New Self-Assembling Polyaspartamide-Based Brush Copolymers Obtained by Atom Transfer Radical Polymerization

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ABSTRACT: A simple and efficient method for the synthesis of polyaspartamide-based brush copolymers using Atom Transfer Radical Polymerization (ATRP) is here presented. The syntheses were performed by using two subsequent steps. In the first step the macroinitiator was obtained by the conjugation of a proper number of 2-bromoisobutyryl bromide (BIB) residues to the α,β-poly(N-2-hydroxyethyl)-D,L-aspartamide (PHEA) side chains, obtaining the PHEA−BIB copolymer. PHEA−BIB copolymer was used as "multi-functional macroinitiator" for the polymerization via ATRP of hydrophilic methacrylic monomers, such as methacrylic acid (MA), obtaining PHEA−IB−poly(MA) copolymer, sodium methacrylate ($MANa^+$), obtaining PHEA−IB−poly($MANa^+$) copolymer, or hydrophobic monomer such as butyl methacrylate (BMA), obtaining PHEA−IB−poly(BMA) copolymer. BMA was also homopolymerized subsequently $MANa^+$ polymerization, extending the poly($MANa^+$) chains by poly(BMA) chains arising PHEA−IB−poly($MANa^+$)-*block*-poly(BMA) copolymer. Different solvent and temperature conditions were used in order to obtain the best ATRP conditions for each monomer in term of high polymerization efficiency in PHEA side chain. All the synthesized PHEA-based brush copolymers were widely characterized and copolymer self-assembling properties in aqueous media were evaluated by turbidimetry measurements, light scattering and SEM analyses. PHEA−IB−poly($MANa^+$) resulted able to form spherical microparticles at pH 2, with a diameter from 1 − 5 μm. PHEA−IB−poly($MANa^+$)-*block*-poly(BMA) copolymer formed particles at elliptic shape and an internal hollow architecture with an outer diameter of 1−4 μm. Finally, PHEA−IB−poly(BMA) copolymer resulted able to produce microfibers in aqueous medium, with an homogeneous tube shape and a thickness ranging from 3 to 5 μm.

Introduction

The need for polymers with specific physical and biological properties has generated continuous interest in novel polymer synthesis. Synthetic biodegradable polymers continue to be developed, to allow finer control of biocompatibility, surface properties, drug release and sensitivity to external stimuli. Recently, the ability of synthetic polymers to self-assemble into micro or nanostructures with particular architectures is playing a crucial role in the drug delivery field.[1,2] More and more frequently, this characteristic is associated to a change of pH as external stimulus, which acts as a driving force for the polymer self-assembling.[3−7]

The objective of producing pH-responsive polymer systems could be gained by utilizing polymers with a combination of hydrophobic and charged portions or chains, either as diblock or branched composition. In general, the hydrophobic portion should control the polymer water solubility, while the charged chains, that are typically extended due to ionic repulsion, can lose the charge as a consequence of pH change and cause the polymer to condense and form new three-dimensional architectures.[3,4] Today, one of the most useful synthetic approach to prepare copolymers with different monomer composition is undoubtedly atom transfer radical polymerization (ATRP).[8,9]

ATRP attracts considerable attention because of advantages of easy and mild polymerization conditions in comparison with other polymerization processes[10] and allows the realization of continuous processes.[11] The ATRP is based on the reversible transfer of a halogen atom from the initial halogenated chain to a transition metal (often Cu^+) complexed by a proper ligand as catalyst, in order to form a radical that can start the polymerization in the presence of proper vinyl monomers. As compared with other living radical polymerizations, ATRP is a versatile technology for preparing block copolymers with well-controlled chemical composition and morphology and it is suitable for monomers with a wide range of polarity.[8−13] This chemical versatility allows to obtain materials with unique mechanical properties, providing additional modes for regulating the release of therapeutic agents and for producing surfaces that are compatible with the in vivo environment.[14] In this field, polymers based on acrylic acid have become increasingly important in application for coatings and biomaterials.[15] However acrylic acid or methacrylic acid are difficult to be directly polymerized by ATRP because of interactions of the carboxylic acid functionalities with the catalyst. Presumably, carboxylic acid react with Cu^{II} species by displacing the halogen atom, resulting in the formation of metal carboxylates which inhibit the activation.[8,16] Additionally, since many of the ligand systems utilized in ATRP are nitrogen molecules, in that protonation of the nitrogen may occur, disrupting its coordination to the Cu center.[8,16] Until now, precursors of poly(acrylic acid), e.g. poly(*tert*-butyl acrylate), were synthesized by ATRP, and subsequently carboxylic acid groups were deprotected yielding well-defined poly(acrylic acid).[17] Ashford and co-workers reported successful ATRP of sodium methacrylate directly in water at 90 °C, using a 2-bromisobutyrylmethoxypoly(ethylene oxide) as the macroinitiator.[18]

In this paper, we present the synthesis and characterization of new polymeric materials with a particular brush structures able to self-assemble in aqueous phase at particular pH condition forming microparticles with different architecture and microfibers. These new copolymers were obtained starting from the biocompatible polymer α,β-poly(*N*-2-hydroxyethyl)-D,L-aspartamide (PHEA)[19,20] used as "multi-functional macroinitiator" for the polymerization in the side chain, via ATRP, of hydrophilic monomers, such as methacrylic acid (MA) or

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sodium methacrylate ($MANa^+$), or hydrophobic monomer such as butyl methacrylate (BMA) alone or in combination with the acid monomer $MANa^+$. The PHEA copolymers so obtained were subjected to different experimental conditions in order to evaluate the possibility to obtain nano- and microstructures useful for biomedical applications including drug delivery.

Experimental Section

Materials and Methods. α,β-Poly(*N*-2-hydroxyethyl)-D,L-aspartamide (PHEA) was prepared and purified according to the previously reported procedure.[19,20] Spectroscopic data (FT-IR and ^{1}H NMR) were in agreement with attributed structure:[19,20] ^{1}H NMR (300 MHz, D_2O, 25 °C, δ): 2.82 (m, 2H, −CH−*CH*$_2$−CO−NH−), 3.36 (t, 2H, −NH−*CH*$_2$−CH$_2$−OH), 3.66 (t, 2H,−CH$_2$−*CH*$_2$−OH), 4.72 (m, 1H, −NH−*CH*−CO−CH$_2$−).

PHEA average molecular weight was 48.0 kDa ($M_w/M_n = 1.66$) based on PEO/PEG standards, measured by size exclusion chromatography (SEC).

Infrared spectra were obtained using a Perkin-Elmer 1720 IR Fourier Transform Spectrophotometer in potassium bromide disks.

The ^{1}H NMR and ^{13}C NMR spectra were recorded in D_2O, DMF-d_7 or DMSO-d_6 (Aldrich) using a Bruker Avance II 300 spectrometer operating at 300 MHz.

Centrifugations were performed using a Centra MP4R IEC centrifuge.

Triethylamine (TEA), SEC polyethylene glycol standards, acetone, diethyl ether, methanol (MeOH) were purchased from Fluka (Switzerland). 2-Bromoisobutyryl bromide (BIB), methacrylic acid, butyl methacrylate (methacrylic derivatives were distilled prior to use in order to remove inhibitors), sodium methacrylate, 2,2′-bipyridine (bpy, 99%), copper(I) bromide (Cu^IBr 99.999%), Dowex 50WX8 100−200 mesh, Sephadex G-15, dimethylacetamide (DMA), and dimethylformamide (DMF) were purchased from Aldrich and were used as received. SpectraPor dialysis tubing was purchased from Spectrum Laboratories, Inc. (Italy).

General Procedure for Synthesis of α,β-Poly(*N*-2-hydroxyethyl)-*co*-[*N*-2-ethylene(2-bromoisobutyrate)]-D,L-aspartamide (PHEA−BIB) Derivatives. PHEA (500 mg; 3.1 mmol of hydroxyethyl−aspartamide repeating units) was dissolved in 10 mL of anhydrous DMA at room temperature followed by the addition of TEA (500 μL; 3.6 mmol). BIB (275 μL; 2.2 mmol) was then added to the mixture of reaction under argon atmosphere and the reaction temperature was maintained at 0 °C. The reaction mixture was then kept at room temperature for 4 h. The reaction mixture was then added dropwise into diethyl ether (150 mL); the resulting white precipitate was isolated by centrifugation and dried under vacuum. The solid residue was solubilized in distilled water and purified by gel permeation chromatography using a Sephadex G-15 packed column. After purification copolymer solution was freeze-dried from water. The pure product was obtained in 80% yield based on theoric yield (110% based on starting PHEA). Obtained copolymer was characterized by ^{1}H NMR and ^{13}C NMR. Molecular weight and polydispersity were estimated by SEC analysis. Weighted average molecular weight of PHEA−BIB was 50.0 kDa ($M_w/M_n = 1.76$) based on PEO/PEG standards.

