable the maintenance and multiplication of cells entrenched in the macromolecular structure made up of more than 90% water are rare. Many teams use hydrogels in order to mimic and to support the growth, multiplication, and differentiation of cells to carry out hybrid regeneration transplants. In tissue engineering, it is crucial to control cell behavior to promote tissue regeneration. For this reason, it is necessary to design suitable biomaterials, able to guide cell response. The nature and the physic chemistry of hydrogels used, controlled the differentiation of stem cells and guide the strategy for tissue regeneration [2]. Today regenerative medicine is moving towards the development of less invasive surgical techniques with the objective of reducing morbidity and the duration of hospitalization. This quest for minimally-invasive surgery has motivated the development of injectable synthetic extracellular matrices. Once implanted, these injectable matrices must also be able to set, acquire the desired form, and present mechanical properties in relation to the tissue to be repaired. Polymers with good viscosifying capabilities in water can be used to make hydrogels via physical, ionic, or covalent cross-linking. In this case, they make veritable 3D macromolecular networks comparable to the extracellular matrices (ECM). Vaccanti *et al.* [3], Miko *et al.* [4] and Mooney *et al.* [5] are amongst the pioneers and the most innovative in the domain of hydrogel physical chemistry for tissue engineering. A lot of polymers and macromolecules, coming from the biology, called

biopolymer, are used in tissue engineering of cartilage. Two types of biopolymers are used to make hydrogels. The first family is macromolecules made of proteins like collagens and the second one family is macromolecules made of polysaccharides like alginate [5], hyaluronic acid [6] or chitin [7].

Among the natural polymers, hydroxypropylmethylcellulose (HPMC) has been one of the most widely studied polymers. HPMC is frequently used as a significant hydrophilic carrier material in many industrial applications such as hydrocarbons research and production [8], and food preparation [9]. Such derivatives are in great demand because of their solubility, gelation properties and their availability in a wide range of molecular weights. Moreover, HPMC possesses appropriate biological properties which make it suitable for biomedical applications [10]. In the biomaterial domain [11], HPMC equally plays a considerable role, one of its most important characteristics being its biocompatibility. In ophthalmic surgery, HPMC and other bioadhesive polymers are used as ophthalmic viscosurgical devices during cataract surgery [12]. For non-invasive surgery and bone repair, HPMC has been used as a matrix in an injectable bone substitute, which has been associated with biphasic calcium phosphate [13].

HPMC is a methylcellulose modified with a small amount of 2-hydroxypropyl groups attached to the anhydroglucose unit of the cellulose. The physicochemical properties of this polymer are highly affected by the methoxyl group content, the hydroxypropyl group content, and the molecular weight.

A novel cellulose-based biohydrogel has been developed for biomedical applications. The polysaccharide used was HPMC (Methocel[®] E4M) grafted with glycidoxypropylsilanes. The first synthesis of silylated polysaccharides was described by Sau *et al.* [14, 15], and Turczyn *et al.* [16] were the first scientists to propose their biomedical applications. The silylated hydroxypropylmethylcellulose (Si-HPMC) appeared as a potential candidate for tissue

engineering [17], as bone [18] and cartilage [19] repair materials. The results of Vinatier *et al.* [20] showed that injectable and self-hardening Si-HPMC was a convenient scaffold for the three-dimensional culture of chondrocytes. This scaffold enabled the growth of phenotypically stable chondrocytes and the synthesis of cartilage extracellular matrix.

The self-hardening principle of the hydrogel is based on the silanes grafted along the Si-HPMC chains (Fig. 1). The dissolution of this product takes place in strong basic media (NaOH), which results in the silane ionization into sodium silanolate ($\text{—SiO}^-\text{Na}^+$). Gel formation is based on the condensation between the silanol groups (—SiOH) by decreasing pH. The Si-HPMC solution is rapidly transformed into Si-HPMC hydrogel with the formation of a three-dimensional network [21] at room or body temperature [17]. Gelation time is an interesting parameter in tissue engineering applications. It is the time needed to manipulate the cells and inject the blend (*i.e.* hydrogel and cells) without a flow character [19]. In case of biomaterials applications, some of them, like endodontic ones need high pH to control acidic pH due to inflammatory process [22].

Cellulose ether solutions are stable up to pH~10, above which viscosity decreases [23]. The literature describes a loss of viscosity when the pH increases due to a slight hydrolysis of the glycosidic linkage, leading to a modification of the macromolecular conformation [24].

The present paper firstly aims at determining the intrinsic viscosity and the rheological properties of Si-HPMC solutions according to pH (NaOH concentration) and the steam sterilization effect. The kinetics of Si-HPMC gelation according to pH and temperature are characterized. Finally, the gelation time is determined by a rheological method and its dependence on pH and temperature further evaluated.

2. Materials and methods

2.1. Polymer synthesis

The hydroxypropylmethylcellulose used in this study is Methocel[®] E4M Premium from the Dow Chemical Company ($M_w=290,000\text{g}\cdot\text{mol}^{-1}$). As specified by the producer, the methoxyl content is 29% and the hydroxypropyl content is 9.7%, corresponding to an average degree of substitution (DS) of 1.9 and molar substitution (MS) of 0.23, respectively. Silane grafting on HPMC involves a Williamson reaction between the hydroxyl function of HPMC and the epoxyde group of silane. The silane used in this study was 3-glycidoxypropyltrimethoxysilane (GPTMS). The principle and procedure of synthesis of Si-HPMC have been described in detail in our previous work [21]. The silane percentage was determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES). The grafted silane percentage (w/w) was calculated at 0.59%.

2.2. Sample preparation

2.2.1. Si-HPMC solutions at different pH

Si-HPMC powders are only soluble at basic pH, the dissolution of the powder being faster when the NaOH concentration is higher. Three different NaOH solutions (0.1, 0.2 and 0.4M) were used. Two different concentrations of HPMC were prepared (2 and 3% w/w) and the different solutions were sterilized by steam at 121°C for 20 min. The pH measurements were performed with a HI 1333B pH electrode (Hanna Instruments[®], UK).

Sterilization was carried out with a 2% concentration even for intrinsic viscosity measurements, after which the sterilized solution was diluted to determine the intrinsic viscosity. The same NaOH solutions were used for dilution as those described above.

2.2.2. Gelation properties of the Si-HPMC hydrogel

Si-HPMC powder at 3% w/w was dissolved in 0.2M NaOH solution (pH~13.1). The resulting Si-HPMC solution was twice dialyzed against NaOH solution (0.09M) using a 6-8kDa D-Tube Dialyzer (Spectra/Por[®], UK). The pH value after dialysis was 12.4. After dialysis, the Si-HPMC solution was steam-sterilized at 121°C for 20 min.

Si-HPMC gelation was performed by decreasing the pH of Si-HPMC with an acid buffer. The final concentration of Si-HPMC was constant. The buffers were formulated in order to control the pH during the gelation step. The formulation of acid buffers was based on 99% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid sodium salt (HEPES) (Aldrich, Germany), NaCl and variable amounts of 0.1M HCl, in order to adjust the final pH.

2.3. Intrinsic viscosity measurements

The intrinsic viscosity ($[\eta]$) was determined experimentally from the measurement of solution viscosity at very low concentration (C) [25] and using a Low Shear 40 rheometer (Contraves, Germany) at 23°C. Since dilute solutions were considered a Newtonian behavior was observed.

Respectively, denoting solution and solvent viscosity as, $\eta_{solution}$ and $\eta_{solvent}$, $[\eta]$ is defined by the following relationships:

$$\eta_{rel} = \frac{\eta_{solution}}{\eta_{solvent}} \quad (1)$$

$$\eta_{sp} = \eta_{rel} - 1 \quad (2)$$

$$[\eta] = \lim_{C \rightarrow 0} \frac{\eta_{sp}}{C} \quad (3)$$

where η_{rel} is the relative viscosity and η_{sp} is the specific viscosity.

