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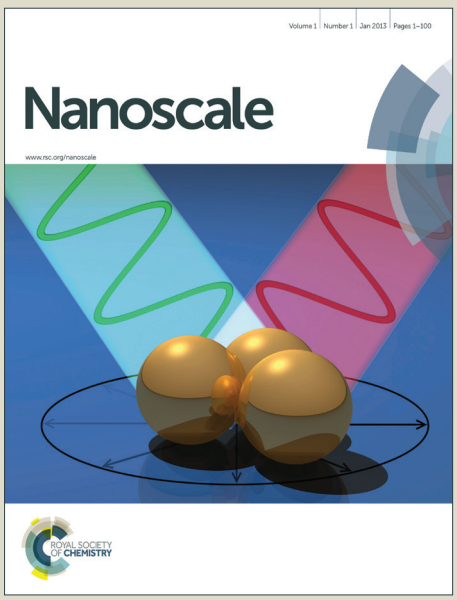

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ARTICLE

Degradable polymeric nanoparticles by aggregation of thermoresponsive polymers and "click" chemistry

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This study describes a novel approach to the preparation of crosslinked polymeric nanoparticles of controlled sizes that can be degraded under basic conditions. For this purpose thermoresponsive copolymers containing azide and alkyne functions were obtained by ATRP of di(ethylene glycol) monomethyl ether methacrylate (D) and 2-aminoethyl methacrylate (A) followed by post polymerization modification. The amino groups of A were reacted with propargyl chloroformate or 2-azido-1,3-dimethylimidazolinium hexafluorophosphate, which led to two types of copolymers. Increasing the temperature of aqueous solutions of the mixed copolymers caused their aggregation into spherical nanoparticles composed of both types of chains. Their dimensions could be controlled by changing the concentration and heating rate of the solutions. Covalent stabilization of aggregated chains was performed by a "click" reaction between the azide and alkyne groups. Due to the presence of the carbamate bond the nanoparticles undergo pH dependent degradation under mild basic conditions. The proposed procedure opens a route to new carriers for controlled release of active species.

Introduction

Polymeric nanoparticles (PNs) have been utilized in an increasing number of fields during the last decades.[1, 2] There are many methods of preparing PNs, either from synthesized polymers or by direct polymerization of monomers.[3, 4] Generally, to produce PNs, techniques such as solvent evaporation, salting-out, supercritical fluid technology, emulsion methods and interfacial polymerization have been used.

Nowadays PNs play a crucial role in different applications such as drug delivery,[5-8] medical imaging,[9] personal care,[10] microfluidics[11] and nanotechnology.[12] Polymeric nanoparticles can serve as carriers of different payloads,[5] some of which can deliver therapeutic agents directly into the intended site of action with superior efficacy.[13] The requirements for designing such a delivery system are: controlled particle size, proper surface character, enhanced permeation, flexibility, solubility, and release of therapeutically active agents in order to attain the target and retain activity of the drug. A polymeric system developed for applications in the biomedical or environmental fields should be free of contaminations, like reactants, surfactants or traces of organic solvents.

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A novel and convenient approach for the preparation of well-defined polymeric nanoparticles is the aggregation of thermoresponsive polymers in aqueous solutions upon heating.[14] Heating aqueous solutions of a thermoresponsive polymer leads to a decrease in polymer solubility in water and polymer precipitation above a certain temperature called the cloud point temperature (T_{CP}). In dilute aqueous solutions above the T_{CP} thermoresponsive chains self-assemble forming spherical, colloidally stable, and equally sized aggregates of the collapsed, dehydrated polymer chains, called mesoglobules.[15, 16] This thermally induced aggregation to mesoglobules has been studied for many thermoresponsive polymers and polymer-peptide conjugates.[14, 17-20] The size of the mesoglobules is controlled by the concentration of polymer solution,[17-19, 21] the rate of heating,[17-19, 21, 22] the molar mass of the polymer[19] and its structure.[19]

Among thermoresponsive polymers the poly(oligoethylene glycol (meth)acrylate)s (POEG(M)A) deserve for special attention. POEG(M)As, due to their amphiphilic structure, hydrophilic oligo(ethylene glycol) side chains (OEG) of different lengths, and hydrophobic backbone (methacrylate main chain) exhibit thermoresponsiveness in a broad range of T_{CP}.[19, 23] The oligo(ethylene glycol) (OEG) pendant groups are responsible for their biocompatibility and permit POEG(M)A-based materials to be used in the biomedical fields.[24]

In spite of the simplicity and environmentally friendly conditions for the preparation of mesoglobules they have not found practical use yet, due to their instability at temperatures below T_{CP} or when some salts are added to the water.

There have been few reports about stabilizing mesoglobules by covering them with an outer crosslinked shell using

nucleated radical polymerization.[18, 25-27] Mesoglobules of poly(glycidol-*co*-ethyl glycidyl carbamate)s,[18] poly(methoxydiethyleneglycol methacrylate)[27] and poly(2-isopropyl-2-oxazoline)[25] were coated with crosslinked poly(N-isopropylacrylamide) (PNIPAM).[25] Mesoglobules of PNIPAM were covered by crosslinked shell of NIPAM or 2-hydroxyethyl methacrylate.[26] The changes in temperature from above to below the T_{CP} caused a reversible response in the nanoparticles' volume. It was shown that the shell played the role of a membrane, allowing the removal of polymer chains from the particle interior by dialysis.[26]

Here we present a new approach for stabilization of mesoglobules achieved by internal crosslinking of collapsed polymer chains. Two thermoresponsive copolymers of diethylene glycol methyl ether methacrylate and 2-aminoethyl methacrylate synthesized by ATRP were modified to introduce alkyne and azide groups. The investigation of thermally induced aggregation of the copolymers and their mixture in water allowed us to establish optimal conditions for the preparation of well-defined mesoglobules. The presence of the alkyne and azide groups suitable for "click" reaction led to stable nanoparticles while the presence of carbamate bonds in the polymer chains allowed for their controlled degradation. [28, 29]

Experimental

Materials

Di(ethylene glycol) methyl ether methacrylate (95%) (D), copper (I) chloride (98%), ethyl α-bromoisobutyrate (EBB, 98%), propargyl chloroformate (96%), 4-dimethylaminopyridin, (99%) (DMAP), 2-azido-1,3-dimethylimidazolinium hexafluorophosphate (97%) (ADMP), (+)-sodium L-ascorbate (≥98%), copper (II) sulfate pentahydrate (≥98%), triethylamine (98%) (TEA), DOWEX MARATHON MSC (H) ion-exchange resin, and Hamilton Duracal buffer solutions (pH 2, 7, 10) were purchased from Sigma-Aldrich. 2-Aminoethyl methacrylate (>98%) (A) was received from Polysciences Inc. (USA). 2,2'-Bipyridine (99%) was purchased from Alfa Aesar (USA). Water (Gradient Grade), methanol (99.8%), ethanol (98%) dichloromethane (99.8%) (DCM), and tetrahydrofuran (99.5%) (THF) were purchased from POCh (Poland). The DCM was distilled before use. THF was distilled over potassium hydroxide before use. The water used to prepare polymer solutions for DLS experiments was purified using a commercial ion exchange system (Hydrolab, Poland). The copolymer solutions were kept overnight at 4 °C prior to use. The other reagents were used as received.