^{1}H NMR (300 MHz, D_2O, 25 °C, δ): 1.90 (s, 6H, $CH_3)_{BIB}$, 2.84 (m, 2H, $CH_2)_{PHEA}$, 3.38 (m, 2H, $CH_2)_{PHEA}$, 3.68 (m, 2H, $CH_2)_{PHEA}$, 4.33 (m, 2H, $CH_2)_{PHEA}$, 4.76 (m, 1H, $CH)_{PHEA}$.^{13}C NMR (300 MHz, DMSO-d_6, 25 °C, δ): 170.8−169.7 $(C{=}O)_{PHEA}$, 59.7 $(-CH_2-O-)_{PHEA}$, 57.3 $(C-Br)_{BIB}$, 50.1 $(-CH-CO)_{PHEA}$, 44.3 $(-HN-CH_2-)_{PHEA}$, 37.5 $(-CH_2-CO)_{PHEA}$, 30.3 $(CH_3)_{BIB}$.

General Procedure for Synthesis of α,β-Poly(*N*-2-hydroxyethyl)-*co*-{*N*-2-ethylene[2-(poly(methacrylic acid))isobutyrate]}-D,L-aspartamide [PHEA−IB−poly(MA)] Derivatives. The atom transfer radical polymerization (ATRP), using PHEA−BIB as the macroinitiator, was carried out under bubbling of argon in a three-necked reaction flask. 100 mg of PHEA−BIB (0.17 mmol of side chain BIB groups) was solubilized in a previously degassed 1:1 DMF/MeOH (v/v) solvent mixture (8 mL); to the flask were then added 150 μL of methacrylic acid (1.75 mmol), Cu^IBr catalyst (25.5 mg, being molar ratio between Cu^IBr and BIB linked group equal to 1), bpy ligand (101 mg, being the molar ratio between bpy ligand and BIB linked group equal to 4) under continuous stirring and argon bubbling; the reaction was kept at room temperature for 24 h, then bubbling of argon was stopped and the reaction mixture left in contact with air oxygen until the complete copper oxidation (the reaction mixture becomes quickly green). The reaction mixture was added dropwise into acetone (100 mL) and the solid residue was washed once in the same solvent. The slightly green residue, obtained after centrifugation, was solubilized in bidistilled water and purified through an acid Dowex 50WX8 column in order to remove any copper traces. Copolymer purification was completed by exhaustive dialysis using a SpectraPor dialysis tubing with a 12 000−14 000 molecular weight cutoff. After dialysis, the solution was freeze-dried from water; the obtained pure product (90% yield based on starting PHEA−BIB copolymer) was characterized by ^{1}H NMR and ^{13}C NMR analysis. Molecular weight and polydispersity were estimated by SEC.

^{1}H NMR (300 MHz, D_2O, 25 °C, δ): 1.18 (m, 3H, $CH_3)_{MA}$, 1.45 (m, 6H, $CH_3)_{IB}$, 1.60 (m, 2H, $CH_2)_{MA}$, 1.94 (s, 6H, $CH_3)_{BIB}$, 2.81 (m, 2H, $CH_2)_{PHEA}$, 3.36 (m, 2H, $CH_2)_{PHEA}$, 3.54 (m, 2H, $CH_2)_{PHEA}$, 3.66 (m, 2H, $CH_2)_{PHEA}$, 4.2−4.4 (m, 2H, $CH_2)_{PHEA}$, 4.72 (m, 1H, $CH)_{PHEA}$.^{13}C NMR (300 MHz, DMSO-d_6, 25 °C, δ): 171.6−169.7 $(C{=}O)_{PHEA}$, 59.4 $(-CH_2-O-)_{PHEA}$, 57.4 $(C-Br)_{BIB}$, 50.1 $(-\mathbf{C}H-CO)_{PHEA}$, 44.3 $(-HN-CH_2-)_{PHEA}$, 37.5 $(-\mathbf{C}H_2-CO)_{PHEA}$, 30.2 $(-CH_2-)_{MA}$, 27.3 $(-\mathbf{C}(CH_3)-CO)_{IB}$, 25.1 $(-CH_3)_{MA}$.

General Procedure for the Synthesis of α,β-Poly(*N*-2-hydroxyethyl)-*co*-{*N*-2-ethylene[2-(poly(methacrylic acid sodium salt))isobutyrate]}-D,L-aspartamide [PHEA−IB−poly(MANa$^+$)] Derivatives. The ATRP polymerization of sodium methacrylate, using PHEA−BIB as the macroinitiator, was carried out using different protocols regarding the reaction temperature, amount of used monomer and reaction time. A typical protocol was the following: 100 mg of PHEA−BIB (0.17 mmol of BIB pendant initiators) was solubilized in a previously degassed 1:1 DMF/water (v/v) solvent mixture (8 mL); to the flask was then added sodium methacrylate (382 mg, 3.5 mmol, being molar ratio between sodium methacrylate and BIB residue equal to 20), Cu^IBr catalyst (25.5 mg, being the molar ratio between Cu^IBr and BIB linked group equal to 1), bpy ligand (101 mg, being the molar ratio between bpy ligand and BIB linked group equal to 4). The reaction was carried out at 50 °C in a water bath under continuous stirring and argon bubbling for 5 h; then reaction flask was left at room temperature under argon atmosphere for 18 h. Reaction was stopped by keeping reaction mixture in contact with air oxygen until the complete oxidation of copper. The reaction mixture was added dropwise into acetone (100 mL) and the resulting solid residue was washed twice by the same solvent. The green residue, obtained after centrifugation, was solubilized in bidistilled water and purified through an acid Dowex 50WX8 column in order to remove any copper traces. Copolymer purification was completed by exhaustive dialysis using a SpectraPor dialysis tubing with 12 000−14 000 molecular weight cutoff. After dialysis the solution was freeze-dried from water; the obtained pure product (90% yield based on starting PHEA−BIB copolymer) was characterized by ^{1}H NMR and ^{13}C NMR analysis. Weighted average molecular weight and polydispersity were estimated by SEC analysis.

^{1}H NMR (300 MHz, D_2O, 25 °C, δ): 1−1.4 [m, 3H, $(CH_3)_{MANa^+}$, 6H, $(CH_3)_{IB}$], 1.62 (m, 2H, $CH_2)_{MANa^+}$, 2.84 (m, 2H, $CH_2)_{PHEA}$, 3.38 (m, 2H, $CH_2)_{PHEA}$, 3.56 (m, 2H, $CH_2)_{PHEA}$, 3.69 (m, 2H, $CH_2)_{PHEA}$, 4.20 (m, 2H, $CH_2)_{PHEA}$, 4.70 (m, 1H, $CH)_{PHEA}$.^{13}C NMR (300 MHz, DMSO-d_6, 25 °C, δ): 172.3−169.7 $(C{=}O)_{PHEA}$, 59.4 $(-CH_2-O-)_{PHEA}$, 50.2 $(-\mathbf{C}H-CO)_{PHEA}$, 44.2 $(-HN-CH_2-)_{PHEA}$, 37.6 $(-\mathbf{C}H_2-CO)_{PHEA}$, 30.2 $(-CH_2-)_{MANa^+}$, 27.3 $(-\mathbf{C}(CH_3)-CO)_{IB}$, 25.1 $(-CH_3)_{MANa^+}$.

General Procedure for the Synthesis of α,β-Poly(*N*-2-hydroxyethyl)-*co*-{*N*-2-ethylene-[2-(poly(methacrylic acid sodium salt))isobutyrate]-*block*-[poly(butyl methacrylate)]}-D,L-aspartamide [PHEA−IB−poly(MANa$^+$)-*block*-poly(BMA)] derivatives. The side chains of poly($MANa^+$) in the ATRP-synthesized PHEA−IB−poly($MANa^+$) were extended by the ho-

mopolymerization of butyl methacrylate monomer. Thus, to the reaction solution of PHEA−IB−poly(MANa$^+$) kept for 24 h at 50 °C, a degassed methanol solution of butyl methacrylate was added (280 μL, 1.75 mmol in 4 mL). The reaction was maintained under continuous stirring and under argon atmosphere for 24 h at room temperature, then reaction was stopped by exposure to air oxygen. The reaction mixture was added dropwise into diethyl ether (100 mL) and the solid residue was washed twice by the same solvent. The crude product, obtained after centrifugation, resulted soluble in water, and it was purified and characterized using the same procedure reported for PHEA−IB−poly(MANa$^+$) copolymer. The obtained pure product yield was 97% based on starting PHEA−BIB copolymer.

