Research Article

Biphasic Equilibrium Dialysis of Poly(N-Isopropyl Acrylamide) Nanogels Synthesized at Decreased Temperatures for Targeted Delivery of Thermosensitive Bioactives

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Hydrogel nanoparticles, referred to also as nanogels, are of special interest for medical and pharmaceutical applications. Due to small size in the range below the diameter of the capillaries, they are proposed as drug delivery carriers. The aim of the study was to estimate the influence of composition and reaction conditions during synthesis of poly-N-isopropyl acrylamide cross-linked by polyethylene glycol diacrylate on the purification rates of the polymer. Six types of thermosensitive nanogels were prepared by surfactant-free dispersion polymerization and assessed in terms of process yield, composition, and size at temperatures below and over volume phase temperature. During the diffusion of impurities, in the course of dialysis, assessed by the conductometric method, the remarkable influence of temperature and initiator concentration on the process was revealed. The release rates varied in the range between $9.63 \cdot 10^{-2}$ and $1.39 \cdot 10^{-1}$ h^{-1} in the first stage of the process, whereas in the second stage they were between $2.09 \cdot 10^{-2}$ and $6.28 \cdot 10^{-2}$ h^{-1}. The evaluated time to obtain acceptable purity of the preparation was estimated to be in the range of 18 days. More detailed research should be directed towards the influence of the structure of obtained material on the purification process.

1. Introduction

Nanogels are of special interest for medical and pharmaceutical applications [1–3]. Due to small size in the range below the diameter of the capillaries, they are proposed as drug delivery carriers. One of the main problems is to overcome the impurity of the product, which is intended for peroral or parenteral applications. With the diameter range between 10 and 1000 nm severe complications arise, when synthesized entities undergo the purification process. Synthesized in different conditions, nanoparticles contain various amounts of residual organic solvents, monomers, comonomers, surfactants, inorganic salts, initiators, accelerators, and noncrosslinked polymer aggregates. The presence of these impurities should be kept at very low level, to avoid adverse reactions within the human organism. Usually, the nanoparticles are purified by dialysis with the use of semipermeable cellulose membranes. With time the rate in impurities diffusion through the membrane decreases to extremely low values, with parallel decrease of impurities concentration in donor solution retentate, during the process. For enhancement of that procedure, various approaches are evaluated, including concentration/diafiltration process membrane filtration, crossflow membrane filtration, filtration

TABLE 1: The feed composition of the substrates, with respective assignments of the nanodispersions.

Component	Percentage [%]	Temperature of reaction [°C]		
		22	38	70
		Assignments of the nanodispersions samples		
TEMED	0.01			
APS	0.02			
PEGDA	0.25	Nanodispersion 22-1	Nanodispersion 38-1	Nanodispersion 70-1
NIPA	0.72			
TEMED	0.02			
APS	0.04			
PEGDA	0.25	Nanodispersion 22-2	Nanodispersion 38-2	Nanodispersion 70-2
NIPA	0.72			

Abbreviations: in the text.

by electrospun nonwoven polymer fibers, purification by spin centrifugation dialysis, and many others [4–11].

Usually the large polymer aggregates may be eliminated by filtration through sintered glass filters. For the research needs, the dialysis using the concentration gradient is acceptable; however the onset of the "clean" product should be specified, that is, by conductometric measurements. For some materials the purification may last for really long time, for example, four weeks, whereas in some cases, the reasonable level of conductivity is obtained within one week [12–14].

Sensitive nanogels, according to their unique environmental responsiveness, have been widely applied in the fields of drug delivery carriers, tissue engineering, biological separation, and enzyme science [15]. Due to the characteristic temperature-responsive activity, the shape, volume, and the swelling ratio of poly-N-isopropyl acrylamide (PNIPA) present specific changes upon reaching its volume phase transition temperature, which is around 32°C in aqueous solution. The polymer absorbs water to a swollen state at a temperature below the transition temperature and shrinks with volume decrease when the temperature increases above the VPTT (volume phase transition temperature). One of the new tasks within the forthcoming aspects of polymer science is synthesis of N-isopropyl acrylamide derivatives in temperatures which do not exceed the temperature of proteins decomposition [16]. The formation of hydrogels as nano- or microstructures was evaluated by numerous authors, whereas Dušek developed in details the problem of so called microsyneresis; in the case of N-isopropyl acrylamide increased temperature plays important role in microgel formation [17–19]. The aim of the study was to estimate the influence of composition and reaction conditions on the estimated dialysis rates. Six nanogels were prepared by surfactant-free dispersion polymerization and assessed in terms of process yield, composition, and size. Within the synthesis process the N,N,N′,N′-tetramethyl-ethane-1,2-diamine (TEMED) was used to accelerate the free radical polymerization process [20]. During the diffusion of impurities, assessed by the conductometric method, the influence of temperature and initiator concentration on the purification process was evaluated. The purification rates were calculated assuming first order release of impurities

