Polymer Bulletin 61, 631–641 (2008)
DOI 10.1007/s00289-008-0987-6

Polymer Bulletin

Stiffness xanthan hydrogels: synthesis, swelling characteristics and controlled release properties

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Received: 16 April 2008 / Revised version: / Accepted: 29 June 2008
Published online: 29 August 2008 –

Summary

New xanthan hydrogels were synthesized at 90°C in water acid media using adipic acid dihydrazide (ADH) as crosslinking agent, in the presence of 1-ethyl-3[3-dimethyl amino] propyl carbodiimide hydrochloride (EDCI) as reagent. In these conditions, xanthan chains are in a predominantly helical conformation and through rheological measurements the influence of the temperature over the helix-coil transition was assessed. Xanthan hydrogels with different concentration in polysaccharide and ADH were obtained and characterised by elemental analysis and swelling properties. High concentration of xanthan (i.e. $25gL^{-1}$) is needed to obtain gel due to the low available carboxylic functions in the helical conformation. During the swelling analysis, it was noted that high temperature (i.e. 90°C) favoured the conformational transitions within the network. The drug loading and releasing properties were estimated using methylene blue as model molecule and different experimental pH and ionic strength conditions.

Keywords: xanthan, ADH, hydrogels, conformation, swelling

Introduction

Natural polymers present important alternative to synthetic ones taking into account their hydrophilicity, ionic character and capacity to be chemically modified through graft functionalisation [1] and hydrophobisation [2, 3]. Biopolymers such as polysaccharides are largely used to prepare hydrogels [4]. Hydrogels consist of polymeric networks that can absorb large quantities of water while remaining insoluble in aqueous solutions due to chemical or physical crosslinking of individual polymer chains [5]. Knowing that the majority of the polysaccharides are biocompatible and/or biodegradable, their use as hydrogels in the biomedical field is consistent [4]. Among the polysaccharides, Xanthan gum (Xan), a microbial exopolysaccharide produced by *Xanthomonas campestris* [6], presents the general properties of the polysaccharides adding a few important ones such as the capacity to thicken, emulsify, stabilize, flocculate, swell and suspend the aqueous solutions forming thus gels, films and membranes [7]. Xanthan has a cellulosic backbone of D-glucose linked in β-1,4 positions. For every alternate glucose unit there is a side chain consisting of β-D-

mannose-(1,4)-β-D-glucuronic acid- (1,2)-α-D-mannose. The terminal mannose may carry pyruvate residues linked to the positions 4 and 6. The internal mannose unit is partially acetylated at C-6 [8]. In aqueous solutions, xanthan presents a conformational transition from an ordered helical shape at physiologically relevant temperatures and salt concentrations to a disordered chain conformation at elevated temperatures and low ionic strength. This transition is more evident in a dilute medium than in a concentrated one. The latest is though the one that leads to a high yield gel synthesis.
Xanthan gels are used in a variety of biomedical applications such as: ophthalmology [9, 10], implantology [11], tissue engineering [12] and controlled drug release systems [13-15]. Xanthan is able to form physical or chemical gels, the physical ones being not as resistant as the chemical ones. In order to form chemical gels xanthan was usually crosslinked with epichlorhydrin [16], but it is known that this crosslinking agent is toxic and carcinogenic [17]. For this reason, the use of non-toxic agents for the hydrogel's synthesis is recommended.
The present study concerns the synthesis and consecutive analysis of swelling behaviour of some xanthan based chemical networks prepared in aqueous acid conditions. The crosslinking agent was adipic acid dihydrazide (ADH), water soluble and non-toxic substance. The synthesis was performed in the presence of 1-ethyl-3[3-dimethyl amino] propyl carbodiimide hydrochloride (EDCI). In this manner, an organic solvent free synthesis was conducted in order to obtain low toxicity hydrogels. The use of ADH as crosslinking agent was reported for obtaining biomaterials based on hyaluronan [18, 19], alginate [20] and pullulan [21]. To our knowledge, it is though the first time when ADH is used for obtaining xanthan chemical networks. The synthesis is conducted in conditions where xanthan is attempted to present helical shape. This seems to be also an original approach. The main objective is to follow the gel properties under conditions where a conformational transition should occur.

