TAILORING THERMORESPONSIVE MICROBEADS BY SYNTHESIS IN SUPERCRITICAL CARBON DIOXIDE

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Thermoresponsive poly(*N*-isopropylacrylamide) copolymers with poly(ethylene glycol) and poly(methacrylic acid) were prepared by dispersion polymerization in supercritical carbon dioxide. The effect of the co-monomer on the structural, morphological and thermal features of the copolymers was evaluated. Microbeads with well-defined morphology were obtained for both PNIPAAm-PEG and PNIPAAm-PMAA copolymers. Furthermore, PNIPAAm-PMAA microbeads were both responsive to temperature and pH and had a higher overall swelling degree than PNIPAAm-PEG, in agreement with a higher hydrophilic character of the PMAA polymer chains. Finally, citotoxicity assays were conducted on the samples, demonstrating the biocompatibility of the microbeads imperative for biomedical applications.

INTRODUCTION

Hydrogels are cross-linked networks of hydrophilic polymers characterized by their three-dimensional physical structure, high water content, biocompatibility and mechanical properties similar to natural biological tissues [1]. These properties have attracted a lot of research on the application of hydrogels in tissue engineering, biomedical implants, drug delivery systems and bionanotechnology [2]. Hydrogels can be in the form of macroscopic networks or confined to smaller dimensions such as microgels.

Poly (*N*-isopropylacrylamide), PNIPAAm, is a thermoresponsive polymer with a low critical solution temperature (LCST) between 30 °C and 32 °C in an aqueous solution, close to body temperature. It dissolves in water below the LCST and precipitates out of the aqueous solution above the LCST due to disruption of hydrogen bonds with water and increasing hydrophobic interactions among isopropyl groups. PNIPAAm hydrogels temperature response has been widely explored for diverse applications, namely drug delivery and cell sheet technology [3, 4].

Copolymerization of NIPAAm with hydrophilic monomers is a possible route to fine tune swelling degree and the thermal and mechanical features of the polymer. Poly(ethylene glycol) (PEG) is a biocompatible hydrophilic polymer that has been widely explored for different biomedical applications due to its resistance to protein adsorption and cell adhesion [5]. Conventional synthesis of PNIPAAm-PEG nanoparticles for drug delivery applications has already been reported in literature, in which the presence of PEG contributes for higher LCST and equilibrium swelling. In principle, that behavior facilitates the incorporation of drugs into the nanoparticles [6]. Polymethacrylic (PMAA) is a biocompatible weak polyelectrolyte. PNIPAAm and PMAA copolymers create systems that exhibit reversible swelling responses to both temperature and pH stimuli. Indeed nano-sized PNIPAAm-PMAA

microgels have been prepared through conventional synthesis and thoroughly studied in their ability to uptake and release different drugs [7].
Synthesis of PNIPAAm microgels in $scCO_2$ has already been reported in literature [8]. The use of supercritical carbon dioxide ($scCO_2$) as a polymerization medium for the preparation of microgels offers many advantages over conventional solvents: CO_2 is nontoxic, nonflammable, inexpensive and readily available in high purity from a variety of sources [9]. Since it is a gas at normal pressure by simply reducing the pressure of the system, it is possible to easily separate the solvent from the polymer, leading to highly pure materials ideal for biomedical applications [10].
Herein the preparation of PNIPAAm-PEG and PNIPAAm-PMAA copolymers by free-radical dispersion polymerization is described. The impact of the different co-monomers on the morphological properties of the hydrogels was evaluated with the main goal of obtaining defined microbeads.

MATERIALS AND METHODS

Materials

N-isopropylacrylamide (NIPAAm; 97% purity), di(ethyleneglycol) dimethacrylate (DEGDMA, 95% purity), poly(ethylene glycol) acrylate (PEGa, average $M_n \approx 375$), methacrylic acid (MMA, ≥ 98.0% purity), (2,2'-azobis(isobutyronitrile) (AIBN, 98% purity), phosphate buffer saline (PBS; 10mM pH 7.4), Accutase™, MTT formazan and MTT solvent (0.1 N HCl in anhydrous isopropanol) were purchased from Sigma-Aldrich. Acetic acid glacial (99.7% purity) was obtained from Panreac and sodium acetate trihydrate (99.5% purity) from Riedel-de Haën. Krytox 157 was purchased from DuPont. Carbon dioxide was obtained from Air Liquide with 99.998% purity. RPMI-1640 (a Roswell Park Memorial Institute medium), trypan blue and fetal bovine serum (FBS) used in cell culture were purchased from Invitrogen. L929 cells were obtained from DSMZ, Germany. All materials were used without any further purification. The deionized water was purified through a Milli-Q system.

