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Development of propolis-loaded block copolymer micelles of superior structural stability and high loading capacity

Petar D. Petrov [a, *], Georgy Grancharov [a], Valeria Gancheva [a], Boryana Trusheva [b], Vassya Bankova [b], Christo B. Tsvetanov [a]

[a] Institute of Polymers, Bulgarian Academy of Sciences, Akad. G. Bonchev Street, Bl.103A, 1113 Sofia, Bulgaria
[b] Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Akad. G. Bonchev Street, Bl.9, 1113 Sofia, Bulgaria

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Dedicated to Prof. Axel H.E. Müller on the occasion of his 70th birthday.

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ABSTRACT

We report on the efficient loading of poplar propolis into core-shell micelles based on amphiphilic poly(ethylene oxide)-*block*-poly(n-butyl acrylate) (PEO-*b*-PnBA) diblock copolymers. The copolymers comprising a hydrophilic PEO block of constant length (113 monomer units) and a soft hydrophobic PnBA block of three different lengths (53, 99 and157 monomer units) were synthesized by atom transfer radical polymerization (ATRP) of n-butyl acrylate initiated by PEO bromoisobutyrate. The study focused on fabrication of stable aqueous colloid systems characterized by complete encapsulation of all bioactive lipophilic propolis constituents with in the micellar carrier. The effects of copolymer/propolis mass ratio, copolymer composition and concentration on system characteristics were examined by dynamic light scattering (DLS), atomic force microscopy (AFM) and turbidity measurements. The results revealed that stable aqueous colloids and complete encapsulation of the lipophilic bioactive compounds into the micelles can be achieved at twofold excess of copolymer to propolis amount.

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1. Introduction

Core-shell polymeric micelles are nano-sized nanoparticles usually formed via self-assembly of amphiphilic block copolymers in selective solvent [1]. In an aqueous media, polymeric micelles are characterized with a hydrophobic core segregated from the aqueous exterior by a hydrated shell. Due to their unique architecture, small size, good biocompatibility, capacity to effectively solubilize hydrophobic compound, high *in vivo* stability, etc., core-shell micelles have been extensively exploited as carriers of poorly water-soluble biologically active substances for biomedical applications [2,3]. Indeed, when properly designed, polymeric micelles encapsulate hydrophobic drugs in their cores, while the hydrophilic shell masks the hydrophobic core from the biological milieu and minimizes protein adsorption on micelles and their cellular adhesion. Thus, polymeric micelles have the ability to evade non-specific capture by the reticuloendothelial system. The size of polymeric micelles is above the threshold for filtration by kidneys and, therefore, they can achieve prolonged circulation in blood stream [4] and can accumulate in solid tumors and sites of inflammation because of the enhanced permeability and retention (EPR) effect [5].

Propolis (bee glue) is the generic name for the resinous substance collected by honeybees from various plant sources and used to seal holes in their honeycombs and to protect the entrance against invaders [6]. Propolis has been used by humans since antiquity for treating diseases. The abundance of chemical compounds in propolis provides multiple biological activities that characterize different geographical samples, such as anesthetic, antimicrobial, antioxidant, antiinflammatory and more recently, antiproliferative and antitumor activity [7]. Propolis is currently incorporated into a wide range of complementary health care products, including tinctures, throat sprays, lozenges, toothpastes, soaps, etc. [8]. The application of propolis in medicines and food additives, however, is restricted since most of the important constituents have poor solubility in water [9]. Furthermore, the information about parenteral administration of propolis formulations is limited to a few papers describing usage of only water-soluble fractions of propolis [10–12].

* Corresponding author.
E-mail address: ppetrov@polymer.bas.bg (P.D. Petrov).