The intrinsic viscosity is therefore obtained by extrapolating data using the Huggins [26] and Kraemer [27] relationships (Eqs. (4) and (5), respectively).

$$\frac{\eta_{sp}}{C} = [\eta] + K_h [\eta]^2 C \quad (4)$$

$$\frac{\ln \eta_{rel}}{C} = [\eta] - K_k [\eta]^2 C \quad (5)$$

where K_h is the Huggins constant and K_k is the Kraemer constant.

2.4. Cloud-point determination of HPMC

Cloud-point determinations were carried out using unmodified HPMC (Methocel[®] E4M). Aqueous solutions of HPMC polymer were prepared with deionized water at different concentrations (from 0.25 to 4% w/w). HPMC solutions contained in sealed cylindrical tubes were heated in a temperature-controlled bath to the required temperature at a rate 0.1°C/min between 25 to 70°C.

The onset of the appearance of slight turbidity was visually determined by comparison with a tube containing the cold solution at the same concentration [28].

2.5. Rheological measurements

Rheological measurements were performed using the Haake MARS rheometer (ThermoHaake[®], Germany) with a titanium cone-plate geometry (60mm diameter, 1° cone angle, 52µm gap).

Steady shear tests were carried out at 25°C on different Si-HPMC solutions (2 and 3% w/w). The operating shear rate ranged from 0.1 to 9000s⁻¹. Different flow curves were fitted and extrapolated to lower shear rates by the Cross equation [29].

Oscillatory measurements were performed at 25°C on different Si-HPMC solutions. The complex viscosity (η^*) was determined as a function of oscillation frequency (ω) under conditions of linear viscoelastic response in the range from 0.6 to 600 rad.s⁻¹, at a constant stress amplitude (1Pa).

An appraisal was made on the gelation properties of Si-HPMC according to buffer pH and temperature using Si-HPMC solution (3% w/w at 0.2M NaOH). The time evolution of the storage (G') and loss (G'') moduli were measured within the linear viscoelastic region at different frequencies of 1, 3.2 and 10Hz, respectively.

The time dependence of G' and G'' of the Si-HPMC hydrogel were firstly measured according to pH at a fixed temperature and secondly according to temperature at a fixed pH. The gelation time (t_{gel}) was calculated as the time at which $\tan\delta (= G''/G')$ is independent of frequency, in accordance with the approach proposed by Winter and Chambon [30, 31].

3. Results and discussion

3.1. Viscosimetric study of Si-HPMC solutions

3.1.1. Intrinsic viscosity

Intrinsic viscosity $[\eta]$ has the dimensions of a specific volume. It is related to the hydrodynamic volume occupied by a single macromolecule, which is closely related to the size and conformation of the macromolecular chain in a particular solvent [32]. In dilute solutions, when comparing the $[\eta]$ values of unsterilized and sterilized Si-HPMC at different NaOH concentrations (Table 1), it is clear that the unsterilized Si-HPMC has systematically a greater intrinsic viscosity than the sterilized samples over the whole investigated range of NaOH concentrations. The difference is higher than the fluctuations in the measurements. It can therefore be deduced that Si-HPMC chains became more “compact” after steam sterilization. This can be attributed either to a decrease in the molecular weight due to polymer degradation at elevated temperatures or to aggregation phenomena of an intra- or intermolecular nature. In addition, the influence of the NaOH concentration on hydrodynamic volume of either unsterilized or sterilized Si-HPMC is small or negligible since the intrinsic viscosity values. $[\eta]$ values fluctuate by less than 5% for unsterilized and sterilized samples.

The values of Huggins (K_h) and Kraemer (K_k) constants were determined for each sample. The Huggins constant is a measurement of the polymer-solvent interaction [33]. Theoretically, K_h should lie between 0.3 and 0.8, and values larger than 1 generally imply that polymer-polymer aggregation or ramification are likely to occur [34]. Reports of Huggins parameter values >1.0 are not uncommon for cellulosic derivatives [35, 36]. Huggins values of 0.16~0.4 and 0.39~0.5 were obtained for unsterilized and sterilized Si-HPMC, respectively, reflecting certain differences in our samples and systematic decreases in K_h with the NaOH concentration. It is of interest to note that the magnitude of K_h was slightly lower than published values (0.53~0.54) for non grafted HPMC (Methocel[®] E4M) [21, 35]. The highest value of K_h (0.5) was obtained for sterilized Si-HPMC at the lowest NaOH concentration (0.1M). The Kraemer constant is a second parameter for the polymer-solvent system. Compared to the Huggins constant, K_k is always lower. Kraemer values of 0.14~0.2 and 0.1~0.13 were obtained for unsterilized and sterilized Si-HPMC, respectively. It can thus be deduced that the addition of even a small proportion of silanolate groups leads to a modification of the interactions between macromolecules, especially at the highest ionic strength.

3.1.2. Flow curves of Si-HPMC solutions

Fig. 2 describes typical flow curves for Si-HPMC solutions (2 and 3% w/w) at 25% and at different pH. In all cases, the behavior is shear-thinning with a Newtonian region in the low shear rate range. As expected, Si-HPMC solutions at 2% w/w were less viscous than Si-HPMC solutions at 3% w/w, for similar concentrations of NaOH. The shear-thinning behavior can be regarded as resulting from modifications of the in-solution macromolecular organization as the shear rate changes and is common in entangled polymer solutions, which corresponds to our concentration conditions ($c[\eta]>1$). At low shear rates, the disruption of entanglements by the imposed shear is balanced by the formation of new ones, thus no net

change in entanglements occurs, leading to a constant value for the viscosity, called the zero shear viscosity (*i.e.* limiting Newtonian viscosity η_0). For higher shear rates, disruption predominates over any formation of new entanglements, molecules align in the direction of flow and the apparent viscosity decreases with increasing shear rate. As chain entanglements increase with concentration, the relaxation time corresponding to the transition from Newtonian to shear-thinning behavior moves to higher values.

Before steam sterilization, the concentration of NaOH did not affect the rheological behavior of the Si-HPMC solutions, regardless of the polymer concentration. However, after steam sterilization, a clear decrease in the viscosity of the solutions was observed, which became even more marked when the NaOH concentration was increased. To quantify this effect, the Cross equation [29] (Eq. (6)) was used to match the rheological behavior of Si-HPMC solutions (Table 2).

$$\eta = \frac{\eta_0}{1 + (\lambda\dot{\gamma})^n} \quad (6)$$

where η (Pa.s) is the viscosity at a given shear rate $\dot{\gamma}$ (s^{-1}); λ is the structural relaxation time (s); n is the exponent in the power law regime and η_0 (Pa.s) is the zero shear viscosity (*i.e.*, limiting Newtonian viscosity).

There was a remarkably good match with the model predictions, as illustrated by the satisfactory values of correlation coefficient ($R \sim 0.99$). Table 2 provides the values of the different parameters as a function of concentration and pH, confirming a variation of about 20% over the pH range of the zero shear viscosity of unsterilized samples, as compared to a factor of 3 for sterilized ones. The values of η_0 increased with the increasing concentration of Si-HPMC (Fig. 3) and the exponent of the power law did not depend on pH, or any sterilization step.

3.1.3. Applicability of Cox–Merz rule of Si-HPMC solutions

For many flexible non-associating polymer solutions, the frequency dependence of complex viscosity (η^*) and the shear rate dependence of apparent viscosity (η) are found to be virtually identical, when the same numerical values of shear rate ($\dot{\gamma}$) and frequency (ω) are compared [37]. This empirical correlation is known as the Cox–Merz rule:

$$\eta^*(\omega) = \eta(\dot{\gamma}) \Big|_{\dot{\gamma}=\omega} \quad (7)$$

The Cox-Merz rule was checked by correlating the dynamic and steady shear properties of Si-HPMC solution. The steady state viscosity versus shear rate and the complex viscosity versus frequency of sterilized Si-HPMC solutions (2 and 3% w/w) were plotted together on Fig. 4. Very clearly, the Cox-Merz rule applies to these solutions and the viscosity under flow is always the same as the complex viscosity. Similar behavior was equally observed for unsterilized Si-HPMC solutions (not shown here), thus it can be concluded that both Si-HPMC (unsterilized and sterilized) samples behave like ordinary polysaccharide solutions [38].

