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ORIGINAL PAPER

Pullulan–STMP hydrogels: a way to correlate crosslinking mechanism, structure and physicochemical properties

Virginie Dulong · Renauld Forbice · Eric Condamine · Didier Le Cerf · Luc Picton

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Abstract Rheological and swelling properties of hydrogels based on pullulan crosslinked with sodium trimetaphosphate (STMP) are explained according to various polymer and crosslinking agent concentrations using ^{31}P-nuclear magnetic resonance study. This method has allowed determining the amount of all the species present in the medium when varying both pullulan and STMP concentrations. We have clearly demonstrated with a good agreement by both ^{31}P-NMR and rheology that a critical STMP concentration occurs which is function of pullulan concentration. This typical crosslinking agent concentration delimitates the maximum of gel structure together with the minimum of swelling.

Keywords Polysaccharide · Crosslinking · Hydrogel · ^{31}P-NMR · Structure–property relation

Introduction

Hydrogel consists of three-dimensional network, where water soluble polymers or copolymers are connected by physical or chemical crosslinks. The resulting

V. Dulong · R. Forbice · D. Le Cerf · L. Picton (✉)
Laboratoire Polymères, Biopolymères, Surfaces, Université de Rouen, CNRS UMR 6270 & FR 3038, 76821 Mont Saint Aignan, France
e-mail: luc.picton@univ-rouen.fr

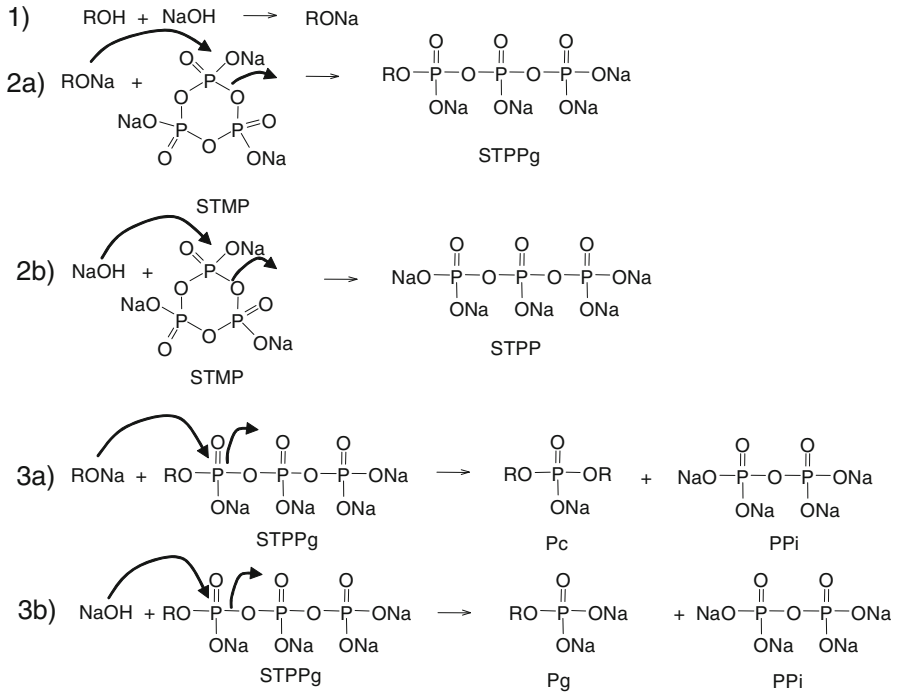
E. Condamine
Institut Fédératif de Recherche Multidisciplinaire sur les Peptides (IFRMP 23), Université de Rouen, CNRS UMR 6014 & FR 3038, 76821 Mont Saint Aignan, France

