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# New hydrogels based on *N*-isopropylacrylamide copolymers crosslinked with polylysine: membrane immobilization systems

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**Abstract**

New hydrogels based on *N*-isopropylacrylamide copolymerized with an activated monomer have been developed as vesicle immobilizing devices. In order to provide a strong and reversible interaction between the solid support and the lipid vesicles, we chose polylysine, a cationic polypeptide as the anchoring element. Our systems are based on *N*-isopropylacrylamide copolymerized with an activated monomer and then crosslinked with polylysine. The innovative feature of this approach is that the polypeptide plays many key roles: (i) it is used as crosslinker; (ii) its positive charges act as anchor sites; and (iii) as an hydrophilic molecule, polylysine improves the swelling properties of the gel and therefore the capacity of vesicle binding. Several hydrogels were synthesized with varying monomer ratios and polylysine lengths. The characterization of the systems includes an estimation of the ability of the gels to immobilize vesicles and of the integrity of the adsorbed vesicles. The most efficient gel is made of a copolymer containing 6 mol% of activated monomer crosslinked with a long polylysine (degree of polymerization of 288). This hydrogel can immobilize up to 1000 μmol of lipids per gram of dry gel. Control experiments show clearly that the nature of the anchoring interaction is electrostatic. As an illustration of the potential applications of such a system, we show that vesicles can be immobilized in a gel-packed column and the release of their content is triggered by an increase of the temperature. 

*Keywords*: *N*-isopropylacrylamide; Polylysine; Lipid vesicle

## 1. Introduction

The immobilization of membranes on solid supports leads to several applications. Solid-supported membranes can be used as containers for controlled drug release systems [1], as a stationary phase for chromatographic devices [2–5], as continuous operating systems performing enzymatic processes [6,7] or as artificial organs [8]. Different methods have been used to immobilize lipid membrane on a solid support. Lipids can be immobilized by covalent bonding [2], by entrapment in a solid matrix [4,8] or by ionic binding. This latter interaction is advantageous in several ways. As an interfacial phenomenon, the electrostatic interaction is less likely to perturb the integrity of the membrane. The binding is usually strong, stable, and reversible; consequently, the solid support can easily be washed and reused. Positively charged matrices are suitable to immobilize cells as biomembranes generally contain negatively charged lipids [9].

We have designed and developed hydrogels based on poly-*N*-isopropylacrylamide crosslinked with polylysine as vesicle immobilizing systems. Poly(*N*-isopropylacrylamide) (PNIPAAm) has been selected for the development of the immobilization system because it is hydrophilic and non-denaturing for many proteins [10]. Aqueous solutions of PNIPAAm have a lower critical solution temperature (LCST) around 30–35°C [10]. Because of their thermal sensitivity, homo- and copolymers of *N*-isopropylacrylamide (NIPAAm) have been used in immunoassays, bioseparations, controlled release systems, and enzyme bioreactors [10–15]. Related NIPAAm hydrogels retain the thermosensitivity [10], and this property can be exploited to control the release from the vesicles immobilized on the gel. A key feature of our systems is that polylysine was used both as the crosslinker and the membrane anchor. It is a water-soluble polycation extensively used for biological application. This polypeptide can interact with ionic sites on vesicles or cell surfaces [16]. It was shown that there is a tight binding between polylysine and negatively charged lipids and this interaction depends on the degree of polymerization (DP) of the peptide [17–19]. The DP may also influence the crosslinking reaction, the swelling of the resulting gels and the

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number of anchoring sites. Therefore, we have examined the effect of the DP of polylysine on the properties of the hydrogels to optimize the performance of the systems.

To crosslink PNIPAAm with polylysine, *N*-acryloxysuccinimide (NAS) was used as the reactive comonomer since its succinimide group is chemically stable but shows high reactivity and selectivity toward amine nucleophiles [11,12,20]. Since the degree of crosslinking may affect the mechanical properties of the hydrogel obtained, various amounts of NAS were used in the preparation of the copolymers to optimize the gel properties.

We report here the preparation of the NIPAAm/NAS copolymers and the related gels. The characterization for the membrane immobilization ability of different gels is also discussed. The most efficient gel is used in an application of thermally controlled release.

## 2. Materials and methods

All chemicals and biochemicals including NIPAAm and acrylic acid (AA) were purchased from Sigma (St. Louis, MO, USA) and Aldrich (Milwaukee, WI, USA), unless otherwise mentioned. AA was purified by distillation in the presence of an inhibitor. Ninhydrin was purchased from Baker Analyzed (Phillipsburg, NJ, USA), sulforhodamine B (SRB) from Molecular Probes (Eugene, OR, USA), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylglycerol (POPG) from Avanti Polar Lipids (Birmingham, AL).

### 2.1. Synthesis of N-acryloxysuccinimide

The coupling of AA with *N*-hydroxysuccinimide (NHS) was carried out as described previously [21]. NHS (0.014 mol) and AA (0.014 mol) were dissolved in 20 ml of anhydrous chloroform. Dicyclohexycarbodiimide (DCC) (0.014 mol) dissolved in anhydrous chloroform (10 ml) was slowly added to this solution. The mixture was stirred for 45 h at room temperature. The insoluble dicyclohexylurea was removed by filtration. NAS was purified by chromatography on a silica gel column. The product displays the expected spectral characteristics [11]: $^1$H NMR ($CDCl_3$) 2.85 (s, 4 H), 6.0–7.0 ppm (m, 3 H); IR (KBr) 1800, 1775, 1735, 1260, 995, 870 $cm^{-1}$.

