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ORIGINAL CONTRIBUTION

A novel amphiphilic pH-sensitive hydrogel based on pullulan

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Abstract Novel polyelectrolyte and amphiphilic hydrogels based on pullulan have been prepared using 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide hydrochloride and N-hydroxysuccinimide. The cross-linking reaction is fast and lead to zero length ester cross-links by the reaction of a carboxylate group with an alcohol function of the polysaccharide. The charge density and the hydrophobic rate of the precursor carboxymethylpullulan (CMP) are controlled during the carboxymethylation of pullulan and the grafting reaction of octyl chains on CMP, respectively. The grafting degree influences the conformation of the hydrophobically modified CMP (HMCMP) in solution and leads to the formation of hydrophobic clusters firstly in the HMCMP solutions and further in the HMCMP hydrogels. The swelling measurements of HMCMP hydrogels at different salt concentrations (0–0.2 M NaCl) and different pH (3–11) shows the ionic strength and pH sensitivity of the gels. The loading of a hydrophobic probe molecule can be controlled by the grafting degree of HMCMP hydrogels.

Keywords Pullulan · Hydrogel · Polyelectrolyte · Amphiphile · pH sensitive

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Introduction

Environment-sensitive hydrogels offer enormous potential in various applications, e.g., controlled drug delivery [1–3], immobilization of enzymes or cells [4], or tissue engineering [5]. They exhibit drastic changes in their swelling behavior and mechanical properties in response to external stimuli such as temperature, pH, or ionic strength. The polymers used to produce environment-sensitive hydrogels are mostly synthetic. Poly(N-isopropylacrylamide) [poly (NIPAM)] or poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) block copolymers (Pluronic®) are well known to show a lower critical-solution temperature and exhibit a temperature sensitivity. The acrylate and methacrylate families of synthetic polymers are usually used to prepare pH-sensitive hydrogels.

However, because of their nature, these synthetic polymers are not biodegradable, and consequently, their applications in the medical field such as drug delivery are limited. Natural polymers (polysaccharides or polypeptides) present higher advantages in this area because they are often biocompatible (the most famous are hyaluronan [6], chitosan [7, 8], alginate [9]) and often biodegradable through enzymatic or catalytic pathways. Recent studies concern pH-sensitive hydrogels prepared from dextran [10–14] or polypeptides [15].

pH-sensitive hydrogels are obtained when acidic or basic functional groups are present on the polymer backbone [2]. The presence of ionizable groups implies a dependency of the swelling with external conditions, e.g., pH, ionic strength, or counterion as the swelling is mainly due to the electrostatic repulsions of the charges present on the polymer. The introduction of hydrophobic groups also influence the swelling by the formation of intra- or

intermolecular associations of the hydrophobic moieties [16, 17] resulting in a decrease in the swelling.

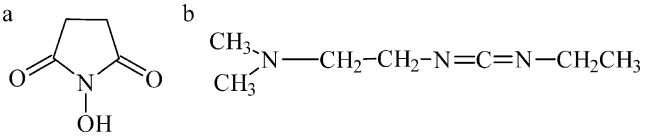
In this paper, we report on the synthesis of novel amphiphilic polyelectrolyte hydrogels based on pullulan prepared by a fast and easy reaction of cross-linking with 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS; Scheme 1). Pullulan is a water-soluble polysaccharide, which is produced by *Aureobasidium pullulans*. Their biological activities can be adjusted by chemical modification [18]. Its linear flexible chain is formed from α-1,4 linked glucose units that are included in a α-1,6 linked maltotriose unit. Various amphiphilic pullulan derivatives have been prepared in our laboratory [19–21]. These hydrophobically modified carboxymethylpullulans (HMCMPs) were shown to develop spontaneous inter- and/or intramolecular interactions in aqueous solution, depending on the content and length of grafted chains [22, 23]. Our purpose was to modify pullulan by a carboxymethylation reaction to introduce acidic groups and to graft octyl chains to the CMP to obtain a range of HMCMPs with various controlled hydrophobic rates and charge densities and finally, to cross-link HMCMPs with EDC and NHS. The cross-linking reaction occurs between the hydroxyl group and carboxylic group of the HMCMP leading to an ester link. EDC and NHS react only as activators of the esterification reaction, and this method was first described by Tomihata and Ikada [24] and further by Zhang et al. [13].