^{1}H NMR (300 MHz, D_2O, 25 °C, δ): 0.90 (m, 3H, $CH_3)_{BMA}$, 1.11 (m, 3H, $CH_3)_{MANa^+}$, 1.35 (m, 6H, $CH_3)_{IB}$, 1.46 (m, 4H, $CH_2)_{BMA}$, 1.62 (m, 2H, $CH_2)_{MANa^+}$, 2.84 (m, 2H, $CH_2)_{PHEA}$, 3.36 (m, 2H, $CH_2)_{PHEA}$, 3.51 (m, 2H, $CH_2)_{PHEA}$, 3.69 (m, 2H, $CH_2)_{PHEA}$, 4.20 (m, 2H, $CH_2)_{PHEA}$, 4.69 (m, 1H, $CH)_{PHEA}$. ^{13}C NMR (300 MHz, DMSO-d_6, 25 °C, δ): 181.2 (C═O)$_{IB}$ 172.1−169.7 (C═O)$_{PHEA}$, 64.6 (−CH_2−O−)$_{BMA}$, 60.0 (−CH_2−O−)$_{PHEA}$, 51.2 (−**C**H−CO)$_{PHEA}$, 43.8 (−CH_2−)$_{BMA}$, 42.5 (−HN−CH_2−)$_{PHEA}$, 37.2 (−**C**H_2−CO)$_{PHEA}$, 31.8 (−CH_2−)$_{MANa^+}$, 30.5 (−CH_2−)$_{BMA}$, 28.3 (−CH_2−)$_{BMA}$, 27.3 (−**C**(CH_3)−CO)$_{IB}$, 25.1 (−$CH_3)_{MANa^+}$, 19.5 (−$CH_3)_{BMA}$, 14.1 (−$CH_3)_{BMA}$.

General Procedure for the synthesis of α,*β*-Poly(*N*-2-hydroxyethyl)-*co*-{*N*-2-ethylene-[2-(poly(butyl methacrylate))-isobutyrate]}-D,L-aspartamide [PHEA−IB−poly(BMA)] Derivatives. The protocol for the ATRP homopolymerization of butyl methacrylate, using PHEA−BIB as the macroinitiator, was essentially the same as that used for methacrylic acid. In detail, 100 mg of PHEA−BIB (0.17 mmol of side chain BIB groups) was solubilized in a previously degassed 1:1 DMF/MeOH (v/v) solvent mixture (8 mL); to the flask were then added 281 μL of butyl methacrylate (1.75 mmol, being molar ratio between butyl methacrylate and BIB residue equal to 10), Cu^{I}Br catalyst (25.5 mg, being the molar ratio between Cu^{I}Br and BIB linked group equal to 1), bpy ligand (101 mg, being the molar ratio between the bpy ligand and BIB linked group equal to 4) under continuous stirring and argon bubbling; the reaction was kept at room temperature for 24 h, then argon bubbling was stopped and the reaction mixture was left in contact with air oxygen until the complete oxidation of copper. The reaction mixture was added dropwise into diethyl ether (100 mL) and the resulting solid residue was washed once in the same solvent. The white residue, obtained after centrifugation, was dispersed in bidistilled water obtaining a homogeneous suspension and purified by exhaustive dialysis using a SpectraPor dialysis tubing with 12 000−14 000 molecular weight cutoff. After dialysis the suspension was freeze-dried from water; the obtained pure product (100% yield based on starting PHEA−BIB copolymer) was characterized by ^{1}H NMR and ^{13}C NMR analysis. Weighted average molecular weight and polydispersity were estimated by SEC analysis.

^{1}H NMR (300 MHz, DMSO-d_6, 25 °C, TMS, δ): 0.77 (m, 3H, $CH_3)_{BMA}$, 0.90 (m, 3H, $CH_3)_{BMA}$, 1.29 (m, 6H, $CH_3)_{IB}$, 1.36 (m, 2H, $CH_2)_{BMA}$, 1.56 (m, 2H, $CH_2)_{BMA}$, 1.88 (s, 6H, $CH_3)_{BIB}$, 2.65 (m, 2H, $CH_2)_{PHEA}$, 3.16 (m, 2H, $CH_2)_{PHEA}$, 3.41 (m, 2H, $CH_2)_{PHEA}$, 3.90 (m, 2H, $CH_2)_{PHEA}$, 4.13 (m, 2H, $CH_2)_{PHEA}$, 4.59 (m, 1H, $CH)_{PHEA}$.^{13}C NMR (300 MHz, DMSO-d_6, 25 °C, δ): 176.2 (C═O)$_{IB}$ 171.1−169.7 (C═O)$_{PHEA}$, 64.6 (−CH_2−O−)$_{BMA}$, 60.0 (−CH_2−O−)$_{PHEA}$, 50.1 (−**C**H−CO)$_{PHEA}$, 43.8 (−CH_2−)$_{BMA}$, 42.5 (−HN−CH_2−)$_{PHEA}$, 37.2 (−**C**H_2−CO)$_{PHEA}$, 30.8 (−CH_2−)$_{BMA}$, 28.3 (−CH_2−)$_{BMA}$, 19.5 (−$CH_3)_{BMA}$, 14.1 (−$CH_3)_{BMA}$.

Size Exclusion Chromatography (SEC) Characterization. The weighted average molecular weights (M_w) and molecular weight distributions (M_w/M_n) of PHEA, PHEA−BIB, PHEA−IB−poly(MA), and PHEA−IB−poly(MANa$^+$) derivatives were determined by size exclusion chromatography (SEC) in aqueous media. The standard SEC protocol involved using two TSK-GEL columns from TOSOH (G4000PW and G3000PW) connected to a Water 2410 refractive index detector. Phosphate buffer solution at pH 8,5 was used as eluent at 37 °C with a flow of 0.8 mL/min and poly(ethylene oxide) standards (range 2740−0.5 kDa) were used for calibration.

In the case of PHEA−IB−poly(BMA), the M_w and M_w/M_n values were determined by SEC in organic media using two Phenogel columns from Phenomenex (10^{4R} and 10^{3R}) connected to a Water 2410 refractive index detector and using a 0.01 M LiBr DMF solution as eluent with a flow of 0.8 mL/min. The column temperature was set at 50 °C. The same poly(ethylene oxide) standards above-mentioned were used for calibration.

pH-Dependent Turbidimetric analysis of PHEA−IB−poly(MA), PHEA−IB−poly(MANa$^+$), and PHEA−IB−poly(MANa$^+$)-*block*-poly(BMA) Brush Copolymers. The turbidity measurements were performed using a UV−vis spectrophotometer UV-2401 PC Shimadzu and recording the turbidity spectrum in the range 350−800 nm. The transmittance % produced by particles were read at 450 nm. For samples preparation, 10 mg of each copolymer were dissolved in 3 mL of bidistilled water and the solution pH was gradually reduced from 5.5 to 2 by the addition of 1 M HCl solution. Turbidity was progressively measured at different pH values.

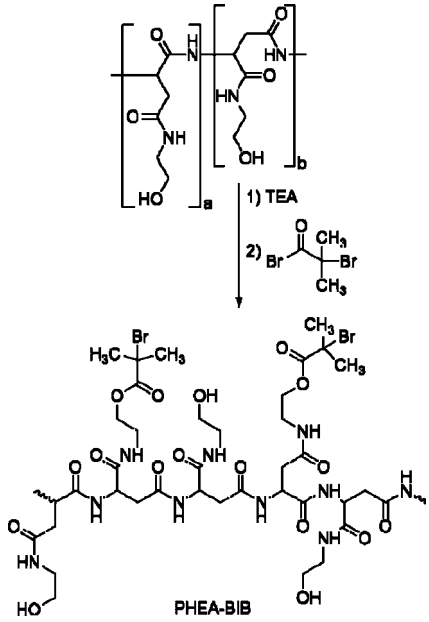
Dynamic Light Scattering Measurement and ζ Potential Analysis of PHEA−IB−poly(MA), PHEA−IB−poly(MANa$^+$) and PHEA−IB−poly(MANa$^+$)-*block*-poly(BMA) Brush Copolymers. Dynamic light scattering studies (DLS) and ζ potential measurements (mV) were performed at 25 °C using a Malvern Zetasizer NanoZS instrument, fitted with a 532 nm laser at a fixed scattering angle of 90°. Aqueous solutions of each copolymer were prepared using distilled water or 0.115 M phosphate buffer solution (PBS) at pH 7.4 (Na_2HPO_4, KH_2PO_4, NaCl) having a final copolymer concentration of 0.5 mg/mL.The copolymer solutions were filtered through a 5 μm cellulose membrane filter before analysis. The intensity-average hydrodynamic diameter and polydispersity index (PDI) were obtained by cumulants analysis of the correlation function. The zeta potential (mV) was calculated from the electrophoretic mobility using the Smoluchowsky relationship and assuming that $Ka \gg 1$ (where K and a are the Debye−Hückel parameter and particle radius, respectively).

PHEA−IB−poly(MANa$^+$) and PHEA−IB−poly(MANa$^+$)-*block*-poly(BMA) Microparticles Preparation. PHEA−IB−poly(MANa$^+$) and PHEA−IB−poly(MANa$^+$)-*block*-poly(BMA) microparticles were prepared according to the following procedure: 100 mg of each copolymer were dissolved in 2 mL of bidistilled water and the obtained aqueous solutions were added to 40 mL of a pH 2 buffer aqueous solution, under stirring. The obtained suspensions were continuously stirred at room temperature for 10 min at 20500 rpm using an Ultra-Turrax T25 homogenizer. The formed microparticles were purified by exhaustive dialysis against bidistilled water for 48 h, using a SpectraPor dialysis tubing with 12 000−14 000 molecular weight cutoff. After dialysis, fine suspensions were freeze-dried from water. Microparticles were obtained with a 80% yield based on the starting copolymer weight.