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Definitely, the total solubilization of all active lipophilic constituents of propolis in aqueous/biological medium is a prerequisite for effective application in therapies requiring intravenous injection of drug formulations. Very recently, a study assessing the ability of polymeric micelles from different poly(ethylene oxide)-*block*-poly(propylene oxide)-*block*-poly(ethylene oxide) (PEO-*b*-PPO-*b*-PEO) block copolymers to solubilize poplar propolis in aqueous environment was reported by our team [13]. Stable colloid solution of propolis was obtained by using PEO_{26}-b-PPO_{40}-b-PEO_{26} (Pluronic P85) at copolymer/propolis (C/P) mass ratio ≥ 2. Micellar propolis was found to exhibit high *in vitro* cytotoxicity at a relatively low concentration. In this paper, we report on the effective solubilization of poplar propolis by core-shell micelles obtained from PEO-b-PnBA diblock copolymers. PEO-b-PnBA copolymer micelles were selected for their small size, low cytotoxicity and excellent resistance upon severe dilution [14]. It was found that such micelles maintain their core-shell structure at a very low concentration (0.0075 g L^{-1}) for days, contrawise to the behavior of the dynamic PEO-b-PPO-b-PEO systems at similar conditions. The present paper is the first study emphasizing the effects of polymer/propolis mass ratio, polymer composition and concentration on the formation of stable aqueous colloids, based on PEO-b-PnBA micelles containing propolis.

2. Experimental

2.1. Materials

Poly(ethylene glycol) monomethyl ether (CH_3-PEO-OH, molar mass 5000 g mol^{-1}, Fluka) was precipitated in cold methanol (−30 °C), filtered and dried under vacuum at 40 °C overnight. n-Butyl acrylate (nBA, Sigma-Aldrich) were passed through silica column just before polymerization. α-Bromoisobutyryl bromide (BIB, Sigma-Aldrich), triethylamine (TEA, Fluka), 1,1,4,7,10,10-hexamethyltriethylenetetramine (HMTETA; Sigma-Aldrich), copper (I) bromide (CuBr, Sigma-Aldrich), ethyl methyl ketone (Merck), SiO_2 (63–200 μm, Merck), ethanol (Merck), methanol (Merck), tetrahydrofuran (Merck) and 1,4-dioxane (Merck) were used as received. Dichloromethane (DCM, Sigma-Aldrich) was stirred overnight on calcium hydride (Merck) and distilled. Poplar propolis raw material was collected near the town of Elena, Bulgaria.

2.2. Synthesis

2.2.1. Synthesis of PEO macroinitiator

Poly(ethylene glycol) monomethyl ether (3 g, 0.6 mmol) was dissolved in 50 mL of toluene and dried by azeotropic distillation. Then, the dry polymer was dissolved in 50 mL of freshly distilled DCM and TEA (0.25 mL, 1.8 mmol) was added via syringe. Then, α-bromoisobutyryl bromide (0.22 mL, 1.8 mmol) was added dropwise and the reaction mixture was stirred at 750 rpm for 24 h at 20 °C. The reaction mixture was filtered to remove the insoluble hydrobromide salt and then DCM was evaporated. The product was dissolved in THF; the macroinitiator was precipitated in cold methanol (−30 °C), recovered by filtration and dried in vacuum at 40 °C. Yield 80%. ^{1}H NMR ($CDCl_3$, δ ppm): 3.7–3.6 (m, -*O*-C$\underline{H}_2$-C$\underline{H}_2$-*O*-), 3.37 (s, C$\underline{H}_3$-*O*-CH_2-), 1.98–1.92 (m, -C-(C$\underline{H}_3$)$_2$Br).