Present Address:
E. Condamine
Laboratoire de Résonance Magnétique Nucléaire, Institut de Biologie Structurale CEA-CNRS-UJF «J.-P. Ebel» (UMR CNRS 5075), 41 rue Jules Horowitz, 38027 Grenoble Cedex 1, France

matrices are capable to swell and retain a large amount of water or biological fluids [1]. This last aspect explains the ability of such structure to mimic natural living tissues opening interesting ways towards biological or pharmaceutical applications [2, 3]. In this context, polysaccharides which are generally biocompatible appear as good candidates available from renewable source (algae, seeds, microorganisms and so on). Focusing on chemical, safe and non-toxic crosslinking agent suitable for polysaccharides matrixes elaboration, the use of sodium trimetaphosphate (STMP) consists in a potentially useful and advantageous way. STMP is accepted by the Food and Drug Administration. For example, STMP is used to prepare food-grade phosphorylated starches [4, 5]. STMP is able to crosslink polysaccharides in strong alkaline conditions. The literature deals mainly with starches [4–13], but some studies reported also such crosslinking on guar gum [14], on carboxymethylcellulose [15] with osteogenic activity, on konjac glucomannan [16] for colon-targeting drug delivery application. In our team, we have developed this type of crosslinking on hyaluronan [17], on xanthan [18] and on pullulan [19, 20]. In suitable conditions, this reaction leads to very consistent pastes, with good resistance to heat, acid and shearing.

Until now, few papers have clearly described the mechanism of this reaction. In 1993, Lim and Seib [8] were the first to describe the crosslinking of starch with a mixture of STMP and sodium tripolyphosphate (STPP) at pH > 9.5. The authors described crosslinking as a two-step reaction: in the first step, the opening of cyclic structure of STMP occurred by reaction of starch alkoxides (in strong alkaline media) with phosphate groups leading to the formation of a tripolyphosphated polymer. In the second step, the addition of a new polymer chain led to the formation of a crosslinked polymer. In 1997, Woo and Seib [9], completed the description of the mechanism and showed that the crosslinking reaction was accompanied by the production of pyrophosphate links. Recently, Seib's team proposed a nice pathway for the reaction between STMP and starch in aqueous sodium hydroxide at pH 9.5–12.5 using ^{31}P-NMR spectroscopy as method of investigation [11]. Thus, by comparison of ^{31}P chemical shifts of inorganic and organic phosphate models they were able to give the structure of the different starch phosphates (distarch monophosphate, monostarch monophosphate and monostarch diphosphate) produced by the reaction between STMP and starch. In quite the same time, we have described the same reaction mechanism of pullulan crosslinking with STMP by using ^{31}P-NMR spectroscopy study [21]. In the first part of this article, we have chosen methyl α-D-glucopyranoside (Glc-OMe) as a simple model molecule with STMP because Glc-OMe is stable at alkaline pH and gives spectra with a better resolution than pullulan. We were thus able to assign the chemical shifts of all the species produced by the reaction between STMP and pullulan by comparison with the model. As a result, we have shown that the mechanism of this reaction can be described as presented on Scheme 1. A brief description of this mechanism is reminded below.

In another previous study [20], we have studied the physicochemical behaviour of pullulan–STMP hydrogels varying by different synthesis conditions, mainly polymer and crosslinking agent concentrations. Swelling properties have been measured together with kinetics of crosslinking and final hydrogel structural data



Scheme 1 Proposed mechanism of the cross-linking reaction of pullulan with STMP [21]

obtained thanks to rheology. Some of the obtained results were atypical and gave rise to hypothesis according to an expected mechanism of the reaction.

The objectives of the present study are to correlate the previously obtained rheological and swelling results [20], to ^{31}P-NMR spectroscopy. In a first step, the ^{31}P-NMR study of the pullulan hydrogels obtained in different experimental conditions (i.e. various pullulan and STMP concentrations) allowed to quantify the different species present in the medium. In a second step, the NMR results have been confronted to the rheological and swelling results to get finally a better understanding of the hydrogels properties. The results of this study could be useful to prepare tailor-made hydrogels with the appropriate properties to suit particular applications as encapsulation and controlled release of functional ingredients [21–23].