Preparation of PNIPAAm-PEG and PNIPAAm-PMAA copolymers

Synthesis of cross-linked PNIPAAm copolymers was carried out on a high-pressure apparatus already described in the literature [8]. In summary, monomers, initiator, cross-linker and stabilizer (Krytox) were loaded into a high-pressure cell (33 mL volume), which was then sealed, purged with CO_2 and tested for leakage. Liquid CO_2 was added to the cell using a high-pressure compressor. The cell was immersed in a thermostatized water bath and additional CO_2 was added until the desired pressure was obtained.
All polymerization reactions were conducted at 65ºC and 28.0 MPa for 24 hours as shown in Figure 1. PEGa and MMA co-monomers were used at different relative molar compositions, as can be observed in Table 1. As the reaction progressed white particles began precipitating inside the cell. The obtained polymers were washed continuously with fresh CO_2 for 1 hour to remove stabilizer and any remaining residues of unreacted monomer or cross-linker, since all these reactants are soluble in CO_2 in those conditions. After venting the resulting polymer was a white, dry, free flowing powder.

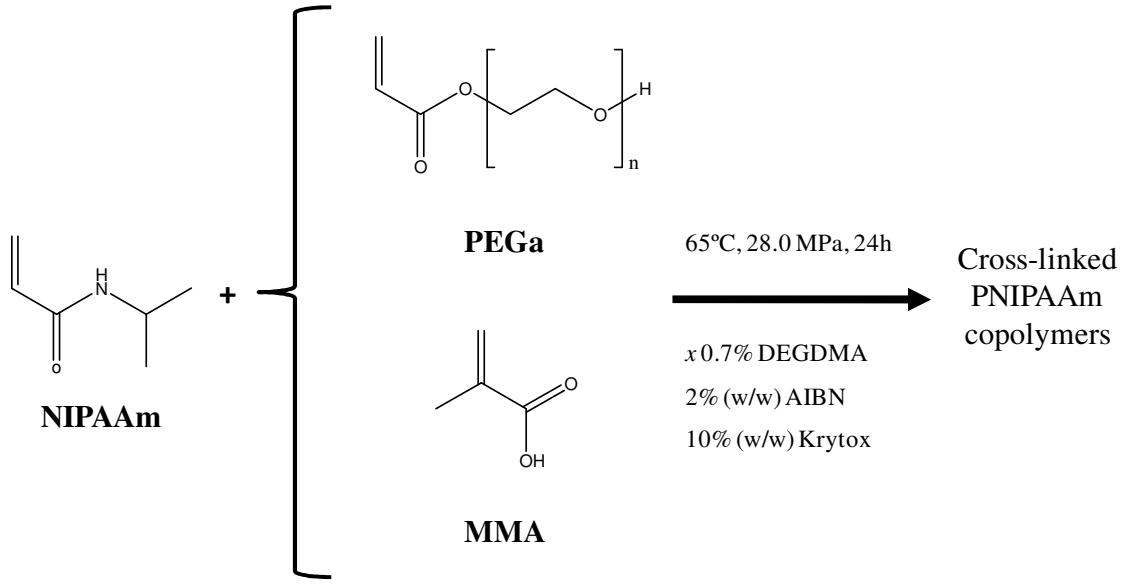

Figure 1: Synthesis of cross-linked PNIPAAm copolymer hydrogels in $scCO_2$.

Polymer structural and morphological characterization

Polymer morphogy was evaluated using scanning electron microscopy (SEM) in a JEOL JSM-7001F, with an accelerating voltage set to 15kV. All samples were gold coated before analysis. Bead size was determined by image analysis using Image J software (NIH). Synthesized cross-linked copolymers were insoluble in solvents usually used as eluents in GPS/SEC columns, thus making impossible to determine the molecular weight of the obtained samples. Polymers were analyzed using Fourier Transform Infrared Spectroscopy (FT-IR) with a Perkin Elmer Spectrum BX (64 scans). Polymer samples were partially solubilized in deuterated chloroform ($CDCl_3$) to perform nuclear magnetic ressonance (NMR) on a Bruker ARX 400 MHz spectrometer. ^{1}H and ^{19}F NMR spectra were recorded demonstrating the synthesis of PNIPAAm with high purity as no residual monomer or stabilizer were detected (data not shown). Elemental analysis was used to determine the molar composition of the cross-linked copolymers. Polymer yield was determined gravimetrically.