from the donor compartment retentate, to the acceptor compartment permeate. Additionally the evaluated time to obtain acceptable purity of the preparation was estimated for the poly-N-isopropyl acrylamide-co-polyoxyethylene glycol diacrylate (PNIPA-co-PEGDA) polymer.

2. Material and Methods

2.1. Materials. N-isopropyl acrylamide (NIPA), polyethylene glycol diacrylate of molecular mass ca. 2000 (PEGDA), and N,N,N′,N′-tetramethyl-ethane-1,2-diamine were supplied by Aldrich (Czech Republic, Prague). Ammonium persulfate (APS) was purchased from Lachema (Czech Republic, Prague). The components obtained from industrial suppliers were used without further purification. A dialysis bag with a molecular weight cut-off of 12000–14000 Da was obtained from Sigma Aldrich (Czech Republic, Prague). Deionized water was obtained from the TKA DI 6000 system (Germany, Niederelbert) and was used in all the following procedures.

2.2. Synthesis of the Thermosensitive Nanoparticles. PNIPA nanogel particles crosslinked by PEGDA were synthesized by surfactant-free dispersion polymerization (SFDP) in deionized water under an inert nitrogen atmosphere according to the procedure evaluated by Pelton and Chibante [21, 22] and developed in the group of Lowe et al., and in other groups [23–25]. The radical initiator, ammonium persulfate, was placed in a 500 mL, three-necked round-bottomed flask and stirred continuously at $120 \, \text{m}^{-1}$. Predissolved NIPA and cross-linking agent PEGDA were dissolved in 20 mL of deionized water with stirring and were then added to the reaction vessel. After 4 hrs, the dispersion was cooled to room temperature and filtered. Dry weight analysis of the nanogels showed the dispersion to have a concentration on the order of 0.1–0.5 wt.%. The starting composition of the substrates is given in Table 1, with respective assignments of the nanodispersions.

2.3. Equilibrium Dialysis Purification. Dialysis-purification by diffusion through semipermeable membrane was performed in the set of glass vessels. The sample of 50 mL of

TABLE 2: The yields of nanogels synthesized in various temperature conditions and different concentrations of initiator.

		Yield [%]	
Concentration of initiator [%]		0.02	0.04
	22	8	16
Temperature [°C]	38	24	89
	70	76	93

nanogel dispersion was closed in dialysis bag and transferred into 300 mL glass vessel. The system was stirred in IKA-VIBRAX-VXR device (Germany), by 100 rpm. The particles were dialyzed against deionized water until the conductivity was less than $1.0\ \mu S \cdot cm^{-1}$ [26]. Within the first 30 hrs the conductivity in the acceptor compartment was measured, and the water remained unchanged in the acceptor compartment. After 30 hrs, the water was replaced in acceptor compartment by fresh deionized water, and the conductivity measurement was taken after 24 hrs. The procedure was repeated every 24 hrs, through 18 days, till the conductivity did not exceed $1.0\ \mu S \cdot cm^{-1}$.

2.4. FTIR Evaluations.
Fourier transformed infrared spectra of the obtained polymers were measured at ambient temperature with a Nicolet Nexus 870 FTIR spectrometer purged with dry air and equipped with a Golden Gate device ATR, with reproducible approximate sample loads of 3 kbar. Samples were measured in the dry state; the spectra were corrected for absorption of the solvent and H_2O vapors.

2.5. Assessment of the Hydrodynamic Diameter.
The hydrodynamic diameter was assessed in aqueous dispersions by dynamic light scattering (DLS). From the ca. 50 mL sample, $50\ \mu L$ was taken and centrifugated within the Eppendorf tube filled with 1.0 mL of prefiltered water, in Roth MicroCentrifuge, at 6000 rpm for 45 min. From the tube $50\ \mu L$ of supernatant were taken and placed into polystyrene-single use fluorimeter cuvettes. Then 1 mL of deionized water, filtered through $0.2\ \mu m$ PVDF Whatman nanofilter, was added to the cuvette and the sample was inserted into Zetasizer Nano with 173° backscatter measurement arrangement, and settings of Mark-Houwink parameters. The duration of measurements was extended for the case of large particles, with relaxation time multiplier of 100000. The measurements settings were automated to seek optimum position; the attenuator selection was also automated.