We describe here the synthesis of HMCMP hydrogels and demonstrate their ionic strength and pH sensitivity. We show the ability of these hydrogels to entrap a hydrophobic probe molecule as a function of the charge density and the hydrophobic rate.

Materials and methods

Materials

Pullulan was purchased from Hayashibara Biochemical Laboratory (Japan). EDC, sodium chloroacetate, Nile red (NR) were purchased from Sigma-Aldrich (France) and NHS from Acros Organics (France) and sodium chloride (NaCl), sodium hydroxide (NaOH), hydrochloric acid (HCl), tetrabutylammonium hydroxide (tBu_4N^+, OH^-) from VWR (France). Water was purified with the milli-Q water reagent system (Millipore). All compounds were used without further purification.



Scheme 1 Structure of **a** NHS and **b** EDC

Synthesis of CMP

The sodium salt of CMP (CMP,Na^+) was synthesized in isopropanol/water medium (2/1 *V/V*) by reacting the hydroxyl groups of pullulan with sodium chloroacetate in the presence of sodium hydroxide, according to the procedure described in previous works [19]. The substitution degree (DS) of carboxylate groups per anhydroglucose unit (AGU) was determined by conductimetric titration [25]. Two different CMPs with two different initial DS_0^i (0.62 and 0.88) were synthesized; the one with $DS_0^i = 0.88$ was used for the syntheses of HMCMPs.

Synthesis of HMCMPs

HMCMPs were prepared according to a method previously described [23] and were obtained by covalent bonding of *n*-bromooctan on carboxylic acid groups of CMP; the sodium salt of CMP was transformed into its acidic form (CMP,H^+) using a cationic resin (Amberlite IRN-77, H^+ form) and then neutralized by tetrabutylammonium hydroxide (tBu_4N^+, OH^-) up to pH 7 to obtain tetrabutylammonium salt of CMP (CMP, tBu_4N^+). After freeze-drying, a known amount of CMP, tBu_4N^+ was dissolved in dimethyl sulfoxide (at a concentration of 100 g l^{-1}) at 40 °C. *n*-Bromooctan was added using a syringe, and the reaction was stirred at 40 °C for 24 h. The amount of *n*-bromooctan was varied to obtain different degrees of substitution in octyl chains. The number of octyl chains (τ) per AGU was determined by gas chromatography analyses after saponification in sodium hydroxide. The codification of the different samples is the following: a HMCMP substituted by τ octyl chains per 100 AGU is coded as CMP-τ-C_8. It should be noted that the charge density (corresponding to the carboxylate groups) decreases as the grafting degree (τ) increases (final $DS_0 = DS_0^i - \tau$). Thus, HMCMP were obtained from a CMP with a higher DS_o to have approximately comparable charge densities between hydrogels based on CMP and based on HMCMP. Table 1 resumes the different CMP-τ-C_8 synthesized.

Cross-linking procedure

CMP and its hydrophobically modified derivatives were cross-linked using carbodiimide chemistry in water in the presence of EDC and NHS (Scheme 2). Hydrogels are formed by reactions between hydroxyl groups and carboxylic groups of the polysaccharide chains leading to ester bonds. The location of the cross-link is unknown. Typically, 500 mg of HMCMP was dissolved in 3 ml of milli-Q water, and then a solution of EDC (160 mg) and NHS (25 mg) in 1 ml of milli-Q water was added while stirring (the different experimental ratios between reactives and CMP are given in Table 1). The gel was formed