PHEA−IB−poly(BMA) Microfibers Preparation. A 100 mg sample of PHEA−IB−poly(BMA) copolymer was solubilized in 2.8 mL of a 2.5/1 v/v PEG_{600}/DMSO mixture. The obtained copolymer solution was then added dropwise to 40 mL of bidistilled water under Ultra-Turrax agitation. The fine dispersion was continuously stirred at room temperature for 10 min at 20500 rpm with the same homogenizer. The formed microfibers were purified by exhaustive dialysis against bidistilled water for 4 days, using a SpectraPor dialysis tubing with 12 000−14 000 molecular weight cutoff. After dialysis, a proper amount of threalose was added to the fine suspensions, and dispersions were subjected to cryogenic freezing by liquid nitrogen immersion, before being freeze-dried. Microfibers were obtained with a 100% yield based on starting copolymer weight.

Scanning Electron Microscopy Analysis. For the morphology studies, freeze-dried PHEA−IB−poly(MANa$^+$), PHEA−IB−poly(MANa$^+$)-*block*-poly(BMA) or PHEA−IB−PBMA samples were visualized using a scanning electron microscope ESEM Philips XL30. Samples were dusted on a double-sided adhesive tape applied

Scheme 1. Synthesis of PHEA−BIB Copolymer



previously on a stainless steel stub. All samples were then sputter-coated with gold prior to microscopy examination.

Results and Discussions

In the past decade, different polymers have been used as macroinitiators for ATRP and several examples of di- and three-block copolymers have been synthesized.[8,21] Multifunctional macroinitiators have also been used in the synthesis of densely grafted copolymers or brush copolymers through different synthetic routes including the so-called "grafting from route",[22] which consists in the contemporaneous polymerization of vinyl monomers, under ATRP conditions, through several initiator residues dislocated alongside a presynthesized polymer backbone. A similar approach was followed to synthesize the new brush copolymers here described, starting from the biocompatible polymer PHEA.

To this aim the PHEA backbone has been modified by conjugating at the polymer side chain a certain number of 2-bromoisobutyryl groups by the reaction of PHEA with 2-bromoisobutyryl bromide (BIB), obtaining the copolymer PHEA−BIB, as shown in Scheme 1.

As shown in Figure 1 the proton NMR (D_2O) and ^{13}C NMR (DMSO) spectra of PHEA−BIB clearly show the signal arising from the BIB methyl hydrogens and carbons respectively. Moreover, the ^1H NMR permitted an easy quantification of PHEA functionalization by comparing the ^1H NMR integral of the peak attributable to the methyl groups of BIB at δ 1.90 (a) to that from to PHEA backbone at δ 2.8 (d). The molar percent of BIB groups covalently linked to PHEA was equal to 35% respect to the hydroxyethyl-aspartamide repeating units. We

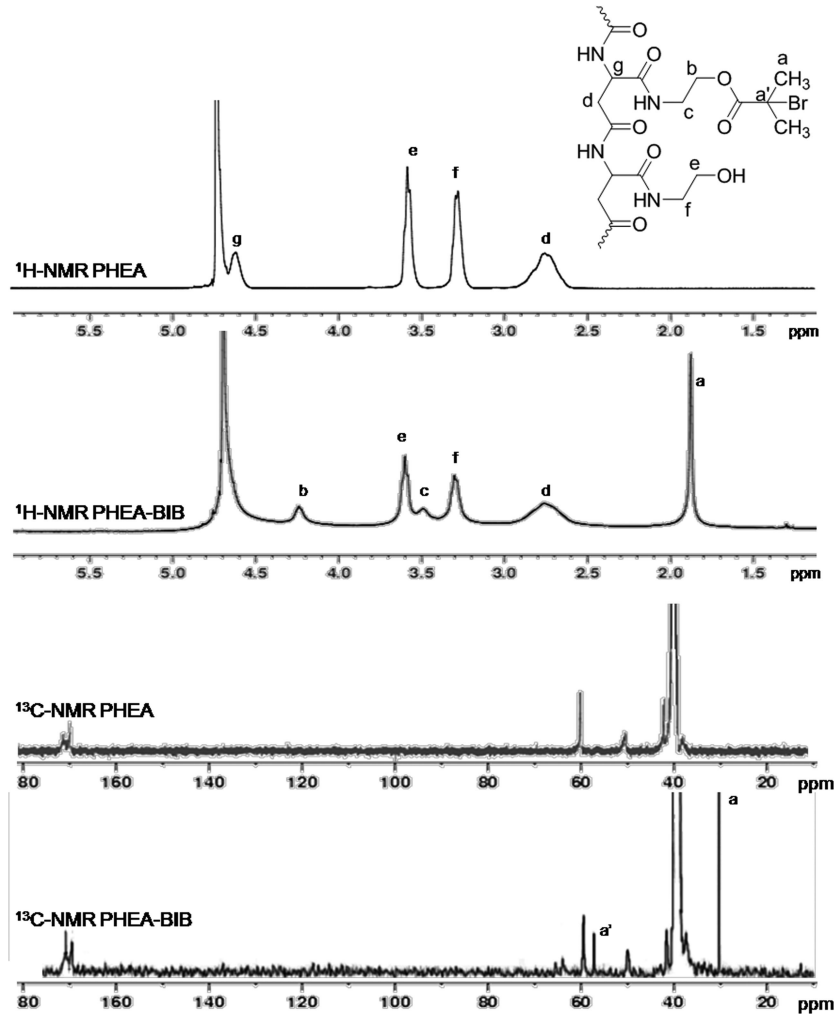


Figure 1. ^1H NMR (D_2O) and ^{13}C NMR (DMSO) spectra of PHEA−BIB copolymer.

Scheme 2. Representation of the Synthetic Pathways of PHEA Brush Copolymers via ATRP, with the Different Monomer Compositions

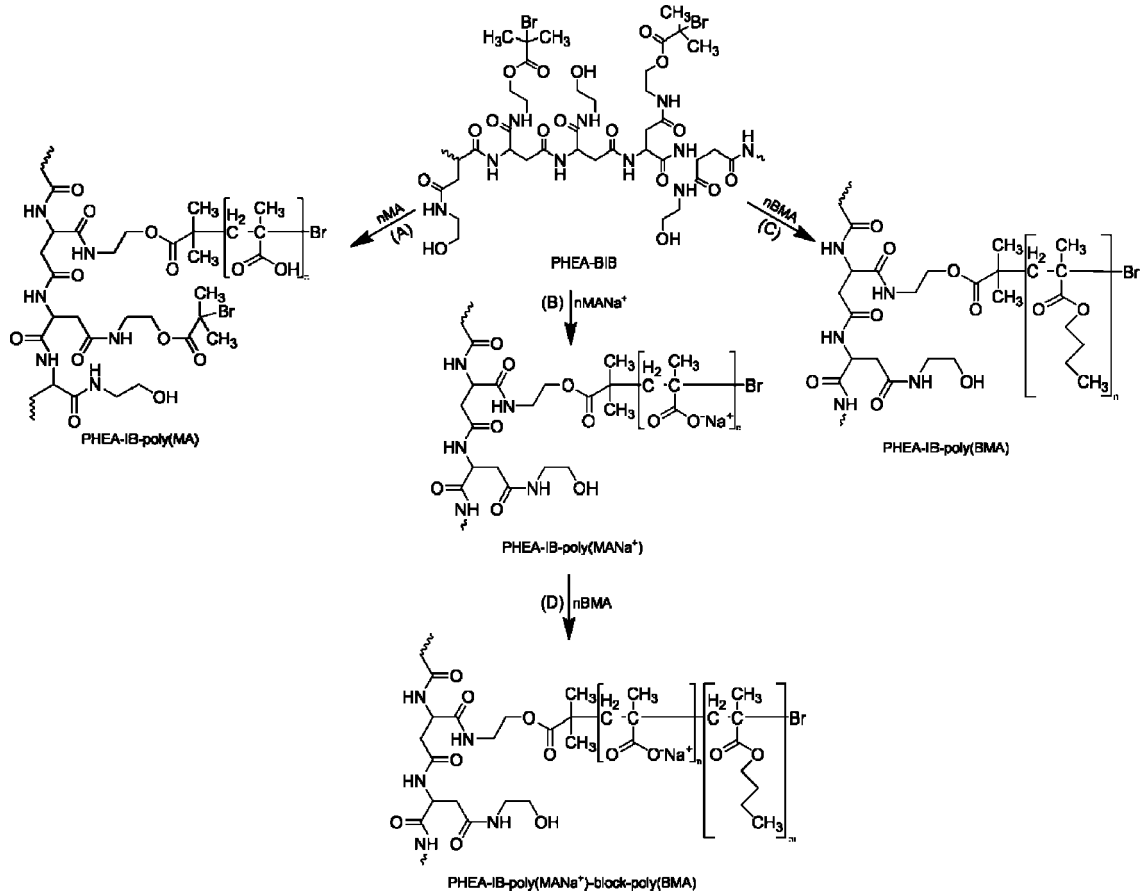


Table 1. Summary of ATRP Conditions and Characterization Data of PHEA−IB−poly(MA) and PHEA−IB−poly($MANa^+$) Copolymers

