Bioresorbable and Nonresorbable Macroporous Thermosensitive Hydrogels Prepared by Cryopolymerization. Role of the Cross-Linking Agent

ARTICLE *in* BIOMACROMOLECULES · DECEMBER 2007

Impact Factor: 5.75 · DOI: 10.1021/bm7007668 · Source: PubMed

Bioresorbable and Nonresorbable Macroporous Thermosensitive Hydrogels Prepared by Cryopolymerization. Role of the Cross-Linking Agent

Paloma Perez,[†] Fatima Plieva,[‡,§] Alberto Gallardo,[†] Julio San Roman,[†] Maria Rosa Aguilar,[†] Isabelle Morfin,[△] Françoise Ehrburger-Dolle,[△] Francoise Bley,[⊥] Sergey Mikhalovsky,[#] Igor Yu. Galaev,[*,§] and Bo Mattiasson[§]

Instituto de Ciencia y Tecnología de Polímeros, CSIC, C/Juan de la Cierva 3, 28006 Madrid, Spain, Protista Biotechnology AB, Ideon, SE-22370 Lund, Sweden, Department of Biotechnology, Center for Chemistry and Chemical Engineering, Lund University, P.O. Box 124, SE-22100 Lund, Sweden, Laboratoire de Spectrométrie Physique, UMR 5588 CNRS-UJF, Domaine Universitaire, F-38402 Saint-Martin d'Hères Cedex, France, Science et Ingénierie des Matériaux et Procédés, UMR 5266-CNRS-UJF-INPG, Domaine Universitaire, 38402 Saint-Martin d'Hères Cedex, France, and School of Pharmacy & Biomolecular Sciences, University of Brighton, Moulsecoomb, Brighton BN2 4GJ, United Kingdom

Received July 11, 2007; Revised Manuscript Received October 3, 2007

Macroporous poly(*N*-isopropylacrylamide) (pNIPA) gels (so-called *cryogels*), cross-linked with different bisacrylic compounds, *N,N′*-methylenebisacrylamide (MBAAm) and dimethacrylate-tyrosine-lysine-tyrosine (DMTLT), were prepared through free-radical polymerization at subzero temperature in dioxane/water media. DMTLT is a hydrolytically degradable cross-linker with relatively hydrophobic character. The effects of different synthesis conditions, namely the concentration of monomers, the cross-linker, and the initiator in the reaction mixture, on the structure of the pNIPA-cryogels have been studied. The equilibrium swelling ratio of the DMTLT cross-linked pNIPA cryogels at temperatures below lower critical solution temperature (LCST) of pNIPA, was over ten times higher than that of the gels synthesized at room temperature from the same feed composition. The MBAAm cross-linked pNIPA cryogels synthesized in water exhibited the highest equilibrium swelling and the fastest response. The critical transition temperature, T_c, was lower ($T_c \approx 31$ °C) for pNIPA-cryogels synthesized in dioxane/water media or cross-linked with DMTLT as compared to MBAAm cross-linked pNIPA cryogels synthesized in water ($T_c \approx 33$ °C). Scanning electron microscopy (SEM) revealed different porous structure and pore surface morphology depending on the cross-linker (MBAAm or DMTLT) and the solvent (water or dioxane/water) used. Gels and cryogels were also characterized by SAXS, showing that the nanostructure of the samples is related to swelling.

1. Introduction

Intelligent polymeric materials, which exhibit response to external stimuli such as temperature,[1] have gathered a great interest in recent years. Poly-*N*-isopropylacrylamide (pNIPA) hydrogels are well-known to exhibit a volume transition at critical temperature (T_c) of about 33 °C in aqueous media.[2,3] Below the T_c, pNIPA hydrogels are swollen, hydrated, and hydrophilic. However, above the T_c, the gels shrink due to the distortion of the hydrophilic/hydrophobic balance in the network structure. One of the important characteristics of pNIPA hydrogels is the reversibility of the shrinking−swelling cycle in response to changes of environmental conditions.[4,5] The rate of response to external temperature changes of traditional pNIPA hydrogels is low due to the formation of a dense "skin layer" of the shrunken gel, which prevents the mass transport of water out of the shrinking gel. The slow response restricts the application of pNIPA hydrogels.

Numerous different approaches have been proposed to prepare fast-responsive macroscopic pNIPA hydrogels. The polymer structure in the gel was modified by graft-polymerization of pNIPA on the cross-linked pNIPA backbone, where the free ends of the grafts acted to accelerate the deswelling response.[6,7] An alternative way to accelerate the response of pNIPA gels consisted in the synthesis of a macropores structure. The macropores served as channels that facilitated convective transport of liquid released during the shrinkage of the gel. One of the methods to form porous pNIPA hydrogels is phase separation polymerization,[8] alternatively pore-forming agents like micronized sucrose[9] or poly(ethylene glycol)[10] have been used.

An efficient way to form porous structures is to use the crystals of a frozen solvent as a porogen avoiding the introduction of new chemicals in the system. Gels obtained at temperatures below the melting temperature of the solvent are called cryogels. At subzero temperatures most of the solvent is frozen, while the dissolved substances are concentrated in small nonfrozen regions, so-called "liquid microphase". As the volume of the nonfrozen liquid microphase is much less than that of the solid phase, the local monomer concentration is much higher than the monomer concentration in the initial reaction mixture.[11]

* Corresponding author. E-mail: igor.galaev@biotek.lu.se. Telephone: 46-46 222-0881. Fax: 46-46 222-7413.
[†] Instituto de Ciencia y Tecnología de Polímeros.
[‡] Protista Biotechnology AB.
[§] Department of Biotechnology, Center for Chemistry and Chemical Engineering, Lund University.
[△] Laboratoire de Spectrométrie Physique.
[⊥] Science et Ingénierie des Matériaux et Procédés.
[#] School of Pharmacy & Biomolecular Sciences, University of Brighton.

10.1021/bm7007668 CCC: $40.75
Published on Web 12/08/2007

The gel formation occurs in this liquid microphase and the crystals of frozen solvents perform like porogen. After melting the ice crystals, a system of large interconnected pores is formed.