Poly(di(ethylene glycol) methyl ether methacrylate) (PD) (M_n=45 000 g/mol, M_w/M_n=1.1) was synthesized according to procedure described previously.[19]

Synthesis of di(ethylene glycol) monomethyl ether methacrylate (D) and 2-aminoethyl methacrylate (A) copolymers

Synthesis of poly((di(ethylene glycol) methyl ether methacrylate)-co-(2-aminoethyl methacrylate)) (P(D-co-A)_1 and P(D-co-A)_2) was performed similarly to a procedure described by Feng et al.[30]

The amounts of reagents used in the syntheses of P(D-co-A)_1 and P(D-co-A)_2 are presented in Table 1.

Table 1 The amounts of reagents used in the syntheses of P(D-co-A)_1 and P(D-co-A)_2

Copolymer	D/A/EBB/CuCl/Bpy[a]	H_2O/MeOH[b]	Monomers concentration [mol/L]
P(D-co-A)_1	50/50/1/1/2	1/2	7.3
P(D-co-A)_2	285/15/1/1/2	1/2	3.8

[a] molar ratio, [b] volume ratio

As an example, the representative method for synthesis of P(D-co-A)_1 is described below.

Monomers (D, 0.5 mL, 2.723 mmol; A, 0.45 g, 2.717 mmol), ligand (Bpy, 17.02 mg, 0.109 mmol), catalyst (CuCl, 5.4 mg, 0.0545 mmol) and 0.75 mL methanol/water mixture were placed in a Schlenk flask equipped with a magnetic stirrer and an argon/vacuum inlet valve. The solution was deoxygenated by three freeze-vacuum-thaw cycles: after the second cycle, the initiator (EBB, 8 µL, 0.0545 mmol) was introduced to the system under argon atmosphere. The polymerization was carried out 24 h at room temperature and was finished by purging the system with air. The copolymer was purified by dialysis with the addition of DOWEX through a membrane (cut-off 3500 Da) against methanol for 5 days. A total of 0.4268 g of P(D-co-A)_1 was obtained.

Synthesis of prop-2-yne carbamate-modified copolymer

According to the procedure reported by Ramesh [31] the P(D-co-A)_2 was reacted with propargyl chloroformate to obtain poly((di(ethylene glycol) methyl ether methacrylate)-co-(2-N-(prop-2-ynyl carbamate)ethyl methacrylate)) (P(D-co-A_Pr).

P(D-co-A)_2 (2.0 g, 0.0465 mmol), triethylamine (1.06 mL, 7.62 mmol,) and 15 mL THF were added to a round-bottom flask equipped with a magnetic stirrer and dissolved. The flask was placed in an ice bath and propargyl chloroformate (0.74 ml, 7.62 mmol) was added dropwise to the mixture. During reaction a white solid of triethylamine chloride was precipitated. After one hour of reaction the flask with the reaction mixture was moved to room temperature and left overnight. The product was purified by dialysis through a membrane (cut-off 3500 Da) against methanol with the addition of DOWEX for 5 days. A total of 1.8869 g of P(D-co-A_Pr) was obtained.

Synthesis of azide-modified copolymer

The amine groups of P(D-co-A)_2 were transferred to azides using a procedure reported by Kitamura.[32]

P(D-co-A)_2 (2.0 g, 0.0465 mmol), 4-dimethylaminopyridine (0.732 g, 5.99 mmol) and 15 mL dichloromethane were introduced to a round-bottom flask equipped with a magnetic stirrer. Then 2-azido-1,3-dimethylimidazolinium hexafluorophosphate (ADMP, 1.708 g, 5.99 mmol) was added to the mixture. The reaction mixture was boiled under reflux for 5 hours and then transferred to room temperature and left

overnight. The obtained product was purified by dialysis with the addition of DOWEX through a membrane (cut-off 3500 Da) against pure methanol for 5 days. A total of 1.1954 g of poly((di(ethylene glycol) methyl ether methacrylate)-co-(2-azidoethyl methacrylate) (P(D-co-A_Az)) was obtained.

Kaiser test

In order to calculate the number of free amine groups present in copolymer chains a reaction with ninhydrin was performed.[33] First, 100 µL of 5% ninhydrin/n-BuOH, 50 µL of 80% phenol/n-BuOH and 50 µL of 0.01 M KCN/pyridine were added to copolymer samples (~1 mg) and heated to 100 °C. After 15 minutes of reaction the absorbance of the post-reaction solutions was measured by UV-vis at 590 nm.

Preparation of mesoglobules

Mesoglobules of P(D-co-A_Pr), P(D-co-A_Az) and their mixture P(D-co-A_Pr)/P(D-co-A_Az) (1:1 weight ratio) were prepared in water by two methods, gradual and abrupt heating.

The first method involve a gradual heating of 2 mL of the copolymer solution (c=0.1, 0.2, 0.5, 1.0 g/L) from 18 °C to 50 °C using a thermocontroller.

In the second method vials containing 4 mL of the copolymer solutions (c=0.1, 0.2, 0.5, 1.0 g/L) were abruptly heated from 8 °C to 55 °C by placing them in a preheated goniometer cell.

Crosslinking of mesoglobules

To crosslink mesoglobules the Huisgen 1,3-dipolar cycloaddition of azides and alkynes catalyzed by copper (II) sulfate in the presence of sodium ascorbate was performed. [34] The crosslinking reactions of mixed P(D-co-A_Pr)/P(D-co-A_Az) mesoglobules (obtained by the abrupt heating method) were performed for a constant concentration (0.2 g/L) using different amounts of catalyst (1/20, 1/2, 1/0.2 and 1/0.02 molar ratios of N_3/Cu) and for a constant amount of catalyst (1/2 N_3/Cu molar ratio) using mesoglobules obtained at different solution concentrations (0.1, 0.2, 0.5, 1.0 g/L).

The general procedure proceeds as follows: to a 4 mL dispersion of mesoglobules in water at 55 °C 10 µL of water containing the proper amount of copper (II) sulfate and 10 µL containing sodium ascorbate (1/10 molar ratio Cu/Na asc) were added. All reactions were performed for 3 h at 55 °C. Next, the mixtures were measured by DLS at 55 °C (above the T_{CP} of the copolymers) and at 18 °C (below the T_{CP} of the copolymers).

Degradation of nanoparticles

For degradation experiments 100 µL of buffer at pH 2, 7 or 10 were added to 0.5 mL of the solution containing nanoparticles. Probes were incubated in an oil bath at 37 °C. At preselected time periods DLS measurements of the nanoparticles were performed at 18 °C, below the T_{CP} of copolymers.

Methods

Proton nuclear magnetic resonance (^{1}H NMR)

^{1}H NMR spectra of copolymers were recorded on a Bruker Ultrashield spectrometer operating at 600 MHz in $CDCl_3$ with TMS as reference or in D_2O referred to signal at 4.64 ppm from water protons.

Fourier transform infrared spectroscopy (FTIR)

The infrared spectra of copolymers were obtained using a Nicolet FTIR 6700 spectrometer working in transmission mode. The infrared spectra were recorded for copolymer films deposited on a KBr crystal. All spectra were acquired between 4000 and 450 cm^{-1} using 64 scan summations and a 4 cm^{-1} resolution. The spectra were evaluated using OMNIC™ software.