2.2.2. Synthesis of PEO-b-PnBA diblock copolymers

In a typical example, a single-neck round-bottom flask equipped with a stir bar was charged with PEO_{113}-Br (2.5 g, 0.4854 mmol, 1 equiv.), Cu(I)Br (0.03479 g, 0.2427 mmol, 0.5 equiv.) and ethyl methyl ketone (8.7 mL). After that, the flask was sealed off with a septum, and the reaction mixture was left stirring at ambient temperature under argon flow for 30 min. Then, degassed monomer nBA (17.39 mL, 0.1214 mol, 250 equiv.) and HMTETA ligand (0.06 mL; 0.2427 mmol, 0.5 equiv) were added via syringe. The polymerization was carried out at 70 °C for 16 h. Then, the reaction mixture was allowed to cool down to ambient temperature under exposure to air. Purification was achieved by precipitation of the reaction mixture in cold methanol (−30 °C) and filtration. The copolymer was redissolved in THF and passed through a silica column to remove the Cu(II) catalyst. Finally, THF was evaporated under vacuum; the copolymer was dissolved in 1,4-dioxane and freeze-dried. Yield 76%. ^{1}H NMR ($CDCl_3$, δ ppm): Signals assigned to PEO - 3.7–3.6 (m, -*O*-C$\underline{H}_2$-C$\underline{H}_2$-*O*-), 3.37 (s, C$\underline{H}_3$-*O*-CH_2-); signals of PnBA - 4.2–3.9 (m, O-C$\underline{H}_2$-CH_2-CH_2-CH_3), 2.4–2.2 (m, -CH_2-C$\underline{H}$(C=O)-), 1.95–1.85 and hidden under the other signals between 1.3 and 1.7 (m, -C$\underline{H}_2$-C(C=O)H-), 1.7–1.55(m, O-CH_2-C$\underline{H}_2$-CH_2-CH_3), 1.45–1.3 (m, O-CH_2-CH_2-C$\underline{H}_2$-CH_3), 1.0–0.9 (t, O-CH_2-CH_2-CH_2-C$\underline{H}_3$).

2.3. Propolis

2.3.1. Preparation of propolis solution

Poplar propolis, grated after cooling, was extracted for 24 h with ethanol/water (70/30 v/v) mixture at room temperature (1:10 w/v). The extract was filtered and dried. Finally, 0.327 g of extract was dissolved in 10 mL ethanol/water (70/30 v/v) mixture and used for further experiment.

2.3.2. Determination of total flavone and flavonol content

2 mL of the propolis solution, 20 mL methanol and 1 mL 5% $AlCl_3$ in methanol (w/v) were mixed in a volumetric flask and the volume was made up to 50 mL with methanol. The mixture was left to rest for 30 min and the absorbance at 425 nm was measured. Calibration was performed using galangin as reference compound.

2.3.3. Determination of total flavanone and dihydroflavonol content

1 mL of the propolis solution and 2 mL of 2,4- dinitrophenylhydrazine (DNP) solution (1 g DNP in 2 mL 96% sulfuric acid, diluted to 100 mL with methanol in a volumetric flask) were heated at 50 °C in 2 mL 96% sulfuric acid, diluted to 100 mL with methanol in a volumetric flask were heated at 50 °C for 50 min (water bath). After cooling to room temperature the mixture was diluted to 10 mL with 10% KOH in methanol (w/v). One mL of the resulting solution was added to 10 mL methanol and was diluted to 50 mL with methanol (volumetric flasks). Absorbance was measured at 486 nm. Calibration was performed using pinocembrin as reference compound.

2.3.4. Determination of total phenolic content

1 mL of the propolis solution was transferred to a 50 mL volumetric flask containing 15 mL distilled water, and 4 mL of Folin-Ciocalteu reagent and 6 mL of a 20% sodium carbonate solution (w/v) were added. The volume was made up with distilled water to 50 mL. The sample was left for 2 h and the absorbance at 760 nm was measured. Calibration was performed using a reference mixture of pinocembrin and galangin, in 2:1 (w/w) for calibration.

2.4. Preparation of micelles

2.4.1. Preparation of propolis–loaded micelles

Poplar propolis (15 mg), dissolved in ethanol/water (70/30 v/v) mixture, were added to 15 mL solutions of PEO-b-PnBA block copolymers (7.5–60 mg) in ethanol. Then, 5 mL of distilled water were added and ethanol was removed from the systems by rotary evaporator under reduced pressure at 40 °C. Following this procedure, three series of samples containing block copolymer and propolis at five different mass ratios: 0.5, 1, 2, 3 and 4 at constant propolis concentration (3 g L^{-1}) were prepared.

2.4.2. Preparation of blank micelles

PEO-b-PnBA block copolymer (30 mg) was dissolved in 15 mL of ethanol; 5 mL of distilled water were added and ethanol was removed from the systems by rotary evaporator under reduced pressure at 40 °C.