3.1.4. The effect of pH and steam sterilization on the properties of Si-HPMC solutions

The intrinsic viscosity ($[\eta]$) and average molecular weight (M_w) can be related according to the Mark-Houwink-Sakurada (M-H-S) relationship [32, 39]:

$$[\eta] = kM_w^a \quad (8)$$

where k is the M-H-S constant and a is the M-H-S exponent.

Parameters k and a depend on polymer, solvent, co-solutes and temperature. Parameter a does not vary with the molecular weight for flexible-chain polymers but it has been reported [32] that a can become a decreasing function of M_w for semi-flexible chains above a critical molecular weight. Both parameters are related to the stiffness of the polymer and almost any value of a ranging from 0.4 to 1.2 has been reported for cellulosic derivatives in aqueous

solution [36]. The parameter k reflects the stereochemistry of the intra-molecular species and cellulosic derivatives are typically described [32] as being comprised of highly expanded coils, leading to high values of k , as a consequence of the 1,4-linkage within the backbone of the polymer chains. The values of the parameters k and a have already been reported for HPMC (Methocel[®] E4M) [39] as 1.86×10^{-4} and 0.82, respectively, for an intrinsic viscosity expressed in dl.g^{-1} and a molecular weight expressed in kDa.

If one assumes that the decrease in intrinsic post-sterilization viscosity is due to a reduction in the molecular weight, the corresponding change can be estimated using the M-H-S equation, provided that the parameters are unchanged for the silane-modified polymer. Table 3 shows that only a moderate change in the molecular weight can be expected (20% for 0.1M NaOH, and dwindling to 8% for 0.4M NaOH). Table 3 also reports the ratio between limiting Newtonian viscosity of unsterilized samples and sterilized ones according to concentration and for various pH values.

Since the viscosity of a concentrated polymer solution depends on the number of entanglements per chain, and thus on the molecular weight, it can be deduced that, the largest drop in the viscosity is expected at the lowest concentration of NaOH, solely on the basis of the molecular weight reduction, which is obviously not observed experimentally, given that the opposite trend is noted. It can therefore be concluded that a decrease in the molecular weight due to the sterilization step, and compatible with the intrinsic viscosity data, does not lead to the observed variations of the zero shear viscosity of the concentrated solutions with the NaOH content.

Phase separation phenomena of Si-HPMC during the sterilization step must also be taken into account and might be responsible for intra or intermolecular associations.

In fact, it has been reported that heating during sterilization leads to a phase separation in the HPMC solution (Methocel[®] E4M), the latter being reversible upon cooling (at least as regards

the clarity of the solutions) [40]. Fig. 5 shows the cloud-point curve (temperature versus concentration) of HPMC solution. The concave shape of the cloud-point curve thus obtained by our procedure indicates that HPMC in water exhibits a lower critical solution temperature (LCST). Below this curve, the system was clear and homogeneous whereas it turned into a homogeneously turbid gel above the curve. This phenomenon was mainly observed for cellulose derivatives [28, 41]. From the minimum in this curve we determined a critical temperature $T_c=50.3^\circ\text{C}$ and a critical polymer concentration $C_c=3\sim 3.5\%$.

The decrease in the intrinsic viscosity, which was measured from the dilution of a 2% steam-sterilized solution, tends to prove that the change involves intramolecular association, although it is carried out in the concentrated area. Such a phenomenon can explain the simultaneous decrease in the viscosity of concentrated post-sterilization solutions, although the detailed role of the pH is still unknown. The determination of the exact nature of the interactions is beyond the scope of this paper and would require long and difficult spectroscopic studies.

Since a sizeable decrease in the viscosity of the concentrated solutions can be observed after sterilization, we could expect a greater decrease in the intrinsic viscosity. It is however possible that the hydrodynamic volume in dilute solution is only slightly affected due to compensation between intermolecular associations which cause it to increase, and intramolecular associations which act in the reverse way.

3.2. Gelation properties of Si-HPMC hydrogel

3.2.1. Gelation study of Si-HPMC hydrogel

The storage (elastic) modulus G' provides information about the elasticity or energy stored in the material during deformation, whereas the loss (viscous) modulus G'' describes the viscous character or energy dissipated on heating. The ratio between the viscous and the elastic

modulus is expressed by the loss tangent $\tan\delta (= G''/G')$ where δ is the phase angle. The loss tangent is a measurement of the ratio of energy lost against energy stored in a cyclic deformation.

Progressive gelation of Si-HPMC solution (3% w/w, at 0.2M of NaOH), mixed with acid buffer (1v0.5) was investigated rheologically by the time evolution of both storage (G') and loss (G'') moduli at 25°C (Fig. 6). Initially, G' was lower than G'' . Both moduli increase with time, however, the faster increase with time of G' compared to G'' prompts a crossover point before reaching a pseudo plateau for both moduli. This dramatic increase in G' is attributed to the initial fast rate of the network formation, facilitated by the high release of acid buffer, which instantly decreases the sample pH from 12.4 to near 7.4. The pH just after the blend of Si-HPMC and the buffer solution was 7.4. Eight minutes after the mixture the pH decrease a little to reach 7.2 and stay stable for more than 3 days.

Fig. 6 clearly indicates that both moduli depend on frequency. After a long period of gelation, G' became independent of frequency but G'' was still slightly dependent on frequency (not shown here). As shown in Fig. 6, the crossover point depends on frequency, and is therefore inappropriate to define gel time (t_{gel}). The Winter and Chambon [30, 31] criterion to determine the gel point, defines t_{gel} as the unique point where the curves of $\tan\delta (= G''/G')$ cross for all investigated frequencies (Fig. 6).

The effects of key parameters, *i.e.* pH and temperature on the gelation of Si-HPMC were investigated via their influence on gelation time (t_{gel}) determined in accordance with the previous method.

3.2.2. Influence of pH on gelation time of Si-HPMC hydrogel

For cartilage tissue engineering applications, when chondrocytes are blended with the Si-HPMC hydrogel the best pH is 7.4 [19]. When this hydrogel is used for dental tissue engineering applications as a biomaterial injected with or without calcium phosphate granules

to fill a tissue defect the pH could be basic to balance the acidic level of the inflammatory process [22]. In case of infected tissue, in specific area like apical zone of teeth we can reach a high pH value like 11.5 or 12 that is equivalent to calcium hydroxide pH used in this indication.

The neutralization behavior of Si-HPMC/buffer solutions is evidently a key characteristic. Gelation time, for example, determined by rheological measurements, is greatly affected by the pH of a prepared Si-HPMC/buffer solution (Fig. 7). To decrease pH of Si-HPMC solutions, different acid buffers (different pH) were used at a final concentration of Si-HPMC (2%). The final pH of post-neutralization hydrogel was measured and gelation time was determined.

The temporal evolution of G' and G'' , measured for different Si-HPMC/buffer, determined the dependence of gelation time on the final pH of the hydrogel. Gelation time decreased when pH increased. Gelation time varied from 872s (pH 7.4) to 11s (pH 11.8). Increasing pH accelerated the gelation process and enabled us to tailor the required gelation time. The rate of silanol condensation was minimum near the neutral pH and increased both at lower (acid) and higher (basic) pH [42, 43].

3.2.3. The influence of temperature on Si-HPMC hydrogel gelation time

The temporal evolution of G' and G'' , measured for a Si-HPMC hydrogel at constant pH (7.4) and various temperatures helped determine the dependence of gelation time on temperature (Fig. 8).

Fig. 8a shows a decrease in gelation time (t_{gel}) with an increase in temperature from 20 to 45°C. The linear relationship between $\ln(t_{gel})$ and $1/T$ depicted in Fig. 8b, suggests that the temperature dependence of gelation can be represented by an Arrhenius equation [44] (Eq. (9)).

$$\ln(t_{gel}) = A + \frac{E_a}{RT} \quad (9)$$

where A is a constant, R the ideal gas constant ($8.314\text{J.K}^{-1}.\text{mol}^{-1}$ of monomeric units), and T the temperature (K).

Calculation of the activation energy (E_a) of the condensation reaction, using the above equation, gives $74.3\text{kJ}.\text{mol}^{-1}$.