Gel permeation chromatography

The molar masses and molar mass dispersities of the copolymers were determined using gel permeation chromatography (GPC-MALLS) with a Dn-2010 RI differential refractive index detector (WGE Dr. Bures) and a DAWN EOS multiangle laser light scattering detector (Wyatt Technologies). GPC was performed in DMF at 45 °C at a nominal flow rate of 1 mL/min using a set of columns: 100 Å, 1000 Å, 3000 Å (Polymer Standard Service). The results were evaluated using ASTRA 5 software (Wyatt Technologies). The refractive index increments of P(D-co-A)_1 (dn/dc=0.082 mL/g) and P(D-co-A)_2 (dn/dc=0.055 mL/g) were independently measured in DMF using a SEC-3010 differential refractive index detector (WGE Dr. Bures) and used to calculate the average molar masses of the copolymers.

Cloud point measurement

The cloud points of the (co)polymers were determined on a Jasco V-530 UV–vis spectrophotometer with a cuvette thermostatted by a Medson MTC-P1 Peltier thermocontroller. The transmittances of the 0.1, 0.2, 0.5 and 1 g/L copolymer solutions were monitored at λ=550 nm as a function of temperature. The cloud points refer to the inflection points of the transmittance curves.

Dynamic light scattering

DLS measurements were performed on a Brookhaven BI-200 goniometer with vertically polarized incident light of wavelength λ=632.8 nm supplied by a He-Ne laser operating at 35 mW and equipped with a Brookhaven BI-9000 AT digital autocorrelator. The scattered light was measured for aqueous copolymer solutions at concentrations of 0.1, 0.2, 0.5, and 1 g/L at an angle of 90°. The autocorrelation functions were analyzed using the constrained regularized CONTIN method to obtain distributions of relaxation rates (Γ). The latter provided distributions of the apparent diffusion coefficient ($D=\Gamma/q^2$), where q is the magnitude of the scattering vector, $q=(4\pi n/\lambda)\sin(\theta/2)$ and n is the refractive index of the medium. The apparent hydrodynamic radius (R_h^{90}) was obtained from the Stokes-Einstein equation (1)

$$R_h^{90} = \frac{kT}{6\pi\eta D} \quad (1)$$

for $\theta=90°$ where k is the Boltzmann constant, η is the viscosity of water at temperature T, and D is the apparent diffusion coefficient. The dispersity of particle sizes was given as $\mu_2/\bar{\Gamma}^2$, where $\bar{\Gamma}$ is the average relaxation rate and μ_2 is its second moment. The cloud point temperatures refer to the inflection points of dependence of the R_h^{90} versus temperature.

Atomic force microscopy

Atomic Force Microscopy (AFM) was applied to visualize obtained nanoparticles. The nanoparticles for AFM analysis were prepared by dropping 10 µL of nanoparticle solution in water on a glass slide and spin coating through 1 h with a rotation speed of 400 rmp/min. The measurements were performed using Multi-Mode with a NanoScope 3D controller (di-Veeco Instruments Inc, USA, CA), which was operated in tapping mode in air with standard 125 mm single-crystal silicon cantilevers (Model TESP, Veeco Instruments Inc., USA). Images 2 µm × 2 µm were obtained using a piezoelectric scanner. Micrographs were recorded using NanoScope Software V531r1 and the most representative images for each sample were selected from three measurements at different surface points.

Results and discussion

Synthesis and characterization of copolymers with alkyne and azide functions

Atom transfer radical polymerization (ATRP) of di(ethylene glycol) methyl ether methacrylate (D) and 2-aminoethyl methacrylate (A) (Fig. 1a) was performed according to the method described by Feng et al.[30] for polymerization of oligo(ethylene glycol)$_{300}$ methacrylate. Two copolymers, P(D-co-A)_1 (D/A, 50/50, mol/mol) and P(D-co-A)_2 (D/A, 285/15, mol/mol), were obtained.

The P(D-co-A)_2 copolymer with the lower A content was chosen for further modifications. The amine groups of P(D-co-A)_2 were reacted with propargyl chloroformate, which led to prop-2-ynyl groups with a terminal alkyne (Fig. 1b), or with 2-azido-1,3-dimethylimidazolinium hexafluorophosphate, which led to azide (Fig. 1c) functions in the copolymer chains.

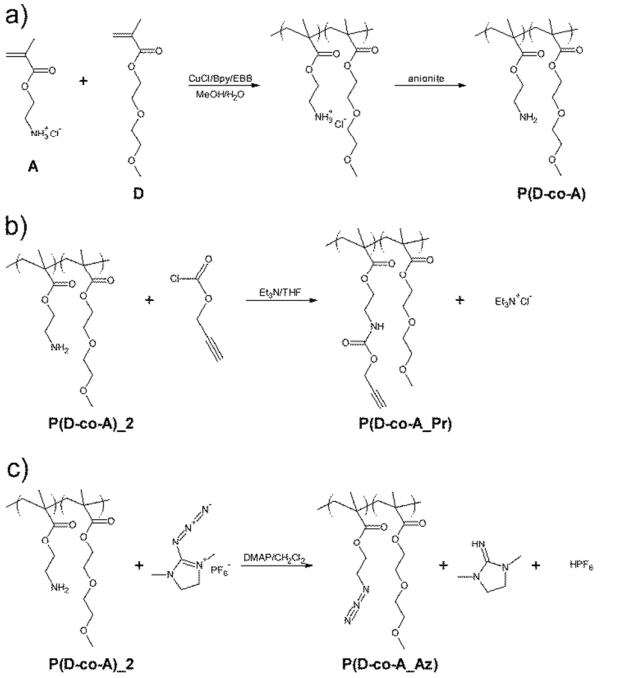

Fig. 1 Schemes of (a) synthesis of P(D-co-A) copolymers by ATRP, (b) reaction of P(D-co-A)_2 with propargyl chloroformate and (c) reaction of P(D-co-A)_2 with 2-azido-1,3-dimethylimidazolinium hexafluorophosphate.

The molar masses and molar mass dispersities of the copolymers were measured using gel permeation chromatography with multiangle light scattering detection (GPC-MALLS). In both cases the chromatograms were monomodal and narrow (Fig. S1, ESI†), and molar mass dispersities (M_w/M_n) were less than 1.2. The number average molar masses were 11 000 and 42 000 g/mol for P(D-co-A)_1 and P(D-co-A)_2, respectively.

In ^{1}H NMR spectra of the copolymers (Fig. 2) signals coming from D units (a-f) as well as from A units (a`, b`, g`, f`) were observed, confirming the structures of the chains.