Experimental

Materials

Pullulan PI20 (M_w 300,000 g mol^{-1}, Ip 1.8) was purchased from Hayashibara Biochemical Laboratory (Okayama, Japan); STMP, sodium hexafluorophosphate ($NaPF_6$) were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France),

sodium hydroxide and sodium chloride from Acros Organic (Halluin, France). Deuterium oxide was purchased from SDS (Peypein, France).

Crosslinking procedure

For rheological and swelling measurements, the hydrogel synthesis was previously described [19]. For NMR measurements, pullulan was dissolved in D_2O at 100, 150 or 200 g L^{-1}, NaOH was then added to obtain a concentration of 0.2 mol L^{-1} and the mixture was stirred together during 1 h. STMP was finally added to the alkaline mixture at different concentrations ($0.05 < [STMP]o < 0.5$ mol L^{-1} or $0.04 < r_2 = [STMP]o/[AGU] < 0.843$) where AGU is anhydroglucopyranose unit. The solution was vigorously stirred before introduction in the NMR tube via a Pasteur Pipette. The higher pullulan concentration used for the NMR experiments was 200 g L^{-1} until a STMP concentration of 0.1 mol L^{-1} because above this value, the solution was too viscous or the gel formation was too rapid leading to an impossible transfer into the NMR tube.

^{31}P-NMR

NMR experiments, previously described [21], were performed on a Bruker ARX 400 spectrometer, equipped with a 5 mm QNP $^{1}H/^{13}C/^{31}P/^{19}F$ probe, interfaced to a Silicon Graphics INDIGO2 workstation using XWINNMR software (Bruker S.A., Wissembourg, France).

Rheology

Rheology measurements (previously described [20]) were performed with an AR2000 rheometer from TA Instruments (New Castle, Delaware, USA) using cone/plate (2°, 4-cm radius cone) geometry. Oscillation frequencies varied from 0.6 to 6 Hz and suitable stresses were applied for each condition (i.e. pullulan and STMP concentrations) to be in the linear viscoelastic domain. We give only G' at 1 Hz in graphs. Temperature control was ensured from a Peltier and a solvent trap was used to avoid any solvent evaporation during measurements.

Results and discussion

The first part of this work consisted in the study of the crosslinking reaction of pullulan with STMP by ^{31}P-NMR according to the experimental condition used during the rheological study. The crosslinking reactions were performed in NMR tubes with pullulan concentrations from 100 to 200 g L^{-1} in D_2O and with varying STMP concentration other parameters being equal ([NaOH], T). An example of ^{31}P-NMR spectra of crosslinked pullulan ([Pullulan] = 100 g L^{-1}) is shown in Fig. 1. The proposed mechanism (Scheme 1) of the reaction between pullulan and STMP in alkaline medium has been already given [21]. It was in very good agreement with