2.5. Measurements

^{1}H NMR spectra were recorded on a Bruker Avance II+ 600 MHz apparatus at room temperature in $CDCl_3$. Size exclusion chromatography (SEC) analysis of block copolymers was performed on a Shimadzu Nexera HPLC chromatograph equipped with a degasser, a pump, an autosampler, a RI detector and three columns: 10 μm PL gel mixed-B, 5 μm PL gel 500 Å and 50 Å. THF was used as the eluent at flow rate 1.0 mL min^{-1} and temperature 40 °C. Sample concentration was 1 g L^{-1} and SEC was calibrated with polystyrene standards. Dynamic and electrophoretic light scattering measurements were performed on a Zetasizer NanoBrook 90Plus Zeta (Brookhaven Instruments Corp.), equipped with a 35 mW red diode laser (λ = 640 nm), at a scattering angle of 90° and temperature of 25 °C without filtration. The ζ potentials were calculated from the obtained electrophoretic mobility. Turbidity of propolis-loaded micelles in aqueous media was recorded using a DU 800 spectrophotometer (Beckman Coulter) at a wavelength of 500 nm at 25 °C. Atomic force microscopy images were obtained with a Bruker Dimension Icon microscope operating in tapping, using microfabricated silicon cantilevers with a spring constant of ~42 N m^{-1}. The image visualization was processed with Nanoscope Analysis v1.5 software. Micellar solutions (0.5 g L^{-1}) were spin coated at 4000 rpm onto freshly cleaned glass substrates. GC–MS analysis was performed with a Hewlett–Packard gas chromatograph 5890 series II Plus linked to a Hewlett–Packard 5972 mass spectrometer system equipped with a 30 m long, 0.25 mm i.d., and 0.5 μm film thickness DB-17HT capillary column. The temperature was programmed from 80 to 320 °C at a rate of 5 °C min^{-1}, and a 10 min hold at 320 °C. Helium was used as a carrier gas at a flow rate of 0.8 mL min^{-1}. The split ratio was 1:10, the injector temperature 280 °C, the interface temperature 300 °C, and the ionisation voltage 70 eV. The identification of the compounds was performed using commercial libraries and comparison of mass spectra and retention times of reference compounds. Semiquantification was carried out by internal normalization with the area of each compound. The addition of individual areas of the compounds corresponds to 100% area.

3. Results and discussion

3.1. Synthesis and characterization of diblock copolymers

A series of poly(ethylene oxide)-*b*-poly(n-butyl acrylate) diblock copolymers was obtained by a synthetic procedure involving preparation of a monofunctional PEO-based macroinitiator and subsequent atom transfer radical polymerization of n-butyl acrylate, as described elsewhere [15]. The ATRP macroinitiator, PEO functionalized bromoisobutyrate (PEO_{113}-Br), was synthesized by reacting commercial CH_3-PEO_{113}-OH with α-bromoisobutyryl bromide in DCM in the presence of TEA. Next, the polymerization of n-butyl acrylate, initiated by PEO_{113}-Br, was carried out in ethyl methyl ketone in the presence of the CuBr/HMTETA catalyst system. The experimental conditions are listed in Table 1.

Both the reaction time and monomer/initiator molar ratio were varied to obtain PnBA blocks of different chain length. In all cases the polymerization of nBA was terminated at a relatively low monomer conversion in order to avoid chain-chain coupling reactions. The purified PEO-PnBA diblock copolymers were analyzed by size exclusion chromatography and proton NMR. SEC chromatograms (Fig. 1) show monomodal molar mass distribution and fairly low dispersity of all polymers studied (Table 2). The peaks of block copolymers are shifted to different extent to lower elution volume as compared to the traces of PEO macroinitiator, which is a direct evidence for extension of PEO chains with PnBA blocks of three different chain lengths. SEC data also indicate a very high efficiency of the PEO_{113}-Br macroinitiator.

Table 1
Experimental conditions for the synthesis of PEO–PnBA diblock copolymers.