Table 1 Characteristics of HMCMPs hydrogels

HMCMP	DS_o [a]	τ [b]	DS_{gel} [c]	R [d] (%)
CMP	0.62	0	0.40	35
CMP-7-C8	0.81	0.07	0.44	46
CMP-12-C8	0.76	0.12	0.42	45
CMP-28-C8	0.60	0.28	0.31	48
CMP-40-C8[e]	0.48	0.40	–	–

Cross-linking reaction conditions: polymer concentration Cp=125 g l^{-1}, [NHS]/[COO^-]=0.15, and [EDCI]/[COO^-]=0.57

[a] DS of carboxylate groups per anhydroglucose unit before cross-linking

[b] τ is number of octyl chains per anhydroglucose unit

[c] DS of carboxylate groups per anhydroglucose unit after cross-linking

[d] R is the yield of cross-linking=$(DS_o-DS_{gel})100/DS_o$

[e] CMP-40-C8 was not cross-linked

in a few minutes and stood for 2 h. Finally, it was dialyzed against milli-Q water and freeze-dried.

The cross-linking degrees (assimilated to the yield of reaction) of the hydrogels were measured by conductimetric titration and were calculated from Eq. 1. The results are given in Table 1.

$$R = \left(DS_0 - DS_{gel}\right)100/DS_0 \quad (1)$$

Rheological measurements

Rheological measurements were performed with a AR2000 rheometer from TA instruments (UK) using a standard-size double concentric cylinders as geometry. A flow procedure with a shear stress ranging from 0.01 to 10 Pa was applied to measure the viscosity of CMP and HMCMP solutions at 25 g l^{-1} as a function of shear rate. These measurements show the influence of the grafting degree on polymer conformation (measurement conditions: steady step flow step 1 at 25 °C from 0.01 to 10 Pa with ten points per decade, sample period=10 s, maximum point time=1 min; steady step flow step 2 at 25 °C from 10 to 0.01 Pa with ten points per decade, sample period=10 s, maximum point time=1 min).

Swelling measurements

A known amount of dried hydrogel was immersed in a solvent (water or NaCl solutions of well-defined pH and concentrations). The swollen gel was then weighed, and the swelling degree was calculated according to Eq. 2 where m is the weight of the swollen hydrogels and m_o, the weight of the dried hydrogels.

$$S = \frac{m - m_0}{m_0} \quad (2)$$

We checked that there was no loss during the swelling and the drying steps by weighting the dried gels at the end of the measurements. The swelling value was averaged using at least three samples. The swelling measurements at different pH were performed in NaCl 10^{-3} mol l^{-1} (to keep a constant ionic strength) by adding a known amount of HCl or NaOH to adjust the pH between 3 and 11.

Entrapment of a probe molecule

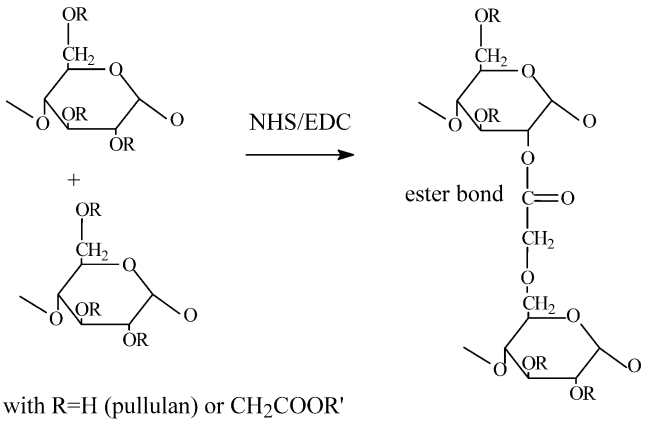
To study the capacity of HMCMP hydrogels to carrier drugs, we chose the probe molecule: NR, a hydrophobic molecule (Scheme 3). Entrapment was followed by UV-Visible analyses NR solutions in which the dialysis bags containing HMCMP hydrogels (or CMP precursors) were immersed. Concentration of NR entrapped was calculated by the difference between the initial concentration and final concentration of solutions containing the dialysis bags. Typically, a dialysis bag containing a known amount of dried hydrogel was immersed in 35 ml of NR solution (5×10^{-5} mol l^{-1} in water/ethanol [2/1 *V*/*V*]) while stirring for 72 h. The concentration of probe molecule in the supernatant was measured by spectrophotometry analysis (Spectrometer UVIKON 860, Kontron Instruments) at λ=580 nm.