Materials and methods

Materials

Xanthan was provided by Rhodia (Rodicare S, France). Its molecular weight (M_w) was determined by size exclusion chromatography with on line multi angle laser light scattering and it was found to be $2 \cdot 10^6$ g/mol. The substitution degrees with acetate and pyruvate, obtained by ^{1}H NMR [22], were determined to be 1 and 0.6 respectively. $LiNO_3$ was purchased from Kelkogel and adipic acid dihydrazide (ADH) from Fluka. The 1-ethyl-3[3-dimethyl amino] propyl carbodiimide hydrochloride (EDCI), the chlorhydric acid (HCl) and the methylene blue (MB) were provided from Sigma-Aldrich. Pullulan (PF20) was purchased from Hayashibara Biochemical Laboratory. The sodium salt of carboxymethylpullulan (CMP) was synthesized in isopropanol/water medium (66%, v/v) by reacting the hydroxyl groups of pullulan with sodium chloroacetate in the presence of sodium hydroxide [2, 21].
All compounds were used without any further purification and during the experiments only Milli-Q water (mQ) was used as solvent.

Rheological measurements

In order to determine the influence of the temperature over the helix-coil transition, rheological measurements were performed on concentrated xanthan solutions, using

a controlled stress rheometer (AR2000, TA Instruments, France) with cone plate geometry (4cm, 2°). The xanthan solution (25gL^{-1}) was obtained by dissolving the suitable quantity of xanthan in $LiNO_3$ 10^{-3} M. pH 3 is fixed by adding HCl (1 M) in order to simulate the synthesis conditions. Oscillatory dynamic experiments were performed after verifying the linearity of viscoelastic properties.

Hydrogels synthesis

Xanthan hydrogels were obtained by a crosslinking reaction with ADH in the presence of EDCI as a reagent. A determined Xanthan quantity was dissolved under magnetical stirring in a 10^{-3}M $LiNO_3$ solution. The pH was fixed at 3 with HCl. After 1 hour the solution was heated up to 90°C and ADH and EDCI were added. The ADH:Xan molar ratios were 2.5, 5 and 10, while the EDCI:Xan molar ratio was kept constant at 1. The obtained solutions were let for 24 fours under magnetical stirring. The obtained gel was purified by successive dialysis in Milli-Q water. Finally, the gels were dried at 40°C. The gel's synthesis yield was calculated according to equation (1):

$$\rho(\%) = \left(\frac{m_h}{m_r}\right) \cdot 100 \quad \textbf{(1)}$$

where ρ is the yield of the crosslinking reaction, m_r is the total weight of the reactants introduced in the reaction and m_h is the weight of the obtained crosslinked dried gel.

The concentrations of carbon (12gmol^{-1}) and nitrogen (14gmol^{-1}) were experimentally measured by the means of elemental analysis and calculated using equations 2 and 3. The experimental crosslinking degree (x) was obtained according to equation 4. Where M_{motif} is the repetition unit weight.

$$\%N = \frac{14(4x)}{M_{motif}} \quad \textbf{(2)}$$

$$\%C = \frac{12(35+6x)}{M_{motif}} \quad \textbf{(3)}$$

$$\frac{\%N}{\%C} = \frac{56x}{420+72x} \quad \textbf{(4)}$$

The CMP (DS=0.58) based gels have been obtained according to the method described by Dulong et al. [21]. The concentration of CMP was 77g.L^{-1}; the ADH:CMP ratio was 0.2 and EDCI:CMP was 1[21].

Swelling tests: swelling degree and kinetics

The swelling degree (Q) of the gels was determined according to Eq. (5):

$$Q(\%) = \frac{m - m_0}{m_0} * 100 \quad \textbf{(5)}$$

Where m is the weight of the swollen gel and m_0 is the weight of the dried gel.