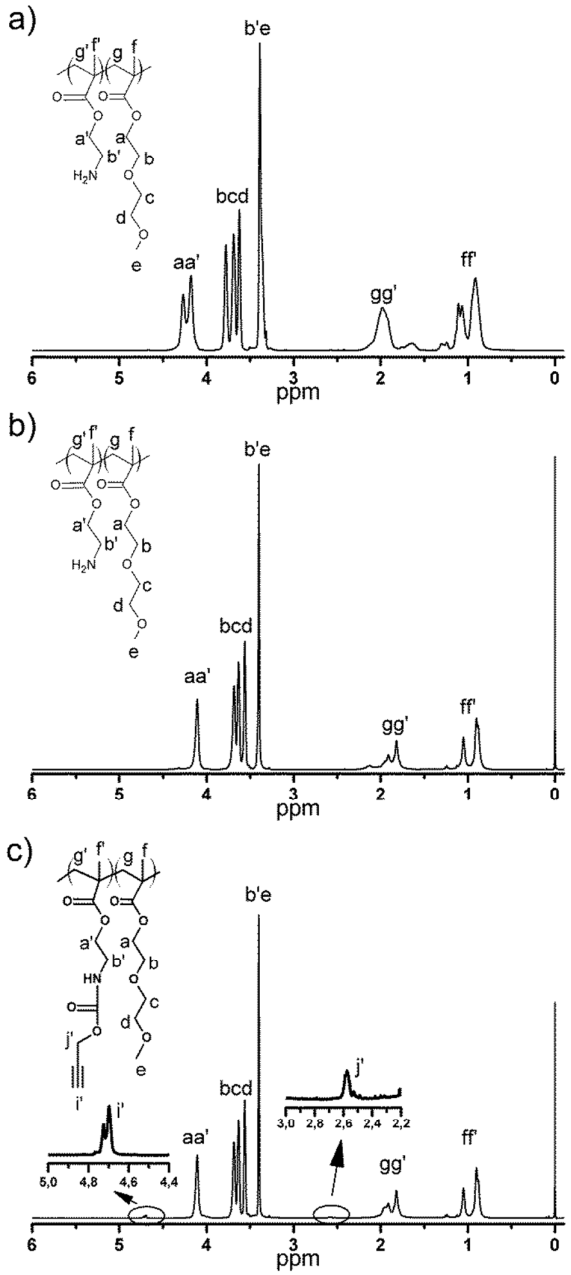

Fig. 2 ^{1}H NMR spectra of (a) P(D-co-A)_1, (b) P(D-co-A)_2 and (c) P(D-co-A_Pr) (600 MHz; a in D_2O and b, c in $CDCl_3$).

The compositions of the copolymers P(D-co-A)_1 and P(D-co-A)_2 were determined from the ratio of peak integrals to calculate the quantity of A using formula (2):

$$\boldsymbol{A}[\%\boldsymbol{mol}] = \left[\frac{I_{aa'} - \frac{I_{bcd}}{3}}{I_{aa'}}\right] \cdot \mathbf{100}\% \qquad (2)$$

where: $I_{aa'}$ is the peak area of signals aa` (δ=4.0 – 4.5 ppm) and I_{bcd} is the peak area of signals bcd (δ=3.5 – 4.0 ppm) present in ^{1}H NMR spectra of P(D-co-A)_1 and P(D-co-A)_2 (Fig. 2a, 2b). The calculated content of A was equal to 45% mol for P(D-co-A)_1 and 7% mol for P(D-co-A)_2. In the ^{1}H NMR spectra of P(D-co-A_Pr) (Fig. 2c) the ≡CH- (j`, δ=2.6 ppm ) and -$CH_2$- signals (i`, δ=4.7 ppm) characteristic of prop-2-ynyl carbamate groups appeared, indicating the presence of these groups in copolymer chains. In the case of P(D-co-A_Az) signals coming from -CH_2- (δ=3.5 ppm) next to azide groups were not visible because they were overlapped by high intensity signals bcd coming from oligo(ethylene glycol) side chains.

FTIR analyses delivered additional information about the chemical structure of the copolymers (Fig. 3). In all spectra characteristic signals appeared from C-O (1245 cm^{-1}) and C=O (1724 cm^{-1}) of the methacrylate ester, C-O (1103 cm^{-1}) of the ether, –C-C- (745 cm^{-1}), C-H stretching (2700–3000 cm^{-1}) and C-H scissoring (1384 and 1448 cm^{-1}) of the -CH_3 and -CH_2- of the methacrylic backbone. For P(D-co-A_Pr) (Fig. 3b) additional signals from the alkyne group -C≡CH (2125 and 3100 cm^{-1}) were observed whereas for P(D-co-A_Az) (Fig. 3c) characteristic signal from an azide group at 2105 cm^{-1} indicated successful modification of P(D-co-A)_2.

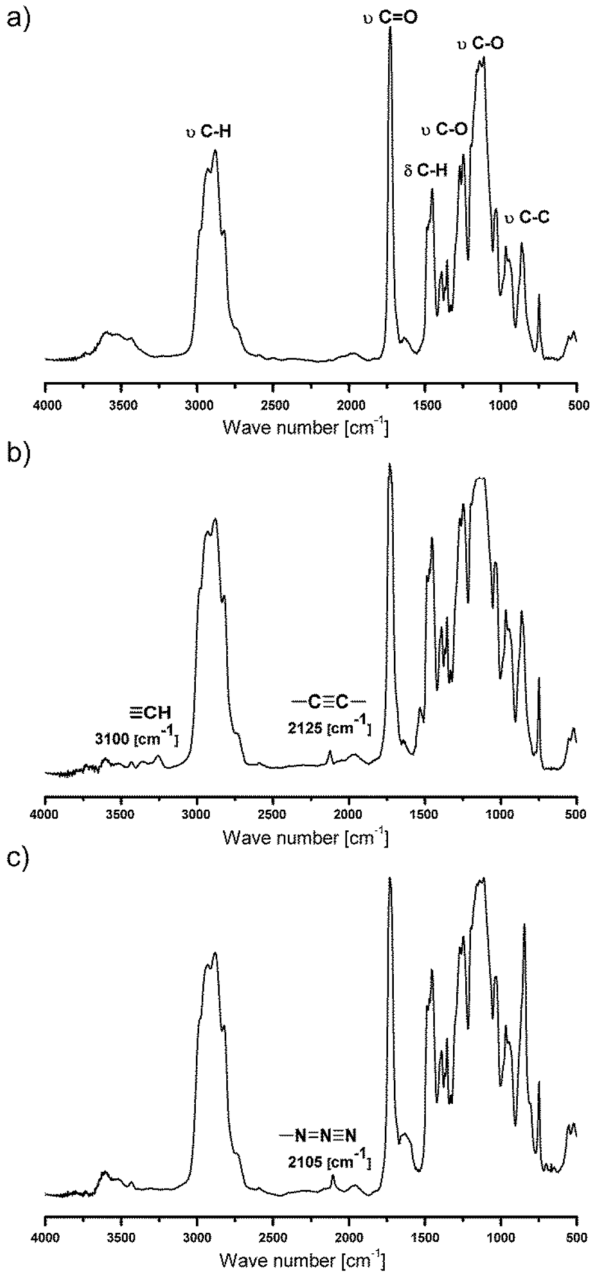

Fig. 3 FTIR spectra of (a) P(D-co-A)_2, (b) P(D-co-A_Pr) and (c) P(D-co-A_Az).

The quantity of A in the copolymers was also determined using ninhydrin reaction (Kaiser test).[33] The absorbance of post-reaction solutions for P(D-co-A)_1, P(D-co-A)_2, P(D-co-A_Pr) and P(D-co-A_Az) measured by UV-vis (Fig. S2, ESI†) were used to calculate the content of free amine groups based on formula:

$$\boldsymbol{A}\,[\%\,\boldsymbol{mol}] = \frac{\boldsymbol{a}\cdot\boldsymbol{V}\cdot\boldsymbol{M_n}\cdot\boldsymbol{DP_n}}{\boldsymbol{\varepsilon}\cdot\boldsymbol{l}\cdot\boldsymbol{m}}\cdot\mathbf{100}\% \qquad (3)$$

where: a - average absorbance, V [dm^3] - sample volume, M_n [g/mol], - polymer molar mass, DP_n- degree of polymerization, ε=5770 [dm^3/(mol·cm)] - extinction coefficient determined in separate experiment, l [cm] - the width of cuvette, m [g] - sample weight.