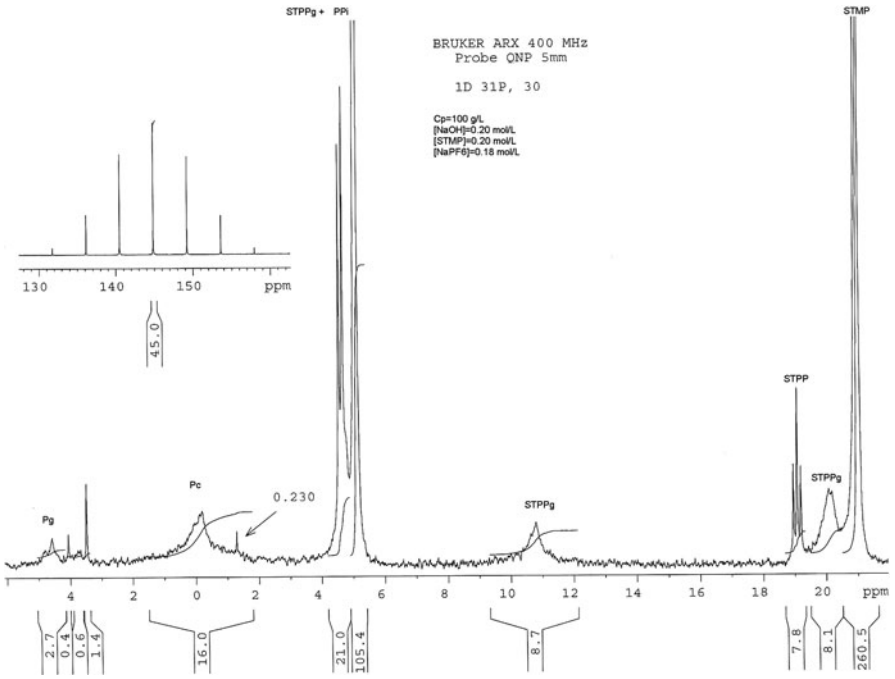


Fig. 1 ^{31}P-NMR spectrum obtained at the end of the reaction between pullulan and STMP in alkaline conditions ([Pull] = 100 g L^{-1}, [STMP] = 0.20 mol L^{-1}, [NaOH] = 0.20 mol L^{-1}, [$NaPF_6$] = 0.18 mol L^{-1}, D_2O, 300 K, 167.97 MHz)

the pathway of the reaction between STMP and starch in aqueous sodium hydroxide [11].

Briefly, in alkaline conditions, STMP reacts with the polymer to give grafted sodium tripolyphosphate (STPPg) (Scheme 1, 2a). STMP also reacts with NaOH to give STPP (Scheme 1, 2b). STPP is the product of the degradation reaction of STMP in alkaline medium, whereas STPPg is the active species leading to the formation of crosslinked chain (Pc) or grafted chain (Pg) together with inorganic pyrophosphate (PPi) (respectively, Scheme 1, 3a and 3b). To sum up only one reaction gives rise to a crosslink with a negative charge (3a), whereas it appears two possibilities of mono-grafted pullulan with 4 and 2 negative charges, respectively, STPPg and Pg (i.e. 2a and 3b). Analysis of NMR spectra for both various polymer and STMP concentrations has permitted to quantify the different species present in the medium. An internal standard, $NaPF_6$, was used to calculate the concentration of each species present in the medium after data integration of the ^{31}P-NMR spectra. Table 1 summarises the data obtained for each spectrum of hydrogel obtained with pullulan and STMP at various concentrations. These data are in agreement with the proposed mechanism.

The main conclusions are the following:

– Amount of unreacted STMP living in the medium is always large. This is more pronounced when the initial STMP concentration is high,

Table 1 Concentrations (from ^{31}P-NMR) of all the species present in the medium at the end of the reaction of pullulan with STMP

[Pull] g L^{-1}	$[STMP]_o^a$ mol L^{-1}	r_2^b	[STMP] mol L^{-1}	[STPP] mol L^{-1}	[PPi] mol L^{-1}	[STPPg] mol L^{-1}	[Pg] mol L^{-1}	[Pc] mol L^{-1}	[Pc]/[AGU]
100	**0.05**	**0.081**	**0.0062**	**0.0054**	**0.0383**	**0.0007**	**0.0056**	**0.0202**	**0.0327**
	0.08	0.130	0.0178	0.0077	0.0552	0.0024	0.0054	0.0277	0.0449
	0.12	0.194	0.0401	0.0092	0.069	0.0022	0.0019	0.0245	0.0397
	0.20	0.324	0.1085	0.0097	0.0659	0.0109	0.0034	0.0201	0.0324
	0.32	0.519	0.2345	0.0084	0.059	0.028	0.0019	0.0212	0.0344
	0.42	0.648	0.3322	0.0096	0.0566	0.0361	0.0030	0.0210	0.0340
	0.52	0.843	0.4397	0.0107	0.0524	0.0525	0.0022	0.0230	0.0373
150	0.05	0.054	0.0095	0.0024	0.0354	0.0031	0.0021	0.0192	0.0207
	0.08	**0.086**	**0.0170**	**0.0037**	**0.0479**	**0.0051**	**0.003**	**0.0221**	**0.0239**
	0.11	0.119	0.0582	0.0033	0.0358	0.0167	0.0018	0.0262	0.0283
	0.21	0.227	0.1161	0.0043	0.0493	0.0324	0.0019	0.0199	0.0215
	0.30	0.324	0.2017	0.004	0.0385	0.0505	0.0005	0.0166	0.0179
	0.41	0.443	0.3281	0.0061	0.0504	0.0532	0.0019	0.0167	0.0180
	0.49	0.529	0.4119	0.0064	0.0489	0.0775	0.0017	0.0164	0.0177
200	0.05	0.040	0.0059	0.0024	0.0395	0.0041	0.0024	0.0255	0.0207
	0.08	0.065	0.0184	0.0026	0.0542	0.0100	0.0014	0.0262	0.0212
	0.10	**0.081**	**0.0214**	**0.0025**	**0.0581**	**0.0116**	**0.0002**	**0.0225**	**0.0182**