For this purpose a weighted quantity of dried gel was added into a large volume of Milli-Q water. At determined time intervals the gel was separated, gently wiped to remove the excess adsorbed solvent and weighted. Using Eq. (5), the swelling kinetic was assessed. The swelling degree (Q) was considered to be maximal (Q_{max}) when identical values of Q were recorded during consecutive measurements (Q=constant). The influence of the parameters such as pH and ionic strength, temperature and ADH:Xan ratio were studied.

Absorption and release of methylene blue

A known amount of dried hydrogel (30mg) was immersed in 30mL of MB solution (2.10^{-5} M) under stirring during 24 h to reach equilibrium. Absorbance of MB in the supernatant was measured by spectrophotometry (Spectrometer UVIKON 860, Kontron Instruments) at λ=665nm. For both entrapment and release, the concentration of MB in the supernatant was estimated from a calibration curve of absorbance versus concentration plotted with MB concentrations varying from 10^{-5} to 3.10^{-5} M.

Results and discussions

Rheological behaviour of concentrated xanthan solutions

The rheological properties of xanthan solution have been determined in the conditions of synthesis. Figure 1a shows dynamical spectra obtained for $25gL^{-1}$, pH 3, LiNO3 10^{-3}M and 90°C. It clearly appears that the storage modulus (G') is largely superior to the loss modulus (G''). This is characteristic of an elastic behaviour suggesting that helical conformation is still present even at 90°C. In order to evaluate the role of pH, we have measured the G' as a function of temperature for pH3 and 7 (figure 1b). For pH3 any variation of G' is observed when the temperature increase until 90°C whereas for neutral pH a significant inflexion point on the G' curve clearly appears above 75°C suggesting the beginning of a transition from helix to coil. Thus rheological results demonstrate that the polysaccharide is in its helical, stiff and ordered conformation in the synthesis conditions.

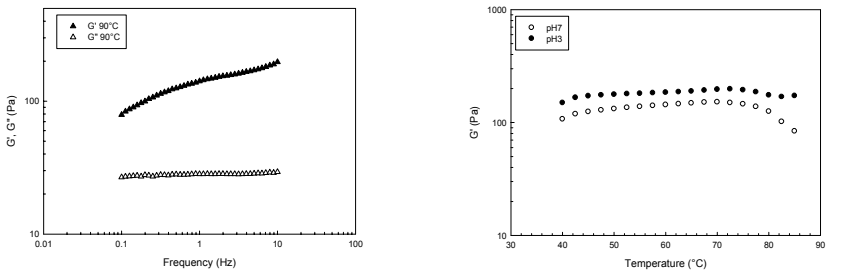


Figure 1. (a) Mechanical spectra of xanthan solutions $25g.L^{-1}$ in LiNO3 10^{-3}M, pH3 at 90°C; (b) Elastic modulus of xanthan solution $25gL^{-1}$ in LiNO3 10^{-3}M as function of temperature and pH

Hydrogels formation

A free organic solvent approach has led to use the water soluble ADH as cross linking agent. This approach has been used already for linear polysaccharides [21], but not for

double helical one, such as xanthan. Since ADH reacts with activated carboxylic groups, xanthane has been activated by reaction with the water soluble non-toxic carbodiimide, EDCI in a solution at pH3. The acid pH favours the protonation of EDCI's nitrogen atoms. In this manner, O-acylurea, an unstable intermediate, is obtained at the first reaction step (figure 2, I). Through a side reaction, O-acylurea is stabilized into N-acylurea (figure 2, II). In the presence of ADH, the amide bonds are formed leading to the gel formation (figure 2, III). Different experimental conditions were used in order to maximize the yield of xanthan gel synthesis. For this reason, polysaccharide concentration, ADH:Xan ratio, reaction temperature, reaction time and stirring conditions were varied. The optimum ratio of EDCI:Xan was previously determined to be 1 [21] since lower ratios did not lead to gel formation. The electrolyte concentration ($LiNO_3$) was constant, 10^{-3} M for stabilizing the helicoidal structure at pH3. Higher $LiNO_3$ concentration induces negative effects over the gel purification time. High ADH quantity was required in order to minimize the N-acylurea formation through reaction (II) [21]. The purification of the gels was conducted by dialysis against pure water until dialysis water presents the conductivity of pure water. In the Table 1 various experimental conditions for the synthesised gels are presented. For further analyses, only the gels obtained through high yields crosslinking reactions were surveyed.