The concentration of NR in the supernatant was estimated from a calibration curve of absorbance vs concentration plotted with NR concentrations varying from 1.25×10^{-5} to 5×10^{-5} mol l^{-1}.

Results and discussion

Syntheses

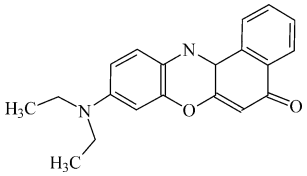
HMCMPs by alkyl chains have been widely studied in our laboratory [19, 20, 22, 23]. The physico-chemical properties in dilute and semidilute regime of HMCMPs exhibit different behaviors depending on intrinsic (molecular



with R=H (pullulan) or CH_2COOR'

and R'= Na+ (CMP) or $(CH_2)_7CH_3$ (HMCMP)

Scheme 2 Cross-linking reaction of HMCMP



Scheme 3 Structure of Nile red

weight, grafting degree) or extrinsic (concentration, pH, salinity) characteristics.

For lower molecular weight (M_n=30,000 g mol^{-1} [23] instead of 160,000 g mol^{-1} [22]), it has been shown that HMCMPs display aggregation because of the existence of hydrophobic intermolecular interactions for all the amount of grafted octyl chains. For HMCMPs of 160,000 g mol^{-1} of molecular weight, we have obtained different conformations and behaviors as a function of the grafted octyl chains, depending on the solvent (pure water or with salt) and the polymer concentration. The transition between dilute and semidilute solution (previously studied by viscometry, by the determination of the critical concentration C_{cr} above which the polymer chains begin to overlap each other) depends on the grafting degree of C8 and Simon et al. [22] showed that in dilute regime, for lower grafting degrees (τ<10), HMCMPs have a conformation close to the CMP precursor, for instance isolated chains with very few aggregates. For 10<τ<30, the intermolecular interactions increase (and C_{cr} decreases), on the contrary when the grafting degree is above 30%, C_{cr} increases, the intramolecular associations predominate, and the conformation of the polymer exhibits a very compact structure inducing very small hydrodynamic volume. Finally, for higher grafting degrees (τ>45%), aggregation occurs, and the polymer becomes insoluble above 50% of octyl chains. In semidilute regime, hydrophobic intermolecular associations occur and permit the connection of amphiphilic chains for lower critical concentration than without hydrophobic grafting.

These hydrophobic associations lead to the presence of hydrophobic clusters in the polysaccharide, which could be used as drug carriers. But without covalent bonding, the three-dimensional structure of these HMCMPs can be disrupted by dilution or shear stress.

The originality of this work was to obtain hydrogels by a very simple cross-linking reaction of HMCMPs in water in the presence of a water-soluble carbodiimide (EDC) and NHS [13] leading to amphiphilic negatively charged hydrogels with zero length ester cross-links. The presence of a zero-length ester cross-link is an advantage because ester links are easily broken and no by-products are formed during the hydrogels' degradation. The presence of carboxylate groups and hydrophobic clusters in HMCMP hydrogels allows entrapment of a wide range of molecules for drug delivery systems. The choice of grafting the CMP by octyl chains (with ester links) was motivated by the fact that their efficiency to entrap hydrophobic molecules had already been proved [23, 25].

Both ionic and hydrophobic characters are controlled by experimental conditions of the carboxymethylation of pullulan and the grafting reaction, respectively.