Traditionally, water has been used for the preparation of macroporous cryogels.[11,12] pNIPA gels with fast response have been prepared through copolymerization of NIPA with hydrophobic[13] (di-*N*-propylacrylamide) or hydrophilic[14] (acrylic acid) comonomers. In these works, the fast responding gels were prepared through two-step procedure in which the initial polymerization was conducted at 18 °C[14] or 20 °C[13] for a short time followed by polymerization at −22 or −28 °C. The authors showed that the response kinetics of these hydrogels can be adjusted according to the polymerization time at 20 °C.[13] Fast responsive pNIPA hydrogels with high swelling ratio were prepared through polymerization in water at −18 °C[15] and by polymerization in organic solvents (DMSO) at −20 °C (below the melting point of DMSO).[16] However, the polymerization reaction in DMSO media was carried out for 72 h and the cryogels were obtained with conversion of 15%.[16]

Here is presented the preparation of pNIPA cryogels cross-linked with MBAAm and DMTLT in aqueous (w-MBAAm/pNIPA) and aqueous-dioxane media (d-DMTLT/pNIPA or d-MBAAm/pNIPA, respectively). As the DMTLT is not soluble in water, the polymerization was performed in dioxane/water medium. The swelling behavior of pNIPA-based cryogels was compared with that of the gels prepared at room temperature from the same feed composition. The morphology of the prepared w- and d-MBAAm/pNIPA and d-DMTLT/pNIPA macroporous gels was visualized by SEM, and nanostructure was studied by small-angle X-ray scattering (SAXS).

2. Experimental Section

2.1. Materials. L-Tyrosine (Aldrich, Steinheim, Germany), lysine diisocyanate methyl ester (Kyowa Hakko), and thionyl chloride (Scharlau, Barcelona, Spain) were used as received. Methacryloyl chloride (Aldrich, Steinheim, Germany), triethylamine, and *N*,*N*′-dimethylacetamide (Scharlau, Barcelona, Spain) were distilled before use. Ethanol and tetrahydrofuran (Scharlau) were dried before use. *N*-Isopropylacrylamide (Kodak, Rochester, NY), *N*,*N*′-methylenebisacrylamide (MBAAm), ammonium persulfate (APS), and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) (Aldrich, Steinheim, Germany) and 1,4-dioxane (T_f = 11.8 °C; Merck, Darmstadt, Germany) were used as received. Phosphate-buffered saline (PBS, Sigma) pH 7.4 composition was 0.01 M PBS, 0.138 M NaCl, 0.0027 M KCl.

2.2. Synthesis of Dimethacrylate-Tyrosine-Lysine-Tyrosine (DMTLT) Cross-linker. DMTLT was synthesized in three steps.[17] Details: in the first step, tyrosine ethyl ester (TEE) was prepared by the reaction of tyrosine (0.276 mol) with thionyl chloride $SOCl_2$ (20 mL) in dried ethanol as solvent.[18] The mixture was heated under reflux for 12 h. After precipitating the product with diethylether, the mixture was filtered and the product was dried under reduced pressure. The product was obtained as chlorohydrate (white powder) with an 81% yield.

In the second step, TEE (0.018 mol) was dissolved in 50 mL of freshly distilled dimethylacetamide. Triethylamine (2.8 ml) was added, and the mixture was stirred for 5 min; after that, 1.12 mL of lysine diisocyanate methyl ester was added. The reaction was carried out at 80 °C for 4 h. The precipitate formed ($Et_3NH^+Cl^-$) was removed by filtration, and distilled water was added to the reaction mixture to precipitate the product, tyrosine-lysine-tyrosine (TLT). After that, the water was decanted and the product was vacuum-dried. TLT was isolated as a yellow powder with a 76% yield.

In the last step, DMTLT was prepared as follows: 0.006 mol of TLT were dissolved in 70 ml of THF. Triethylamine (2.65 ml) was added, and the solution was cooled in an ice bath to about 0 °C. To this solution, 5 mL of freshly distilled methacryloyl chloride was added dropwise under N_2 atmosphere. The solution was stirred for 45 min at 0 °C followed by 12 h at room temperature. The precipitated material was removed by filtration, and the final product was precipitated in a NaOH (1% w/v) dissolved in distilled water. The solid powder was separated by filtration, washed with cold distilled water, and vacuum dried. Yield: 72%.

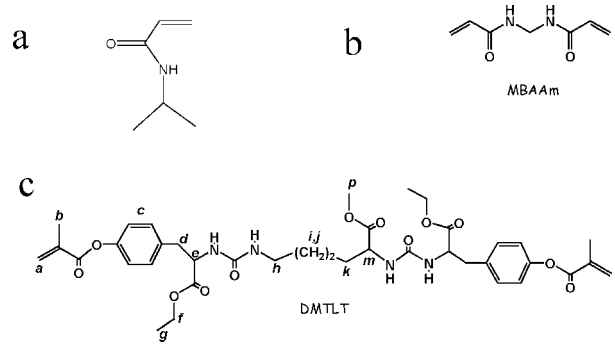

Figure 1. Chemical structure of NIPA (a) and the cross-linkers MBAAm (b) and DMTLT (c).

DMTLT was characterized by ^{1}H NMR (Varian Unity-500), using DMSO-d_6 as solvent (δ 2.54), giving the following chemical shifts (the assignments of the peaks correspond to Figure 1): DMTLT ^{1}H NMR (500 MHz): δ 1.11 (t, 6h, g), 1.25 (dm, 4H, i,j), 1.55 (dm, 2H, k), 1.97 (s, 6H, b), 2.91 (m, 6H, d,h), 3.58 (s, 3H, p), 4.02 (q, 4H, f), 4.08 (m, 1H, m), 4.36 (m, 2H, e), 5.86 (s, 2H, a), 6.06 (t, 1H, NH), 6.13 (d, 1H, NH), 6.24 (s, 2H, a), 6.31 (d, 1H, NH), 6.55 (d, 1H, NH), 7.06 (dd, 4H, c), 7.19 (dd, 4H, c).

2.3. Preparation of Macroporous Gels at Subzero (Cryogels) and Room (Conventional Gels) Temperatures. The macroporous pNIPA-based cryogels were prepared in a dioxane:water mixture (70:30 v/v). The monomers (NIPA and DMTLT) were dissolved to give a total monomer concentration of 0.5 M, and the DMTLT/NIPA ratio was 1/40 mol/mol. The reaction mixture was bubbled with nitrogen for 10 min after addition of the initiator pair APS/TEMED (2% to total mol of NIPA + cross-linker) (the minimum concentration of initiator system needed to induce gelation at room temperature after 30 min), the reaction mixture was poured into glass columns (7 mm inner diameter) with sealed bottoms and was frozen at −20 °C for 1 h and then at −12 °C overnight. After that, the cryogels were thawed at room temperature and washed first with water, then with acetone, and finally again with water. Monolithic cryogels were obtained by this procedure. Control gels were prepared from the same monomer solutions at room temperature. The cryogels and gels were dried first at room temperature for 24 h and then at 60 °C for 24 h.

Cryogels with different total monomer concentrations (1 and 2 M) and different cross-linker/monomer molar ratios (DMTLT/NIPA 1/100, 1/20, 1/10) were prepared (Tables 2 and 3). pNIPA-based gels and cryogels cross-linked with MBAAm (MBAAm/NIPA molar ratio was 1/40) with total monomer concentration 0.5 and 1.0 M were prepared both in water and dioxane:water (70:30 v/v) mixture.