The thermal features of the hydrogels were investigated through differential scanning calorimetry (DSC), using a Setaram (Model DSC 131) instrument. The analyses were performed using approximately 10 mg of sample from 0 °C to 80 °C at 3 °C.min^{-1}, under a dry nitrogen atmosphere (20 mL.min^{-1} flow rate).

Calibration for the temperature and heat flow ranges was carried out using a pure indium standard.

Swelling measurements

Swelling degree (W(%)) of the synthesized copolymers was determined as referred in equation (1):

$$Swelling\ degree\ \big(W\ (\%)\big) = \frac{W_t - W_d}{W_d} \times 100 \qquad (1)$$

where W_d is the weight of the dry polymer and W_t is the weight after reaching equilibrium hydration (7 hours) in 10 mM PBS pH 7.4 buffer (for both copolymers) and in 10 mM acetate pH 4 buffer (for pH sensitive PNIPAAm-PMAA copolymers).

Dynamic Mechanical Behavior of PNIPAAm-PMAA microgels

PNIPAAm-PMAA 90:10 microgels were left in equilibrium with 10 mM PBS pH 7.4 buffer and 10 mM Acetate pH4 buffer overnight at 20 ºC. Oscillatory measurements were performed in a Bohlin Gemini HR^{nano} rotational rheometer at a frequency from 1 to 100 $rad.s^{-1}$ and 1% strain to determine the complex dynamic modulus G* at these conditions. G* consists of a real component, the storage modulus G', related to the elastic behavior of the material, and an imaginary component, the loss modulus G'' that translates the viscous component.

Citotoxicity assays

PNIPAAm copolymers were tested for cytotoxicity following the ISO 10993-5 guidelines. Briefly, triplicates of the polymers were placed in polystyrene tubes at a concentration of 10 mg/ml in RPMI – 1640 media with 10% (v/v) of FSB and kept in an incubator (37ºC, 5% CO2, fully humidified) for 1 day. The liquid extracts were diluted to 1 mg/ml and 0.1 mg/ml concentration and used to culture L929 mouse fibroblasts (initial density $10x10^4$ cells/mL) in 24-well plates for 36 hours. The cell metabolic activity was determined by analyzing the conversion of MTT (yellowish color) to its formazan derivative (purple – absorbance at 570 nm after a 3 hour incubation at 37ºC) using a MTT-based Cell growth determination kit. The results were normalized to the negative control for cytotoxicity (fresh RPMI medium).

RESULTS

Polymerization of PNIPAAm-PEG and PNIPAAm-PMAA in $scCO_2$

PNIPAAm hydrogels cross-linked with *N,N*-methylenebisacrylamide (MBAm) had already been prepared through precipitation polymerization in supercritical CO_2, yielding slightly aggregated particles [8]. In order to obtain larger particles, NIPAAm was copolymerized at a higher concentration (6.1% (w/v) to CO_2 ratio) than previously described, using a commercially available perfluoropolyether, Krytox, as a polymerization stabilizer. For PNIPAAm-PEG synthesis, polymerizations were performed a 5% molar fraction of PEGa, relative to NIPAAm. It was observed that the initial feed was not totally homogeneous at 65ºC and 28.0 MPa, which may have impaired polymerization yield and lead to a final molar composition different from the initial mixture (Table 1). After 24 hours a white, dry, free-flowing polymer was obtained.
PNIPAAm-PMAA copolymer was synthesized at a NIPAAm concentration of 3.8% (w/v) and a 10% molar fraction of MAA. In the beginning of the polymerization a homogenous mixture was observed. In agreement with that observation higher yields were obtained for PNIPAAm-PMAA copolymer than for PNIPAAm-PEG.

Table 1: Effect of the initial co-monomer fraction on the obtained PNIPAAm copolymers.