A range of amphiphilic polyelectrolyte hydrogels with different grafting degrees (0, 7, 12, and 28) have been synthesized (Table 1). The very high polymer concentration (125 g l^{-1}) used during the cross-linking reaction suppose that the critical concentration is reached and that intermolecular hydrophobic associations occur leading to clusters. Simon et al. [22] showed that these associations depend on

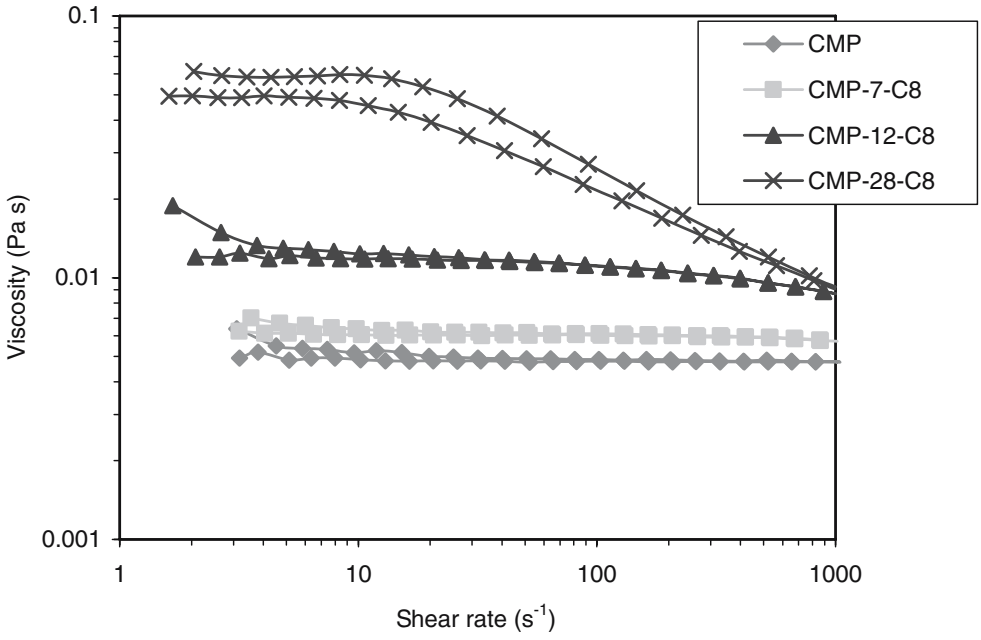


Fig. 1 Viscosity versus shear rate for aqueous solutions of HMCMP at 25 g l^{-1}

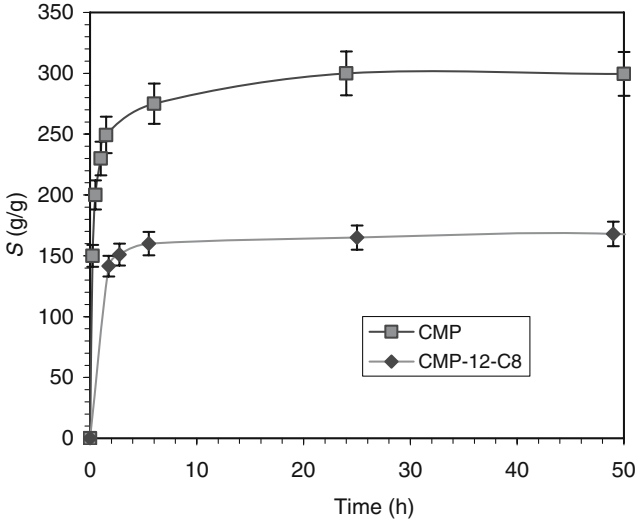


Fig. 2 Swelling kinetic in water of CMP and CMP-12-C8 hydrogels

the grafting degree. To check this behavior on our HMCMPs, we measured their viscosities vs shear rate. The rheological behavior of CMP-τ-C8 (measurements were conducted at 25 g l^{-1} instead of 125 g l^{-1}, by supposing that HMCMPs are above the critical concentration) shows an increase in the viscosity if τ increases (Fig. 1) as a consequence of an increase in the intermolecular hydrophobic associations. A slight thixotropy is observed for the CMP-28-C8 in the measurement conditions. So the quantity of hydrophobic clusters present in HMCMP hydrogels becomes higher if τ increases. Thus, the amphiphilic properties of the hydrogels can be adapted to specific applications.