The results of the Kaiser test indicated 50% mol A in P(D-co-A)_1 and 8% mol A in P(D-co-A)_2 copolymers. The obtained values are consistent with values obtained from NMR and the assumed theoretical composition of copolymers. For further calculation value of 8% mol of 2-aminoethyl methacrylate in P(D-co-A)_2 was used. In the case of P(D-co-A_Pr) and P(D-co-A_Az) no absorption at 590 nm was observed, proving quantitative conversion of amine to azide or prop-2-ynyl carbamate groups.

The NMR, FTIR and Kaiser test confirmed the expected copolymer structure. The introduction of azides and prop-2-ynyl carbamate groups into the polymer chain led to copolymers capable of "click" reaction.

Thermoresponsiveness of copolymers and their aggregation in water solution

Poly(di(ethylene glycol) methyl ether methacrylate) (PD) belongs to the group of thermoresponsive polymers.[19, 35] It is well known that the addition of hydrophilic moieties to thermoresponsive polymer chains causes an increase of their phase transition temperature while hydrophobic moieties shift it to lower values.[19, 35-37]

As shown in the transmittance curves (Fig. 4a), the presence of 8% hydrophilic 2-aminoethyl methacrylate groups in P(D-co-A)_2 chain shifted the T_{CP} of the copolymer to 37 °C i.e. 10 °C higher compared to PD of similar molar mass (T_{CP}=27 °C). The modification of the amine groups of P(D-co-A)_2 to prop-2-ynyl carbamate P(D-co-A_Pr) and azide P(D-co-A_Az), with less affinity for water than the initial amines, resulted in a decrease in T_{CP}. The prop-2-ynyl carbamate groups shifted T_{CP} to 19.5°C whereas azide groups moved it to 33 °C.

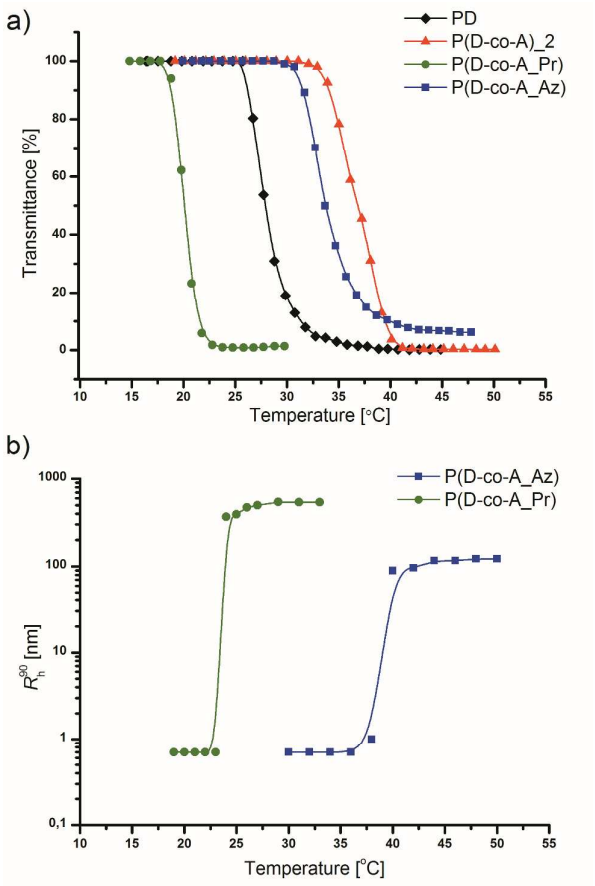

Fig. 4 (a) Transmittance versus temperature of PD, P(D-co-A)_2, P(D-co-A_Pr), and P(D-co-A_Az) in water at concentration of 1 g/L, (b) The apparent hydrodynamic radius (R_h^{90}) of particles as a function of temperature for water solutions of P(D-co-A_Pr) and P(D-co-A_Az) at a concentration of 0.2 g/L.

As is observed for other thermoresponsive polymers, the T_{CP} of P(D-co-A_Pr) and P(D-co-A_Az) decreased with increasing solution concentration (Table 2). Besides T_{CP} determined from transmittance curves, Table 2 contains the respective values obtained from DLS when measuring the hydrodynamic radii of particles in solution during their gradual heating (Fig. 4b).

Table 2 Cloud points temperatures for water solutions of P(D-co-A_Az) and (P(D-co-A_Pr) at different concentrations

	T_{CP} [°C]							
	c=0.1 g/L		c=0.2 g/L		c=0.5 g/L		c=1.0 g/L	
Polymer	UV-vis	DLS	UV-vis	DLS	UV-vis	DLS	UV-vis	DLS
P(D-co-A_Pr)	24	25	22.5	23	21	20	19.5	18
P(D-co-A_Az)	42	38	40	36	38	34	33	31

As discussed in the introduction, in dilute water solution thermoresponsive polymers aggregate above the phase transition temperature, forming spherical particles called mesoglobules[38, 39] of sizes determined by the solution concentration, the molar mass and composition of the (co)polymer and the heating rate of the solution.[19, 38-42]

Here two heating procedures were explored - gradual and abrupt (see experimental). The changes in apparent hydrodynamic radii R_h^{90} of particles formed by P(D-co-A_Pr) and P(D-co-A_Az) in 0.2 g/L solutions subjected to gradual heating are shown in Figure 4b. Non-associated individual chains of ca. 1 nm radius aggregated at the T_{CP} in a narrow temperature range (1-3 °C), to mesoglobules of sizes dependent of copolymer composition. The sizes of nanoparticles were stable above the phase transition.

The copolymer solutions were also heated abruptly. At the same concentration abrupt heating led to the formation of smaller mesoglobules than gradual heating. The dispersity of particles' sizes were between 0.15 and 0.30 for the mesoglobules obtained by gradual heating, whereas mesoglobules obtained using an abrupt heating method have considerably smaller dispersities between 0.005 and 0.20. The dispersity of particle sizes was given as $\mu_2/\bar{\Gamma}^2$.

The values of R_h^{90} for P(D-co-A_Pr) and P(D-co-A_Az) mesoglobules obtained in both heating procedures at different solution concentrations (0.1, 0.2, 0.5 and 1 g/L) are compared in Table 3.

Table 3 Apparent hydrodynamic radii R_h^{90} of mesoglobules formed by P(D-co-A_Pr) and P(D-co-A_Az) at different solution concentrations obtained by gradual and abrupt heating

	R_h^{90} [nm]							
	Gradual heating				Abrupt heating			
Polymer	c=0.1 [g/L]	c=0.2 [g/L]	c=0.5 [g/L]	c=1.0 [g/L]	c=0.1 [g/L]	c=0.2 [g/L]	c=0.5 [g/L]	c=1.0 [g/L]
P(D-co-A_Pr)	330	550	620	840	80	100	110	160
P(D-co-A_Az)	130	170	215	330	20	50	60	100

The results contained in Table 2 confirmed the dependence of the mesoglobules' sizes on concentration and the heating rate of the polymer solution. Regardless of the applied heating method the radii of the mesoglobules of both P(D-co-A_Pr) and P(D-co-A_Az) were larger at the higher copolymer concentrations. For both copolymers abrupt heating resulted in the formation of significantly smaller mesoglobules than gradual heating. These dependencies have also been observed for other thermoresponsive polymers.[17, 22, 38]

Mixtures of copolymers - thermoresponsiveness and aggregation

In order to obtain well-defined mesoglobules composed of both copolymers, necessary for further crosslinking, it was essential to investigate the aggregation process in solution of mixed copolymers and to determine the structure of the resulting mesoglobules.