### 2.2. Synthesis of poly(N-isopropylacrylamide-co-N-acryloxysuccinimide)

The copolymer was prepared by dissolving NIPAAm and NAS (total weight about 1 g) in 15 ml of a mixture of anhydrous tetrahydrofuran (THF) and anhydrous toluene 1/3 (v/v); the NAS proportions were 2 or 5 mol%. 2,2′-Azoisobutyronitrile (AIBN) (1 mol% of the monomers) was added as the initiator for the polymerization, performed under nitrogen at 55°C for 24 h. THF (60 ml) was added to the mixture to solubilize the copolymer. The mixture was then added drop-wise into 500 ml petroleum ether to precipitate the copolymer. The precipitate was filtered under vacuum and dried at 42°C overnight [22]. The homopolymer of NIPAAm was synthesized similarly in toluene.

### 2.3. Crosslinking of the copolymers by polylysine

A long poly(L-lysine) with a DP of 288 (PL$\mathcal{L}$) and a short one with a DP of 19 (PLs) were used as crosslinkers. 200 mg of the copolymer were swollen in 1 ml of dimethylformamide. Polylysine (about 50 mg) in 10 ml of 2-*N*-morpholinoethanesulfonic acid (MES) buffer (50 mM, pH 7.4) was added to the polymer and incubated at 10°C during 48 h with constant agitation at 40 rpm [14] and then at room temperature overnight. The resulting gels were repeatedly washed with water and then lyophilized.

### 2.4. Characterization of the copolymers

The molecular weights of the linear copolymers were determined by size exclusion chromatography (SEC) using THF as the mobile phase and polystyrene as standards. The LCST was determined by measuring the turbidity at 500 nm of a solution containing 0.2% (w/w) copolymer in water as the sample was heated up.

The succinimide group content in the copolymer before and after crosslinking was monitored by FTIR spectroscopy. The NAS content was calculated from the ratio of the intensity of a band associated with the succinimide group at 1735 $cm^{-1}$ to that of the band at 2876 $cm^{-1}$ corresponding to the acrylamide monomer. Calibration curves were made from spectra of NAS and NIPAAm mixtures with various ratios. A quadratic function was used to fit the baseline in both regions prior to the determination of the intensity. The samples were included in KBr pellets and the spectra were recorded on a Bio-Rad FTS-25 FTIR spectrometer equipped with a mercury–cadmium–telluride detector. For each spectrum, 100 scans were collected with a nominal resolution of 2 $cm^{-1}$.

### 2.5. Characterization of the synthesized gels

The succinimide content was determined by FTIR as described above. The unreacted polylysine in the wash was assayed with the Lowry protein assay [23]. The polylysine immobilized in the hydrogel was estimated as the difference between the amount used as reactant and the recovered quantity in the wash.

The swelling ratio (SR) of the gels was measured gravimetrically. The gels were equilibrated in water at room temperature overnight. The SR was determined by

$$\mathrm{SR} = \frac{w_S - w_D}{w_D} \quad (1)$$

where $w_S$ and $w_D$ are the dry and the swollen weights of the gel, respectively.

### 2.6. Acetylation of hydrogel amino groups

The gel was swollen in an aqueous solution of sodium acetate (450 mg/ml, pH 7.5) and stirred for 5 h. Acetic anhydride (60 times in excess) was then added to the mixture. The acetylation was carried out between 0 and 4°C during 3 h while the pH was maintained at 7.5 [24]. Several hours were needed for the reaction, because of the limited diffusion of the reactants in the gel. The sample was then centrifuged, washed with water several times and lyophilized. The amount of amino-group substitution was measured by the ninhydrin procedure of Brown and Green [25], using PL*s* as the standard. To a known weight of acetylated gel were added 75 μl of a ninhydrin solution in ethanol (50 g/l), 100 μl of a solution of phenol in ethanol (4 g/ml) and 75 μl of a KCN solution in pyridine (20 μM). The mixture was well vortexed and heated at 100°C for 5 min. The absorbance was measured in the supernatant at 568 nm. The result was compared to that obtained on PNIPAS6–PL$\ell$ before acetylation.

### 2.7. Interaction of the free and grafted PL with the vesicles

First, the binding of free PL$\ell$ and PL*s* to lipid vesicles was determined by a centrifugation assay [26]. The polylysine was dissolved in a MES buffer and added to a suspension of POPC:POPG (85:15) multilamellar vesicles (MLV). The samples were then freeze-and-thawed five times and centrifuged to isolate the free polylysine from that bound to the vesicles. Polylysine concentration in the supernatant was measured by the Lowry protein assay [23].

Two aspects were examined for the immobilization of lipid vesicles on the crosslinked gels: the amount of lipids retained by the gel, and the integrity of the immobilized lipid vesicles. In order to perform these assays, dye-containing vesicles were prepared by hydrating the lipids with a buffer (50 mM HEPES, 5 mM ethylenediaminetetraacetic acid (EDTA), 10 mM NaCl, pH 7.4) containing SRB (80 mM SRB) [27]. The lipid suspension (ca. 30 mM) was then freeze-and-thawed and extruded to obtain vesicles of 100 nm in diameter. The SRB-containing vesicles were separated from the free SRB by SEC using a column filled with Sephadex G-50 fine gel swollen in an isosmotic external buffer (50 mM HEPES, 5 mM EDTA, 134 mM NaCl, pH 7.4). The dry gel (between 2 and 7 mg) was swollen in 2 ml of external buffer and vigorously stirred for an hour to grind the gel. The size distribution of the resulting particles depends on the mechanical properties of the gel, as discussed later. The lipid vesicles were added to the gel particles and the samples were incubated at 25°C for 1.5 h with constant agitation. The samples were then centrifuged to pellet the gel. The quantity of immobilized lipids was determined by measuring the phospholipid concentration in the supernatant using the Fiske–SubbaRow phosphorus assay [28]. A control experiment showed that all the lipids remained in the supernatant after incubation and centrifugation in the absence of the gel.