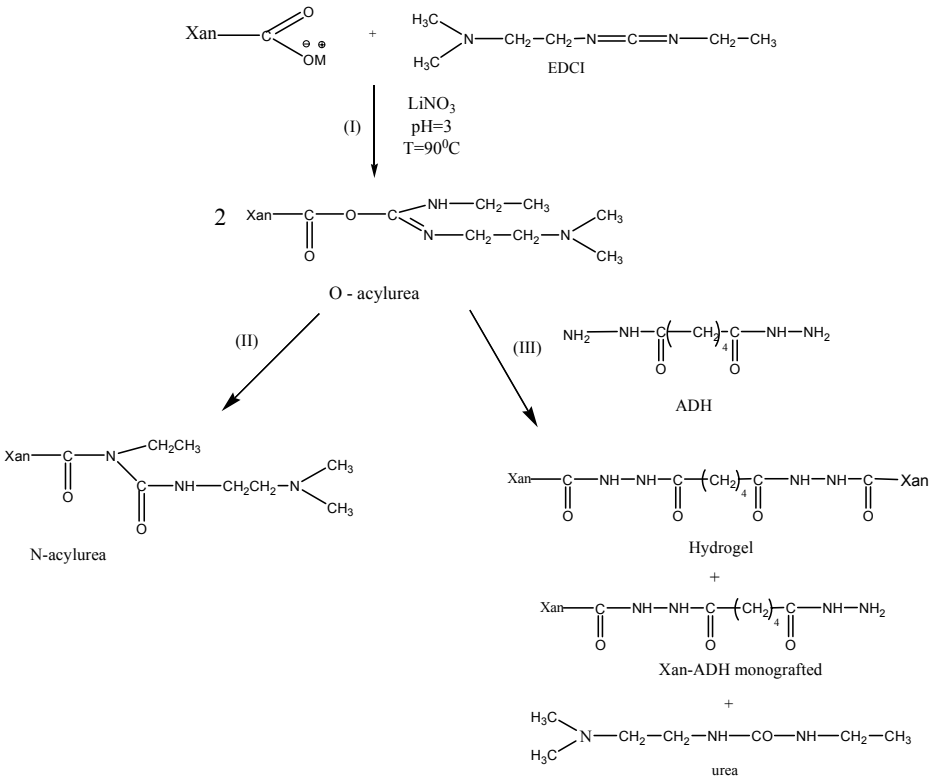


Figure 2. (I) Reaction of xanthan with EDCI, (II) rearrangement of O-acylurea, (III) cross-linking reaction of xanthan with ADH [21]

From Table 1 it can be observed that high Xanthan concentrations, high temperature, strong stirring lead to the highest yields. It is not surprising to observed improved gel formation when increase the xanthan concentration. This has been already shown with other polysaccharide [21, 23]. Logically, crosslinking needs entangled polymer solution to occur. Nevertheless, the new information here concerns the very high level of xanthan concentration needed for gel formation (bad yield gel below $10gL^{-1}$ then largely above C*). This should be explained by the poor accessibility of carboxylic groups in the helical conformation of xanthan. In other terms, only few carboxylic functions seem available for reacting. In agreement with that, we can notice that an increase of ADH concentration doesn't induce a better yield of gel formation. Table 1 clearly evidences that any gel is obtained at 25°C on the contrary with 90°C. This should be interpreted by the necessity of sufficient polymer chain movements needed to ensure the second step of crosslinking reaction. Probably for the same reasons, higher stirring leads to better gel yields (Table 1).