The transmittance (Fig. 5a) and R_h^{90} values (Fig. 5b) reflected behavior of P(D-co-A_Pr)/P(D-co-A_Az) mixture (1:1 weight ratio) at different total concentrations in water under gradual heating.

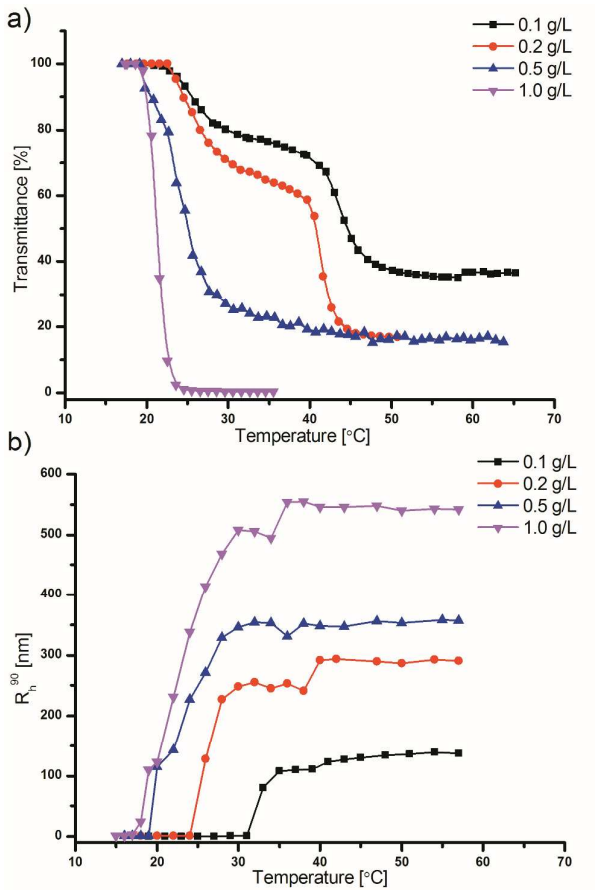

Fig. 5 a) Transmittance and b) apparent hydrodynamic radius (R_h^{90}) of particles versus temperature of P(D-co-A_Pr)/P(D-co-A_Az) at a 1:1 weight ratio for 0.1, 0.2, 0.5 and 1.0 g/L total concentrations.

In the diagram (Fig. 5a) two phase transitions appeared for 0.1 and 0.2 g/L solutions of the copolymers. They occurred at T_{CP}s characteristic for individual components (Tab. 2). At higher solution concentration 0.5 and 1 g/L only one phase transition at the T_{CP} corresponding to the T_{CP} of P(D-co-A_Pr) was observed. For 1 g/L the second phase transition could not be visible due to the competing decrease in transmittance after the phase transition of the P(D-co-A_Pr) copolymer.

In the R_h^{90} vs. temperature diagram two phase transitions were noticeable also for higher concentrations (Fig. 5b). The observed alterations between the results obtained from UV-vis and DLS are caused by experimental limitations because the

heating rate for the two techniques differs. However, both experiments provided complementary information about aggregation processes.

The values of R_h^{90} for mixed mesoglobules composed of P(D-co-A_Pr) and P(D-co-A_Az) obtained by gradual and abrupt heating at different total solution concentrations (0.1, 0.2, 0.5 and 1 g/L) are compared in Table 4.

Table 4 Sizes of mesoglobules formed by gradual and abrupt heating methods in P(D-co-A_Pr)/P(D-co-A_Az) mixtures at different total concentration

Concentration [g/L]	R_h^{90} [nm]	
	Gradual heating	Abrupt heating
0.1	130	60
0.2	260	70
0.5	350	80
1.0	520	85

It should be emphasized here that in both heating procedures only one population of particles was formed in solutions of copolymer mixture (Fig. 6).

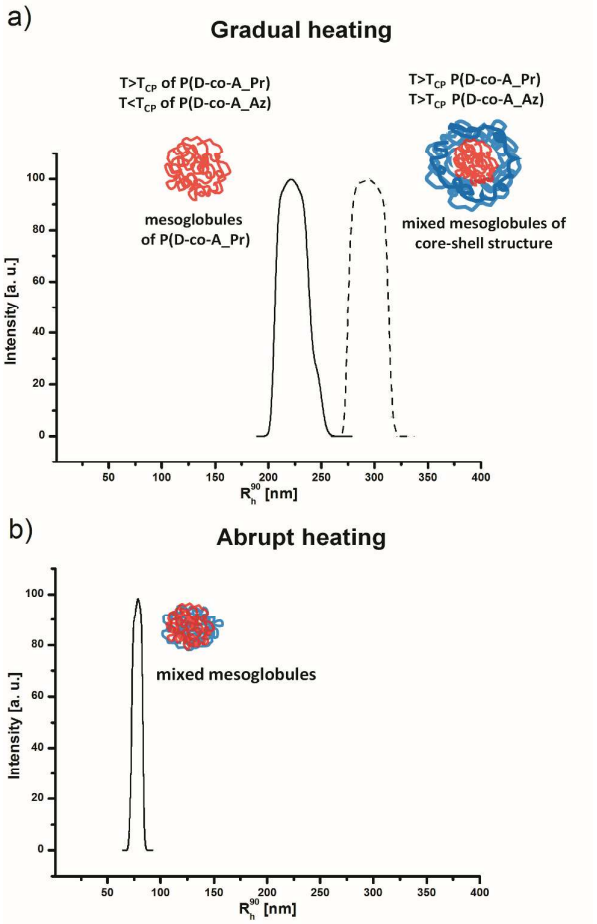

Fig. 6 The size distributions of the mesoglobules formed in aqueous solutions of P(D-co-A_Pr)/P(D-co-A_Az) (1:1) at total concentration 0.2 g/L by (a) gradual and (b) abrupt heating.

Similarly as was observed for solutions of individual copolymers, the abrupt heating led to significantly smaller particles and a narrower size distribution than gradual heating.

DLS and UV-vis proved that during gradual heating of P(D-co-A_Pr)/P(D-co-A_Az) solutions at temperatures exceeding the T_{CP} of P(D-co-A_Pr), the chains of these copolymer aggregated. During further heating the size of the obtained nanoparticles was maintained until the T_{CP} of the second copolymer P(D-co-A_Az) was reached (Fig. 6a). Above this temperature the R_h^{90} of the nanoparticles increased rapidly, and still one population of particles was present in solution. This indicated that above the T_{CP} of P(D-co-A_Pr), but below the T_{CP} of P(D-co-A_Az), the formed mesoglobules are composed mainly of a single copolymer - P(D-co-A_Pr). Further heating caused the nucleation of the P(D-co-A_Az) chains on mesoglobules of P(D-co-A_Pr) or collapse of these P(D-co-A_Az) chains, which were entangled in P(D-co-A_Pr) particles. This indicate that mesoglobules with a core-shell structure are formed.