2.6. Evaluation of the Conductivity Data from Purification Experiment.
We assumed that the observed process of the release of impurities from the donor compartment may be considered as the first order process. The calculations were performed using obtained data, assuming one-stage process of the release of impurities and two-stage process of the release of impurities. The fitted functions of one-stage, first order kinetics model and two-stage, first order kinetics model were compared by using the correlation factors (r^2). The assumption was made that the conductivity parameter
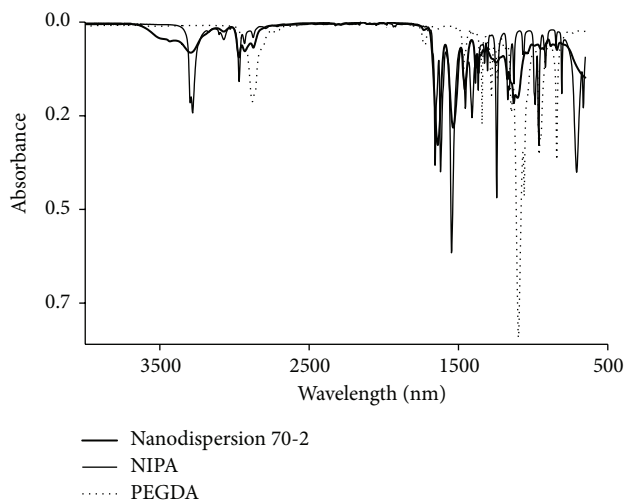

FIGURE 1: The FTIR spectra of N-isopropyl acrylamide (NIPA), polyethylene glycol diacrylate of molecular mass ca. 2000 (PEGDA), and freeze-dried Nanodispersion 70-2.

reflects the concentration of impurities within the diluted nanodispersions.

3. Results and Discussion

Due to the FTIR assessments within all the polymeric entities, the PNIPA polymer was synthesized in all the cases with implemented PEGDA cross-linker—on Figure 1, there is exemplification of the spectrum of NIPA, PEGDA, and freeze-dried nanodispersion.

The yield of the reaction varied, depending on the applied conditions and compositions. In general, the yield of nanogel, calculated on the basis of gravimetric assessment, was in the range between 8% and 93%. The yields for nanogel are presented in Table 2.

Within the first 30 hrs of the purification process, the conductivity was measured every 1 hr. The conductivity in all the assessed cases decreased in the donor compartment with time, and course of the obtained function adhered, with high correlation factors, to the logarithmic function, which reflected the first order reaction, as it is presented on (1), where C and C_0 are the actual conductivity in the donor compartment and initial conductivity in the donor compartment, respectively. The k and t denominate the process rate and the actual time:

$$C = C_0 \cdot e^{-kt}. \tag{1}$$

This fact supports the idea of first order process diffusion which is triggered by the concentration gradient in the assessed copolymers. However we observed some variety between the evaluated nanogels, in terms of measured conductivity. For quantitative evaluation of the results, we treated the obtained data by the method usually applied for the release experiments. Due to the experimental data, the course of the conductivity increases and can be divided into two stages. In the first stage the increasing is faster than in the

TABLE 3: Correlation factors (r^2) calculated for course of the function time versus conductivity, assuming 1-stage or 2-stage release of impurities from the donor compartment, during purification process of synthesized nanogels, where the A and B are the theoretically evaluated starting values of conductivity, which reflect the initial concentrations for the first and for the second stage, with respective α and β rates of the process [28].

Model type		1 stage		2 stages
Equation		$C = C_0 \cdot e^{-kt}$		$C = A \cdot e^{-\alpha t} + B \cdot e^{-\beta t}$
	Nanodispersion 22-1	0.8413	1	0.9736
			2	0.9944
	Nanodispersion 38-1	0.8554	1	0.9799
			2	0.9930
	Nanodispersion 70-1	0.8277	1	0.9696
			2	0.9893
Preparation	Nanodispersion 22-2	0.9383	1	0.9921
			2	0.9886
	Nanodispersion 38-2	0.8797	1	0.9795
			2	0.9797
	Nanodispersion 70-2	0.7202	1	0.9489
			2	0.9585

second stage. Higher values of correlation factors confirmed the 2-stage course idea. In Table 3 the correlation factors for the 1-stage and 2-stage models are presented, according to equations developed in earlier work [28].