The self-quenching property of the entrapped dye was used to estimate the integrity of the immobilized vesicles. The fluorescence of SRB inside the vesicles is quenched because of its high concentration [29]. If the vesicles permeability is affected by the interaction with the gel, SRB would leak out and the internal concentration of the probe would decrease: this dilution would lead to an increase of fluorescence. This perturbation can be probed by the self-quenching efficiency ($Q$) of the remaining entrapped SRB. This parameter is calculated according to:

$$Q = \left(1 - \frac{I_0}{I_T}\right)100 \tag{2}$$

where $I_0$ is the initial fluorescence intensity of the SRB after vesicle immobilization and $I_T$ the total fluorescence intensity measured after complete disruption of all the vesicles by Triton X-100 (0.1 vol%). The $Q$ values were calculated for the pellet (i.e. on the bound-vesicles) as well as for the remaining free vesicles in the supernatant. In the case of the pellet, an aliquot of the vesicle-containing gel was re-suspended in the external buffer in a fluorescence cell.

The fluorescence measurements were performed at 25°C on a SPEX Fluorolog-2 spectrometer equipped for sample stirring. The fluorescence intensity of SRB was monitored with an excitation wavelength of 565 nm, an emission wavelength of 586 nm and a response time of 0.3 s. The excitation and emission bandpath widths were set at 1.5 and 0.5 nm, respectively.

### 2.8. Controlled release with temperature

The SRB containing vesicles were prepared and immobilized on the swollen gel as described above. The vesicle-containing gel (about 750 mg) was packed in a glass column (10 × 7 mm) thermostated by a water bath. The column was connected to a flow-through cuvette placed in a Hitachi Model 100–60 UV–VIS Spectrophotometer and the absorbance was measured at 565 nm to detect the SRB release. For some collected fractions, the self-quenching of the SRB was determined as described above to determine whether the released SRB is free or still encapsulated in vesicles. The phospholipid concentration in the collected fractions was determinate using the Fiske–SubbaRow phosphorus assay [28]. The flow rate was 25 ml/h. The mobile phase was the external buffer (50 mM HEPES, 5 mM EDTA, 134 mM NaCl, pH 7.4). The gel was first washed at room temperature for 20 min with buffer, the temperature of the column was then increased to 50°C. At the end of the experiment, when a flat baseline was recovered, the column was washed by ethanol.

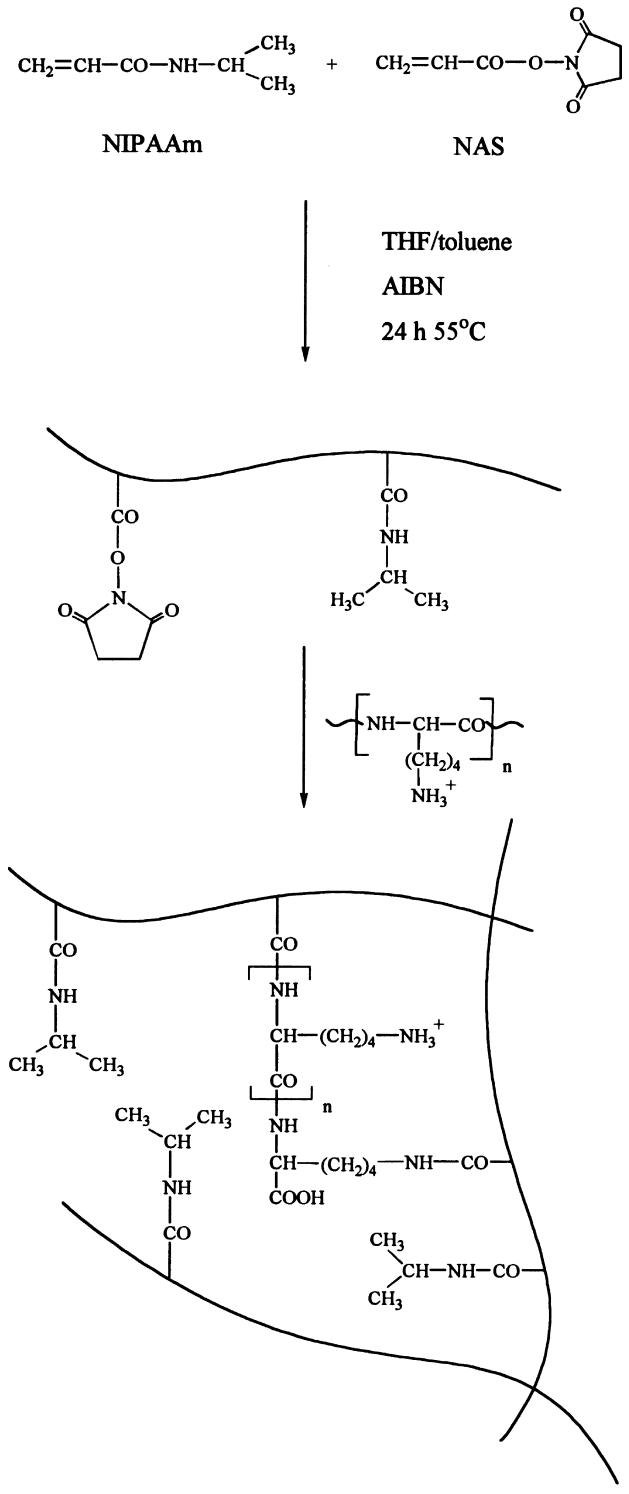


Fig. 1. Synthetic schemes of the copolymers (PNIPAS) and the crosslinking of PNIPAS by polylysine.