[a] Initial concentration of STMP

[b] $[STMP]_0/[AGU]$

[Pull] = 100 g L^{-1}; [AGU] = 0.617 mol L^{-1}; [$NaPF_6$] = 0.18 mol L^{-1}; [NaOH] = 0.20 mol L^{-1}

[Pull] = 150 g L^{-1}; [AGU] = 0.926 mol L^{-1}; [$NaPF_6$] = 0.20 mol L^{-1}; [NaOH] = 0.20 mol L^{-1}

[Pull] = 200 g L^{-1}; [AGU] = 1.234 mol L^{-1}; [$NaPF_6$] = 0.19 mol L^{-1}; [NaOH] = 0.20 mol L^{-1}

- Concentrations of Pg are always very low,
- Concentrations of PPi are high whatever the experimental conditions,
- Concentration of STPPg increases when the initial concentration of STMP increases.

Influence of pullulan concentration Cp

In Fig. 2 are reported the rheological results [20]. It appears that when r_2 ([STMP]o/[AGU]) is constant, both elastic and viscous moduli (respectively, G' and G'') increase when polysaccharide concentration increases. In the same time, the swelling ratio (Q) decreases logically. This result clearly shows that a more structured network is established when polymer concentration is increased. Usually, the final mechanical properties depend only on the density of chemical crosslink but according to this result, two hypotheses may be pointed on.

On one hand, it can be supposed that an increase of polymer concentration favours an increase of chemical crosslink (even if the ratio r_2 is constant) thanks to a shorter length between chains as instance.

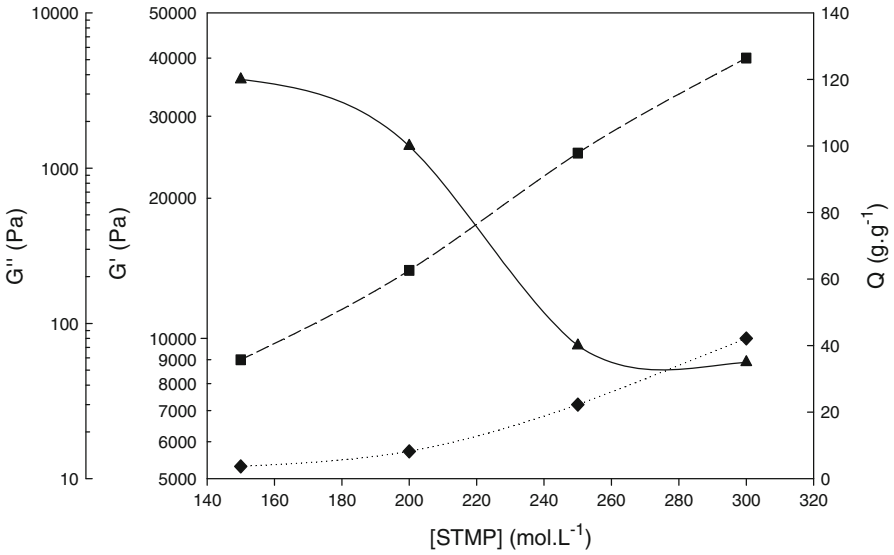


Fig. 2 Plots of G' (*filled square*), G'' (*filled diamond*) and Q (*filled triangle*) versus pullulan concentration ($T = 24$ °C, [NaOH]/[AGU] = 0.15, $r_2 = 0.22$, $t = 48$ h) [20]