At pH 7.4, we demonstrated the decrease of the gelation time by increasing the temperature. This behavior could be explained by catalytic action of temperature on the silanol condensation[45]. It might also be possible that, at high temperature (45°C or higher) additional mechanisms, such as the association of the hydrophobic zones in the polysaccharide chain, can favor the essential network formation process due to the silanol condensation (pH effect). Indeed, this temperature range is close to the LCST curve and associations between polymer segments are likely to occur.. Hussain *et al.* have already demonstrated that for HPMC (Methocel[®] E4M) the water solution clouding point was observed at 42°C and phase separation occurred at 55°C , confirming physical gel formation [40]. In our case, phase separation of the HPMC water solution was observed at 50.3°C , but this macroscopic phenomenon is only observed when the size of the aggregates allows light scattering. Furthermore; silanisation of HPMC could probably lower the temperature of this phenomenon but further detailed investigations are required on this subject.

4. Conclusions

In the present work the Si-HPMC self-hardening hydrogel was studied. The hydrogel was formed by condensation of silanol moieties using acid buffers as neutralizing agents. Si-HPMC solutions were firstly studied without initiating the crosslinking reaction to characterize the influence of pH and sterilization. Intrinsic viscosity measurements revealed that hydrodynamic volume decreased after steam sterilization with a weak effect of pH. The

influence of pH and steam sterilization for the concentrated solutions was also studied. Although at a fixed Si-HPMC concentration, the limiting viscosity of unsterilized samples was stable according to NaOH concentration; this was no longer the case after sterilization, where a drop (up to factor 3) in viscosity was observed, which was extended by increasing NaOH concentration. This change has been attributed to the formation of both intra and intermolecular associations during the phase separation process.

The study of the Si-HPMC gelation properties showed a steadfast influence of the pH and temperature on gelation time. In general, gelation accelerates with an increased pH of the Si-HPMC hydrogel. Gelation time decreased as temperature increased from 20 to 45°C due to an increased rate of the condensation process. In conclusion, pH and temperature are important parameters in the gelation process and their influence is a key factor in controlling gelation time.

Acknowledgements

This present work was supported by the regional program BIOREGOS (Pays de la Loire, France). Authors wish to express their sincere thanks to Colorcon Limited for the supply of the polymer Methocel[®] E4M.

References

- [1] Drury JL, Mooney DJ. Hydrogels for tissue engineering: scaffold design variables and applications. *Biomaterials*. 2003;24(24):4337-51.
- [2] Hwang NS, Varghese S, Elisseeff J. Controlled differentiation of stem cells. *Adv Drug Deliv Rev*. 2008;60(2):199-214.
- [3] Vacanti CA, Langer R, Schloo B, Vacanti JP. Synthetic polymers seeded with chondrocytes provide a template for new cartilage formation. *Plast Reconstr Surg*. 1991;88(5):753-9.
- [4] Temenoff JS, Mikos AG. Injectable biodegradable materials for orthopedic tissue engineering. *Biomaterials*. 2000;21(23):2405-12.
- [5] Alsberg E, Anderson KW, Albeiruti A, Rowley JA, Mooney DJ. Engineering growing tissues. *Proc Natl Acad Sci U S A*. 2002;99(19):12025-30.
- [6] Borzacchiello A, Mayol L, Ramires PA, Pastorello A, Bartolo CD, Ambrosio L, et al. Structural and rheological characterization of hyaluronic acid-based scaffolds for adipose tissue engineering. *Biomaterials*. 2007;28(30):4399-408.
- [7] Chenite A, Chaput C, Wang D, Combes C, Buschmann MD, Hoemann CD, et al. Novel injectable neutral solutions of chitosan form biodegradable gels in situ. *Biomaterials*. 2000;21(21):2155-61.
- [8] Sovilj VJ, Petrovića LB. Influence of hydroxypropylmethyl cellulose–sodium dodecylsulfate interaction on the solution conductivity and viscosity and emulsion stability. *Carbohydrate Polymers*. 2006;64(1):41-9.
- [9] Rosella CM, Foegeding A. Interaction of hydroxypropylmethylcellulose with gluten proteins: Small deformation properties during thermal treatment. *Food Hydrocolloids*. 2007;21(7):1092-100.
- [10] Hardya IJ, Windberg-Baarup A, Neria C, Bywaya PV, Boothia SW, Fitzpatrick S. Modulation of drug release kinetics from hydroxypropyl methyl cellulose matrix tablets using polyvinyl pyrrolidone. *International Journal of Pharmaceutics*. 2007;337(1-2):246-53.
- [11] Fatimi A. Rheological behavior of biomaterials for osteoarticular and dental tissue engineering: Synthetic extracellular matrix and calcium phosphate suspensions. *IRBM*. 2009;(doi:10.1016/j.irbm.2008.11.001).
- [12] Maltese A, Borzacchiello A, Mayol L, Bucolo C, Maugeri F, Nicolais L, et al. Novel polysaccharides-based viscoelastic formulations for ophthalmic surgery: rheological characterization. *Biomaterials*. 2006;27(29):5134-42.
- [13] Weiss P, Gauthier O, Bouler JM, Grimandi G, Daculsi G. Injectable bone substitute using a hydrophilic polymer. *Bone*. 1999;25(2 Suppl):67S-70S.
- [14] Sau AC. Self-crosslinkable sialated polysaccharides. American Chemical Society, Polymer Preprints, Division of Polymer Chemistry. 1990;31(1):636-7.

- [15] Sau AC, Majewicz TG. Cellulose ethers: self-cross-linking mixed ether silyl derivatives ACS Symp Ser 1992;476:265-72.
- [16] Turczyn R, Weiss P, Lapkowski M, Daculsi G. In situ self hardening bioactive composite for bone and dental surgery. *J Biomater Sci Polym Ed.* 2000;11(2):217-23.
- [17] Fatimi A, Axelos MAV, Tassin JF, Weiss P. Rheological characterization of self-hardening hydrogel for tissue engineering applications: gel point determination and viscoelastic properties. *Macromolecular Symposia.* 2008;266(1):12-6.
- [18] Laïb S, Fellah BH, Fatimi A, Quillard S, Vinatier C, Gauthier O, et al. The in vivo degradation of a ruthenium labelled polysaccharide-based hydrogel for bone tissue engineering. *Biomaterials.* 2009;30(8):1568-77.
- [19] Vinatier C, Gauthier O, Fatimi A, Merceron C, Masson M, Moreau A, et al. An injectable cellulose-based hydrogel for the transfer of autologous nasal chondrocytes in articular cartilage defects. *Biotechnol Bioeng.* 2009;102(4):1259-67.
- [20] Vinatier C, Magne D, Weiss P, Trojani C, Rochet N, Carle GF, et al. A silanized hydroxypropyl methylcellulose hydrogel for the three-dimensional culture of chondrocytes. *Biomaterials.* 2005;26(33):6643-51.
- [21] Fatimi A, Tassin JF, Quillard S, Axelos MAV, Weiss P. The rheological properties of silated hydroxypropylmethylcellulose tissue engineering matrices. *Biomaterials.* 2008;29(5):533-43.
- [22] Enkel B, Dupas C, Armengol V, Akpe Adou J, Bosco J, Daculsi G, et al. Bioactive materials in endodontics. *Expert Rev Med Devices.* 2008;5(4):475-94.
- [23] Bourges X, Weiss P, Daculsi G, Legeay G. Synthesis and general properties of silated-hydroxypropyl methylcellulose in prospect of biomedical use. *Adv Colloid Interface Sci.* 2002;99(3):215-28.
- [24] Bohic S, Weiss P, Roger P, Daculsi G. Light scattering experiments on aqueous solutions of selected cellulose ethers: contribution to the study of polymer-mineral interactions in a new injectable biomaterial. *J Mater Sci Mater Med.* 2001;12(3):201-5.
- [25] McMillan DE. A comparison of five methods for obtaining the intrinsic viscosity of bovine serum albumin. *Biopolymers.* 1974;13:1367-71.
- [26] Huggins ML. The viscosity of dilute solutions of long-chain molecules. IV. Dependence on concentration. *Journal of the American Chemical Society.* 1942;64:2716-8.
- [27] Kraemer EO. Molecular weights of celluloses and cellulose derivatives. *Industrial and Engineering Chemistry.* 1938;30:1200-3.
- [28] Chevillard C, Axelos MAV. Phase separation of aqueous solution of methylcellulose. *Colloid and Polymer Science.* 1997;275(6):537-45.
- [29] Cross MM. Rheology of non-Newtonian fluids: a new flow equation for pseudoplastic systems. *J Colloid Sci.* 1965;20:417-26.