reaction conditions	monomer type	R^a	solvent	reaction temperature [°C]	reaction time [h]	DD [%^{b}]	$M_w{}^c$ [kDa]	M_w/M_n
a	MA	10	DMF/MeOH 1:1	25	4	7	48.4	1.8
b	MA	10	DMF/MeOH 1:1	25	24	10	68.3	1.9
c	MA	10	DMF/H_2O 1:1	25	24	12	70.0	1.9
d	$MANa^+$	10	DMF/MeOH 1:1	25	4	31	52.5	1.9
e	$MANa^+$	10	DMF/MeOH 1:1	25	24	33	70.2	1.8
f	$MANa^+$	10	DMF/MeOH/H_2O 4:4:1	25	24	38	73.8	1.9
g	$MANa^+$	10	DMF/H_2O 1:1	50	4	39	74.9	1.8
h	$MANa^+$	10	DMF/H_2O 1:1	50	24	43	75.5	1.7
i	$MANa^+$	20	DMF/H_2O 1:1	50	24	52	86.8	1.7

[a] R = monomer/BIB molar ratio. [b] DD% = (linked MA or $MANa^+$ residues/PHEA repeating units) × 100 (mol/mol). Calculated by ^{1}H NMR. [c] Evaluated by SEC in aqueous phase.

calculated that the number of BIB residues covalently linked to PHEA was about 110 for each polymeric chain.

The synthesized PHEA−BIB copolymer was used as multifunctional macroinitiator for the homopolymerization of: methacrylic acid (MA) to obtain PHEA−IB−poly(MA) graft copolymer (pathway A of Scheme 2); sodium methacrylate ($MANa^+$) to obtain PHEA−IB−poly($MANa^+$) graft copolymer (pathway B of Scheme 2); butyl methacrylate (BMA) to obtain PHEA−IB−poly(BMA) graft copolymer (pathway C of Scheme 2). Moreover, the polymerization of sodium methacrylate was continued by the subsequent homopolymerization of BMA, creating the PHEA−IB−poly(MA)-*block*-poly(BMA) graft copolymer (pathway D of Scheme 2). Actually, the acid and salt monomers were chosen in order to confer pH-sensitivity to the PHEA copolymers in aqueous medium. On the other hand, the hydrophobic BMA was selected to confer to the PHEA copolymer a particular hydrophilic/hydrophobic balance, thus to influence its water solubility and self-assembling behavior. The pathway of reactions are collected in Scheme 2.

In the case of pathway A, three different experimental conditions were tested as reported in Table 1. All copolymers were characterized from the chemical and molecular point of view by ^{1}H NMR and SEC analyses in aqueous medium. The MA derivatization degree (DD) of PHEA−IB−poly(MA) copolymers was calculated by comparing the ^{1}H NMR integral of the peak attributable to the methylenic groups of MA at δ 1.60 to that from PHEA backbone at δ 2.8 and then it expresses

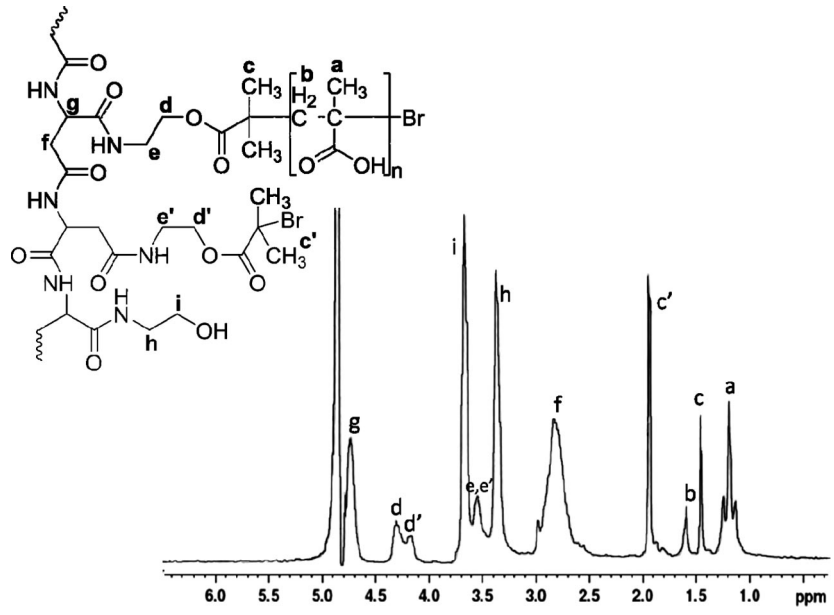


Figure 2. [1]H NMR spectrum of PHEA−IB−PMA obtained by condition c.

the ratio between moles of linked methacrylic acid groups and repeating units of PHEA.

We started by a protocol involving a system DMF/MeOH (1:1) as solvent and mild conditions such as room temperature for 4 h, considering that these experimental conditions were successful used for other hydrophilic monomers in the literature.[23−26] But under these experimental conditions the extension of MA polymerization was rather low, considering that $DD\%_{MA}$ was equal to 7.7% mol/mol that corresponds in average to 1 molecule of MA for each 5 BIB residue. Only a slight increase of extension of MA polymerization was observed by increasing reaction time from 4 to 24 h (condition b) or using a DMF/H_2O (1:1) solvent system (condition c) (Figure 2).

The relatively low polymerization efficiency of methacrylic acid, by using Cu/bpy as catalyst system, in not unusual in ATRP reactions[16] and has been attributed to the fast reaction of this monomer with the metal complexes to form metal carboxylates that deactivate ATRP catalysts.[8,16] To overcome this drawback subsequent reactions were performed by using sodium methacrylate ($MANa^+$) as monomer (experimental conditions d−i). In general, our reactions were carried out under experimental conditions milder than that reported in the literature, with temperature ranging from 25 to 50 °C, in order to avoid any degradation of PHEA backbone. As showed in table 1, when $MaNa^+$ was used, the polymerization efficiency of resulting PHEA−IB−poly($MANa^+$) copolymer was always greater than that obtained with MA in resulting PHEA−IB−poly(MA). In particular by using the same experimental monomer/BIB molar ratio ($R = 10$) and the same solvent system (DMF/MeOH 1:1) the DD% values increase from 8 and 10% at 4 h and 24 h respectively for MA to 31 and 33% for $MANa^+$ at the same reaction time. Weighted average molecular weights of resulting PHEA−IB−poly($MANa^+$) copolymers were in agreement with the increased polymerization efficiency. A further improvement of the polymerization efficiency (and then of degree of derivatization) was obtained by adding water to the solvent system achieving a DD% of about 38, corresponding to $n \sim 1.1$ (conditions f); using DMF/H_2O as solvent system and increasing temperature from 25 to 50 °C we observed a significant temperature-dependent increment of DD% and M_w (samples g and h). Finally, the highest DD%, equal to 52%, was obtained for PHEA−IB−poly($MANa^+$) copolymer at 50 °C using a $MANA^+$/BIB molar ratio of 20 (sample i) corresponding to an average

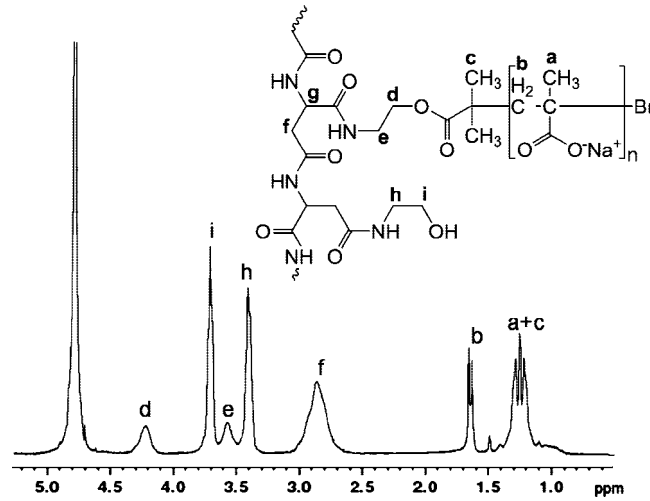


Figure 3. [1]H NMR spectrum of PHEA−IB−$PMANa^+$; $n \sim 1.5$.

polymerization number of 1.5. The [1]H NMR of PHEA−IB−poly($MANa^+$) copolymer is reported in Figure 3.