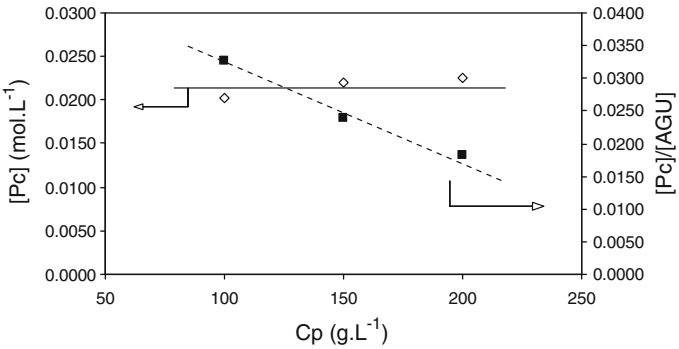


Fig. 3 Concentration of crosslinked pullulan [Pc] and yield of crosslinking [Pc]/[AGU] versus pullulan concentration at constant degree of crosslinking ($r_2 = 0.08$)

On the other hand, it can be proposed that such system can be represented as a semi-interpenetrated polymer network (semi-IPN) model with a sensible contribution of the entangled polymers in the chemical crosslinked polymer network.

^{31}P-NMR results give us the opportunity to choose between these two possibilities. Particular attention was paid to the results obtained with the same degree of crosslinking (i.e. $r_2 = 0.08$) for various pullulan concentrations (bold characters in Table 1). We thus have plot the crosslinking phosphate concentration (Pc) and the [Pc]/[AGU] ratio as a function of pullulan concentration at this constant degree of crosslinking (Fig. 3). It clearly appears that an increase of polymer concentration does not lead to an increase of chemical crosslink. More than that, the

ratio [Pc]/[AGU] decreases with polymer concentration indicating a diminishing of the yield of crosslink. This can be explained by the spectacular increase of STPPg amount when polymer concentration increases (see bold characters in Table 1). As a matter of fact, the negative charges brought by STPPg should prevent, by electrostatic repulsion, the second step of crosslinking reaction (i.e. [Pc]).

This result seems to invalidate the first hypothesis and should confirm the contribution of the entangled polymer interpenetrated in a chemical crosslinked network.

Influence of the initial concentration of STMP ([STMP]o)

In our previous study [20], we have also reported the mechanical behaviour (G', G'' and swelling ratio Q) of various gels differing by the amount of crosslinking agent (i.e. STMP). The results (Fig. 4) evidenced atypical trends. The whole behaviour can be separated into three specific ones:

- For the lowest amount of STMP (from 0 to about 0.12 M), we can observe an increase of elastic modulus together with a logical decrease of the swelling ratio when [STMP] increase. It should correspond to the expected result: more is the crosslinking agent amount, and more should be the crosslinking density (i.e. increase of [Pc]).
- For the middle amount of STMP (from 0.12 to 0.3 M), one can observe a plateau of elastic modulus together with a strong increase of the swelling ratio. It is an atypical behaviour that has been explained previously by a possible increase of negative charges brought by monolinked phosphorus (i.e. increase of [STPPg]