## 3. Results and discussion

### 3.1. Synthesis and characterization of the copolymers

NAS was copolymerized with NIPAAm to introduce reactive groups towards the primary amine groups of polylysine (Fig. 1). The degree of crosslinking should depend on the amount of NAS residues in the copolymer. We have used 2 and 5 mol% of NAS in the feed to examine the impact of this parameter. The complete polymerization was confirmed by NMR spectroscopy, inferred by the disappearance of protons associated with the C=C double bond. In addition, the inclusion of succinimide groups in the copolymers was established by the presence of three peaks at 1814, 1784 and 1739 $cm^{-1}$ in the IR spectra (Fig. 2D). These bands were observed in the NAS monomer spectrum (Fig. 2B) but absent in that of the PNIPAAm homopolymer (Fig. 2C). In order to estimate the proportion of NAS in the copolymer, we have developed a method based on band intensity ratios from the IR spectra. The band at 2876 $cm^{-1}$ is due to the symmetric stretching vibration of the methyl group of *N*-isopropylacrylamide (Fig. 2A and C). Its intensity was used to probe the NIPAAm content, the very small contribution of NAS at this wave number (Fig. 2B) being neglected. Three bands were observed in the carbonyl stretching region of the NAS monomer spectrum (Fig. 2B). These bands, at 1800, 1775 and 1735 $cm^{-1}$, were also well resolved in the spectra of the copolymer but slightly shifted upon polymerization (Fig. 2D). The vibrational coupling of the two cyclic carbonyl should lead to two bands in this region, corresponding to the antisymmetric and symmetric coupled C=O stretching vibration [30]. The third band could be attributed to the ester carbonyl of the NAS group. As proposed previously [14], we have used the band at 1735 $cm^{-1}$ to probe NAS, since it is the most intense. A calibration curve was established from the intensity ratio of the band associated with the succinimide group at 1735 $cm^{-1}$ and the band associated with the acrylamide monomer at 2876 $cm^{-1}$ (Figs. 3 and 4). Using this curve, we have estimated that the NAS/NIPAAm ratios of the two copolymers prepared were 4 and 6 mol% of NAS; these copolymers are referred to as PNIPAS4 and PNIPAS6, respectively (Table 1). These ratios are consistent with the reactivity ratios of the monomers (0.277 for NIPAAm and 1.934 for NAS [22]). The content of NAS has also been estimated by determining the concentration of the NHS anions generated by the reaction of succinimide group of the polymer with isopropylamine, measuring its absorbance at 259 nm [11,22,31]. This method was found very sensitive to the reaction conditions (pH, reaction time), especially in the case of gels where the reactants have to diffuse in a polymer matrix. In this case, systematic underestimation of the content of NAS was observed. We believe that the IR method proposed here provides more reliable estimates of the NAS proportions.

Table 1
Characterization of the copolymers

| Copolymer | NAS (mol%) | Yield (%) | $M_n$ (g/mol) | $M_w/M_n$ | LCST (°C) |
|---|---|---|---|---|---|
| PNIPAS4 | 4 | 81 | 12,000 | 3.2 | 33.5 |
| PNIPAS6 | 6 | 73 | 32,000 | 2.5 | 34.1 |