Temperature profile (freezing curve) was measured by placing a thermocouple (T-type) directly into the reaction mixture (5 ml) in a glass column (i.d. 10 mm). The thermocouple was placed at the center of the glass column at half of the height of the reaction mixture. The sample was placed in a chamber of a low temperature thermostat LAUDA cooled until −20 °C was reached. The temperature at the sample was measured using a digital thermometer Dual LogR. Temperature changes in the sample depending on the cooling time were recorded.

2.4. Characterization of the Macroporous Cryogels. Swelling dependence on temperature was measured in the interval 4−40 °C. Dried gels and cryogels (cylindrical shape; diameter: 5–6 mm; height: 3–4 mm) were incubated in PBS, pH 7.4, at different temperatures for at least 24 h, to achieve the swelling equilibrium at the given

Table 1. Cryogels Produced in Different Solvents

solvent	solvent:water ratio, v/v	macroscopic appearance[a] (cryogel yield, %) MBAAm	DMTLT
water		spongy and elastic cryogel (77%)	
DMSO	100:0	no cryogel formation	no cryogel formation
formamide:water	70:30	weak cryogel (23%)	no cryogel formation
dioxane:water	70:30	spongy and elastic cryogel (72%)	spongy and elastic cryogel (61%)

[a] Experimental conditions: total monomer concentration was 0.5 M, molar ratio cross-linker/NIPA was 1/40. Cryogels were prepared via freezing at −20 °C for 1 h followed by storage in frozen state for 16 h.

Table 2. Influence of APS/TEMED Content in the Reaction Mixture on Cryogel Preparation

APS/TEMED content, % of total mol of monomers	d-DMTLT/NIPA[a]		d-MBAAm/NIPA[a]	
	gel at room temperature	cryogel at − 20 °C	gel at room temperature	cryogel at − 20 °C
0.4[b]	no gel formed	no cryogel formed	no gel formed	no cryogel formed
0.8	no gel formed	no cryogel formed	elastic gel	elastic and spongy cryogel
2.0	weak gel	elastic spongy cryogel	too fast gelation (20–30 s)[c]	too fast gelation (20–30 s)[c]

[a] Total monomer concentration 0.5 M, ratio cross-linker/NIPA 1/40, mol/mol. [b] Elastic and spongy w-MBAAm/NIPA cryogels were prepared when APS/TEMED content in the reaction mixture was 0.4%. [c] Estimated gelation time obtained by visual inspection.

Table 3. Influence of Monomer Concentration on d-DMTLT/NIPA Preparation

monomer concentration, M[a]	gel (at room temperature)	cryogel (at − 20 °C)
0.25	no gel	no cryogel
0.5	weak gel	elastic and spongy cryogel
1.0	elastic gel	elastic and spongy cryogel
2.0	rigid gel	rigid elastic cryogel

[a] The molar ratio DMTLT/NIPA was 1/40.

temperature. Swollen gels and cryogels were quickly removed from the media, blotted with filter paper to remove any excess surface moisture, and weighed. This procedure was performed at least in triplicate. Swelling ratio was defined as $\%S = (W_s - W_o)/W_o$, where W_s is the weight of the swollen sample and W_o is the weight of the dried sample. T_c was determined from the swelling over temperature curves as the temperature at which the cryogel showed an increase in 0.2 in swelling ratio from the baseline value above the transition temperature.

T_c of gels and cryogels was also determined by differential scanning calorimetry (DSC) using a Perkin-Elmer DSC 7 calorimeter with a N_2 flow of 50 mL/min. Samples were swollen in PBS buffer pH 7.4 for 2 days at 4 °C and were then sealed in 50 μL aluminum capsules. Thermograms were recorded between 5 and 50 °C at 3 °C/min. All samples were incubated at 5 °C for 5 min before the experiment. T_c was defined as the maximum of the endothermic peak that presented the thermogram of each sample.

Swelling kinetics were measured gravimetrically at two different temperatures, 23 and 37 °C (below and above T_c). Dried samples of cryogels were immersed into PBS pH 7.4 at fixed temperature and were weighed at different time intervals. Before weighing, the excess surface moisture was removed with filter paper. Swelling ratio was calculated as mentioned above.

The pulsed behavior in swelling of these cryogels was measured by alternating immersion of cryogels into 0.1 M phosphate buffer pH 7.4 at two different temperatures, 19 or 37 °C, (below and above T_c), respectively, and weighing them at different time intervals. The samples were transferred between baths maintained at either 19 or 37 °C for swelling−deswelling studies. The swelling ratio was determined as described above.

w-MBAA/pNIPA and d-MBAA/pNIPA cryogel samples for scanning electron microscopy (SEM) were fixed in 2.5% glutaraldehyde in PBS, pH 7.4, at 4 °C for 36 h. The samples were dehydrated in ethanol (0–20–40–70–90–99.5%) and critical point dried. The dried samples were coated with gold/palladium (40/60) and examined using a JEOL JSM-5600LV scanning electron microscope.

d-DMTLT/pNIPA cryogel samples were analyzed by environmental scanning electron microscopy (ESEM) technique using the ESEM Philips XL30 microscope with tungsten filament. Micrographs were taken in the ESEM mode (wet cryogels, low vacuum) and in the SEM mode (lyophilized cryogels, high vacumm). Freeze-drying was carried out in a Lyoalfa-10 machine, at a pressure of 0.1 bar and samples at –20 °C.

SAXS experiments were performed at the European Synchrotron Radiation Facility (ESRF) [http://www.esrf.eu/], Grenoble, France, on the French CRG Beamline D2AM. The incident energy was set to 15.193 keV ($\lambda = 0.77$ Å). The detector, an indirect illumination CCD camera (Princeton Instruments), with pixel size equal to 50 μm, was located at distances of 206.4 and 35.1 cm from the sample. These configurations provided data for wave vectors q ($q = (4\pi/\lambda)\sin(\theta/2)$) ranging between 5×10^{-3} and 1 $Å^{-1}$. Data obtained from the detector were corrected by taking into account the flat field and the dark current. The beam-stop was a small pillar (2 mm diameter lead wire). The swollen samples were placed in stainless steel holders closed by two mica windows 1 mm apart. For these samples, scattering of the same thickness of pure water was subtracted from the total intensity. No mica windows were used for the dry samples. The images were processed by means of the software *bm2img* available on the beamline [http://www.esrf.fr/exp_facilities/bm2/bm2.html].