Polymer	*x* co-monomer[a]	Yield (%)	Diameter (μm)[b]	LCST (°C)[c]
PNIPAAm-PEG 95:5	3.6	69	2.70±0.37	33.2
PNIPAAm-PMAA 90:10	7.5	92	3.02±0.70	31.4

[a] as determined through elemental analysis
[b] as determined from image analysis of SEM micrographs: average diameter ± standard deviation for 100 particles.
[c] as determined from DSC analysis.

Effect of comonomer on PNIPAAm copolymers structural and morphological characteristics

The presence of PEG and PMAA in the composition of the respective copolymers was further confirmed in the FT-IR spectra (Figure 1).
PEG showed a band corresponding to the carbonyl stretch mode (υ(C=O) at 1720 cm^{-1}) while PNIPAAm was characterized by two amide bands (υ(C=O) at 1650 cm^{-1} and υ(N-H) at 1540 cm^{-1}). For the PNIPAAm-PMAA copolymer, PMMA exhibited a band corresponding also to the carbonyl stretch mode (υ(C=O) at 1722 cm^{-1}).

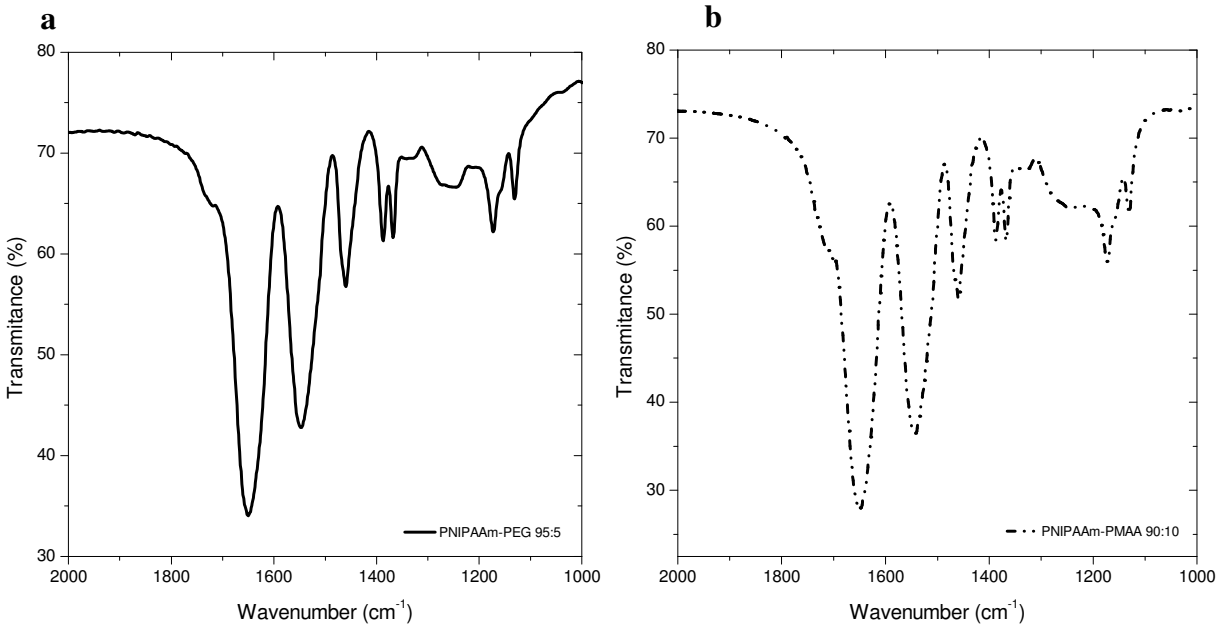

Figure 2: FT-IR spectra for (a) PNIPAAm-PEG 95:5and (b) PNIPAAm-PMAA 90:10 copolymers.

In regards to the copolymers morphology it was clear that the dispersion polymerization approach lead to the *in situ* formation of well defined PNIPAAm-PEG and PNIPAAm-PMAA microbeads, as shown in Figure 3.