Due to the experimental data, the two-stage process seems to be more probable than the one-stage. The data presented in Figure 2 depict the course of conductivity increasing in the acceptor compartment. The upper straight lines on the graph represent the upper limit of the conductivity of the nanodispersion, measured in parallel samples: after 30 hrs, but before purification process—this value reflects the highest content of impurities in the sample. The values of conductivity in the first stage of the purification were in agreement with the simple predictions. In the case of high initiator level, the donor compartment released faster impurities than in the case of lower initiator level in the reactor. Also the level of conductivity was higher, compared to that of nanogels synthesized with low feed content of initiator—Figure 2.

The maximal level, due to the prediction equation within the first step of the release of impurities, should be obtained after ca. 14 hrs, 42 hrs, and 48 hrs for the nanogels 70-1, 38-1, and 22-1, respectively, as it is assigned by dashed circles in Figure 2(a). Increased concentration of initiator in the feed composition influenced the level of conductivity during the purification but we did not identify any obvious tendency in the process. The estimated times of final equilibrium, due to the estimations from the first step equation, are between 23 and 28 hrs. From the data gathered in Table 3, as it was mentioned, the course of the impurities release investigated by the conductivity measurements should be bi-phasic. The second stage of conductivity increase is presented in Figure 3, both for the low and high feed content of the initiator in the reacting mixture.

The evaluation of the second stage of the purification reveals that the 70-1 sample will be finally equilibrated after almost 100 hrs, whereas the sample 70-2, with double amount

TABLE 4: The rates of the increase in the conductivity, within the acceptor compartment calculated for course of the function of time versus conductivity, assuming two-stage release of impurities from the donor compartment, during purification process of synthesized nanogels.

Rate in	1st stage [h^{-1}]	2nd stage [h^{-1}]
Nanodispersion 22-1	$9.63 \cdot 10^{-2}$	$2.65 \cdot 10^{-2}$
Nanodispersion 38-1	$1.03 \cdot 10^{-1}$	$2.95 \cdot 10^{-2}$
Nanodispersion 70-1	$1.25 \cdot 10^{-1}$	$3.28 \cdot 10^{-2}$
Nanodispersion 22-2	$1.39 \cdot 10^{-1}$	$6.28 \cdot 10^{-2}$
Nanodispersion 38-2	$1.26 \cdot 10^{-1}$	$4.21 \cdot 10^{-2}$
Nanodispersion 70-2	$1.27 \cdot 10^{-1}$	$2.09 \cdot 10^{-2}$

of initiator in the feed composition, will not reach equilibrium even after double time of, that is, 200 hrs. Oppositely, the samples 22-2 and 38-2 should reach the equilibrium just after around 50 hrs, whereas the samples 22-1 and 38-1 will be still in the course of dialysis due to the equation, describing the second stage of conductivity increase. The calculated rates are gathered in Table 4.