3. Results and Discussion

3.1. Preparation of Macroporous Cryogels and Homogeneous Gels.

pNIPA-based cryogels were prepared using MBAAm (Figure 1b) and DMTLT (Figure 1c) as cross-linkers. MBAAm is a common nondegradable cross-linker, used for the preparation of the acrylamide based gels for electrophoresis,[19] and DMTLT has been introduced recently as a new biodegradable acrylic cross-linker.[20] DMTLT is a pseudopeptide composed by tyrosine-lysine-tyrosine amino acids linked through urea bonds and containing two methacrylic groups, one at each end of the molecule, making this compound an excellent cross-linker for polymerization reaction and for obtaining new biodegradable materials. In addition, the main products of the biodegradation of DMTLT in physiological conditions are tyrosine and cynnamic acid, which makes porous hydrogels very interesting as drug delivery supports and, scaffolds for cell growth and tissue regeneration. According to previous biodegradation studies, DMTLT is sensitive to the hydrolysis in aqueous media, giving rise to tyrosine and cynnamic acid as main products,[17,20] which is very interesting for the preparation of biodegradable and

Table 4. Influence of Cross-Linker (DMTLT) Content on d-DMTLT/NIPA Preparation

molar ratio DMTLT/NIPA (mol/mol) [a]	gel (at room temperature)	cryogel (at − 20 °C)
1/100	no gel	weak cryogel
1/40	weak gel	elastic and spongy cryogel
1/20	elastic gel	elastic cryogel
1/10	dense elastic gel	dense elastic cryogel

[a] Total monomer concentration was 1 M.

bioresorbable hydrogels and their application in the biomedical field. As DMTLT is poorly soluble in water, three organic solvents (DMSO, formamide, and dioxane with melting temperatures of 18, 2, and 11 °C, respectively) were chosen for the preparation of the gels at −20 °C (Table 1). Dioxane/water and formamide/water systems with 70% v/v of dioxane or formamide were found as minimal compositions allowing for complete dissolution of DMTLT. No cryogels were formed in pure DMSO after 48 h of polymerization, whereas weak cryogels were formed in 70% v/v formamide/water (Table 1). Zhang et al.[16] prepared pNIPA-based cryogels in DMSO media after 72 h of polymerization. However, we found that it was too time-consuming and the conversion was rather low.[16] As strong DMTLT-cross-linked pNIPA cryogels, with conversion higher than 60%, were formed in 70% v/v dioxane/water (Table 1), this medium was selected for the further experiments.

When preparing cryogels, it is important to accomplish freezing of the solvent before the gelation reaction occurs. Then cryopolymerization proceeds in a nonfrozen microphase, resulting in macroporous structure. Otherwise, when the solvent crystallizes within the already formed gel, the crystals will simply tear apart the gel structure, as was shown for polyacrylamide based macroporous cryogel monoliths.[21] To ensure the freezing of the solvent before the gelation, the content of the initiator system (APS/TEMED) was kept close to 0.4% (% total mol of monomers) for the w-MBAAm/pNIPA, 0.8% (% total mol of monomers) for d-MBAAm/pNIPA, and 2% (% total mol of monomers) for d-DMTLT/pNIPA cryogels. Under these conditions, gelation of the cryogels with total monomer concentration 0.5 M, proceeded within 15–20 min at room temperature (Table 2).

Elastic, spongy, and mechanically stable cryogels were prepared at total monomer concentration of 0.5–1.0 M (Table 3) and DMTLT/NIPA molar ratios 1/40 (Table 4). However, d-DMTLT/pNIPA cryogels were elastic but not spongy for ratios DMTLT/NIPA 1/20 and 1/10 (Table 4), which correspond to the cryogels with higher cross-linking density.

Analysis of the cooling curves allows for the determination of phase and structural changes in the system during the cooling.[22,23] For example, temperature jump or change of slope on freezing curve indicates the phase changes in the system.[23] Freezing thermograms obtained for freezing of the reaction mixtures when preparing w- and d-MBAAm/pNIPA and d-DMTLT/pNIPA cryogels, respectively, indicated that freezing was accomplished (i.e., the final temperature −20 °C has been reached) within 8 min in all cases (Figure 2). The freezing thermograms showed overcooling up to −9 °C for 1 min in dioxane/water medium for both d-MBAAm/NIPA and d-DMTLT/NIPA systems (Figure 2, dashed and dashed−dotted lines, respectively). Interestingly, the overcooling was much less pronounced for cryopolymerization in pure water as compared to dioxane−water medium (Figure 2, full line). The differences in the freezing process during the cooling of the reaction

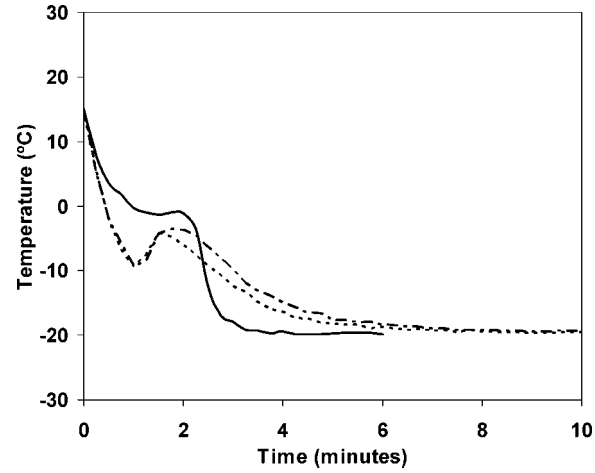

Figure 2. Freezing curves for the w-MBAAm/pNIPA (smooth line), d-MBAAm/pNIPA (dash−dotted line), and d-DMTLT/pNIPA (dashed line) cryogels produced at –20 °C. Experimental conditions for cryogel synthesis: total monomer concentration 0.5 M, molar ratio cross-linker/NIPA 1/40.

mixtures in water and dioxane−water media resulted apparently in different structures of the crystals formed and hence resulted in different porous structures of the cryogels.

Porous structure of w- and d-MBAAm/pNIPA cryogels was visualized by SEM (Figure 3). w-MBAAm/pNIPA cryogel had a macroporous structure with large (1–150 μm) interconnected channel-like pores surrounded with thin (1–2 μm) nonporous walls (Figure 3 a,b) and presented a spongelike morphology with good elasticity that allowed fast reswelling after drying and fast swelling−deswelling kinetics (see details below in Section 3.2). It was possible simply to squeeze mechanically most of the water out of these cryogels by compressing the monolith sample. d-MBAAm/pNIPA cryogels, prepared in dioxane/water medium at the same monomer concentration and cross-linker content, presented a fundamentally different porous structure (Figure 3 c,d). Large (5–20 μm in size) round shapes and closed pores were surrounded with thick (10–40 μm) walls were visualized. These cryogels were also elastic but not spongy. Hence, the swelling of d-MBAAm/pNIPA cryogel was slower as compared to that of the w-MBAAm/pNIPA cryogel.