Table 1. Experimental conditions for xanthan's gel synthesis

Gel	Xan [g/l]	ADH : Xan	T [°C]	time [h]	stirring (±)	ρ [%]
$10X2A\text{-}25_{48\pm}$	10	2	25	48	±	-
$10X2.5A\text{-}90_{4+}$	10	2.5	90	4	+	10
$10X2.5A\text{-}25_{48+}$	10	5	25	48	+	-
$10X5A\text{-}90_{4+}$	10	5	90	4	+	25
$10X5A\text{-}90_{24+}$	10	5	90	24	+	56
$10X5A\text{-}90_{72+}$	10	5	90	72	+	47
$15X5A\text{-}90_{24+}$	15	5	90	24	+	71
$20X5A\text{-}90_{24+}$	20	5	90	24	+	72
$25X2.5A\text{-}25_{48\pm}$	25	2.5	25	48	±	-
$25X2.5A\text{-}90_{24+}$	25	2.5	90	24	+	78
$25X2.5A\text{-}90_{24-}$	25	2.5	90	24	-	-
$25X5A\text{-}90_{24+}$	25	5	90	24	+	74
$25X10A\text{-}25_{48\pm}$	25	10	25	48	±	-
$25X10A\text{-}90_{24+}$	25	10	90	24	+	63

Notation: nXpA-$T_{z\pm}$: n - xanthan concentration, p – ADH : Xan ratio, T - reaction temperature, z - reaction time, ± - stirring presence (+) or absence (-), ρ (%) – yield.

According the results from Table 1, we have focused our attention on the serie 25X-ADH-90_{24+}. For these samples, the values of C and N elemental analysis and the experimental crosslinking degree (x) are presented in table 2.

Table 2. C and N elemental analyses values

Element [%]	Xan	25X2.5A	25X5A	25X10A
C	35	36	39.8	37.6
N	-	2.70	3.04	2.87
x	-	0.62	0.63	0.63

By comparison to the Xanthan precursor, the nitrogen concentration increased significantly for all the gels demonstrating the efficacy of the crosslinking reaction.

Regarding the accuracy of nitrogen content (+/- 0.2), any clear differences are noted between the 3 samples varying by the ADH:Xan ratio. This is fully confirmed by the values of x. As EDCI quantity remains constant, these results seem to confirm that only few COOH are available in Xanthan in helical shape. In other words, the lowest ADH:Xan ratio (i.e. 2.5) seems sufficient to ensure the reaction between all accessible activated acid functions. While on the basis of this results, it is not possible to affirm that the crosslinking density is the same in each sample. Incorporated ADH may be mono or bi grafted (crosslinking). Let us note that in the case of mono incorporation, positive charge should appear in acidic media.

Swelling behaviour of gels

Swelling of hydrogels from the previous serie 25XADH have been measured (after 1 hour at 25°C needed to reach equilibrium) in different media: pH 3, 7 and 13 (Figure 3). Logically, the swelling degree is expected to depend on crosslinking density and/or on electrostatic repulsions.

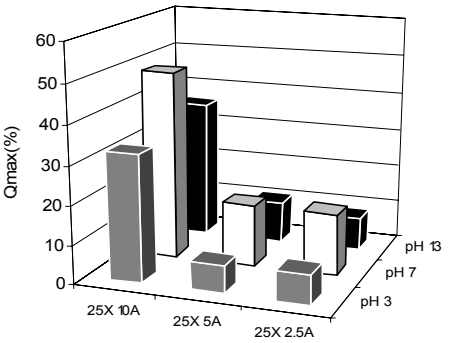


Figure 3. Variation of maximum swelling degree as a function of pH for: 25X10A, 25X5A, 25X2.5A