The yield of cross-linking reactions was determined by the measurement of the substitution degree of carboxylate groups by conductimetric titration. These measurements also allowed the comparison of the charge density of hydrogel. As shown in Table 1, DS_{gel} (corresponding to the charge density of hydrogel) is almost constant except for CMP-28-C8 in which the charge density is logically slightly lower (0.3 instead of 0.4). The yield is almost the same for the cross-linking reactions of the HMCMPs but is lower for CMP. In the presence of hydrophobic graftings, there is a physical attraction of the polymer chains, facilitating the reaction between the two reactive functions.

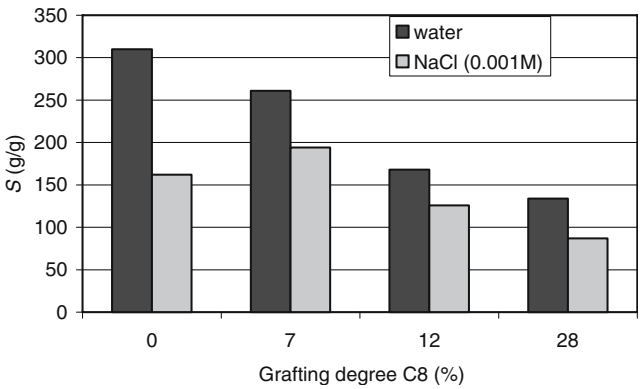


Fig. 3 Swelling of HMCMP hydrogels in water or NaCl 10^{-3} mol l^{-1} vs the grafting degree of octyl chains

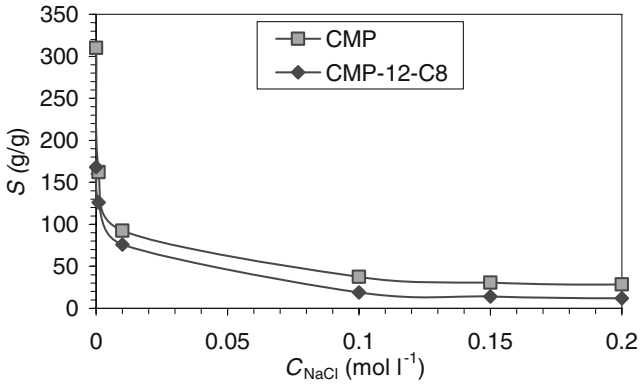


Fig. 4 Swelling of CMP and CMP-12-C8 hydrogels vs NaCl concentration

Swelling

The swelling of HMCMP hydrogels has been studied in water and in the presence of NaCl at different concentrations and pH to evaluate the capacity of these gels to carrier drugs.

Effect of ionic strength on swelling

The initial rate of the swelling of CMP hydrogel (Table 1—entry 1) in water is very high, and the maximum of swelling is reached within about 10 h (Fig. 2). In comparison, the initial rate of swelling of a grafting CMP (CMP-12-C8) is lightly lower because of the presence of intermolecular hydrophobic interactions that slow down the polymer chain movement.

The grafting degree influences the maximum swelling of HMCMP hydrogels in pure water, which decreases if τ increases (Fig. 3). The higher the grafting degree, the more the hydrophobic interactions occur and influence the