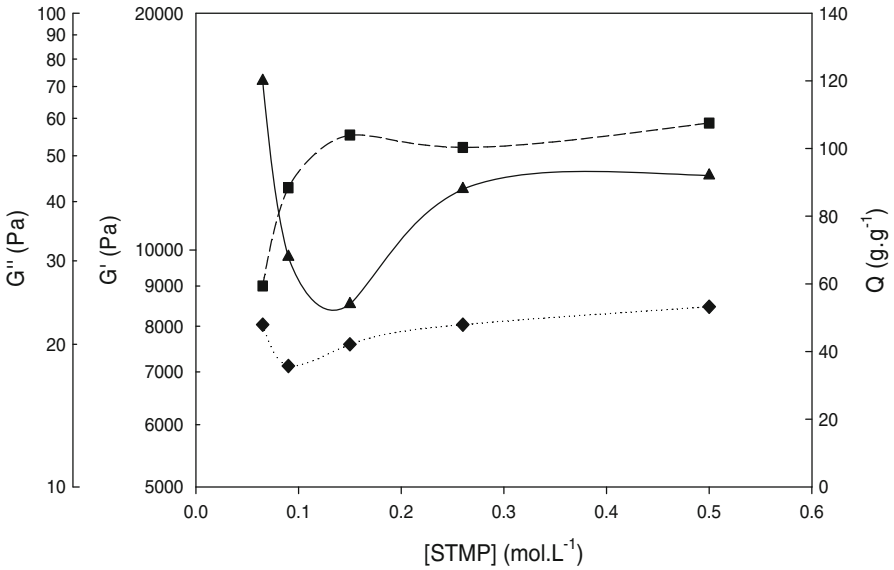


Fig. 4 Plots of G' (*filled square*), G'' (*filled diamond*) and Q (*filled triangle*) versus initial concentration of STMP ($T = 24$ °C, [NaOH]/[AGU] = 0.15, Cp = 200 g L^{-1}, $t = 48$ h) [20]

and/or [Pg]). It results an increase of electrostatic repulsions that should induce both an increase of swelling and a decrease of the second crosslinking step yield (i.e. decrease of [Pc]).

– For higher amounts of STMP (from 0.3 to 0.5 M), the system does not change anymore as also mentioned [23].

From these results, it appears that a critical STMP concentration occurs which modifies the mechanical behaviour of the gels.

In order to corroborate the rheological results and the accompanying hypothesis, we have plotted the amount of Pc and the global amount of charges brought by STPPg and Pg as function of initial concentration of STMP (respectively, Figs. 5, 6). At both studied pullulan concentrations (i.e. 100 and 150 g L^{-1}), the concentration of crosslinked pullulan (Pc) starts to increase when increasing [STMP]o, then reach a maximum at a critical concentration of STMP and finally decreases to reach a plateau (Fig. 5). Let us notice that for the upper pullulan concentration (i.e. 200 g L^{-1}) data are not shown because of the poor quality of the NMR spectra when STMP concentration becomes higher than 0.1 M. This is due to strong viscosity and too rapid crosslinking as evocated above. As a result, it appears that a critical STMP concentration ([STMP]c) can be observed by both rheological and NMR methods. On Fig. 7, we have plotted the obtained [STMP]c as a function of pullulan concentration (from NMR for the lowest ones and from rheology for the higher ones [20]). It is interesting to note that a good correlation seems evidenced between the two methods.

The second part of the Fig. 4 (i.e. swelling up together with G' stable when increase STMP amount) was explained by the hypothesis of an increase of negative charges. The results evidenced on Fig. 6 show an important increase of charged species as a function of initial STMP amount whatever the pullulan concentration.

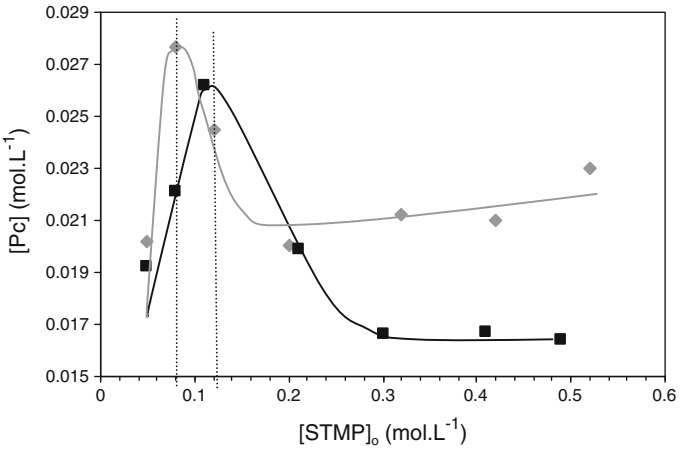


Fig. 5 Concentration of crosslinked pullulan [Pc] versus initial concentration of STMP during the reaction of STMP with pullulan at two pullulan concentrations: 100 (*filled diamond*) and 150 g L^{-1} (*filled square*)