From the main course of the purification process in first 30 hrs it may be seen that the release of impurities from the donor compartment is faster in the first stage of the process, and one raw slower, after ca. 10 hrs. The increase in temperature of the reacting mixture, in the case of low feed content of initiator, resulted in faster release of impurities in the purification procedure. This tendency was observed both in the first and in the second stage of the purification process as it is presented in Table 4. However the rates of release of the impurities, measured by the conductivity phenomena, were ordered actually in a decreasing way, when the high amount of initiator was applied. The kinetics of impurities release was measured in the period of first 30 hrs, without change of the water. Then, the data were collected in 24 hr intervals,
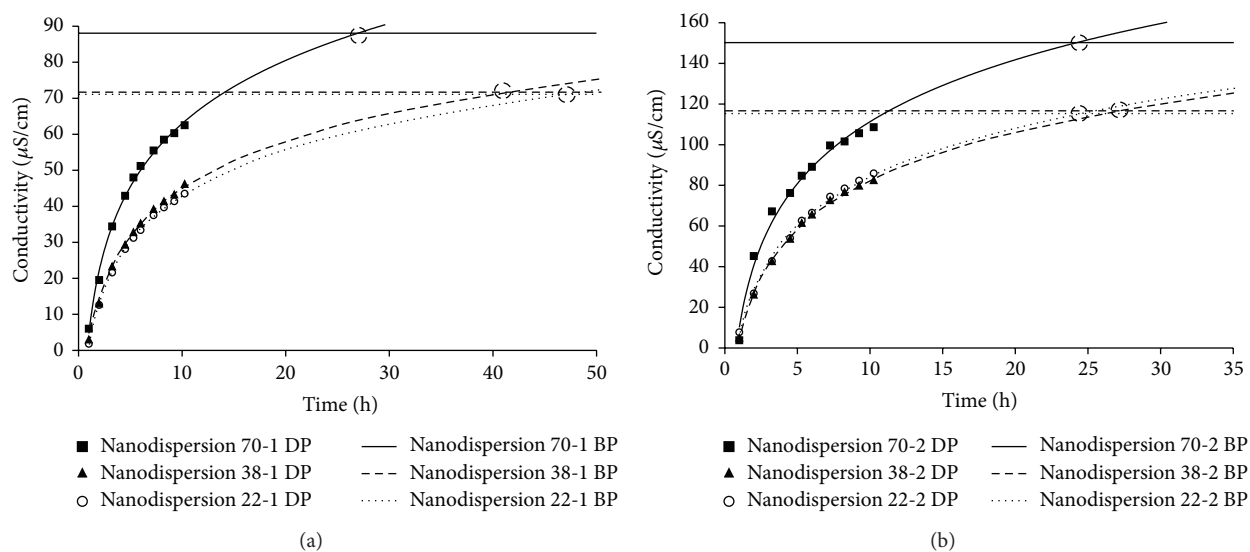
(a)

(b)

FIGURE 2: The first stage of purification of aqueous dispersion of a nanogel, measured in terms of conductivity, for nanogels synthesized with low (a) and high (b) feed content of initiator. The dashed circles represent the intersection of the conductivity curve with a straight line which represents the upper possible level of conductivity in specified samples. DP refers to the data from the purification process in acceptor compartment—the lines are the guides for the eye. BP refers to the data of the same concentration and volume, not purified—straight lines, explanation in the text. Other acronyms which are given in Table 3 refer to the feed composition of the nanogels.
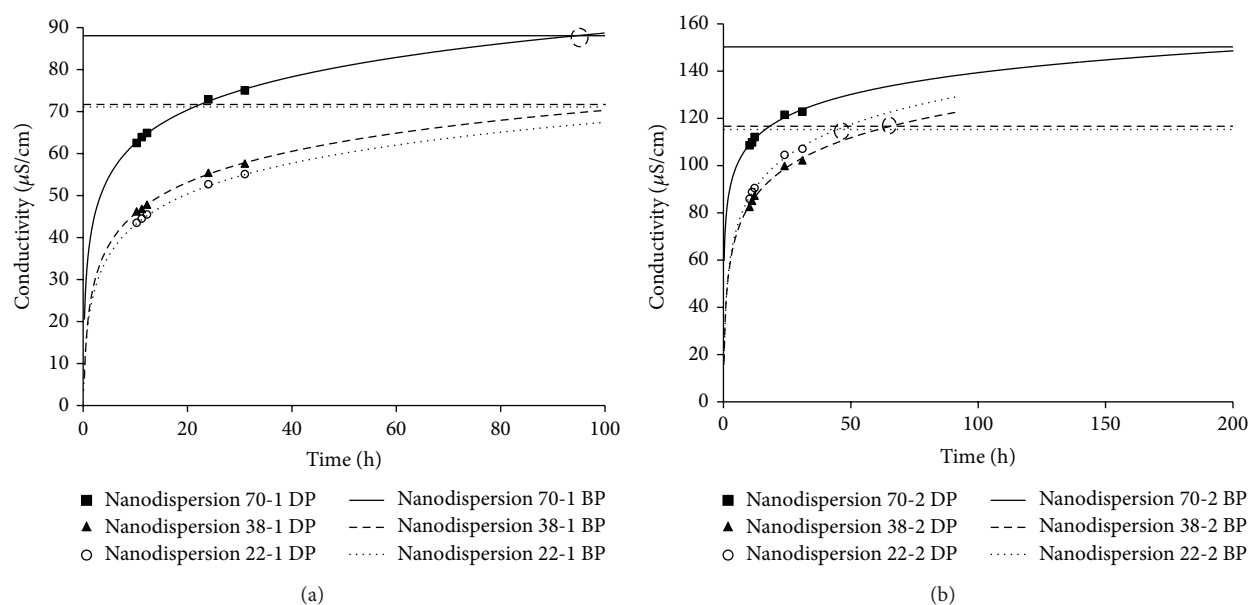