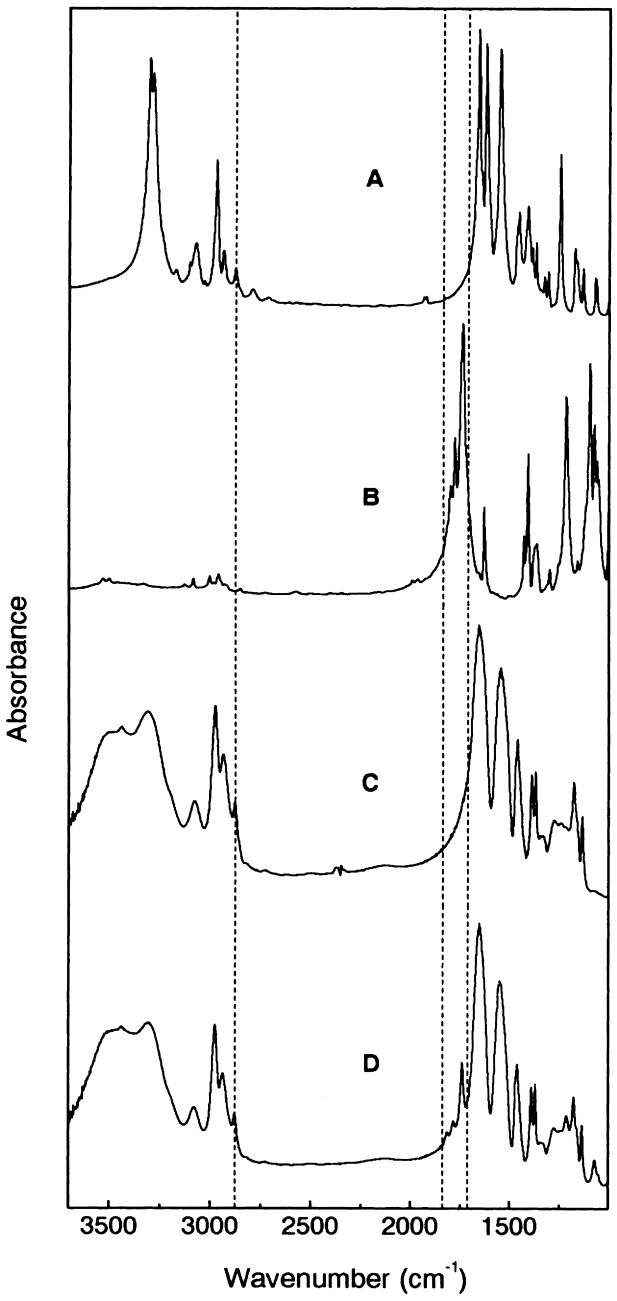


Fig. 2. IR spectra of the monomers, NIPAAm (A) and NAS (B), and the polymers, PNIPAAm (C) and PNIPAS6 (D). The dashed lines (– – – –) indicate the band at 2876 $cm^{-1}$ associated with the symmetric stretching vibration of the methyl group of NIPAAm and the region between 1710 and 1837 $cm^{-1}$ including the bands associated with the succinimide group.

The copolymers have lower molar mass ($M_n$) than the homopolymer of NIPAAm. The $M_n$ was 12,000 and 32,000 for PNIPAS4 and PNIPAS6, respectively, with identical copolymerization conditions. The same phenomenon was observed by Chen et al. [32] for copolymerization with various NAS/NIPAAm ratios: the $M_n$ obtained was in the same range as ours, and increased with increasing NAS/NIPAAm ratio. We have tried to improve the $M_n$ of PNIPAS4 by varying the THF/toluene ratio used for the polymerization since THF has a high chain transfer activity [11,20]. The optimal ratio was 1/3 (v/v) in agreement with Yang et al. [22]. The changes of the initiator concentration and the initial concentrations of the monomers did not lead to any significant improvement of the $M_n$. The LCSTs of the copolymers were around 33–35°C (Table 1). The small difference between the LCSTs of PNIPAS4 and PNIPAS6 may be due to the chemical composition and the molar mass of the copolymers [20].

### 3.2. Crosslinking of the copolymers with polylysine

Two polylysines with different DPs were used as crosslinking agents (Fig. 1). Table 2 summarizes the characteristics of the synthesized gels.

One gel was made with PNIPAS4 copolymer crosslinked by PL$\ell$ and is referred to as PNIPAS4–PL$\ell$. In this case, all the succinimide groups reacted during the crosslinking reaction as inferred from the complete disappearance of the IR bands at 1814, 1784 and 1739 $cm^{-1}$. It was calculated that 250 mg of polylysine were bound per gram of dry gel. The charges introduced by the lysine residues increase considerably the hydrophilicity of the polymer matrix and can explain the high SR observed: the gel can take up water, as much as 42 times of its dry weight. However, the resulting gel is soft, sticky and difficult to manipulate. The low molecular weight of the copolymer PNIPAS4 (12,000 g/mol) may explain the softness of the gel.

The gels PNIPAS6–PL$\ell$ and PNIPAS6–PL*s* resulted from the crosslinking of PNIPAS6 with PL$\ell$ and PL*s*, respectively. Both gels were resilient and easy to grind. Based on the IR determination, about 50% of the initial NAS have reacted in both cases. The presence of unreacted NAS is likely due to the slow diffusion of polylysine in the gels as the crosslinking proceeded. For PNIPAS6–PL$\ell$, 270 mg of PL$\ell$ per gram of dry gel was grafted. As expected, the swelling was high because of the insertion of the polylysine charges in the gel. The gel PNIPAS6–PL*s* contains 125 mg of short polylysine per gram of dry gel; this is about 50% of the polylysine content compared to PNIPAS6–PL$\ell$. In parallel, the swelling of this gel was smaller by a factor of 2, probably as a result of the limited charge content.

### 3.3. Immobilization of vesicles

The ability of the different gels to immobilize vesicles was evaluated (Table 2). The absolute quantity of immobilized lipids is reported in μmol of immobilized lipids per gram of dry gel. The measurement of the self-quenching efficiency $Q$ of a fluorescent probe encapsulated in the vesicles was used to verify the integrity of the immobilized membranes. The experiments were performed with membranes containing 15% of POPG, a negatively charged lipid, and 85% of POPC, a zwitterionic lipid. For all these experiments, the gels were ground in small particles in order to maximize the gel surface accessible for interacting with lipids.