Target composition	[M]:[I]:Cu(I):[L]	Reaction temperature (°C)	Reaction time (h)	nBA conversion $^{1H-NMR}$ (%)
PEO_{113}-b-$PnBA_{50}$	250: 1: 0.5: 0.5	70	16	21
PEO_{113}-b-$PnBA_{100}$	325: 1: 0.5: 0.5	70	20	30
PEO_{113}-b-$PnBA_{150}$	425: 1: 0.5: 0.5	70	23	37

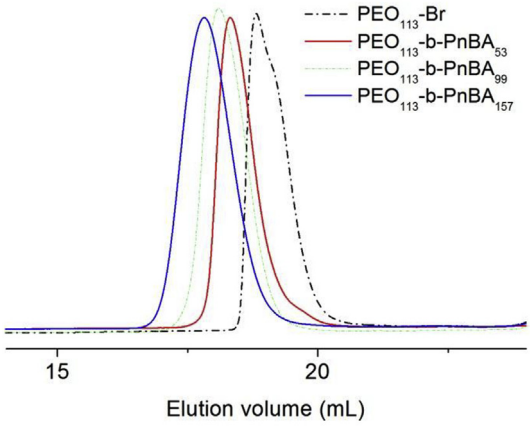


Fig. 1. SEC chromatograms of PEO-Br macroinitiator and PEO-b-PnBA diblock copolymers.

Proton NMR analysis was employed to determine the number-average degree of polymerization (DP_n) of nBA and the copolymer molar mass, respectively (Table 2). DP_n of PnBA was calculated by comparing the integrals assigned to the methylene protons of PEO at 3.65 ppm to the methylene protons of PnBA at 4.03 ppm, assuming that the peak integral of PEO at 3.65 ppm corresponds to 452 protons (Fig. 2).

The fact that the calculated DP_n values of nBA were very close to the target ones, in combination with the narrow molar mass distribution of block copolymers, is a proof for good control over the polymerization of nBA and formation of copolymers of well-defined composition and structure.

3.2. Propolis

Poplar propolis, extracted from the row bee product with ethanol/water (70/30 v/v) mixture, is a dark brown resinous solid.

Table 2
Molecular characteristics of PEO-Br macroinitiator and PEO-b-PnBA diblock copolymers.

Copolymer composition $^{1H\ NMR}$	Mn $^{1H\ NMR}$ (g mol^{-1})	Mn ^{GPC} (g mol^{-1})	Dispersity^{GPC}
PEO_{113}-Br	5150	4250	1.09
PEO_{113}-b-$PnBA_{53}$	11 800	6980	1.16
PEO_{113}-b-$PnBA_{99}$	17 700	9140	1.18
PEO_{113}-b-$PnBA_{157}$	25 100	12 500	1.31

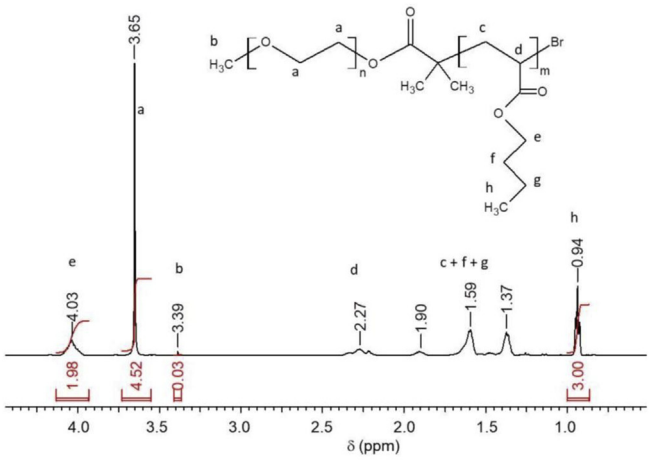


Fig. 2. Proton NMR spectrum of PEO_{113}-b-$PnBA_{99}$ diblock copolymer in $CDCl_3$.

The extract is composed of over 50 individual compounds, as confirmed by GC-MS analysis (Table 3) [13]. The percentage of the components was determined by internal normalization, considering their areas as percentage of the total ion current. Major constituents of the extract were the flavonoids chrysin, galangin, pinobanksin acetate, pinocembrin, pinobanksin, and the esters benzyl caffeate and phenethyl caffeate (CAPE). It should be mentioned that the ion current generated depends on the characteristics of the individual compound and is not a true quantification.