(a)

(b)

FIGURE 3: The second stage of purification of aqueous dispersion of a nanogel, measured in the terms of conductivity, for nanogels synthesized with low (a) and high (b) feed content of initiator. The dashed circles represent the intersection of the conductivity curve with a straight line which represents the upper possible level of conductivity in specified samples. DP (during purification) refers to the data from the purification process in acceptor compartment—the lines are the guides for the eye. BP (before purification) refers to the data of the same concentration and volume, not purified—straight lines, explanation in the text. Other acronyms which are given in Table 3 refer to the feed composition of the nanogels.

preceded by change of the water in acceptor compartment. The data are gathered in Figure 4.

The complete purification of the nanogel dispersion was confirmed after the eighth to the tenth day, with final values close to $1.0\,\mu\text{S}\cdot\text{cm}^{-1}$. However, after keeping the dispersion in isolated environment, the conductivity increased in acceptor compartment to ca. $5\,\mu\text{S}\cdot\text{cm}^{-1}$, but after consequent dialysis purification through four days it decreased to the value of $1.0\,\mu\text{S}\cdot\text{cm}^{-1}$ and maintained for prolonged period. As the synthesized nanogels are intended for drug delivery, with the
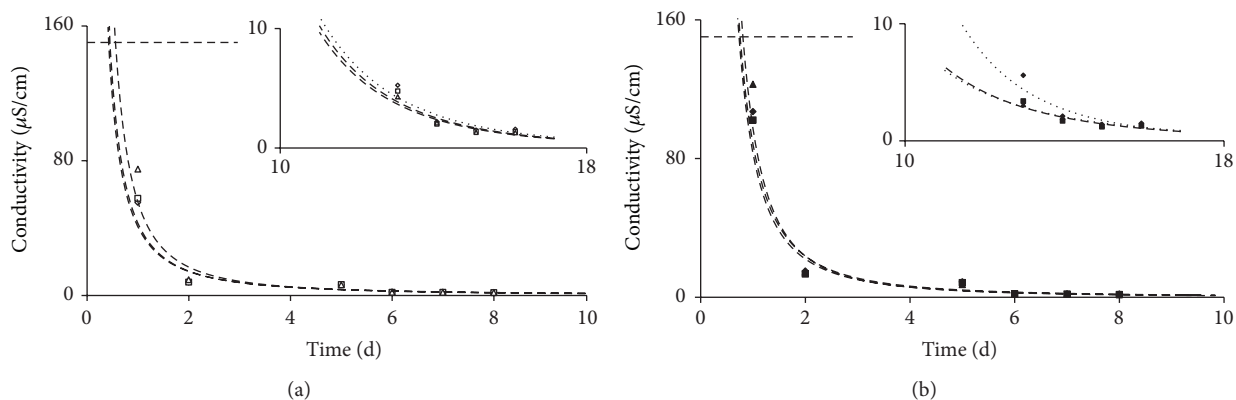
(a)

(b)