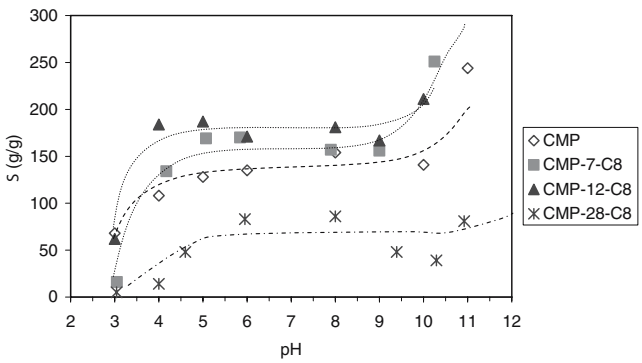


Fig. 5 Swelling of HMCMP hydrogels in NaCl 10^{-3} mol l^{-1} vs pH

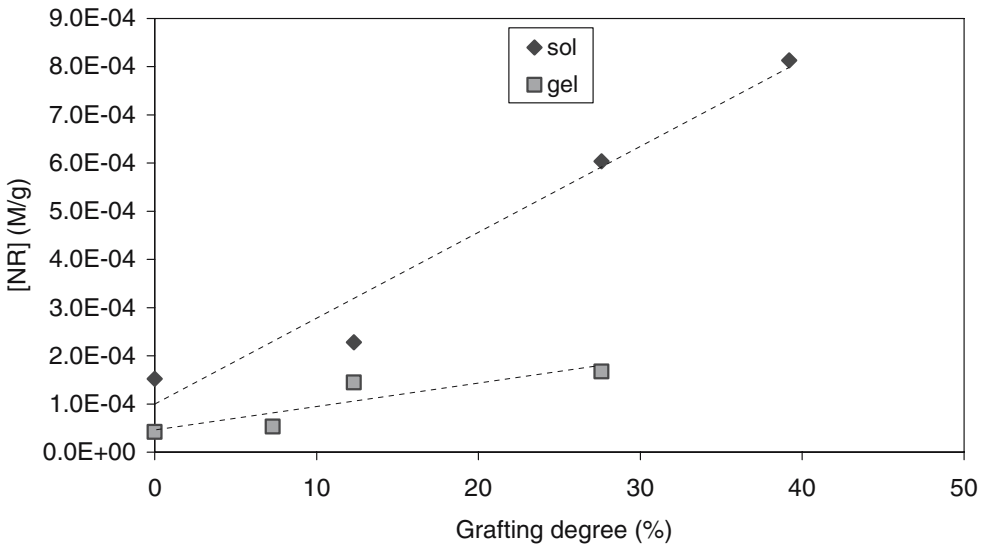


Fig. 6 Concentration of NR loaded in HMCMPs solutions and hydrogels vs the grafting degree of octyl chains

swelling by compacting the polymer chains. There is also a correlation between the swelling and the yield of cross-linking reaction; the swelling degree decreases by increasing the yield of cross-linking reaction, and the results are in agreement with the experimental values of the yields.

If NaCl is added, the swelling degree of all hydrogels highly decreases (Fig. 3) and reaches a minimum value at about 0.1 mol l^{-1} in salt (Fig. 4). In the presence of NaCl, the negative charges are screened so the electrostatic repulsions decrease and permit a contraction of the three-dimensional network. We can suppose that all the charges are screened at about 0.1 mol l^{-1}, which can explain why the NaCl concentration does not affect anymore the swelling above this value. It can be noticed that the effect of ionic strength on swelling is more important on precursor CMP hydrogel (no hydrophobically modified): S is almost divided by 2 between NaCl 0 and 10^{-3} mol l^{-1} for CMP although, it is only divided by about 1.3 for CMP-τ-C8 (Fig. 3). In the absence of intermolecular hydrophobic interactions, the mobility of the polymer chains is larger, leading to an easily tightening of the meshes. The effect of ionic strength on the swelling of HMCMP hydrogels presents a reversible character because if the gels are replaced in pure water, the swelling increases again.

Effect of pH on swelling

The swelling of HMCMP hydrogels was measured between pH 3 and 11 (in NaCl 10^{-3} mol l^{-1} to avoid the effects of salt when we adjust the pH by adding the appropriate amount of HCl or NaOH 0.1 mol l^{-1}). The presence of carboxylate groups in HMCMP hydrogels supposes a pH-dependent swelling. At lower pH (under 5), the carboxylic groups (COOH) are protonated, and the electrostatic repulsions decrease as a consequence, leading to a decrease in swelling. Between pH 5 and 8, the COOH are dissociated, and the swelling should be constant if the ionic strength is also fixed. And finally if the pH is increased up to 10, two phenomenon can appear: on one hand, the ester bonds that constitute the cross-links probably start to degrade themselves and the three-dimensional structure is lost little by little, and on the other hand, the ester bonds of the hydrophobic grafts probably also start to degrade themselves, so there are less and less hydrophobic interactions in hydrogels, and the swelling increases as a consequence of both phenomenon.