The porous structure of d-DMTLT/pNIPA cryogels (prepared in the same conditions as d-MBAAm/pNIPA cryogel) was analyzed by ESEM technique that allows for analysis of porous structure both in the hydrated state (low vacuum) and in the dried state (high vacuum). The macro- and microporous structure and morphology of d-DMTLT/pNIPA cryogels is shown on Figure 4. Figure 4a corresponds to a micrograph of the cryogel in the wet (hydrated) state, obtained by ESEM at low vacuum. The macro- and microporous structure of the d-MBAAm/pNIPA cryogel is well detected, which demonstrates that the porosity is kept after the hydration of the cross-linked polymeric matrix. Parts b and c of Figure 4 show the porous structure of the cryogel after drying the cryogel. An interconnected macro- and microporosity is shown clearly with homogeneously distributed pores in the polymeric matrix.

3.2. Characterization of Macroporous Cryogels. Swelling ratio at temperatures above T_c of w-MBAAm/pNIPA cryogels, was much higher compared to that of d-MBAAm/pNIPA and d-DMTLT/pNIPA cryogels (Figure 5). Both w- and d-MBAAm/pNIPA cryogels reached higher swelling ratios at temperatures below LCST for pNIPA than d-DMTLT/pNIPA, which was probably due to the more hydrophobic nature of DMTLT as

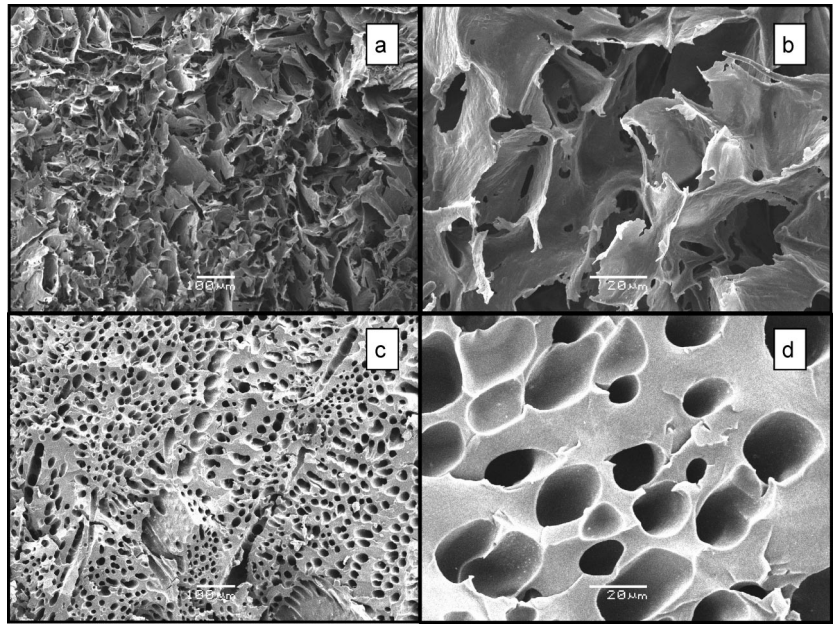

Figure 3. SEM of w-MBAAm/pNIPA cryogels (a,b) and d-MBAAm/pNIPA cryogel (c,d). Experimental conditions for cryogel synthesis: total monomer concentration 0.5 M, molar ratio MBAAm /NIPA 1/40; freezing at −20 °C for 1 h followed by storage in frozen state for 16 h.

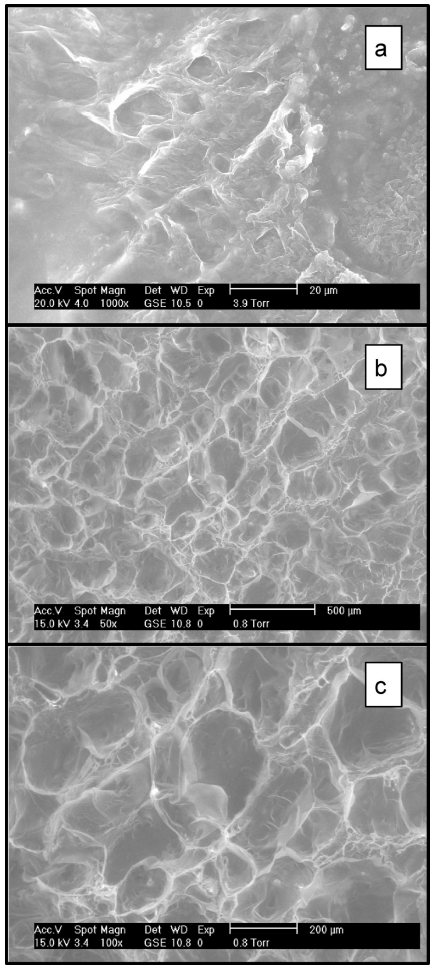

Figure 4. ESEM of d-DMTLT/pNIPA cryogels in hydrated state (low vacuum) (a) and in dried state (high vacuum) (b,c). The d-DMTLT/pNIPA cryogels were prepared from feed with total monomer concentration 0.5 M and molar ratio DMTLT/NIPA 1/40.

compared to MBAAm. The T_c for cryogels prepared in water was higher (around 33 °C) as compared to cryogels prepared in dioxane/water media (around 32 °C) (Figure 5). Moreover,

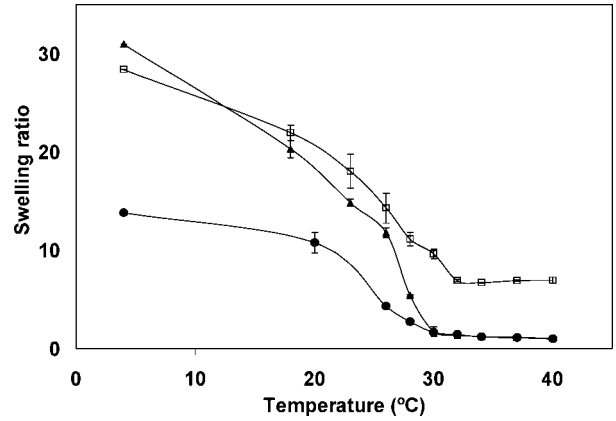

Figure 5. Equilibrium swelling at different temperatures for the w-MBAAm/pNIPA (open squares), d-MBAAm/pNIPA (closed triangles), and d-DMTLT/pNIPA cryogels (closed circles). Experimental conditions for cryogel synthesis: total monomer concentration 0.5 M; molar ratio cross-linker/NIPA 1/40; freezing at −20 °C for 1 h followed by storage in frozen state for 16 h.

the T_c for d-DMTLT/pNIPA cryogels (around 31 °C) was less compared to the d-MBAAm/pNIPA, again due to the hydrophobic nature of the DMTLT. It is known that the T_c of pNIPA hydrogels decreases with the introduction of a more hydrophobic comonomer into the polymeric chain.[24]

Rigidity of d-DMTLT/pNIPA cryogels increased with increasing the total monomer concentration in the reaction mixture. The higher total monomer concentration of d-DMTLT/pNIPA cryogels, the lower the swelling ratios at temperature below T_c were. d-DMTLT/pNIPA cryogels swelled more compared to ordinary d-DMTLT/pNIPA gels produced at room temperature. Swelling was strongly dependent on the content of the cross-linker in the reaction mixture: the higher the DMTLT content, the lower the swelling degree of the prepared cryogels and ordinary gels was in all the temperature ranges studied. The highly cross-linked cryogels d-DMTLT/pNIPA with DMTLT/NIPA molar ratio 1/10 did not show any thermoresponsive properties over the temperature range (4−40 °C). The lower concentration of the cross-linker (DMTLT), the higher the swelling degree and the thermoresponse were.