FIGURE 4: The conductivity in acceptor compartment, after 24 hr period of static dialysis by deionized water. The assignments are as follows: nanodispersion 22-1 (◇), nanodispersion 38-1 (□), nanodispersion 70-1 (△), nanodispersion 22-2 (♦), nanodispersion 38-2 (■), and nanodispersion 70-2 (▲), with straight line on the large graph representing the initial conductivity in the donor compartment. The inserted small graphs give information on the conductivity after the tenth to the eighteenth day of static dialysis.
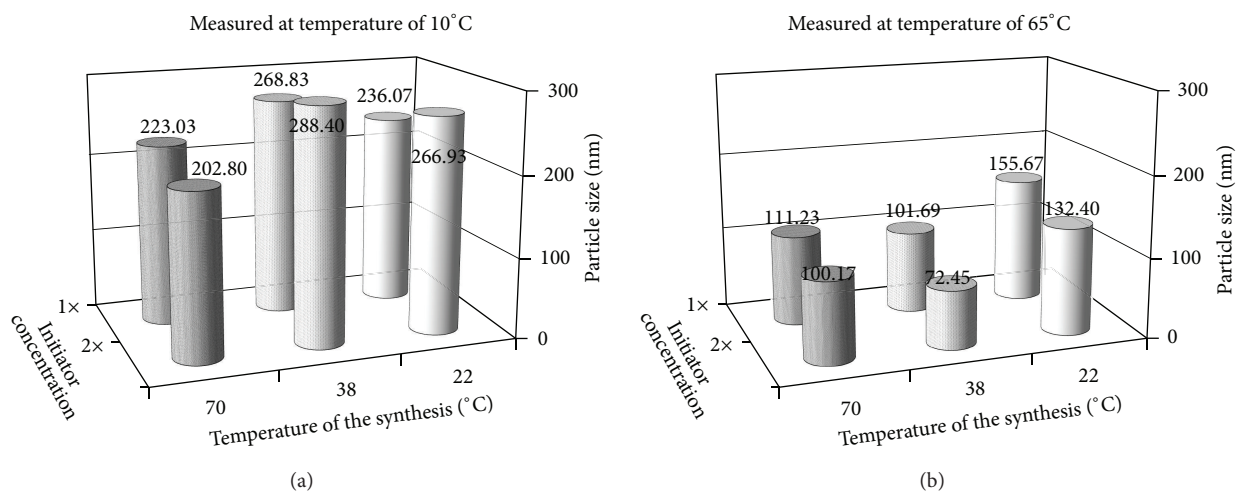

(a)

(b)

FIGURE 5: The hydrodynamic diameters, measured by DLS at temperatures of 10°C and 65°C. The concentration of the initiator (Init. conc.) was varied in experiments between 0.01% (1x) and 0.02% (2x). Particle size refers here to the hydrodynamic diameter.

trigger factor, temperature, the most interesting issue is the thermosensitivity of obtained structures. The hydrodynamic diameters measured in the DLS device are in the range between 72.45 nm and 155.67 nm when measured at 65°C; however the hydrodynamic diameter is much higher, when the assay is performed at decreased temperature of 10°C, that is, between 202.80 nm and 288.40 nm. We gathered the data within the graph in Figure 5, with respect to the concentration of the initiator and to the temperature of the synthesis.

Obtained yield values presented in Table 2 indicate that, with the increase in the temperature in the course of the reaction polymerization, the initiator seems to be more active in the system. The applied concentration of accelerator TEMED seems to be acceptable for the initial growth of the polymer molecules; however at decreased temperatures also the polymerization rate seems to decrease. The elevated level of the main initiator APS favors the reaction course. The data

from DLS measurements performed at elevated temperatures suggest that the application of increased amount of initiator leads to the increase in number of particles in the system, what is in agreement with observations of Chiu and Lee [29, 30] as well as with some theoretical predictions [31, 32].

The differences in dialysis course are connected to the remains of the unreacted components within the reaction mixture. However, the results obtained in the conductometric measurements may indicate also the influence of product structure on the course of release of the impurities gathered in the nanogel. According to the development of small-angle neutron scattering (SANS) technique the evaluation of the substructures of microgels became more feasible [33]. The concept of two compartments in the structure of microgels and nanogels, derivatives of N-isopropylacrylamide, was initially developed by Wu et al.; due to theirs observations, the monomers distribution was more dense within the center of the synthesized microgels [34]. This idea was confirmed by
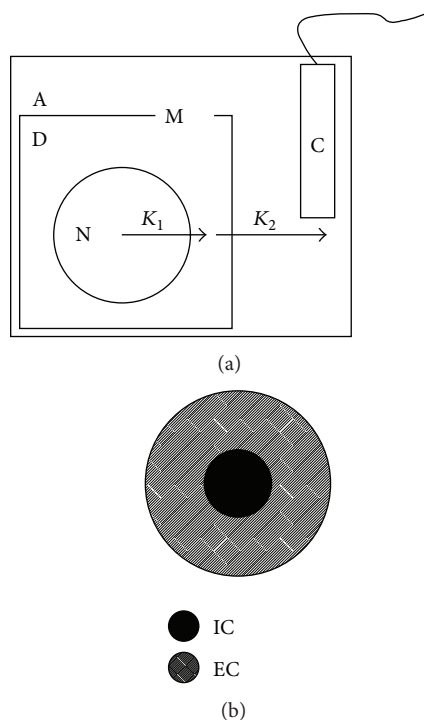
(a)