The quantity of the major bioactive constituents of propolis extract was further determined by means of UV-vis absorption measurements. The amount of total phenolics, total flavones/flavonols and total flavanones/dihydroflavonols was 65.3, 16.3 and 12.2%, respectively. It is important to note that investigations on individual propolis constituents showed that the total extract has a greater activity than any single propolis component [16,17]. Therefore, propolis has general pharmacological value as a natural mixture and finding ways to prepare water-soluble form containing all the important bioactive constituents of propolis is an important task.

3.3. Preparation of blank and propolis-loaded micelles

Amphiphilic block copolymers composed of a hydrophilic PEO block and a hydrophobic PnBA block are able to form various aggregates by self-assembly in aqueous media, depending on copolymer composition and experimental conditions. In a previous study, we discovered that nano-sized core-shell micelles can be readily obtained from copolymers comprising up to 223 nBA units at a fixed PEO length (MW 5000 g mol^{-1}) [14]. In this study, propolis-loaded and blank micelles based on PEO_{113}-b-$PnBA_m$ (m = 53, 99 or 157) diblock copolymers were prepared by the so-called solvent evaporation method. Copolymers, dissolved in ethanol, and propolis, dissolved in ethanol/water (70/30 v/v) mixture, were mixed at different mass ratio (0.5; 1; 2; 3; 4) under stirring at 25 °C. Then, water was added and ethanol was removed from the system by rotary evaporator, yielding three series of yellowish aqueous colloids ($C_{propois}$ = 3 g L^{-1}). Blank polymeric micelles (without propolis) were prepared by the same method. The colloid solutions obtained were studied by turbidity measurements, dynamic light scattering and atomic force microscopy.

3.4. Effect of copolymer/propolis mass ratio

The samples obtained at C/P mass ratio ≥2 were slightly opalescent colloids, while those containing the smallest amount of polymer were completely opaque. Turbidity measurements of micellar suspension revealed a sharp increase of transmittance with the increase of C/P ratio from 0.5 to 2 in the three copolymer series. After that, a constant transmittance, depending on copolymer composition, was reached (Fig. 3).

Next, we monitored the colloid stability of the systems with time. All samples obtained at C/P mass ratio ≥2 exhibited excellent stability without any visible precipitation for a period of 40 days. In the most turbid suspensions (C/P 0.5), a significant portion of

Table 3
Chemical composition of ethanol extract of poplar propolis [13].

Flavanones and dihydroflavonols (37.3*)	Flavones and flavonols (22.6*)	Phenolic acid esters (13.0*)	Aromatic acids (6.6*)	Chalcones (1.6*)	Others (4*)
Pinobanksin (5.2)	Galangin (8.2)	Pentenyl coumarate (0.1)	Benzoic acid (0.6)	Pinocembrin chalcone (1.0)	Phenylethyl alcohol (0.1)
Pinocembrin (10.4)	Chrysin (7.6)	Pentenyl coumarate (isomer) (0.2)	Cinnamic acid (0.1)	Trihydroxymonomethoxy chalcone *m/z* = 502 (0.2)	Hexadecanoic acid (0.1)
Sakuranetin (0.2)	Dihydroxymonomethoxy flavone (0.8)	3-Methyl-3-butenyl ferulate (0.1)	*p*-Hydroxybenzoic acid (0.1)	Pinobanksinacetate chalcone (0.4)	Sugars (3.8)
Dihydroxymethoxyflavanone (2.1)	Kaempferol (0.9)	3-Methyl-3-butenyl caffeate (1.5)	*p*-Methoxycinnamic acid (0.2)		
3-Acetylpino-banksin (14.4)	Quercetin (1.1)	2-Methyl-2-butenyl ferulate (0.1)	*p*-Coumaric acid (0.6)		
Pinobanksin butanoate (0.7)	Kaempferol methyl ether (1.3)	3-Methyl-2-butenyl ferulate (0.9)	Dimethoxycinnamic acid (1.1)		
Pinobanksin propanoate (1.6)	Quercetin-methyl ether (1.2)	2-Methyl-2-butenyl caffeate (0.6)	Ferulic acid (Z) (0.8)		
Pinobanksin pentanoate (1.8)	Quercetin-methyl ether (isomere) (0.7)	3-Methyl-2-butenyl caffeate (1.9)	Ferulic acid (E) (0.8)		
Alpinone (0.2)	Quercetin-methyl ether (isomere) (0.6)	Phenylethyl *p*-coumarate (0.1)	Caffeic acid (Z) (0.2)		
Pinobanksin pentenoate (0.7)	Dihydroxymonomethoxy flavone (0.2)	Benzyl caffeate (2.3)	Caffeic acid (E) (2.1)		
		Benzyl ferulate (0.2)			
		Cinnamyl caffeate (1.3)			
		Phenethyl caffeate (CAPE) (3.7)			