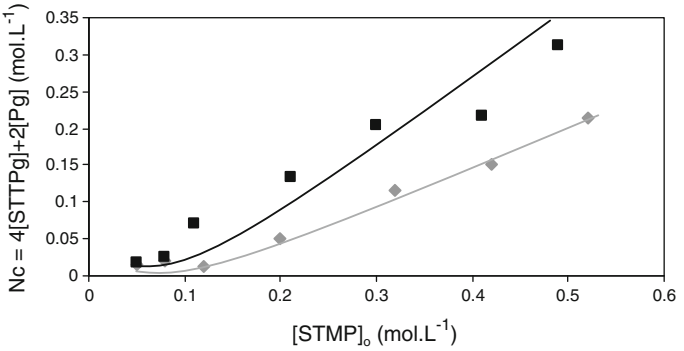


Fig. 6 Concentration of negative charges versus initial concentration of STMP during the reaction of STMP with pullulan at two pullulan concentrations: 100 (*filled diamond*) and 150 g L^{-1} (*filled square*)

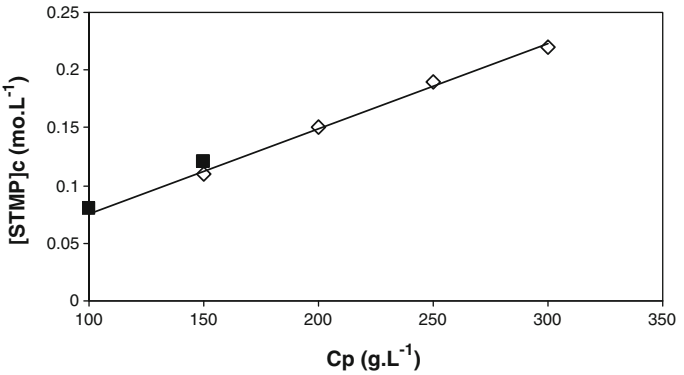


Fig. 7 Critical concentration of STMP ([STMP]c) versus pullulan concentration, from rheology (*open diamond*) and NMR (*filled square*)

This fully confirms the above mentioned hypothesis according to the effect of electrostatic repulsions that prevent crosslinking and lead to an increase of swelling.

Conclusion

The atypical behaviour of the hydrogels (from rheological point of view and swelling results) observed in a previous study when changing both polymer and STMP concentration has led us to propose some hypothesis according to an expected mechanism of crosslinking. Now, thanks to ^{31}P-NMR spectroscopy study of the crosslinking reaction of pullulan with STMP, we are able to correlate the rheological behaviour and the swelling properties of pullulan hydrogels with the mechanism we previously proposed for the crosslinking reaction.

We showed that the formation of the crosslinked pullulan is accompanied by the formation of negatively charged species which prevent the crosslinking reaction because of electrostatic repulsion. A critical concentration of STMP was highlighted

by this NMR study, which is in a very good agreement with those obtained by swelling and rheological measurements. This critical concentration is correlated with the maximum yield of the crosslinking. From then on, when the critical STMP concentration was exceeded, more negative charged species were formed. Consequently, we recommend using the critical concentration of STMP to obtain the best yield of crosslinking in polysaccharide hydrogels. Finally, STMP is a cheap and non-toxic crosslinking agent. It reacts with many (probably all) polysaccharides via a mechanism which we explained and correlated to the physicochemical behaviour of the gels. The better understanding of this reaction could encourage the industries to use STMP as crosslinker agent of polysaccharides of grade food.

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