(b)

FIGURE 6: The scheme of dialysis purification of obtained nanogels (a) and schematic representation of two compartmental microgel structures (b), where N is the nanogel, D is the donor compartment with retentate, A is the acceptor compartment with permeate, M is the semipermeable membrane of MWCO 12–14 kDa, C is the conductivity probe, K_1 is the diffusion rate of impurities from the internal nanogel structure, K_2 is the diffusion rate of impurities from the solution into the donor compartment, IC is the internal compartment and EC is the external compartment of the nanogel due to the evaluation in reference [27].

other researchers in optical measurements [35], as well as in several SANS studies [36–38]. The detailed evaluation of the topic was given later by Sanders—there is still open question, if the internal and external compartments of the microgel origin from ongoing synthesis or following agglomeration and cross-linking [27]. Due to the fact that almost 90% of the microgel in water consists of water, the soluted impurities should be distributed nonhomogenously within the polymeric matrix [39]. Partially the impurities may be distributed within the core, and partially within the shell which is less dense [40]. We suggest in our case two-step purification process, due to the scheme presented in Figure 6(a). In the first phase the purification course is dominated by the diffusion of the impurities from the external water phase, that is, $K_2 > K_1$, which reflects the impurities in the aqueous environment and the external compartment of the nanogel. During the second phase the process is ruled mainly by the diffusion of the impurities from the internal nanogel phase, internal compartment, and $K_1 > K_2$. The respective values for theoretical estimation of the acceptable purification time may be red from the graphs in Figure 3. The initial level of impurities was in the highest in the case of nanodispersion

70-2, and the time for sufficient purification is the longest. The relatively short time for acceptable purification course is estimated in the case of nanodispersion 38-2 and nanodispersion 22-2. nanodispersion 70-1, nanodispersion 38-1, and nanodispersion 22-1 may be characterized by similar, rather long purification rate. As it was mentioned above, the important factors affecting the impurities release from the nanoparticles may include both the level of reactants, and the internal structure of the nanogel. To evaluate the topic we calculated the rates of impurities release from the donor compartment, implementing the idea of two-stage and first order kinetics. The calculations confirm the idea of influence of structure on the purification time of the nanodispersions. In the first stage the impurities release is comparable within the group of low starting values of initiator: $9.63 \cdot 10^{-2}$, $1.03 \cdot 10^{-1}$, and $1.25 \cdot 10^{-1} \, h^{-1}$. Also in the group of nanodispersions produced with high amount of initiator, the purification rates calculated on the basis of conductometric assays are similar: $1.39 \cdot 10^{-1}$, $1.26 \cdot 10^{-1}$, and $1.27 \cdot 10^{-1} \, h^{-1}$. However in the second stage of the purification process, the rates increased for samples prepared with low initiator concentration, with the temperature of reaction. Oppositely the rates in the second stage decreased in samples prepared with higher amount of initiator—Table 4. By high initiator concentration, at elevated temperatures higher amount of components was used to form polymer particles. When low concentration of initiator was applied the structures of nanoparticles were characterized by higher size—at elevated temperature the particles were able to preserve high amount of impurities, which were released with lag time from the core-shell nanostructures, as it is presented in Figure 6(b). Evaluation of the impurities release from the bicompartmental microgel structure in the terms of bi-phasic kinetic process should have remarkable impact on the future experiments, for example, in the field of controlled drug release. From practical point of view the acceptable purity is in our case acquired after 18 days of dialysis through semipermable membrane.

4. Conclusion

Both, composition and reaction, conditions influence the acceptable time for purification of nanogel dispersions obtained with N-isopropyl acrylamide-*co*-polyethylene glycol diacrylate. The purification course may be divided into two kinetic stages. At low concentrations of initiator the residual impurities seem to be at lower level. When high concentration of initiator is applied, the respective impurity level is higher and the purification time should be elapsed. After 18 days of the process of purification, the nanogel dispersions were cleaned within acceptable level of conductivity, not higher than $1.0 \, \mu S \cdot cm^{-1}$. More detailed research should be directed towards the influence of the structure of obtained material on the purification process, as the bicompartmental structure of the nanogel may be crucial for the process. The optimal dialysis time for the synthesized nanoparticles was estimated to be 18 days.

Conflict of Interests

None of the authors has any conflict of interests to disclose in this work.

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