Studies on the reaction kinetics were performed at 25 °C and different monomer/BIB ratio in 1/1 DMF/water solvent system in order to have a further quantitative estimation of the different polymerization efficiency of MA and $MANa^+$ used as monomers. By reporting the M_w/M_{w0} (were M_w is the weighted average molecular weight of copolymer at fixed polymerization reaction time and M_{w0} is the weighted molecular weight of the starting PHEA−BIB) as a function of reaction time (see Figure 4), a more rapid increase of molecular weight was detected for PHEA−IB−poly($MANa^+$) in comparison with PHEA−IB−poly(MA); this result is in agreement with a greater polymerization efficiency by ATRP for $MANa^+$ in comparison with MA. This occurs because, in the case of MA, the protonation of the nitrogen of bpy ligand prevents the coordination complex formation between Cu^+ and bpy ligand, making slower the polymerization reaction. Moreover an expected dependence of the polymerization widening on the molar ratio between monomer $MANa^+$/BIB was observed.

PHEA−IB−poly($MANa^+$)-*block*-poly(BMA) graft copolymer was obtained by subsequent polymerization reaction of $MANa^+$ by using PHEA−$BIB_{35\%}$, followed by polymerization

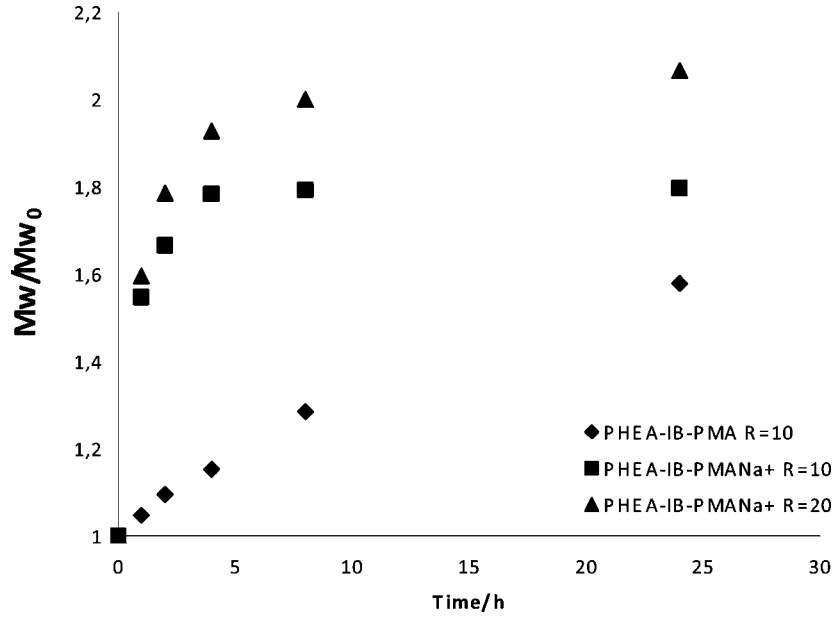


Figure 4. Plots of M_w/M_{w0} (M_w = copolymer molecular weight at fixed polymerization reaction time; M_{w0} = starting PHEA−BIB molecular weight) as a function of time. R = monomer/BIB molar ratio.

Table 2. ATRP Conditions and Molecular Parameters of PHEA−IB−poly(MANa$^+$)-*block*-poly(BMA) and PHEA−IB−poly(BMA) Copolymers

monomer type	R^a	solvent	reaction temperature [°C]	reaction time [h]	DD [%]^b	M_w [kDa]^c	M_w/M_n
MANa$^+$/BMA	10/10	DMF/H_2O 1:1	25	24/24	$65_{[MANa^+]}$ $25_{[BMA]}$	92.0	1.9
BMA	10	DMF/MeOH 1:1	25	24	$100_{[BMA]}$	184	2.1

^{a} R = monomer/BIB molar ratio. ^{b} Calculated by ^{1}H NMR. ^{c} Evaluated by SEC in aqueous phase, excepting for PHEA−IB−poly(BMA) (DMF).

of butyl methacrylate (BMA), performed by direct addition to the reaction mixture of PHEA−IB−poly(MANa$^+$) the proper amount of butyl methacrylate. The degree of derivatization in butyl methacrylate groups of the PHEA−IB−poly(MANa$^+$)-*block*-poly(BMA) copolymer was calculated by comparing the integral of the peak attributable to CH_3 of butyl groups at δ 0.9 to that of NH-CH-CH_2-CO at δ 2.8 attributable to PHEA backbone. Reaction conditions and molecular parameters of resulting PHEA−IB−poly(MANa$^+$)-*block*-poly(BMA) copolymer are reported in Table 2.

Finally, the homopolymerization of BMA as single monomer starting from PHEA−BIB was performed in DMF/methanol (since BMA monomer was only partially soluble in DMF/water) at room temperature in order to obtain PHEA−IB−poly(BMA) graft copolymer. In Table 2 are reported reaction conditions and molecular parameters of PHEA−IB−poly(BMA) copolymer.

Basically, comparing the $DD\%_{BMA}$ of PHEA−IB−poly(BMA) with that obtained for the PHEA−IB−poly(MANa$^+$)-*block*-poly(BMA), we observed a considerable difference in the polymerization efficiencies (25% mol/mol against 100% mol/mol). In fact, the DD_{BMA} of PHEA−IB−poly(MANa$^+$)-*block*-poly(BMA) is 4-fold lower than that of PHEA−IB−poly(BMA) at the same reaction time, as shown in Table 2. In this case we speculate that the presence of water, necessary for the MANa$^+$ homopolymerization, influences negatively the subsequent BMA polymerization during diblock synthesis because of the low water solubility of the monomer. Moreover, we cannot exclude that the steric hindrance produced by the polymerized MANa$^+$ chains on PHEA−IB−poly(MANa$^+$) copolymer, can be a further cause of the low polymerization of BMA monomer. Similar considerations were reported by Haddleton and coworkers.[27]

Self-Assembling Properties. In order to evaluate the copolymer ability to self-aggregate in aqueous medium forming three-dimensional systems, like nano- or microparticles, the synthesized graft copolymers were subjected to turbidimetry measurements and dynamic light scattering (DLS) analysis in dependence of pH. In particular turbidimetry and DLS analyses were carried out using diluted polymer solution filtered through 5 μm cellulose filters of: PHEA−IB−poly(MA) with DD_{MA} of 10% (sample b of table 1), PHEA−IB−poly(MANa$^+$) with DD_{MANa^+} of 52% (sample i of table 1) and PHEA−IB−poly(MANa$^+$)-*block*-poly(BMA) with DD_{MANa^+} of 65% and DD_{BMA} of 25%, respectively. Actually, the absence of particles with big diameters permitted to evaluate the self-assembling process without interferences on the light scattering.

Turbidimetry is a simple indirect optical technique for measuring particle size[28] and a valid method to evaluate the particle formation.[29] In our experiments the sample dispersions containing a note amount of each copolymer in aqueous medium at a previously adjusted pH value, was illuminated by a light beam at a fixed wavelength, and the percentage transmittance (T%) of the emerging beam was detected at 450 nm. In the same way, the transmittance of the medium was taken as reference. Definitely, when the light beam course is obstructed by particles suspended in the medium the transmittance decreased as a consequence of the light extinction produced by particles. This effect is strictly correlated to the particles size and abundance.

In Table 3 are reported the T % values obtained by spectrophotometric analysis and average diameter values detected by DLS.

As shown in Table 3, by decreasing pH from 5.5 to 2 we observed the concomitant reduction of T % of copolymer dispersion. This phenomenon was relevant for PHEA−IB−poly(MANa$^+$) and PHEA−IB−poly(MANa$^+$)-*block*-poly(BMA) brush copolymers, in fact, their solution transmittance value decrease respectively until about 28% and 48% at pH 2, and dispersions resulted opalescent. The observed T % reduction can be attributed to the formation of particles as a consequence

Table 3. Turbidimetry (T %) and DLS (*Z*-Average, PDI = Polydispersity Index, Z-Potential) Data of PHEA−IB−poly(MA), PHEA−IB−poly(MANa$^+$), and PHEA−IB−poly(MANa$^+$)-*block*-poly(BMA) brush Copolymers Aqueous Solutions (0.5 mg/mL) at Various pH Values[a]

copolymer	pH	T %	*Z*-average [nm]	PDI	*Z*-potential [mV]
PHEA−IB−poly(MA) (sample c)	5.5	100	75	0.62	−14.3
	3	99.5	194	0.28	−8.9
	2	99.2	265	0.46	−3.5
PHEA−IB−poly(MANa$^+$) (sample i)	5.5	91.2	245	0.43	−30.8
	3	90.5	560	0.38	−19.3
	2	27.8	691	0.67	−1.5
PHEA−IB−poly(MANa$^+$)-*block*-poly(BMA)	5.5	92.0	218	0.38	−29.6
	3	91.7	480	0.42	−15.8
	2	48.1	624	0.68	−0.8

[a] All sample solutions were filtered on 5 μm filters before DLS analysis.