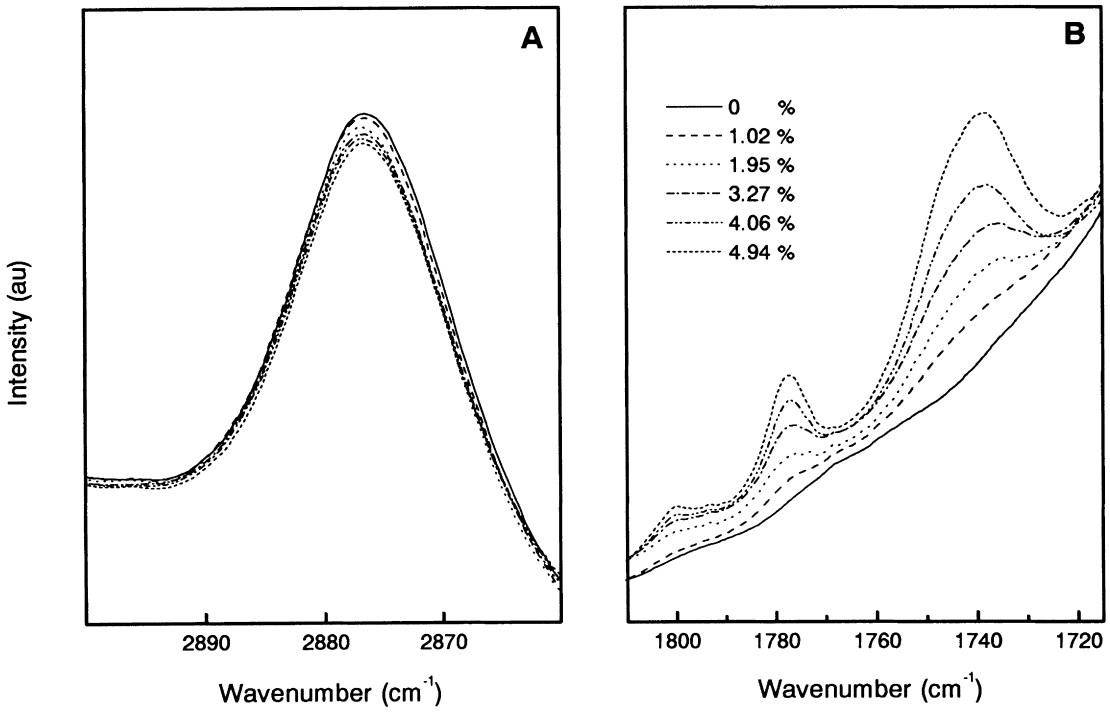


Fig. 3. IR spectra of standard mixtures of NAS/NIPAAm monomers with defined ratio. (A) Band at 2876 $cm^{-1}$ associated with NIPAAm and used to normalize the spectra. (B) Bands at 1800, 1775 and 1735 $cm^{-1}$ associated with the succinimide group.

Because of the poor mechanical properties of PNIPAS4–PL$\ell$, this gel could not be properly ground and very coarse heterogeneous particles were obtained in this case.

Our results indicate clearly that the hydrogels are suitable for vesicle immobilization (Table 2). The best performing gel is PNIPAS6–PL$\ell$. In this case, about 1000 ± 100 μmol of lipids can be immobilized per gram of dry gel (this corresponds to 21 μmol of lipid/ml of swollen gel). If fewer lipids were present during the incubation step, all the lipid vesicles were immobilized illustrating the high anchoring efficiency of the gel. The maximal load for our gel is in the same range as that reported for lipids immobilized sterically by reverse phase evaporation on Sephacryl S-1000 beads [4]. The very good ability of PNIPAS6–PL$\ell$ to immobilize vesicles is directly linked to the presence the polylysines since they provide strong anchoring sites and ensure a good swelling of the gel, and therefore a good accessibility to the sites. The main anchoring force of the vesicles on the hydrogel seems to be of electrostatic nature, as demonstrated by two findings. First, the quantity of the neutral POPC vesicles immobilized is much lower (a reduction of 97%) than that of vesicles containing negatively charged lipids (Table 2). Secondly, the gel has been chemically modified to neutralize the positive charges of polylysine by acetylation. The resulting acetylated gel is referred to as PNIPAS6–PL$\ell$Ac. This gel showed very poor swelling and immobilized a very limited quantity of lipids. These results highlight the prime importance of the lysine charges in our designed gel.

In order to characterize the accessibility of the binding sites to the 100 nm LUV, we have characterized the stoichiometry of the free amino groups per accessible POPG in our gels (Table 3). The free amino group content is defined as the total number of amino groups on polylysine in the gel minus those that are involved in the crosslinking (deduced from the amount of NAS that reacted). The accessible POPG molecules were associated to the POPG on the external leaflet of the membrane, assuming homogeneous lipid

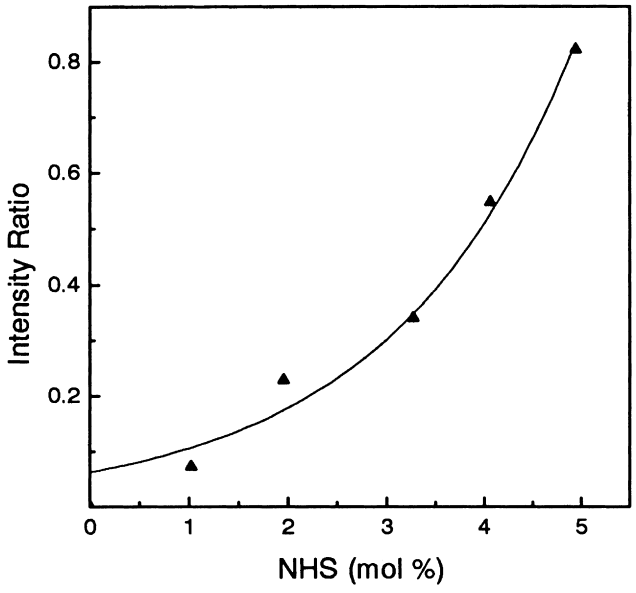


Fig. 4. Calibration curve obtained from the variation of the intensity ratios of the band at 1735 $cm^{-1}$ assigned to the succinimide group to the NIPAAm band at 2876 $cm^{-1}$ in the IR spectra of NAS/NIPAAm monomer mixtures.