- [30] Winter HH, Chambon F. Analysis of linear viscoelasticity of a crosslinking polymer at the gel point. *Journal of Rheology*. 1986; 30:367-82.
- [31] Chambon F, Winter HH. Linear viscoelasticity at the gel point of a crosslinking PDMS with imbalanced stoichiometry. *Journal of Rheology*. 1987;31:683-97.
- [32] Lapasin R, Prici S. Rheology of polysaccharide systems. *Rheology of industrial polysaccharides: Theory and applications*. Glasgow: Blackie; 1995. p. 250-494.
- [33] Morris ER, Cutler AN, Ross-Murphy SB, Rees DA. Concentration and shear rate dependence of viscosity in random coil polysaccharide solutions. *Carbohydrate Polymers*. 1981;1(1):5-21
- [34] Lai LS, Chiang HF. Rheology of decolorized hsian-tsoa leaf gum in the dilute domain. *Food Hydrocolloids*. 2002;16:427-40.
- [35] Nilsson S, Sundelöf L-O, Porsch B. On the characterization principles of some technically important water soluble non-ionic cellulose derivatives. *Carbohydrate Polymers*. 1995;28(3):265-75.
- [36] Brandrup J, Immergut EH. *Polymer handbook*. 3 ed. New York: Wiley-Interscience; 1989.
- [37] Cox WP, Merz EH. Correlation of dynamic and steady viscosities. *Journal of Polymer Science*. 1958;28:619-22.
- [38] Ross-Murphy SB, Shatwell KP. Polysaccharide strong and weak gels. *Biorheology*. 1993;30(3-4):217-27.
- [39] Keary CM. Characterization of METHOCEL cellulose ethers by aqueous SEC with multiple detectors. *Carbohydrate Polymers*. 2001;45(3):293-303.
- [40] Hussain S, Keary C, Craig DQM. A thermorheological investigation into the gelation and phase separation of hydroxypropyl methylcellulose aqueous systems. *Polymer* 2002;43(21):5623-8.
- [41] Desbrières J, Hirrien M, Ross-Murphy SB. Thermogelation of methylcellulose: rheological considerations. *Polymer*. 2000; 41 2451-61.
- [42] Michael A. *Silicon in organic, organometallic, and polymer chemistry*. New York: Brook John Wiley & Sons; 2000.
- [43] Rytger-Kricheldorf H. *Silicon in polymer synthesis*. Berlin, Heidelberg: Springer-Verlag; 1996.
- [44] Nickerson MT, Patel J, Heyd DV, Rousseau D, Paulson AT. Kinetic and mechanistic considerations in the gelation of genipin-crosslinked gelatin. *Int J Biol Macromol*. 2006;39(4-5):298-302.
- [45] Bourges X, Weiss P, Coudreuse A, Daculsi G, Legeay G. General properties of silylated hydroxyethylcellulose for potential biomedical applications. *Biopolymers*. 2002;63(4):232-8.

Figure captions

Fig. 1. Reactions of grafting and neutralization for the synthesis of Si-HPMC.

Fig. 2. Viscosity (η) versus shear rate ($\dot{\gamma}$) at 25°C for different Si-HPMC solutions (2 and 3% w/w) before (NS) and after (S) steam sterilization. The full line represents predictions of the Cross model.

Fig. 3. Limiting viscosity (η_0) versus concentration of NaOH (0.1, 0.2 and 0.4M) at 25°C for Si-HPMC solutions (2 and 3% w/w) before (NS) and after (S) steam sterilization.

Fig. 4. Cox-Mertz representation of sterilized Si-HPMC solutions (2 and 3% w/w) obtained from steady-state flow data (0.1M (Δ), 0.2M (\diamond) and 0.4M (\circ)) and dynamic data (0.1M (\blacktriangle), 0.2M (\blacklozenge) and 0.4M (\bullet)).

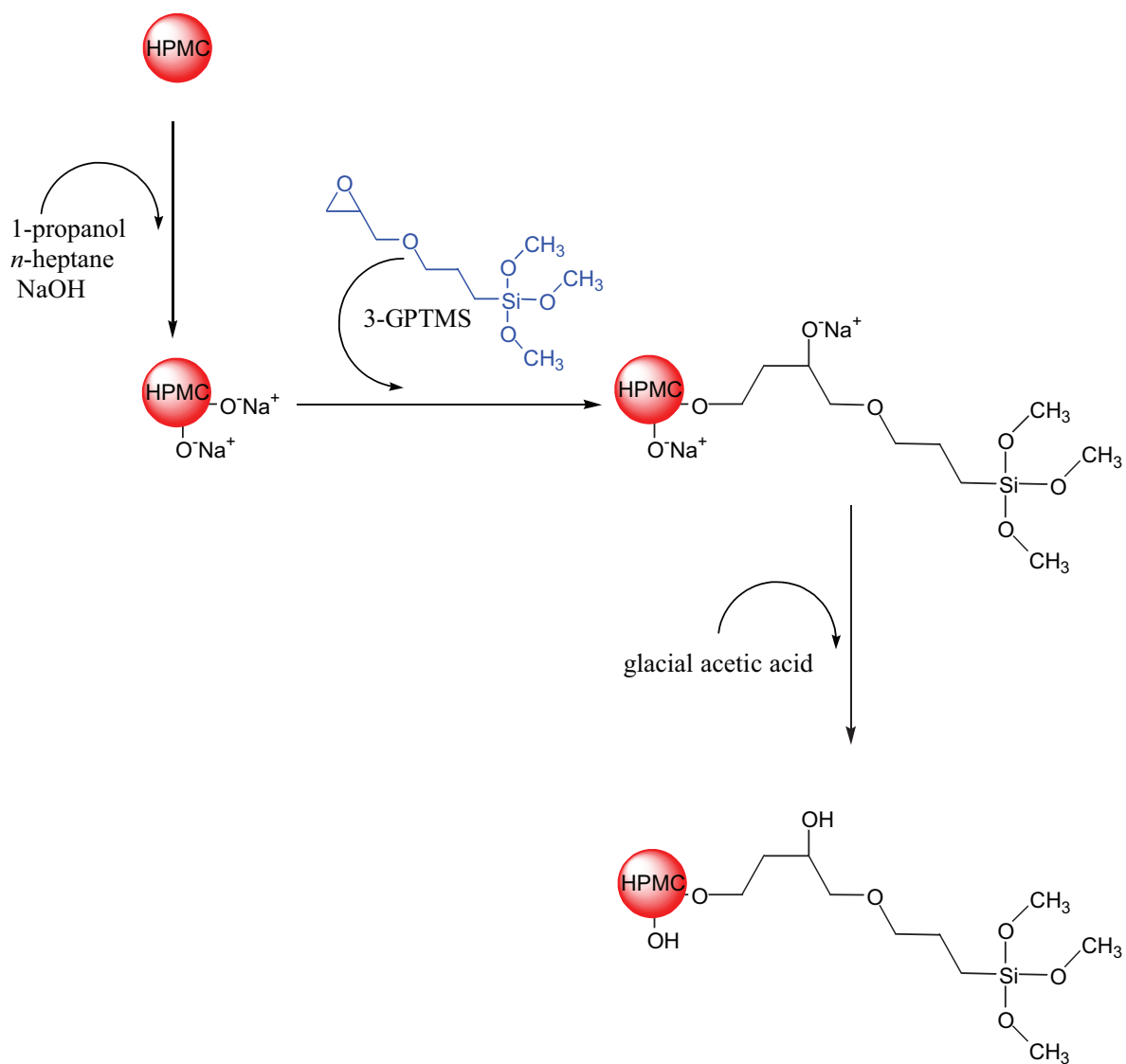
Fig. 5. Cloud-point as a function of the HPMC concentration in water. HPMC solutions contained in sealed cylindrical tubes were heated in a temperature-controlled bath to the required temperature at a rate 0.1°C/min between 25 to 70°C.