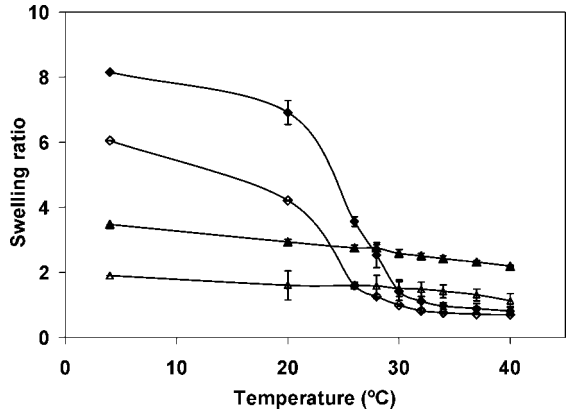

Figure 6. Equilibrium swelling of d-DMTLT/pNIPA cryogels (closed symbols) and ordinary gels prepared at room temperatures (open symbols). Experimental conditions for gel and cryogel synthesis: molar ratio DMTLT/NIPA 1/40 (diamonds) and 1/10 (triangles); freezing at −20 °C for 1 h followed by storage in frozen state for 16 h.

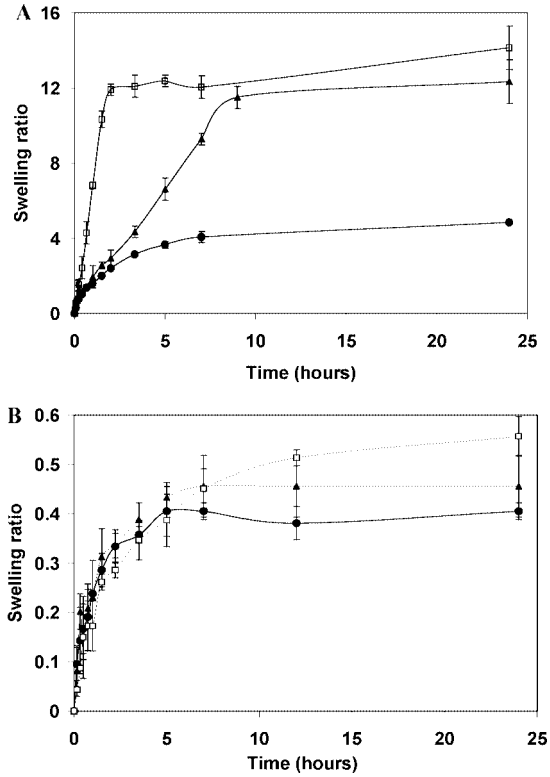

Figure 7. Swelling kinetics of w-MBAAm/pNIPA cryogels (open squares), d-MBAAm/pNIPA (closed triangles) and d-DMTLT/pNIPA cryogels (closed circles) at 23 °C (a) and 37 °C (b). Experimental conditions for cryogel synthesis: total monomer concentration 0.5 M, ratio cross-linker/NIPA 1/40, mol/mol; freezing at −20 °c for 1 h followed by storage in frozen state for 16 h.

w-MBAAm/pNIPA cryogels swelled fast at 23 °C (below T_c of pNIPA), with equilibrium swelling achieved within 1 h (Figure 7 a). Whereas d-MBAAm/pNIPA cryogels swelled much more slowly, the equilibrium swelling was practically independent of the solvent (water or dioxane/water medium) used for the cryogel preparation. However, d-DMTLT/pNIPA cryogels swelled much less and much more slowly than MBAAm-cross-linked cryogels. Apparently, the equilibrium swelling of cryogels with NIPA macromolecules in an expanded state is defined predominantly by the nature of the cross-linker and is practically independent of the porous structure. However, the kinetics of swelling is defined by the porous structure of

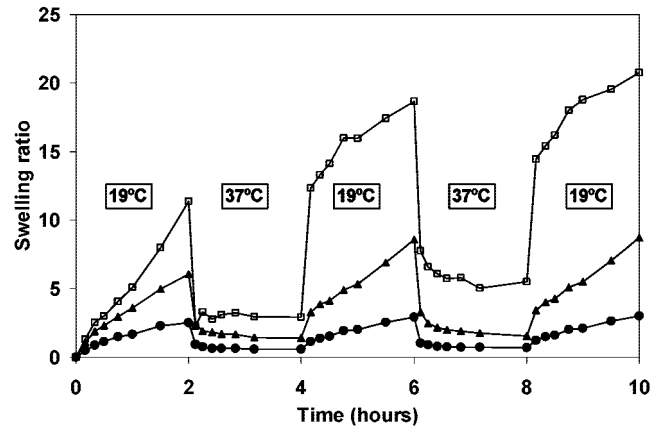

Figure 8. Swelling−deswelling kinetics of w-MBAAm/pNIPA cryogels (open squares), d-MBAAm/pNIPA cryogels (closed triangles) and d-DMTLT/pNIPA cryogels (closed circles) in response to temperature change from 19 to 37 °C in 0.1 M Na-phosphate buffer pH 7.4. Experimental conditions for cryogel synthesis: total monomer concentration 0.5 M, molar ratio cross-linker/NIPA 1/40; freezing at −20 °C for 1 h followed by storage in frozen state for 16 h.

Table 5. T_c Values of Cryogels (Prepared at −20 °C) and Ordinary Gels (Prepared at Room Temperatures)[a]

	T_c cryogel, °C	T_c gel, °C
w-MBAAm/pNIPA	30.2	32.8
d-MBAAm/pNIPA	32.0	32.0
d-DMTLT/pNIPA	31.8	31.1

[a] Total monomer concentration 0.5 M, molar ratio cross-linker/NIPA 1/40.

the cryogels. At 37 °C (above T_c of pNIPA) both the swelling kinetics and equilibrium swelling were very similar for all three cryogels (Figure 7 b) independently of the porous structure and the nature of the cross-linker, indicating that, under these conditions, pNIPA collapsed macromolecules define both the kinetics and equilibrium swelling.

Cryogels were exposed to temperature cycles between 19 and 37 °C in order to study their swelling−deswelling behavior (Figure 8). w-MBAAm/pNIPA cryogels showed rapid and high amplitude response. The dried cryogel samples reswelled slowly from the dried state during the first cycle, however, the sequential swellings were much faster and reversible for all three cryogel samples.