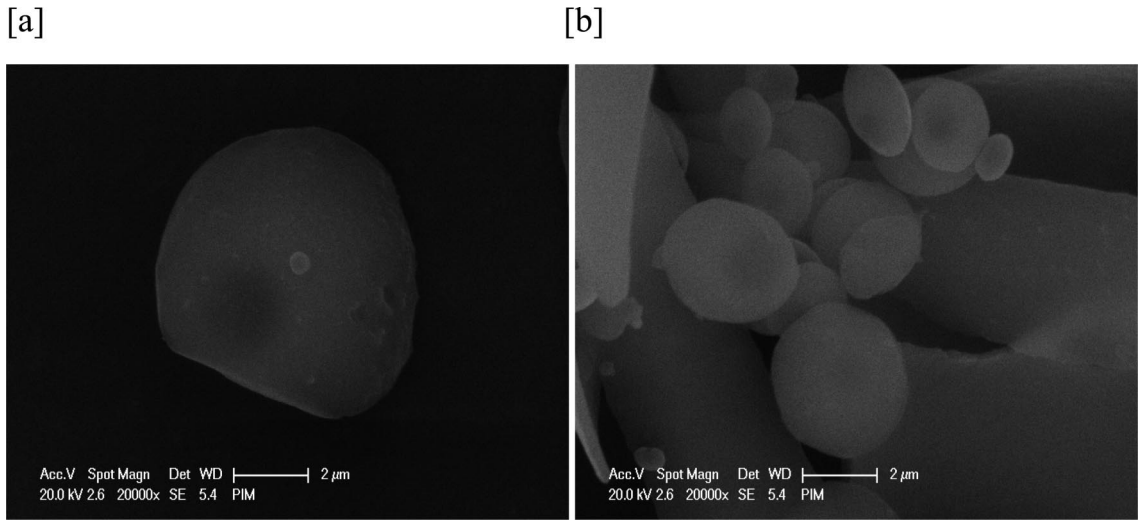


Figure 5. (a and b) SEM images showing the shape and morphology of PHEA−IB−poly(MANa$^+$) microparticles obtained at pH 2.

of the phase separation induced in the polymer dispersions by a drop of the pH. This phase separation can be associated to the protonation of the carboxylic groups in the copolymers side chains (as confirmed by the *Z*-potential reduction shown in table 3) thus increasing copolymer hydrophobicity. This phenomenon induces the copolymer self-assembling into particles.[4]

DLS analysis of the same copolymer dispersions revealed the presence of particles with average diameters growing with pH reduction. In particular, at pH 2 we observed particles having an average diameter of about 265 nm for the PHEA−IB−poly(MA) copolymer, 690 nm for the PHEA−IB−poly(MANa$^+$) copolymer and 620 nm for PHEA−IB−poly(MANa$^+$)-*block*-poly(BMA) copolymer. In agreement with the higher T% reduction, greater particles were obtained in the presence of PHEA−IB−poly(MANa$^+$) and PHEA−IB−poly(MANa$^+$)-*block*-poly(BMA) copolymers. In addition the effect of polymer concentration on the self-assembling properties of obtained copolymers was preliminarily tested performing DLS and turbidimetry analyses also at lower copolymer concentration (2 mg/mL versus 3.3 mg/mL). Experimental results showed no significant differences in the transmittance values as well as in the Z-average values measured under the same experimental conditions. This result evidence that in this range of concentration (2−3.3 mg/mL) tested copolymers exhibit analogue self-assembling behavior. Particles obtained at pH 2 starting from these two copolymers were characterized by Scanning electron microscopy (SEM) analysis. SEM images obtained from the sample PHEA−IB−poly(MANa$^+$) (Figure 5, parts a and b) showed particles having spherical shape and diameter ranging from 1 to 5 μm. Microparticles provide an attractive form of drug delivery due to their ease of fabrication, simplicity of administration, and possible use in localized and targeted delivery. They represent a good example of controlled release system for an improvement of drug bioavailability and for a better controlling of drug delivery.[30,31]

Different shapes were observed for the PHEA−IB−poly(MANa$^+$)-*block*-poly(BMA) microparticles (Figure 6a−d); in fact, particles showed an elliptic shape and the presence of a hole in the central region of the particle surface, as it is easy to note in the particulars of Figure 6, parts c and d. The outer diameter was in this case comprised between 1 and 4 μm.

At this stage, it is difficult to understand the exact correlation between the particular particle shape and the copolymer composition, but we think that the presence of the hydrophobic BMA portions at the end of poly(MANa$^+$) chains induces a particular disposition of the macromolecules in aqueous phase, in which the hydrophobic BMA portions are oriented toward the internal side of the particle, whereas the hydrophilic PHEA−IB−poly(MANa$^+$) chains constitute the surface portions.[32]

PHEA−IB−polyBMA copolymer was investigated in order to evaluate its ability to form particles in aqueous medium as well. Given its intrinsic hydrophobicity and water insolubility (independent of pH for the absence of carboxylic acid groups in side chain), some attempts were carried out to prepare microsystems by dispersing PHEA−IB−poly(BMA) organic solutions (using ethanol, DMSO, chloroform as solvents) into aqueous phase and using dispersing agents such as poly(ethylene glycol) (PEG). In effect, by dispersing a 2.5/1 v/v DMSO/PEG$_{600}$ PHEA−IB−poly(BMA) solution in water we observed the formation of microsystems having shape of fiber as demonstrated by the SEM analysis of freeze-dried dispersion. In Figures 7a and b are illustrated the SEM images of a PHEA−IB−poly(BMA) microfibers prepared in the presence of PEG$_{600}$.

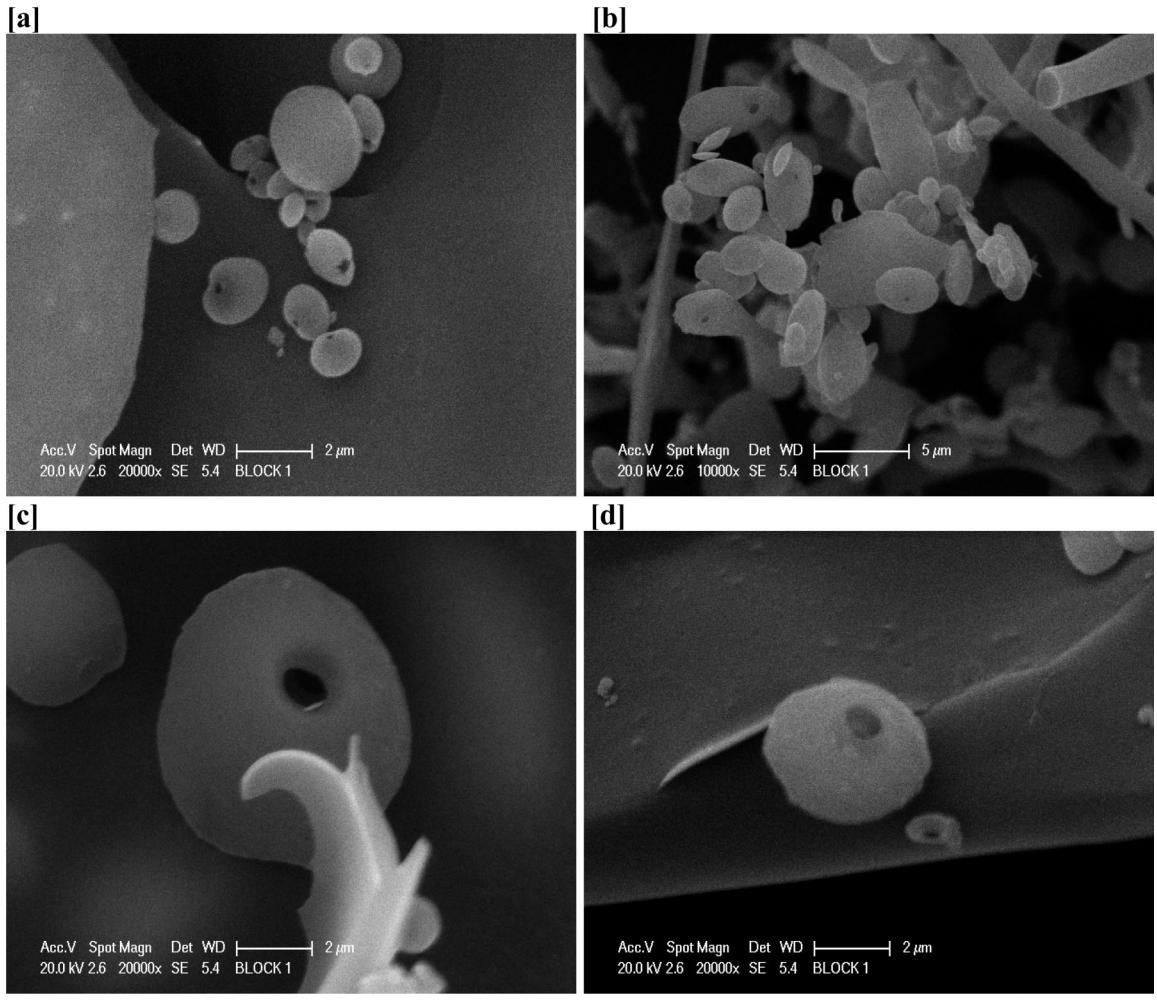


Figure 6. (a−d) SEM images showing the shape and morphology of PHEA−IB−poly(MANa$^+$)-*block*-poly(BMA) microparticles obtained at pH 2, at different magnifications.