Fig. 6. Time evolution of G' (closed symbols) and G'' (open symbols) at 25°C for Si-HPMC (3%) hydrogel at pH 7.4. Three frequencies were applied (1, 3.2 and 10Hz) at fixed total shear stress (1Pa). The $\tan\delta$ is shown in the inset versus the frequency for Si-HPMC hydrogel where gel time (t_{gel}) is indicated.

Fig. 7. Gelation time as a function of pH of the Si-HPMC solution (Si-HPMC 3% w/w; NaOH 0.2M) at 25°C. The pH differences were generated by altering the pH of buffer solution used to dissolve a constant amount of Si-HPMC.

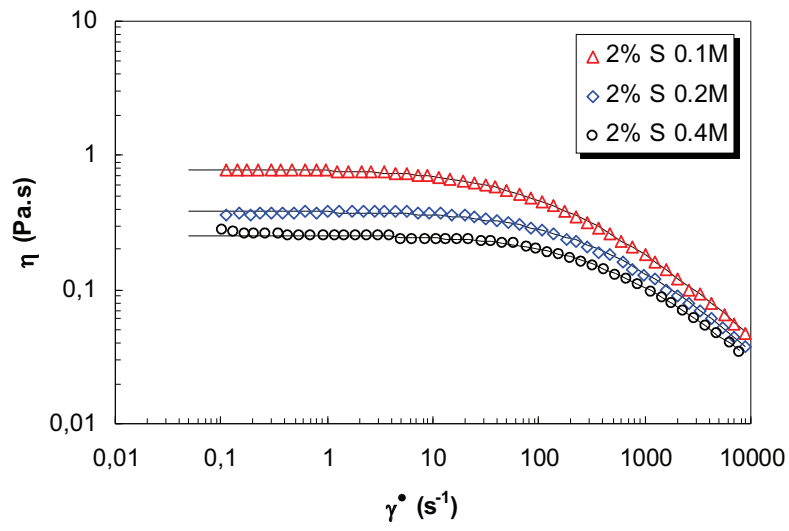
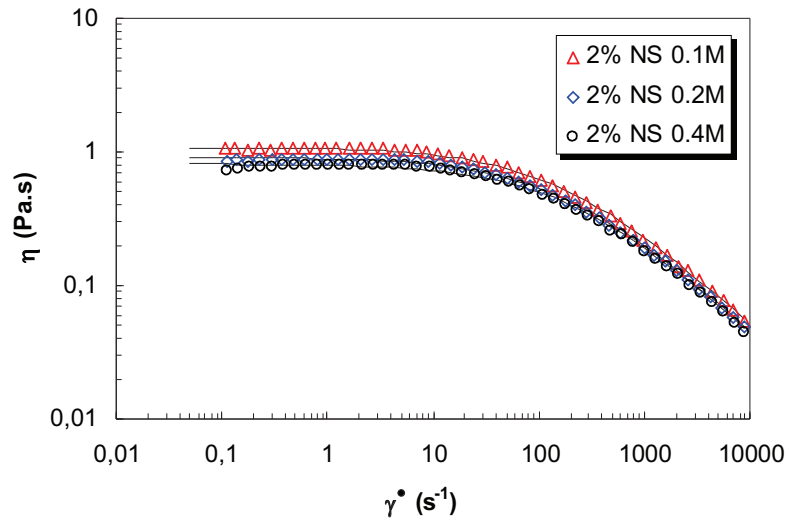
Fig. 8. Variation of gelation time (t_{gel}) as a function of temperature for Si-HPMC for Si-HPMC (3%) hydrogel at pH 7.4: (a) decrease in t_{gel} with the temperature. and (b) linear relationship of $\ln(t_{gel})$ versus $1/T$.

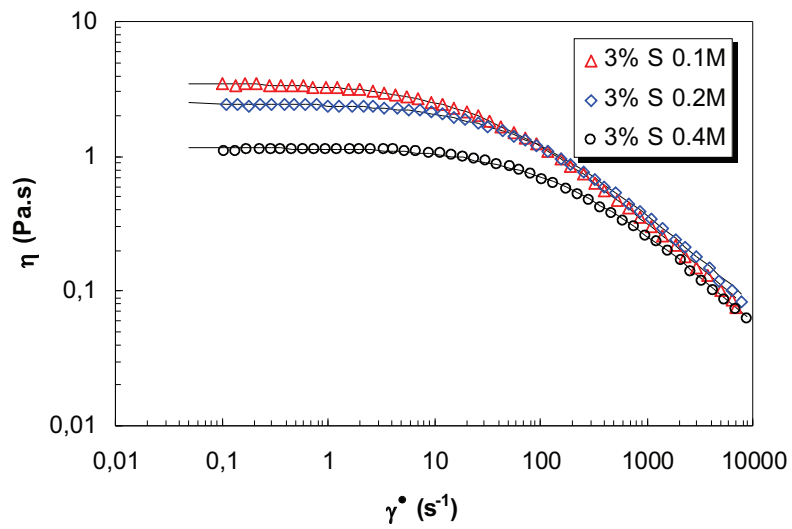
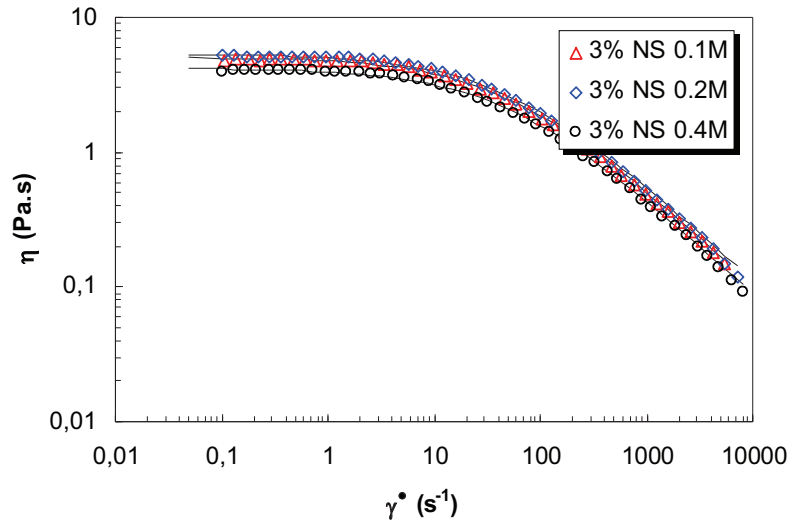
Figure 1



Reactions of grafting and neutralization for the synthesis of Si-HPMC.