3.3. DSC of Gels and Cryogels. DSC thermograms of d-DMTLT/pNIPA cryogels prepared from DMTLT/NIPA feeds with different total monomer concentration, 0.5, 1.0, and 2.0 M, with molar ratio DMTLT/NIPA 1/40, showed that T_c of d-DMTLT/pNIPA was independent of the total monomer concentration in the feed composition, as T_c appeared in all cases at 31.8 °C (Table 5). These results were in agreement with the swelling curves obtained from the experiments carried out at different temperatures that showed the same T_c when increasing the total monomer concentration.

DSC analysis of d-DMTLT/pNIPA, d-MBAAm/pNIPA, and w-MBAAm/pNIPA showed T_c values of 31.8, 32.0, and 30.2 °C, respectively (Table 5). T_c values for d-DMTLT/pNIPA and d-MBAAm/pNIPA were in a good agreement with the results obtained by the swelling experiments, while T_c for w-MBAAm/pNIPA was quite low. Such an unusually low value of T_c (which was expected from the swelling curves to be somewhat higher, around 33 °C) might be explained by the presence of two transitions, i.e., one microscopic and the other macroscopic.[25] As pore size in w-MBAA/pNIPA cryogels is much larger

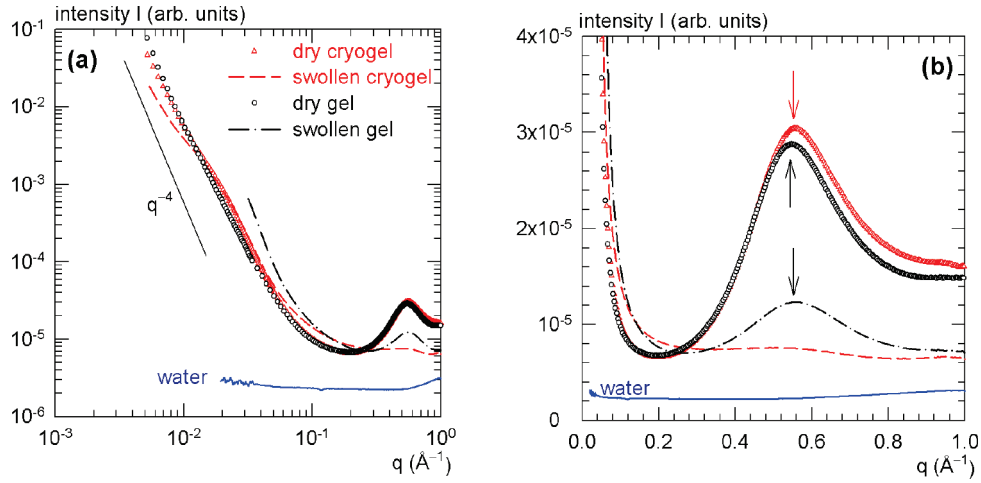

Figure 9. Comparison of the SAXS curves obtained for a d-DMTLT/pNIPA cryogel and an ordinary gel prepared in the same chemical conditions ([DMTLT]/[NIPA]1/40); (a) log–log plots; (b) linear plots showing the high-q domain.

(compared to the other cryogels), the real total monomer concentration (NIPA + MBAAm) in the pore walls is much higher. Thus increased local concentration of the cross-linker (MBAAm) in the pore walls resulted in a decreased value of T_c, observed by DSC. The microscopic transition is detected by DSC as the first rearrangement that takes place in the cryogel structure when increasing temperature, but this transition is not yet observable at the macroscopic scale. The macroscopic transition, which appears at 33 °C, is observed in the swelling diagrams. The results were confirmed by the DSC curves obtained for the ordinary gels (d-DMTLT/pNIPA, d-MBAA/pNIPA, and w-MBAA/pNIPA) prepared at the same conditions but at room temperature (Table 5). As the ordinary gels have isotropic homogeneous porous structure (compared to cryogels with anisotropic heterogeneous structure), the T_c values should be higher for w-MBAA/pNIPA gels than for d-MBAA/pNIPA and d-DMTLT/pNIPA gels. Indeed, T_c value for w-MBAA/pNIPA gels were higher (32.8 °C) compared to d-MBAA/pNIPA gel (32.0 °C) and d-DMTLT/pNIPA gel (31.1 °C).

Thus the decreased value of T_c for cryogels, prepared in water as compared to the same gels prepared at room temperature (measured by DSC), was due to the pronounced cryoconcentration of reagents in nonfrozen zones (or "liquid microphase") where the polymerization reaction proceeded.[11] As the formed ice crystals were big, the volume of this "liquid microphase" was much lower compared to the volume of bulk solution.

T_c values for cryogels and ordinary gels prepared in dioxane media were practically the same (Table 3). The porous structure of cryogels formed in dioxane media is fundamentally different from that of cryogels formed in water (see SEM images). Because of the smaller and isolated solvent crystals formed during freezing, the macroporous structure with large but not interconnected pores was formed. As a result, the volume of the "liquid microphase" where polymerization took place was greater, making the reagents (NIPA + MBAA or NIPA + DMTLT) less concentrated than in the case of cryogels prepared in water. In dioxane, cryoconcentration was not sufficient to result in detectable T_c differences between cryogels and gels.

3.4. SAXS Characterization. Figure 9a shows the SAXS curves obtained for a gel and a cryogel d-DMTL/pNIPA (d-d) in the dry and the swollen state, at room temperature (r.t.). For the dry samples, the curves are nearly similar, yet the cryogel displays a slightly larger intensity between 1.5×10^{-2} and 5×10^{-2} Å^{-1} than the gel. Below 10^{-2} Å^{-1}, the intensity scales nearly as q^{-4}, as expected for a sharp boundary in the dry samples. Above this q value, the decrease of the intensity is smoothed as a result of an internal structure that appears as a bump for the dry cryogel. In the high-q domain, the two samples show a broad peak located at 0.549 and 0.567 Å^{-1} for the gel and the cryogel, respectively (Figure 9b). The corresponding characteristic length, d, in the real space, can be obtained as a first approximation, by mean of the Bragg relation: $d = 2\pi/q$, yielding 11.44 and 11.09 Å for the gel and the cryogel, respectively. For the swollen gel, the intensity of the peak is significantly reduced. For the swollen cryogel, the peak nearly vanishes. Interestingly, the swelling ratio of the cryogel is larger than for the gel synthesized at room temperature in the same chemical conditions (Figure 6). For the two other cryogels investigated by SAXS, the peak observed in the dry state also vanishes in the swollen cryogels (Figure 10a,b) despite the fact that the swelling ratio at room temperature is significantly smaller for DMTLT/pNIPA (d-d) than for w-MBAA/pNIPA (w-m) and d-MBAA/pNIPA (d-m) (Figure 7a). To investigate the structure of a cryogel above the T_c, the sample DMTLT/pNIPA (d-d), swollen in water, was heated at 60 °C and cooled down to room temperature (r.t.). Figure 11 shows that the high-q SAXS curves are nearly identical for the dry cryogel and the swollen cryogel at a temperature above the critical temperature T_c, corresponding to the volume phase transition (VPT). It follows that the nanostructure is probably the same after deswelling by evaporation of water and by heating above T_c. Sugiyama et al.[26] came to a similar conclusion, yet at the mesoscopic scale, the observed peak being located at 0.026 Å^{-1}. Kosik et al.[27] observed a peak located at a similar q value (around 0.57 Å^{-1}) in MBAAm/pNIPA gels above T_c. This peak also vanishes below T_c.