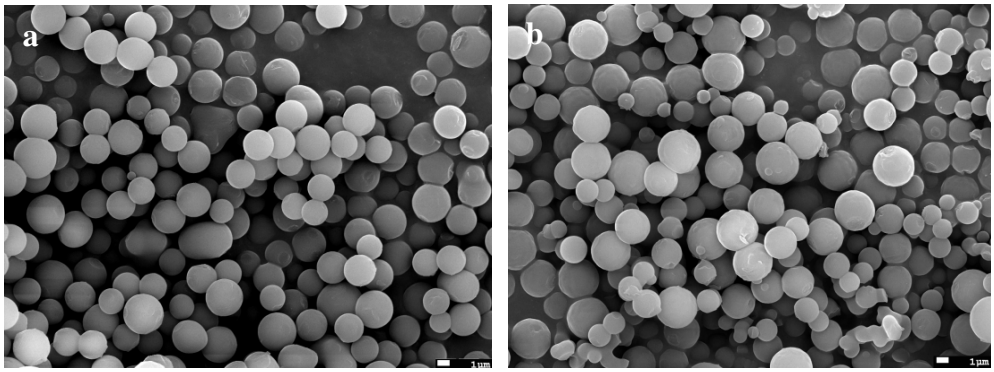

Figure 3: Scanning electron microscopy images of PNIPAAm-PEG hydrogels cross-linked with 0.74% DEGDMA: (a) PNIPAAm-PEG 95:5; (b) PNIPAAm-PMAA 90:10. Scale bar: 1μm.

The swelling behavior of the obtained thermoresponsive copolymers is shown in Figure 4. Below the LCST the polymer is able to uptake water due to H-bonding between water molecules and hydrophilic polymer chains. Water molecules become trapped inside the polymeric network leading to swelling. Above the LCST H-bonding is disrupted as hydrophobic interactions between the isopropyl groups of PNIPAAm become predominant and the polymer chains collapse, releasing water.

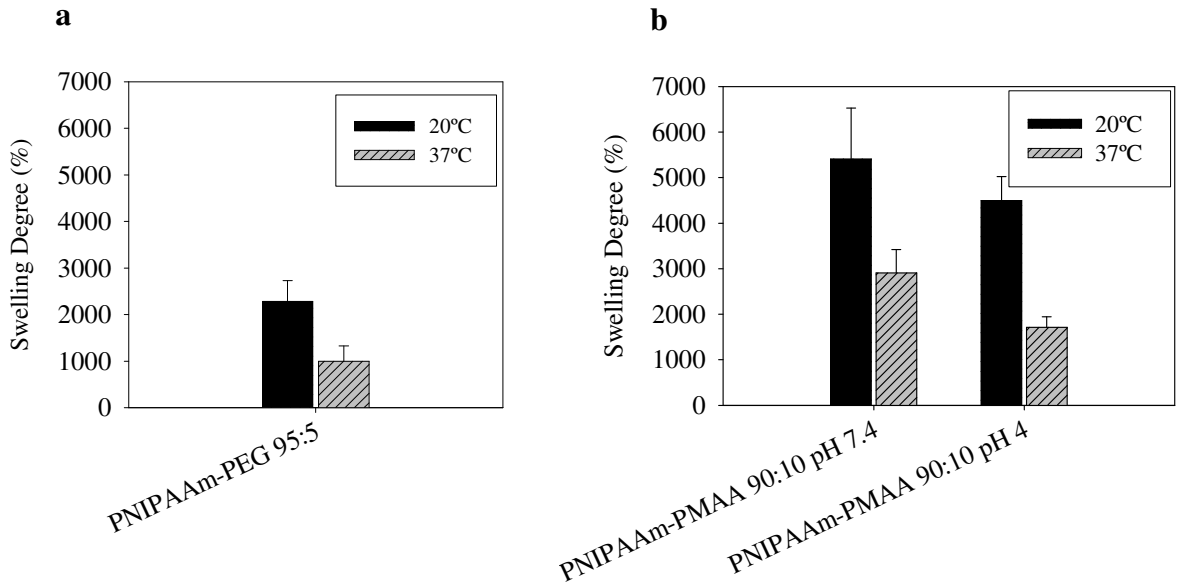

Figure 4: Equilibrium swelling at 20°C and 37°C for (a) PNIPAAm-PEG hydrogels in 10mM PBS buffer pH 7.4 and (b) PNIPAAm-PMAA 90:10 hydrogels in 10 mM PBS buffer pH 7.4 and 10 mM Acetate buffer pH 4.

Swelling of PNIPAAm-PMAA copolymers was higher than PNIPAAm-PEG in 10 mM PBS pH 7.4 buffer, demonstrating a higher hydrophilic character of PMAA. In fact the carboxylic groups of PMAA may establish stronger hydrogen bonds with water molecules than the ether group of PEG, enhancing water uptake, regardless of the lower LCST (as determined from DSC measurements) [11].