Table 2
Characterization of the gels and immobilization and permeability of the vesicles at 25°C

| Gel | Unreacted NAS (mol%) | Amount of PL ($mg/g_{dry\ gel}$) | SR | Vesicle composition POPC/POPG (mol/mol) | $Q_{pellet}$ (%) | $Q_{supernatan}$ (%) | Immobilized lipids ($\mu mol/g_{dry\ gel}$) |
|---|---|---|---|---|---|---|---|
| PNIPAS4–PL$\ell$ | 0 | 250 | 42 | 85/15 | 88 ± 2[a] | 85 ± 2 | 70 ± 30 |
| PNIPAS6–PL$\ell$ | 2.6 | 270 | 46 | 85/15 | 93 ± 6 | 80 ± 10 | 1000 ± 100 |
| PNIPAS6–PL$\ell$ | 2.6 | 270 | 46 | 100/0 | 57 ± 9 | 92.0 ± 0.6 | 13 ± 6 |
| PNIPAS6–PL$\ell$Ac | – | 270 | 1 | 85/15 | 79 ± 8 | 92 ± 4 | 4 ± 2 |
| PNIPAS6–PL*s* | 3.0 | 125 | 21 | 85/15 | 83 ± 6 | 94.8 ± 0.3 | 6 ± 4 |

[a] The uncertainties represent the standard deviation on at least three measurements.

distributions and that 60% of the lipids were on the outer leaflet. As a preliminary characterization, we estimate the $NH_3^+$/POPG stoichiometry for free polylysine in solution. The long polylysine binds to POPG-containing vesicles in a stoichiometric manner since we find approximately one lysine per accessible POPG. In the case of the short polylysine, there is only 0.1 lysine per POPG molecule (Table 3). As polylysine was added in excess in the centrifugation assay, we conclude that PL*s* has a smaller affinity for POPC/POPG (85/15) vesicles than PL$\ell$. The affinity of the polypeptide for these vesicles is very dependent on the peptide length. Different behaviors for long and short polylysine were associated with conformation effects [17,19]. Once grafted on the polyacrylamide, a ratio of 20 amino groups per accessible POPG was found for PNIPAS6–PL$\ell$. This ratio, which is lower than that obtained with the free PL$\ell$, suggests that not all the amino groups are accessible to the vesicles and the vesicle concentration is probably not homogeneous throughout the gel.

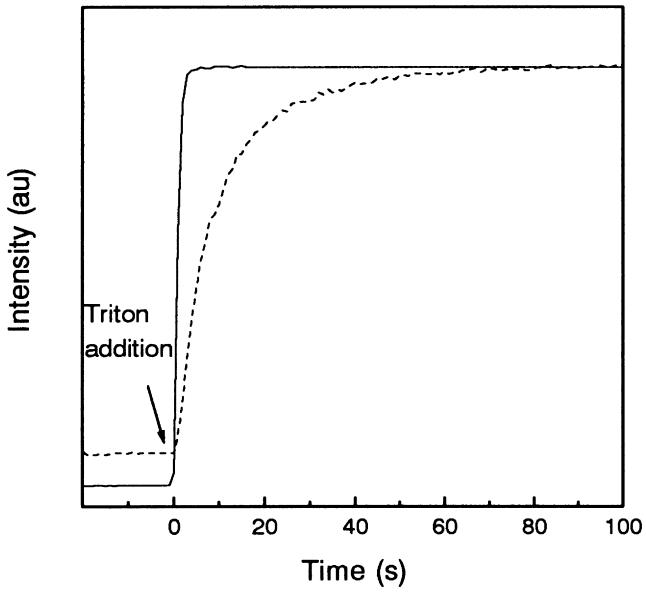


Fig. 5. Fluorescence quenching measurements: the SRB release induced by the addition of Triton-X as a function of time for free POPC/POPG vesicles (—) and for the same vesicles after immobilization on PNIPAS6–PL$\ell$ (– – –).

The preservation of the membrane integrity during the immobilization process was also examined by looking at the potential release of a dye (Table 2). For all the POPC/POPG vesicles, the *Q* value of the SRB entrapped in lipid vesicles remained high for both the immobilized vesicles and those remaining free in the supernatant. This indicates that the interaction between polylysine and the lipids does not affect the permeability of the vesicles. Therefore, our designed system presents the advantage that the immobilized vesicles can be used as small containers. During the measurement of *Q*, the immobilized vesicles were emptied from their content and, interestingly, different kinetics of SRB-release induced by the addition of Triton-X could be observed for free and immobilized vesicles (Fig. 5). In the case of the free vesicles, a rapid and complete release of the fluorophore occurred within the first 2–3 sec following the addition of the detergent. Conversely, the SRB release was much more progressive for the gel-immobilized vesicles and extended over one minute. In this case, the detergent needs to diffuse in the matrix to disrupt the vesicles and its diffusion coefficient modulates the efflux from the vesicles. Therefore, this hydrogel system offers the possibility of controlled release by choosing different disrupting agents.