The presence of a peak in samples collapsed either after dehydratation or after heating above T_c suggests the existence of a preferred distance d between correlated scattering objects. For the dry DMTLT/pNIPA gel, d is slightly larger ($d = 11.4$ Å) than for the corresponding dry cryogel ($d = 11.3$ Å) (Figure 9b). Also, for this dry cryogel, d is slightly smaller than for the MBAAm/pNIPA cryogels ($d = 11.5$ Å for the cryogels synthesized in water or in dioxane). Finally, for the DMTLT/pNIPA cryogel, d increases from 11.1 Å (above T_c) to 11.3 Å during cooling, while the peak flattens.

The values of d are much smaller than correlation lengths between nanodomains.[26,28] They are too large to be the separation distance between adjacent isopropyl groups along the polymer chain.[27] Because the distances d obtained in this

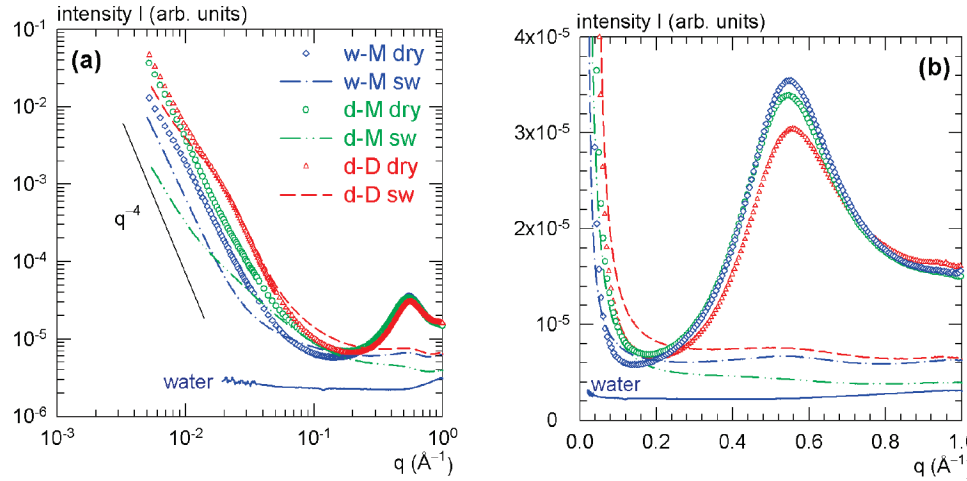

Figure 10. Comparison of the SAXS curves obtained for w-MBAAm/pNIPA (w-m), d- MBAAm/pNIPA (d-m) and d-DMTLT/pNIPA(d-d) cryogels prepared with the same amount of cross-linker (1/40); (a) log–log plots; (b) linear plots showing the high-q domain.

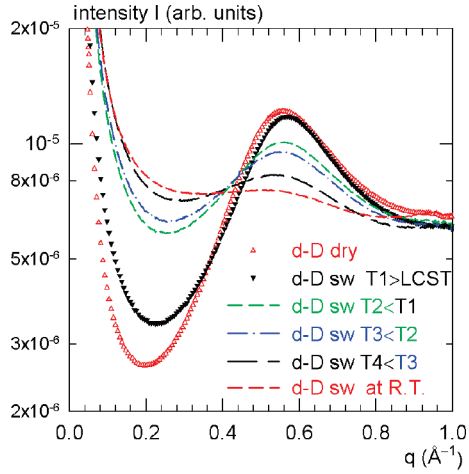

Figure 11. Comparison between the SAXS curves obtained for d-DMTLT/pNIPA (d-d) cryogels during cooling down from a temperature above T_c and for the dry cryogel.

experimental work are close to twice the length of the side groups, it may be assumed that d is a mean distance between main chains when interactions between neighbor side groups are no longer screened by water molecules.[29] Thus, vanishing of the peak in the swollen cryogels at temperatures below T_c indicates complete swelling. It follows that the existence of a small intensity peak in swollen gels below T_c suggests the presence of polymer-rich domains that cannot swell. This feature could be related to the difference between swelling ratios of gels and cryogels prepared in the same chemical conditions. Meanwhile, it is difficult at present to relate the presence of unswollen domains in gels to the fact that dried gels can hardly be reswollen, unlike dried cryogels.

4. Conclusions

The structure of NIPA-based macroporous cryogels prepared at subzero temperatures depends on the nature of the cross-linker (MBAAm or the more hydrophobic DMTLT) and the solvent used. MBAAm cross-linked cryogels produced in aqueous medium have an open pore structure that endows these cryogels with spongelike appearance, elasticity, and fast swelling−deswelling kinetics. The cryogels prepared in dioxane/water medium have, however, closed pore structure with thick pore walls. The differences in the structure are clearly reflecting different development of cryopolymerization in aqueous and organic−aqueous media with pronounced overcooling occurring in dioxane/water medium. The equilibrium swelling of the produced cryogels under the conditions where pNIPA chains are expanded depended on the chemical composition of the polymer network rather than the pore structure, whereas the latter defines the kinetics of swelling. Above T_c, all cryogels with the same total monomer concentration have nearly the same swelling kinetics and equilibrium swelling independently of the cross-linker or solvent used. SAXS experiments have shown that the nanostructure of the sample is related to the degree of swelling of gels or cryogels. Particularly, it is shown that, unlike cryogels, ordinary gels synthesized at room temperature exhibit unswollen domains below T_c.

Acknowledgment. This work was financially supported by Protista Biotechnology AB (Lund, Sweden), the Swedish Foundation for Strategic Research, the Swedish Foundation for International Cooperation in Research and Higher Education (STINT, IG2003-2089), the Swedish Institute (Visby program, projects 2886/2002 and 01211/2004), Royal Swedish Academy of Sciences. Financial support from the grant MAT 2004-01654 from the CICYT and the NoE EXPERTISSUES from the EC is acknowledged. We are grateful to the ESRF, Grenoble, for access to the French CRG Beamline D2AM (proposal no. 0201702) and we acknowledge the help of its technical staff, J. F. Berar, N. Boudet, B. Caillot, and S. Arnaud.

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BM7007668