The main result concerns the higher ADH:Xan ratio (i.e. 25X10A) for which a strong increase of swelling is evidenced as compared to both 25X5A and 25X2.5A that evidence quite the same swelling. It seems indicate that 25X10A present a lower crosslinking density than the others. Crosslinking reaction results from 2 reacting steps. As the density of activated sites is constant, increasing ADH concentration lead to increase the proportion of mono grafted ADH (1^{st} step). Then, an increase of the kinetic of percolation should be expected which may prevent the 2^{nd} step of the reaction. This should be amplifying by the low available carboxylic functions and the stiffness of xanthan. Based on that, a high level of heterogeneity in the polysaccharide gel matrix should also be expected.

Whatever the gel sample, the effect of pH on the swelling is not so drastic. This is probably due to the low amount of effective charge in xanthan helical and to the stiffness of the xanthan in the matrix that limit possible electrostatic repulsions. We just can notice that the higher swelling degree is obtained for neutral pH as compared to acidic or alkaline media. As a consequence, alkaline media seems not capable to induce a helical toward coil transition (known for dilute xanthan concentration) within the matrix.

In order to study a possible influence of helical/coil transition within the matrix, we have measured the swelling at 90°C in pure water of both xanthan (helical) and CMP (coil) based matrix. Results are presented figure 4.

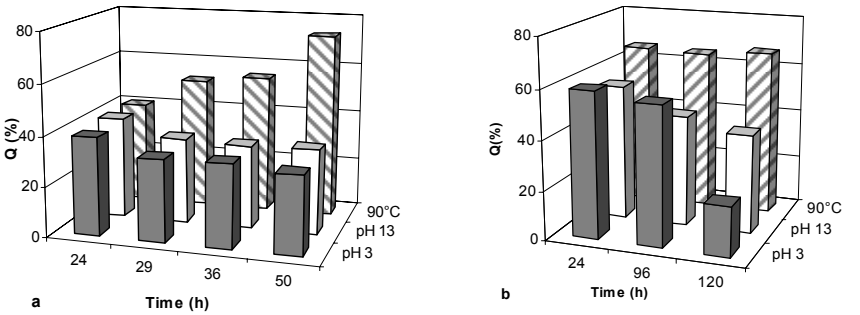


Figure 4. pH and temperature dependence of the swelling degree as a function of time: **a** - 25X10A gel, **b** – ADH crosslinked carboxymethylpullulan.

The Figure 4a confirms the previous result about the pH influence. For acid pH (synthesis conditions), xanthan is having a predominantly helical conformation within the crosslinked networks. This explains the lowest values of Q registered in the case of a pH3 (figure 4a) with a low time variation. In the case of pH13, even though the coil conformation is favoured, the Xan chains are linked through strong amide bonds and determine a swelling degree similar to the one measured in acid conditions (figure 4a). On the contrary a constant time increasing of Q was noticed when the swelling behaviour was monitored at 90°C in Milli-Q water (figure 4a). This phenomenon may have two possible explanations: a temperature triggered xanthan degradation or the presence of a conformational transition. In order to check the validity of the first hypothesis, similar swelling tests were performed using ADH crosslinked carboxymethylpullulan (figure 4b). This is a linear polysaccharide without helical conformation. During several days, a constant Q decrease may be observed both in pH3 and 13 and this can be related to the CMP degradation [24] (figure 4b). The same network shows a constant Q at 90°C in Milli-Q water. In these conditions, the absence of the xanthan degradation may be assumed. Based on this and considering also the rheology results presented above, it is correct to assume that this swelling behaviour may be determined by a helix-coil temperature triggered conformational transition.

Methylene blue: loading and releasing properties

In order to evaluate xanthan gels capacity to entrap and release different biologically active molecules, methylene blue (MB) was chosen as a model (figure 6). MB is a cationic molecule with a high affinity for negatively charged polymers.