Unlike in gradual heating, during abrupt heating the formation of mesoglobules happens in one step (Fig. 6b). By shock heating, a mixed solution of P(D-co-A_Pr) and P(D-co-A_Az) is taken to a temperature that is well above the T_{CP}s of the individual copolymers, and the formed mesoglobules are composed of both copolymers and their structure is expected to be homogeneous.

Recently, we described similar behavior [43] for a mixture of poly(N-isopropylacrylamide) and poly(2-isopropyl-2-oxazoline). Depending on the heating procedure which involved abrupt or gradual heating, the obtained mixed mesoglobules were homogenous or had a core-shell structure, respectively.

In order to prepare crosslinked nanoparticles only an abrupt heating method leading to mesoglobules of homogenous structure composed of both copolymer chains P(D-co-A_Pr) and P(D-co-A_Az) was adopted.

Crosslinking of mesoglobules

Stabilization of the structure of homogenous mesoglobules of P(D-co-A_Pr) and P(D-co-A_Az) containing azide and alkyne groups in the copolymer chains, can be achieved by their crosslinking using copper catalyzed Huisgen 1,3-dipolar cycloaddition. This reaction proceeds under mild conditions in aqueous solution with high selectivity and efficiency [44, 45] which makes it very attractive for the stabilization of polymeric nanoparticles.

The mixed mesoglobules formed using abrupt heating of a P(D-co-A_Pr)/P(D-co-A_Az) solution at a concentration of 0.2 g/L were crosslinked at 55 °C using different amounts of catalyst to establish the optimal reaction conditions (samples denoted as N_1_0.2 - N_5_0.2). For all samples, prior to the crosslinking reaction, the initial hydrodynamic radius of the mesoglobules was approximately 70 nm. After crosslinking the R_h^{90} of the obtained nanoparticles were measured at 55 and 18 °C i. e. above and below the T_{CP} of both copolymers (Table 5).

Table 5 Parameters of nanoparticles obtained by crosslinking of mixed mesoglobules P(D-co-A_Pr)/P(D-co-A_Az) (abrupt heating, 0.2 g/L) using different amounts of catalyst

Sample	N_3/Cu mol/mol	R_h^{90} [nm] before reaction 55 °C	R_h^{90} [nm] after reaction 55 °C	R_h^{90} [nm] after reaction 18 °C	Swelling ratio V_{18}/V_{55}
N_1_0.2	1:20	70	67	81	1.77
N_2_0.2	1:2	70	68	101	3.28
N_3_0.2	1:0.2	70	67	1-30*	-
N_4_0.2	1:0.02	70	66	1-10*	-
N_5_0.2	no Cu	70	69	1**	-

*several populations of particles, ** dissolved individual chains

Samples N_1_0.2 and N_2_0.2, crosslinked using a N_3/Cu ratio above 1:2, retained their integrity; even cooled below the T_{CP} of P(D-co-A_Pr), they merely swelled. Decreasing the copper concentration (N_3_0.2 and N_4_0.2) made the crosslinking incomplete, and the nanoparticles disintegrated when cooled to unimers or poorly defined small aggregates. When no crosslinking occurred (N_5_0.2) the particles dissolved completely when cooled below the T_{CP} of P(D-co-A_Pr) (18 °C).
Crosslinking reactions with the use of an optimal amount of catalyst (1:2 molar ratio N_3/Cu) were also performed for mixed mesoglobules formed using abrupt heating of P(D-co-A_Pr)/P(D-co-A_Az) at different total concentrations of 0.1, 0.2, 0.5, 1.0 g/L (Table 6).

Table 6 Parameters of nanoparticles obtained by crosslinking of mixed mesoglobules P(D-co-A_Pr)/P(D-co-A_Az) (abrupt heating, 0.1, 0.2, 0.5 and 1.0 g/L) using a 1:2 molar ratio of N_3/Cu catalyst

Sample	Concentration [g/L]	R_h^{90} [nm] before reaction 55 °C	R_h^{90} [nm] after reaction 55 °C	R_h^{90} [nm] after reaction 18 °C	Swelling ratio V_{18}/V_{55}
N_2_0.1	0.1	59	55	111	8.22
N_2_0.2	0.2	70	68	101	3.28
N_2_0.5	0.5	86	88	118	2.41
N_2_1	1	87	91	102	1.41

After crosslinking at the temperature 55 °C nanoparticles with R_h^{90} close to the initial value were observed. Measurements below the T_{CP} of P(D-co-A_Pr) (18°C) showed that a single population of stable nanoparticles with increased R_h^{90} were present in solution for all samples. By changing the total concentration of P(D-co-A_Pr)/P(D-co-A_Az) mixture it was possible to obtain crosslinked nanoparticles of different sizes.
AFM images confirmed the results obtained by DLS (Fig. 7). The radii of N_2_0.2 nanoparticles dried on the surface are around 110 nm, which is in good agreement with the DLS results.

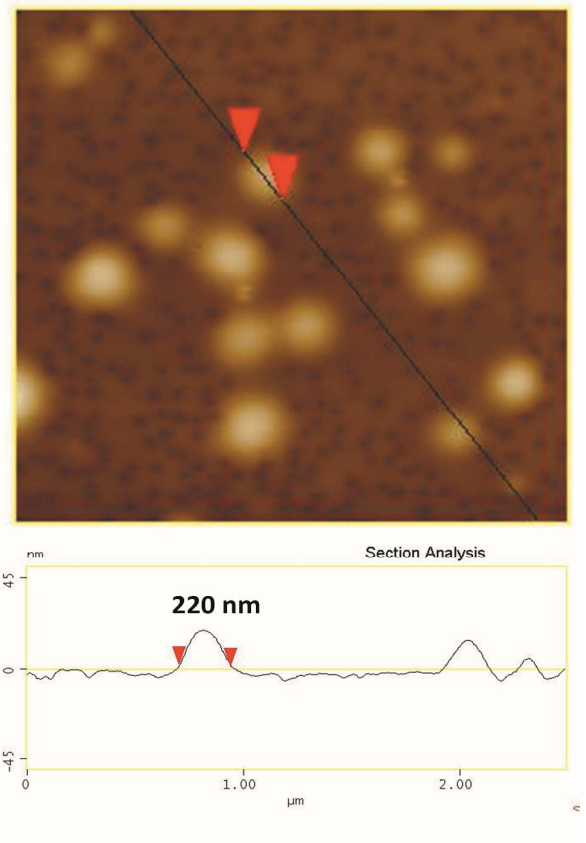

Fig. 7 An AFM image (2 µm×2µm) and its cross section for crosslinked nanoparticles (sample N_2_0.2).

Assuming a spherical morphology, which was proven by AFM, the hydrodynamic volumes of the particles at 55 and 18 °C (V_{55} and V_{18}, respectively) as well as the swelling ratio V_{18}/V_{55} were calculated (Table 5 and Table 6).
The crosslinked nanoparticles retained their stability when subjected to heating/cooling cycles. As an example, the variation in R_h^{90} of N_2_0.2 particles with abrupt temperature changes is shown in Fig. 8. These reproducible results indicate the full reversibility of the process.