It was found that the gel prepared from a copolymer containing less NAS monomer (here 4%) was less suitable than PNIPAS6–PL$\ell$. PNIPAS4–PL$\ell$ was soft and sticky, and therefore was difficult to grind. Despite a good swelling and a reasonable content of polylysine, this gel could immobilize fewer lipids than PNIPAS6–PL$\ell$. This lower efficiency was related to the bigger size of the gel particles leading to a limited effective surface for vesicle anchoring. This poor immobilization ability of the gel is illustrated by

Table 3
Stoichiometry of the immobilization of vesicles by free and bound polylysine

| Free polylysine | | Bound polylysine | |
|---|---|---|---|
| Polylysine | $NH_3^+$/POPG | Gel | $NH_3^+$/POPG |
| PL$\ell$ | 1 | PNIPAS4–PL$\ell$ | 210 |
| | | PNIPAS6–PL$\ell$ | 20 |
| PL*s* | 0.1 | PNIPAS6–PL*s* | 1090 |

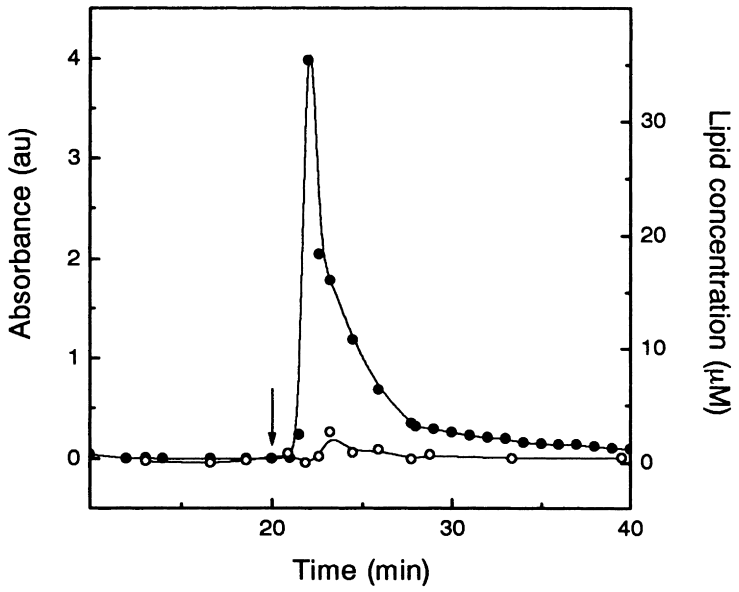


Fig. 6. Elution profile determined by measuring the SRB absorbance (●) and lipid concentration (○) as a function of time. At an elution time of 20 min (↓), the temperature of the column is raised to 50°C. Both *y* axes have been normalized so they relate directly the SRB absorbance to the lipid concentration according to the measurement on intact vesicles.

the high value of free amino groups per POPG (Table 3). It should be noted that our anchoring approach preserves also in this case, the integrity of the vesicles after immobilization as indicated by the high self-quenching coefficient of the entrapped fluorophore (Table 2).

Our results also indicate that long polylysine is more suitable as a crosslinking agent. Few lipids were immobilized on PNIPAS6–PL*s*, the gel made from the crosslinking of PNIPAS6 with PL*s*. Only a small quantity of lysine was found in this gel. This leads to a limited number of anchoring sites as well as a reduced swelling. Therefore, the gel could immobilize only very few lipid vesicles. Actually, the immobilization of lipids by this gel is comparable to the acetylated gel.

### *3.4. Thermally controlled release*

As an illustration of the potential use of PNIPAS6–PL$\ell$ as a thermosensitive support, we show that the content of immobilized vesicles can be released in a control manner using a thermal trigger. First, a column was filled with PNIPAS6–PL$\ell$. The vesicle immobilization step was done in suspension by mixing gel particles and POPC/POPG (85/15) vesicles. The vesicle-containing gel was then packed in a thermostated column. Afterwards, a 20 min elution at room temperature did not release any lipids or SRB (Fig. 6). After 20 min, the temperature of the column was raised to 50°C, over the transition temperature of the gel. An increase of the absorbance at 565 nm was then observed on the elution profile (Fig. 6), probing for the release of SRB. Since almost no lipids were detected in the collected fractions, we concluded that the released SRB was free. This was confirmed by a very low SRB self-quenching in the collected fractions. After 10 min, the baseline was recovered. Therefore, the thermosensitivity of the gel can be efficiently used for thermal controlled release. We believe that upon heating, the gel shrinks, and the reorganization perturbs the immobilized vesicles which release a fraction of their SRB content. After the elution, the column was washed by ethanol and the immobilized lipids and remaining SRB could be recovered in the washing.

## 4. Conclusion

A family of hydrogels based of on polyacrylamide and polylysine were synthesized and characterized to develop a system for the immobilization of lipid vesicles. Among the hydrogels tested, PNIPAS6–PL$\ell$ is the most suitable. PNIPAS6–PL$\ell$ enables the immobilization of a considerable amount of negatively charged vesicles without apparent perturbation of the vesicle permeability. The polylysine content appears to be a crucial factor for the properties of the gel since the charges borne by the polypeptide provide the anchoring sites and allow the gel to display an appropriate swelling to make the charges accessible to lipid vesicles. PNIPAS6–PL$\ell$ has interesting mechanical properties since it is firm enough to be easily ground in small particles that can be used in suspensions or in columns. In addition, the thermosensitivity of the gel can be efficiently used to have a controlled release of the vesicle content.

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