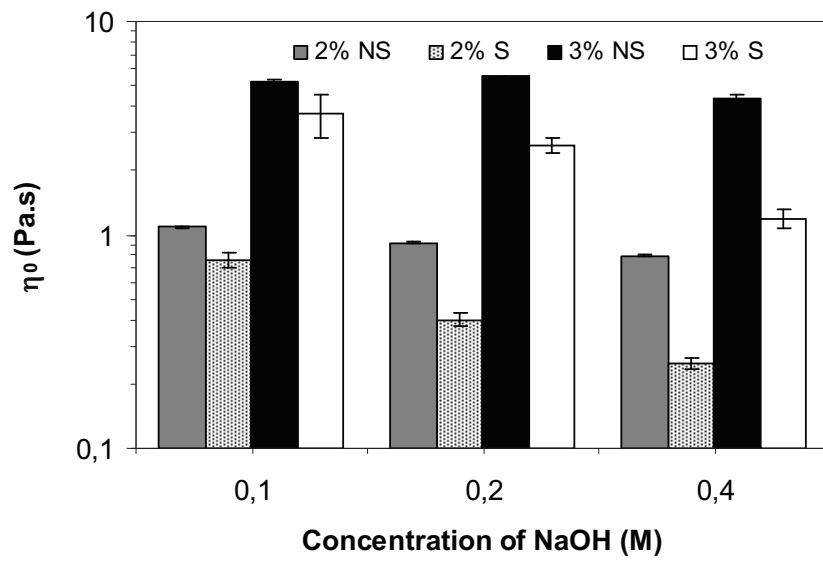
Figure 2





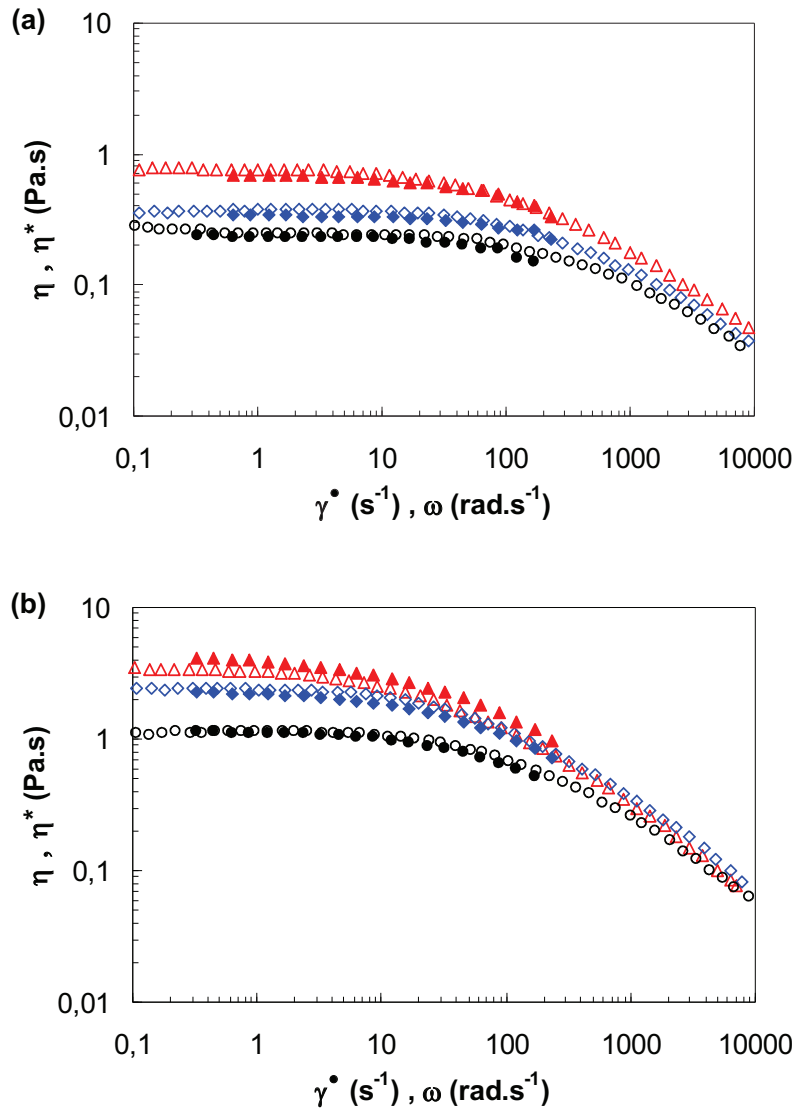
Viscosity (η) versus shear rate ($\dot{\gamma}$) at 25°C for different Si-HPMC solutions (2 and 3% w/w) before (NS) and after (S) steam sterilization. The full line represents predictions of the Cross model.

Figure 3



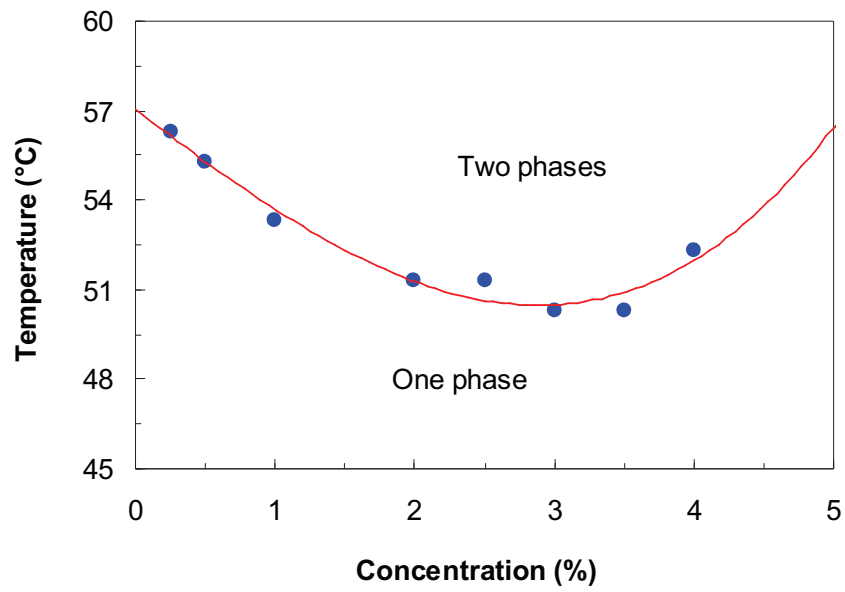
Limiting viscosity (η_0) versus concentration of NaOH (0.1, 0.2 and 0.4M) at 25°C for Si-
HPMC solutions (2 and 3% w/w) before (NS) and after (S) steam sterilization.

Figure 4



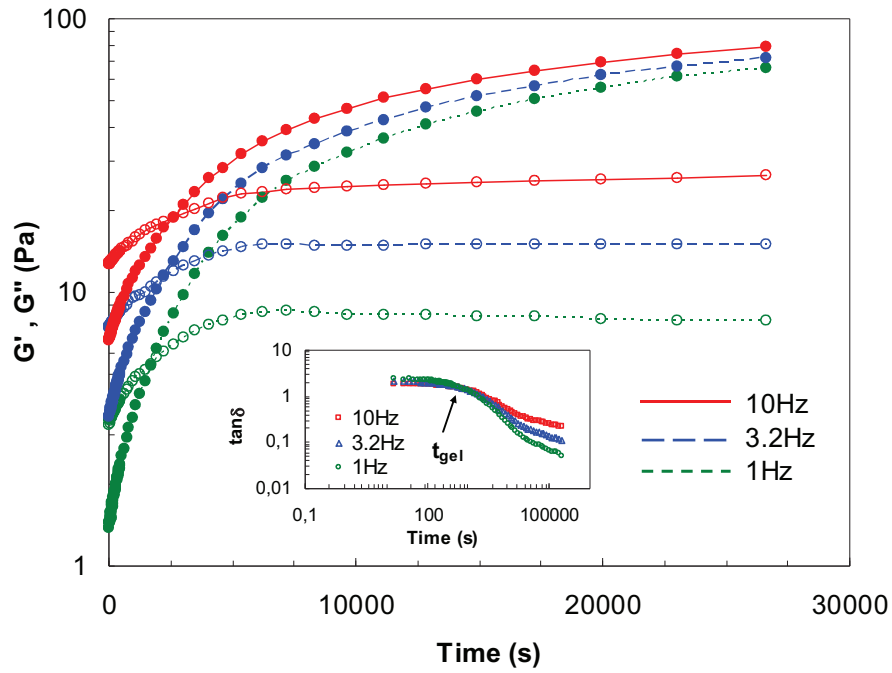
Cox-Mertz representation of sterilized Si-HPMC solutions (2 and 3% w/w) obtained from steady-state flow data (0.1M (Δ), 0.2M (\diamond) and 0.4M (\circ)) and dynamic data (0.1M (\blacktriangle), 0.2M (\blacklozenge) and 0.4M (\bullet)).