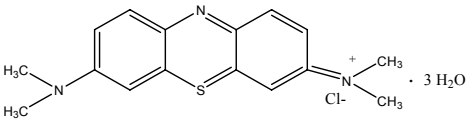
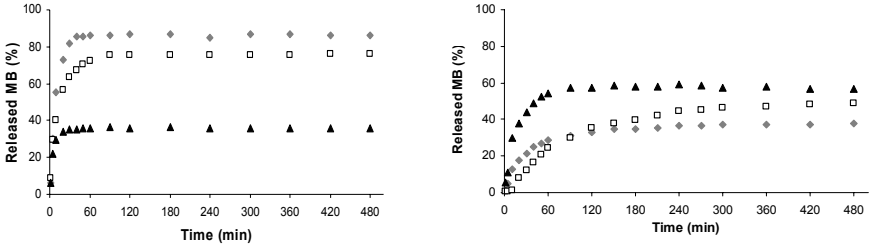


Figure 6. Chemical structure of methylene blue

In order to assess the maximum loading capacity, each of the analyzed weighted dried gels was immersed in a MB solution of known concentration (20 $\mu molL^{-1}$) and the variation of the characteristic peak absorbance (664 nm) was measured. The final value of the absorption was registered after 24 hours. Approximately 98% of MB was absorbed in each case. For investigating the releasing kinetics, two different media mimicking human body fluids were considered: a 1.5 pH solution (HCl adjusted) similar to the gastric fluid and a 0.1 M NaCl solution similar to physiological serum (figure 7a and b).



a **b**

Figure 7. Methylene blue releasing kinetics studied in: **a** – pH = 1.5, **b** = NaCl (0.1 M) (Legend: rhombus – 25X2.5A, square – 25X5A and triangle – 25X10A).

At pH 1.5, a high slope kinetic drives the releasing process for all the considered gels (figure 7a). After approximately 60 minutes, the release MB reaches a constant value which is different according to the gel. From the released percentage point of view, we have obtained the following order: 25X10A < 25X5A < 25X2.5A. Surprisingly, for 0.1M NaCl, the plateau release values are in the opposite order: 25X2.5A<25X5A<25X10A (Figure 7b) and the kinetic is lower than for pH1.5. It seems to indicate that ionic interaction between matrix and MB does not constitute the main mechanism to explain these results. This is in agreement with the low level of available carboxylic function into xanthan in its helical conformation. Beyond these results, it appears possible to control the release of entrapped molecule by changing the synthesis conditions of xanthan matrix formation.

Conclusions

In the present work we report the original synthesis in aqueous media of matrix based on xanthan in helical conformation thanks to ADH crosslinking reagent. This new xanthan networks should be suitable to be used as substrates for controlled drug release systems. A preliminary rheological study has determined the helical conformational structure of Xan in the synthesis conditions. Elemental analysis and aqueous time stability measurements confirmed the efficacy of the crosslinking reaction. Very high concentration of xanthan is needed to ensure the percolation. This has been attributed to the low level of available carboxylic function present in xanthan in its helical structure. As a consequence, the amount of incorporated ADH does not change when ADH concentration increases. But when ADH concentration is too high, the swelling increases due to a probable competition between the percolation kinetic and the movement of the chain within the matrix. This may lead to a probable increase of mono grafted ADH in the matrix whan ADH concentration increases. According to

this last remark, the crosslinking reaction (2 steps) needs strong stirring and high temperature (i.e. 90°C) to occur. Acid and alkaline conditions do not strongly affect the swelling of such gel. On the contrary, high temperature (i.e. 90°C) leads to a progressive increase of swelling. Thanks to a comparison with CMP-ADH gel, this has been attributed to a conformation change of xanthan within the matrix from helical to coil. Entrapment and controlled release of Methylene Blue (MB) as a model molecule has been evidenced from xanthan gels using acidic condition or ionic strength.

Acknowledgements. Dr. Virginie Dulong (FRE3101 CNRS, Université de Rouen, France) is acknowledged for her valuable help with the CMP synthesis and characterization.

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