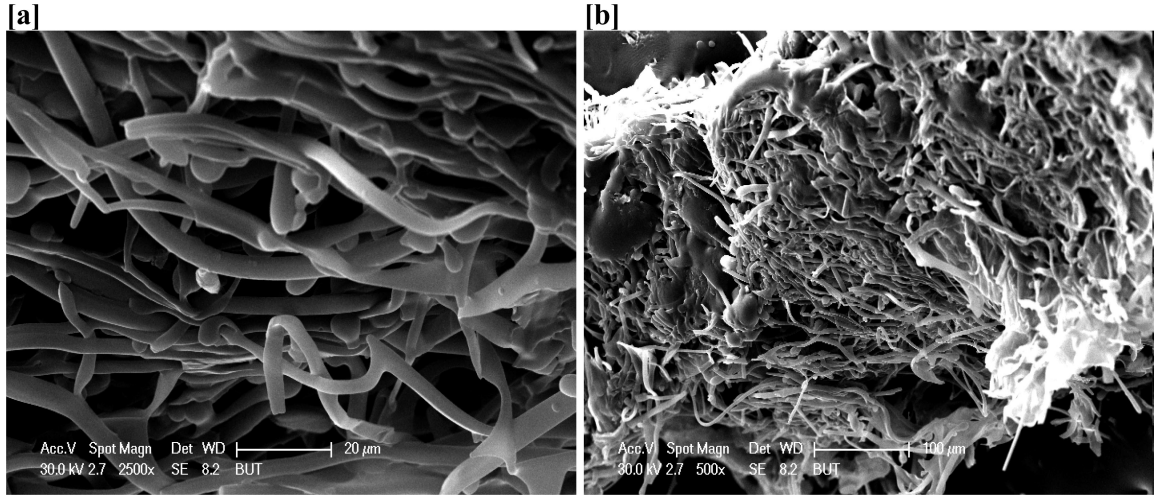


Figure 7. (a and b) SEM images showing the morphology of PHEA−IB−poly(BMA) microfibers at different magnifications.

The fibers showed a tubular shape and a thickness comprised between 3−5 μm as judged by SEM images. Moreover these fibers seem to be longer than 100 μm and produced a white spongy structure after freeze-drying, making this material useful for the production of scaffolds for biological or industrial application.

In Figure 8 were schematically represented the self-assembling behaviors of PHEA−IB−poly(MANa$^+$), PHEA−IB−poly(MANa$^+$)-*block*-poly(BMA) and PHEA−IB−poly(BMA) copolymers in aqueous media.

Conclusions

In summary, we describe the synthesis of new PHEA−based brush copolymers with different self-assembling behavior in aqueous media, obtained by polymerization of MA, MANa$^+$ and/or BMA monomers on the PHEA side chain using ATRP.

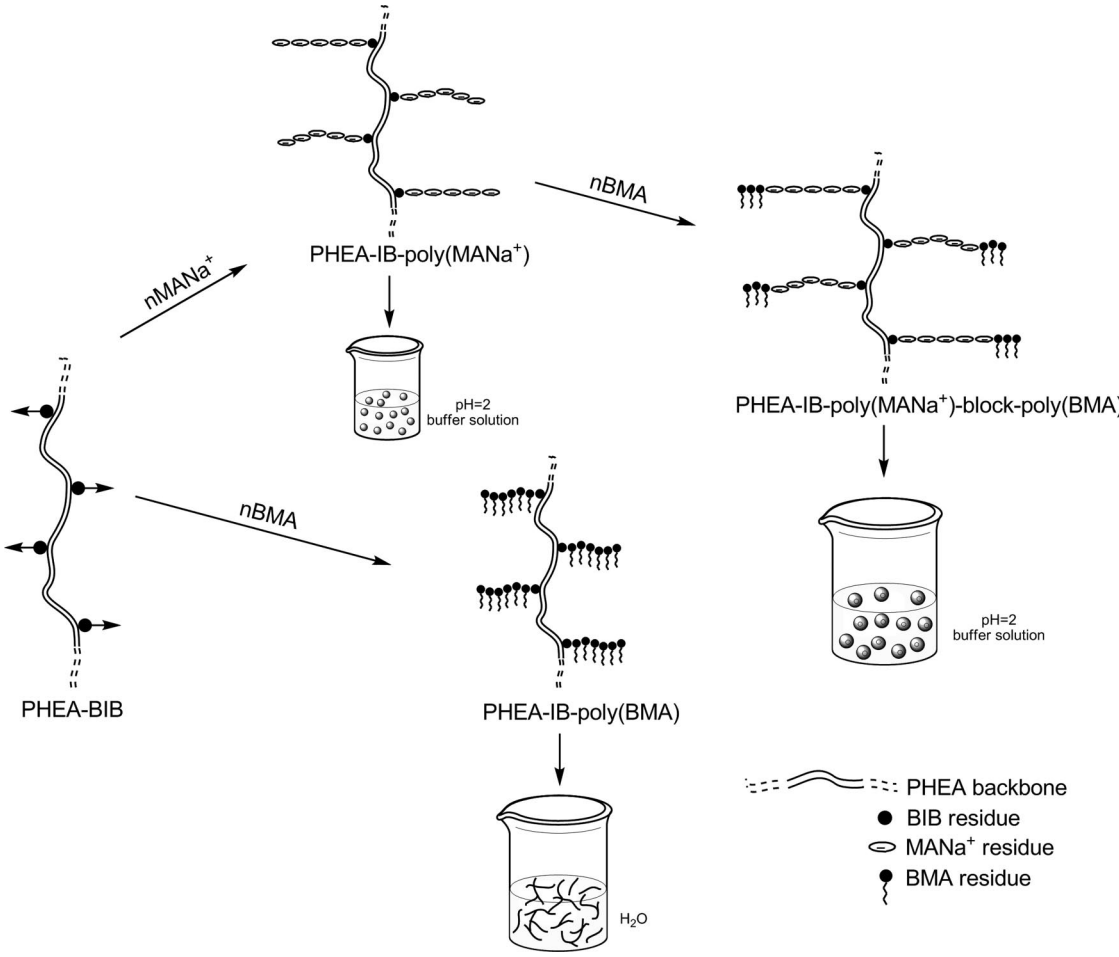


Figure 8. Representation of different self-assembled microsystems based on PHEA brush copolymers.

To this aim we used a PHEA−BIB copolymer as "multifunctional macroinitiator" for the polymerization, via ATRP, of methacrylic acid (MA), obtaining PHEA−IB−poly(MA) copolymer, sodium methacrylate ($MANa^+$), obtaining PHEA−IB−poly($MANa^+$) copolymer and butyl methacrylate (BMA), obtaining PHEA−IB−poly(BMA) copolymer. BMA was also homopolymerized subsequently $MANa^+$ polymerization, extending the poly($MANa^+$) chains by poly(BMA) chains arising PHEA−IB−poly($MANa^+$)-*block*-poly(BMA) copolymer. Synthesized PHEA−based brush copolymers showed self-assembling properties in aqueous media, as evaluated by performing turbidimetry measurements and light scattering analysis. In particular PHEA−IB−poly($MANa^+$) resulted to have a pH-depending water solubility and the ability to form spherical microparticles at pH 2, with a diameter ranging from 1 to 5 μm. PHEA−IB−poly($MANa^+$)-*block*-poly(BMA) copolymer formed "pierced" particles at pH 2 with an elliptic shape and an outer diameter of 1−4 μm. Finally, PHEA−IB−poly(BMA) copolymer resulted insoluble in water and able to produce microfibers in water, with an homogeneous tube shape and a thickness ranging from 3 to 5 μm.

Therefore, the versatility of the synthetic approach used in this paper, starting from PHEA−BIB copolymer as multifunctional macroinitiator for ATRP, supports the use of this technique of polymerization for the synthesis of new graft copolymers useful as starting materials for biomedical application. In particular the possibility to obtain microparticles from PHEA−IB−poly($MANa^+$) and PHEA−IB−poly($MANa^+$)-*block*-poly(BMA) by simple self-assembling of these copolymers at acid pH, make these materials useful for obtaining new drug delivery systems. Moreover, the ability to form microfibers by PHEA−IB−poly(BMA) at entangled structure make these materials interesting for the production of scaffold for tissue engineering.

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Supporting Information Available: A figure showing the proton−carbon bidimensional NMR spectrum. This material is available free of charge via the Internet at http://pubs.acs.org.

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