Figure 5



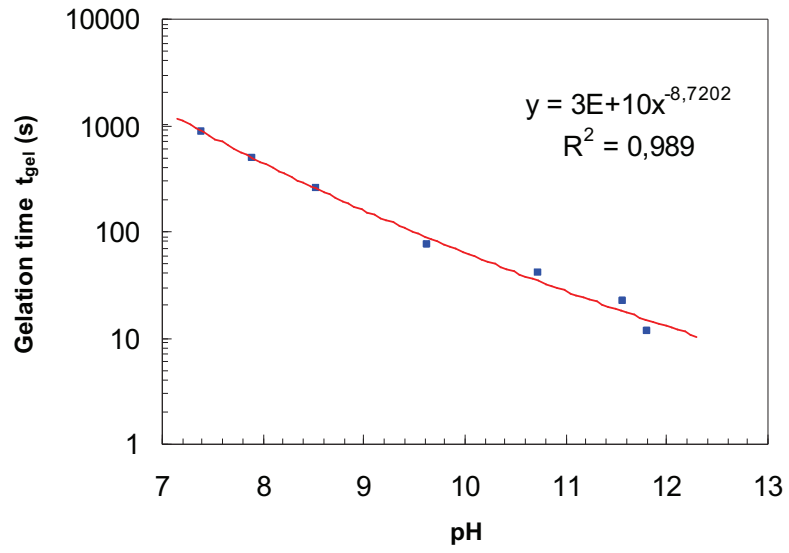
Cloud-point as a function of the HPMC concentration in water. HPMC solutions contained in sealed cylindrical tubes were heated in a temperature-controlled bath to the required temperature at a rate $0.1^{\circ}\text{C}/\text{min}$ between 25 to 70°C .

Figure 6



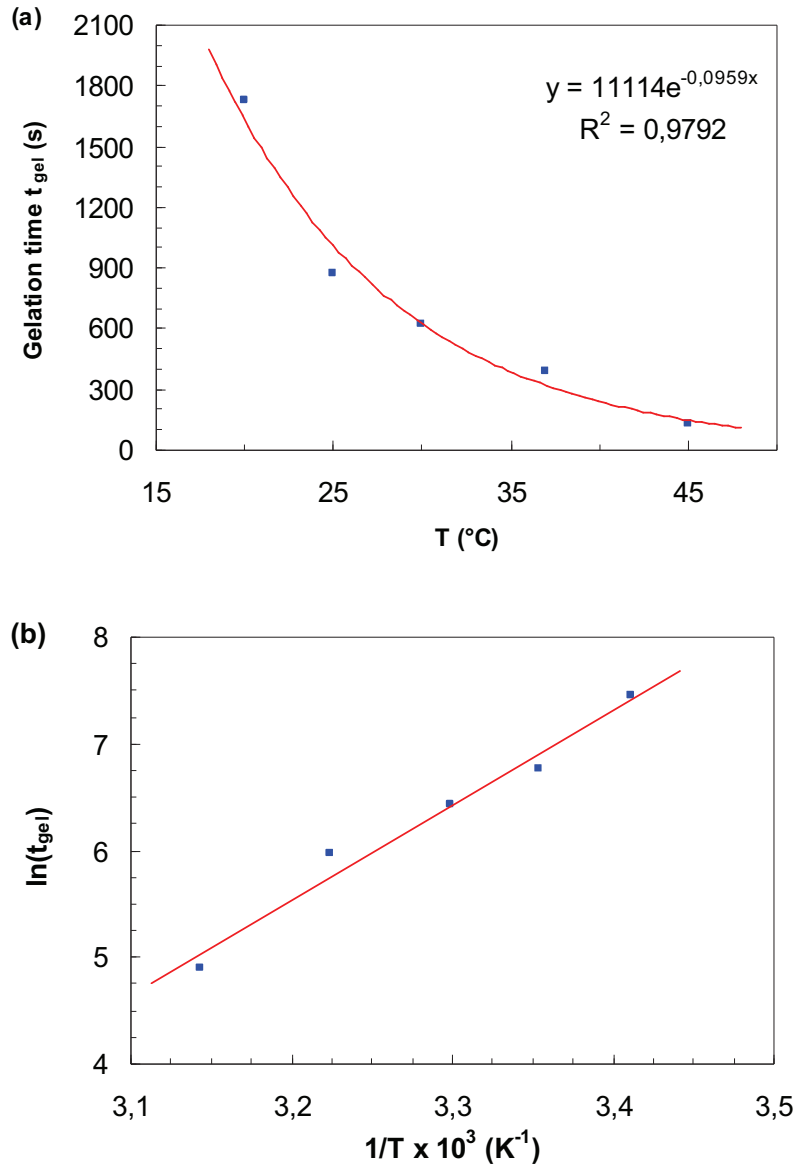
Time evolution of G' (closed symbols) and G'' (open symbols) at 25°C for Si-HPMC (3%) hydrogel at pH 7.4. Three frequencies were applied (1, 3.2 and 10Hz) at fixed total shear stress (1Pa). The $\tan \delta$ is shown in the inset versus the frequency for Si-HPMC hydrogel where gel time (t_{gel}) is indicated.

Figure 7



Gelation time as a function of pH of the Si-HPMC solution (Si-HPMC 3% w/w; NaOH 0.2M) at 25°C. The pH differences were generated by altering the pH of buffer solution used to dissolve a constant amount of Si-HPMC.

Figure 8



Variation of gelation time (t_{gel}) as a function of temperature for Si-HPMC for Si-HPMC (3%)

hydrogel at pH 7.4: (a) decrease in t_{gel} with the temperature. and (b) linear relationship of

$\ln(t_{gel})$ versus $1/T$.

Table 1

Intrinsic viscosity obtained for different Si-HPMC samples before and after steam sterilization.

Sterilization	NaOH (M)	pH	$[\eta]$ (dl.g ⁻¹)	K_h	K_k
Unsterilized	0.1	12.8	6.85 ± 0.08	0.40	0.14
	0.2	13.1	6.55 ± 0.03	0.35	0.15
	0.4	13.4	6.52 ± 0.10	0.16	0.20
Sterilized	0.1	12.8	5.96 ± 0.08	0.50	0.10
	0.2	13.1	5.92 ± 0.03	0.39	0.14
	0.4	13.4	6.12 ± 0.02	0.39	0.13

Table 2

Magnitudes of the Cross model parameters obtained for unsterilized and sterilized Si-HPMC at different concentrations and pH. Results are expressed as mean \pm standard error of triplicate determinations.

Si-HPMC (% w/w)	Sterilization	NaOH (M)	η_0 (Pa.s)	$\lambda \times 10^{-3}$ (s)	n
2	Unsterilized	0.1	1.09 \pm 0.01	6.29 \pm 0.14	0.72 \pm 0.00
		0.2	0.92 \pm 0.01	5.83 \pm 0.11	0.72 \pm 0.00
		0.4	0.80 \pm 0.02	5.04 \pm 0.26	0.72 \pm 0.00
	Sterilized	0.1	0.76 \pm 0.03	5.23 \pm 0.72	0.69 \pm 0.01
		0.2	0.40 \pm 0.02	2.36 \pm 0.08	0.72 \pm 0.00
		0.4	0.25 \pm 0.01	1.56 \pm 0.15	0.73 \pm 0.02
3	Unsterilized	0.1	5.19 \pm 0.17	22.29 \pm 1.39	0.72 \pm 0.00
		0.2	5.54 \pm 0.19	23.18 \pm 2.13	0.72 \pm 0.00
		0.4	4.35 \pm 0.16	21.08 \pm 0.88	0.72 \pm 0.00
	Sterilized	0.1	3.67 \pm 0.19	24.72 \pm 1.65	0.71 \pm 0.01
		0.2	2.63 \pm 0.12	12.46 \pm 1.09	0.72 \pm 0.01
		0.4	1.19 \pm 0.03	5.53 \pm 0.04	0.74 \pm 0.01

Table 3

Different relative values (intrinsic viscosity, limiting viscosity and molecular weight) of Si-HPMC samples.

NaOH (M)	pH	$[\eta]$ ratio [‡]	M_w ratio [‡]	η_0 ratio [‡] at 2%	η_0 ratio [‡] at 3%
0.1	12.8	1.15	1.19	1.43	1.42
0.2	13.1	1.11	1.13	2.30	2.11
0.4	13.4	1.06	1.08	3.19	3.66

[‡]Between values of unsterilized and sterilized samples (NS/S).