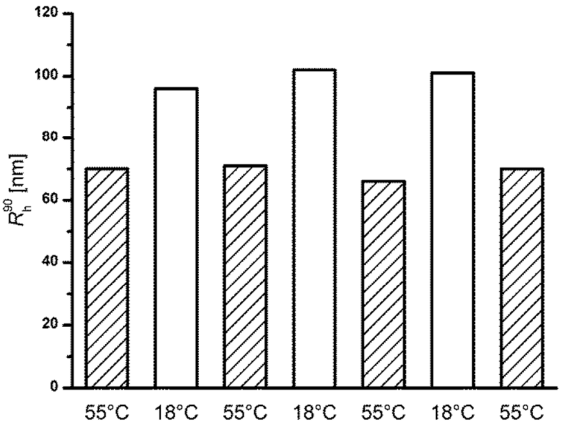

Fig. 8 R_h^{90} of N_2_0.2 nanoparticles during heating and cooling cycles (c=0.2 g/L).

Degradation of nanoparticles

It is known that carbamate bonds undergo hydrolysis at basic pH to an hydroxyl, a carbon dioxide, and an amine while under acidic conditions they are stable.[28, 29] Due to the presence of carbamate bonds in their structure the crosslinked nanoparticles may be expected to undergo disintegration under basic conditions (Fig. 9a).

We followed the degradation process of N_2_0.2 nanoparticles in buffered solutions at pH 2, 7 and 10 at 37 °C, measuring their sizes by DLS (Fig. 9b).

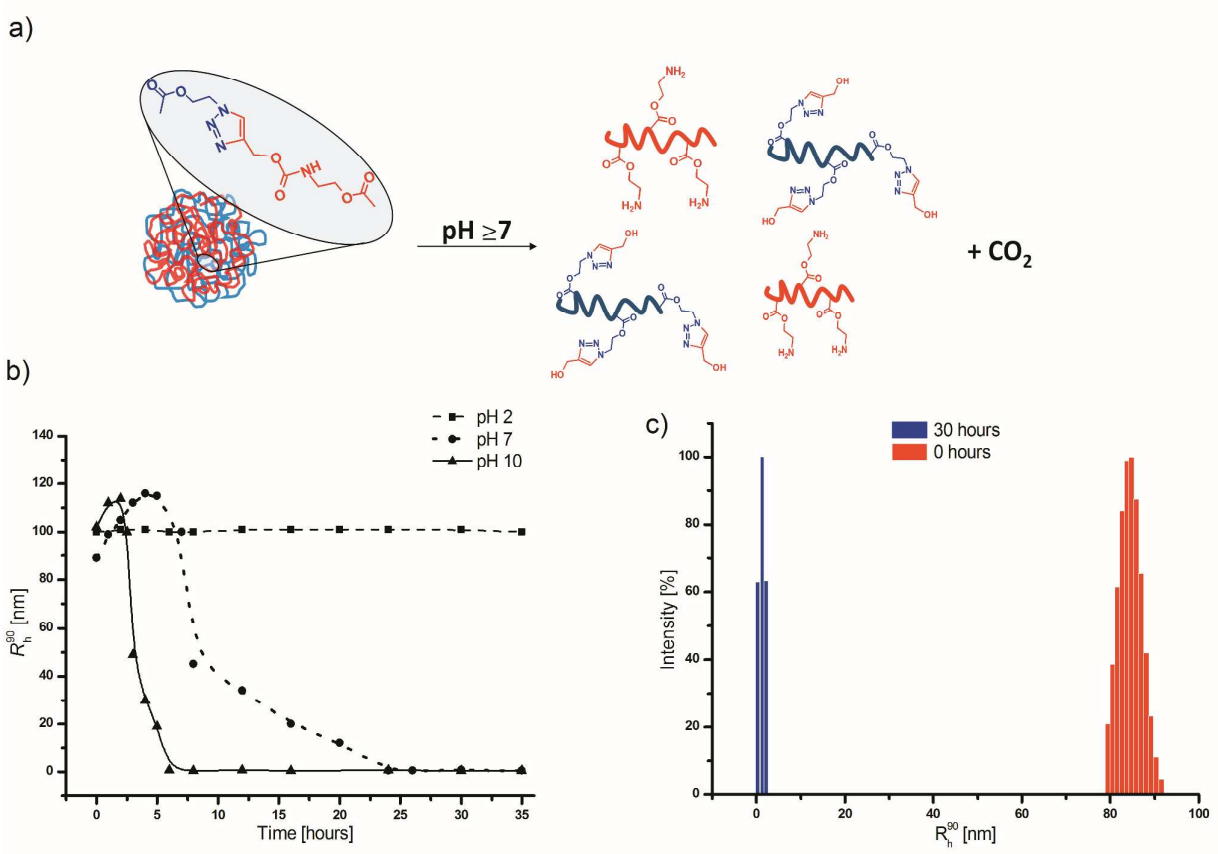

Fig. 9 (a) Scheme of the degradation of mesoglobules, (b) changes in N_2_0.2 nanoparticle sizes during degradation at different pH, (c) size distribution of N_2_0.2 before and after degradation at pH 10.

No degradation of nanoparticles was observed at pH 2. The sizes of the mesoglobules were stable over a month. At pH 7 and 10 rapid degradation occurred. The process began with swelling of the particles. In both buffers their radii increased by about 20 nm. Then a rapid decrease in the particles' sizes was observed. The size distribution of N_2_0.2 nanoparticles before and after degradation at pH 10 are shown in Figure 10c. At pH 10 degradation was faster than at pH 7. Complete disintegration of nanoparticles was observed after 6 hours at pH 10 and after 24 hours at pH 7. The nanoparticles degraded to a species of around 1 nm, the size characteristic of individual chains dissolved in solution.

Conclusions

Here we presented a new and convenient approach leading to degradable polymeric nanoparticles built from thermoresponsive copolymers of di(ethylene glycol) methyl ether methacrylate. The applied synthesis method with involved ATRP led to well-defined copolymers of di(ethylene glycol) methyl ether methacrylate and 2-aminoethyl methacrylate containing reactive amine groups that were quantitatively transferred to azides and prop-2-ynyl carbamate groups capable of "click" reactions. The obtained copolymers exhibited thermoresponsiveness at T_{CP}s differing by about 14 °C. In dilute solutions above their T_{CP} individual copolymers aggregated into mesoglobules. In mixed copolymer solutions, regardless of heating protocol, only a single population of mesoglobules was present. The size of mesoglobules can be controlled by changing the total concentration of mixed solutions. The heating method influences mesoglobules' structure. During gradual heating nanoparticles with a core-shell structure were formed. Abrupt heating caused simultaneous collapse of both copolymer chains and the

creation of mixed mesoglobules with a homogenous structure. Mesoglobules composed of both copolymer chains, containing azides and prop-2-ynyl carbamate groups, mixed homogenously could be easily crosslinked using copper catalyzed Huisgen 1,3–dipolar cycloaddition of azides and alkynes. The minimal amount of Cu sufficient for effective crosslinking was determined. The obtained nanoparticles were stable below the phase transition temperatures of the copolymers. In response to cyclic changes in temperature from below to above the phase transition temperature of the copolymers, the nanoparticles reversibly changed their sizes. The presence of carbamate bonds in the structure of the nanoparticles allowed for their degradation above pH≥7. The rate of degradation increased with the pH of the solution. The proposed procedure opens a route to new polymeric nanocarriers for controlled delivery and release of therapeutics.

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