One interesting feature of the PNIPAAm-PMAA copolymer was its temperature and pH responsive behavior. In fact by comparing the swelling behavior in PBS pH 7.4 and in acetate

pH 4 buffers it was clear that swelling decreased at pH 4. Having in consideration that the pK_a of linear PMAA is between 5 and 6, the carboxylic groups of PMAA are fully protonated at pH 4, inducing a compact conformation possibly due to predominant hydrophobic interactions of PMAA methyl groups and/or intramolecular H-bonding between PMAA carboxyl group and PNIPAAm amide [11, 13]. On the other hand at a pH above the pK_a electrostatic repulsions between anionic groups enhanced swelling [11].

PNIPAAm-PMAA 90:10 microgels showed a similar rheological behavior in pH 7.4 and pH 4, as determined from oscillatory measurements in a rheometer (G^* = 63.9 Pa to 243.8 Pa at pH 4 and G^* = 75.3 Pa to 230.1 Pa at pH 7.4). Apparently, the difference in water uptake at different pH did not have an impact in terms of the microgel mechanical properties.

Biocompatibility of PNIPAAm-PEG and PNIPAAm-PMAA microbeads

The use of the synthesized microbeads in biomedical applications requires a citotoxicity evaluation to assess the interactions between cells and the material. Figure 5 presents the metabolic activity of L929 fibroblast cells (normalized to control) after 36 h of culture with liquid extracts of the copolymers. No considerable cytotoxic effect is observed for any polymer nor any trend in terms of the concentrations of polymers extracts present in the media. Therefore these copolymers may potentially be used in biomedical settings.

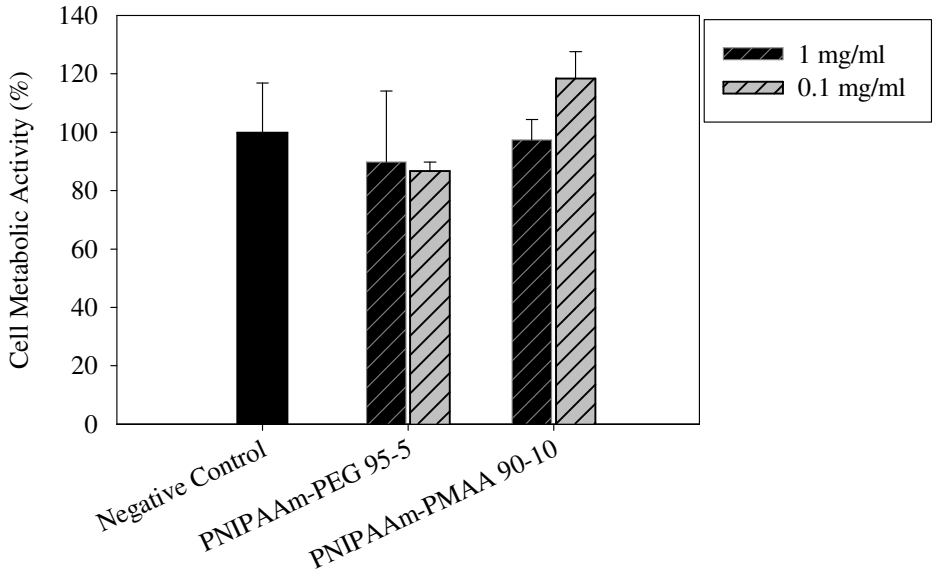

Figure 7: Citotoxicity tests of PNIPAAm-PEG and PNIPAAm-PMAA microgels according to the ISO standards for biomaterials. Cell metabolic activity is normalized to the negative control (tissue culture plate control).

CONCLUSION

Biocompatible PNIPAAm copolymers were synthesized in supercritical carbon dioxide yielding well-defined microbeads. By choosing different compositions in monomers, the morphology, thermal response and swelling degree of the obtained microbeads was tuned. The successful copolymerization of PNIPAAm-PMAA yielded temperature and pH responsive beads with an interesting potential for biomedical applications. Moreover these microbeads exhibit reactive groups that may be further functionalized. The synthesis strategy may also be optimized in future studies in order to obtain microbeads for other copolymer compositions.

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