As expected, Fig. 5 shows that the swelling of each gel is almost constant between pH 5 and 8. Before pH 5, the swelling is lower, and above pH 10, it is higher. At pH 3, the hydrogels were replaced in pure water, and their swelling increased again indicating the reversibility of the pH dependence.

HMCMP hydrogels are ionic strength and pH sensitive, and the swelling is also dependent of the grafting degree; the control of the charge density and the grafting degree during

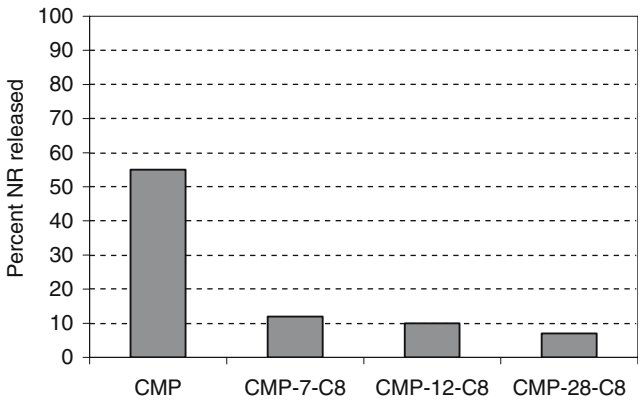


Fig. 7 Percentage of NR released from the HMCMPs hydrogels in NaCl 0.5 mol l^{-1}

the syntheses allowed the adjustment of the hydrogels swelling properties to specific applications.

Loading of a probe molecule: NR

NR is a hydrophobic molecule that is usually used as a stain in cellular biology.

Measurements were first made on solutions of CMP-τ-C8 (not cross-linked) to check the proportionality of NR loading with the grafting degree τ [25]. Kinetics of loading were followed for each sample during 7 days, and as shown in Fig. 6, the amount of NR absorbed in the solutions of CMP-τ-C8 was proportional to τ. The method used to absorb NR (dialysis bag immersed in NR solutions) involves that NR can diffuse even in no HMCMP, and so, the straight lines in Fig. 6 do not go through the origin.

Measurements were then done with the HMCMP hydrogels, and as expected, the amount of NR loaded was also proportional to τ but was slightly smaller than with linear HMCMP (Fig. 6). Hydrophobic clusters are certainly smaller and less numerous in a gel than in a solution because of a chain movement (contributing to the formation of the hydrophobic associations) restricted by the ester cross-links.

So, HMCMP hydrogels are able to load hydrophobic molecules, and it is possible to adjust the quantity of entrapped drug by adjusting the grafting degree during the synthesis of HMCMP.

The release of NR was performed in 0.5 mol l^{-1} NaCl solution and was very low (Fig. 7) probably because of a strong affinity of NR with the hydrophobic clusters in the hydrogels. Similar results were observed with multilayer films based on amphiphilic pullulan [26].

Conclusion

This study shows that novel amphiphilic and polyelectrolyte hydrogels based on pullulan can be easily prepared by cross-linking of CMP modified by τ octyl chains using EDC and NHS and leading to a zero-length ester cross-link. The density of charge (carboxylate groups) and the grafting degree are controlled by the carboxymethylation of pullulan and the grafting reaction of octyl chains onto CMP, respectively. These HMCMP hydrogels are ionic strength and pH sensitive because of the presence of the negative carboxylate groups in the structure. The loading of a hydrophobic molecule is controlled by the grafting degree of hydrophobic octyl chains. The amphiphilic character and the ionic strength and pH sensitivity of these amphiphilic hydrogels confer on it a large domain of specific applications, and they could be regarded as macromolecular carrier of hydrophobic molecules.

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