*% of total ion current.

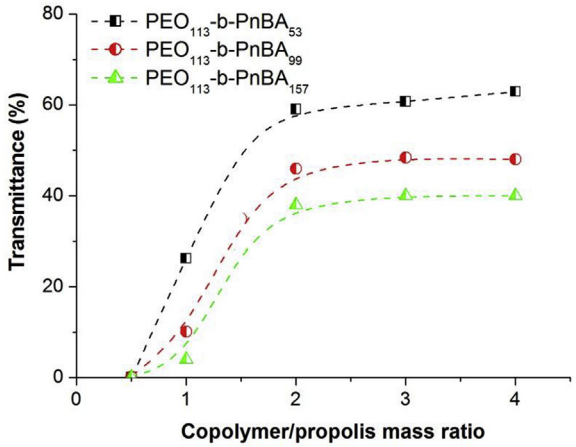


Fig. 3. Turbidity measurements of colloid systems based on different PEO-b-PnBA diblock copolymers and propolis.

material precipitated after 2 days of storage, similarly to the behavior of propolis dispersed in water without any copolymer. In the samples containing equal amounts of copolymer and propolis (C/P 1) relatively small but still visible precipitate was detected.

DLS measurements of blank and propolis-loaded PEO-b-PnBA micelles (C/P mass ratio from 1 to 4) were performed at 25 °C at a scattering angle 90°. The concentration of propolis was kept constant (3 g L-1), while copolymer amount was varied in the 3–12 g L^{-1} concentration range. The samples were studied without filtration. In the case of blank polymeric micelles, a bimodal distribution of the intensity-weighted hydrodynamic diameter (D_h), obtained by cumulant analysis of the autocorrelation function, was registered. Fig. 4A (black line) illustrates a typical plot of PEO_{113}-b-$PnBA_{53}$ micellar solution. The dominant peak, detected at a fast relaxation time, corresponds to micellar species with $D_h = 33 \pm 2$ nm, while the less intensive peak at a low relaxation time indicates existence of a second population of larger aggregates ($D_h = 197 \pm 15$ nm). Considering that a larger particle scatters light much more intensely than smaller one, and taking into account the area of the two peaks, the contribution of the second population can be practically neglected. This assumption was further supported by the fact that only one peak appears in the volume-weighted size distribution plot (Fig. 4A, red line), overlapping the position of the dominant one from the intensity-weighted plot. Similar plots were obtained for PEO_{113}-b-$PnBA_{99}$ and PEO_{113}-b-$PnBA_{157}$ -based systems, confirming formation of micelles with hydrodynamic dimeter of 43 ± 2 and 51 ± 3 nm, respectively. The presence of a second peak in intensity-weighted plots, however, obstructed the correct calculation of dispersity values.

DLS measurement of propolis-loaded micelles showed identical results as compared to the blank micelles, except the samples prepared at C/P mass ratio 1 (Fig. 4B and C). Indeed, the hydrodynamic diameters of micelles formed at C/P mass ratio 2, 3 and 4 were very close to each other and did not differ significantly from D_h of the analogous blank micelles (Fig. 5). In contact, the position of the dominant (micelles) and second (larger aggregates) peaks of the samples obtained at C/P mass ratio 1 were shifted to higher diameters, and the intensity of the low relaxation time peak were increased. This tendency was observed for all polymer systems studied.

Both DLS and turbidity measurement showed a strong influence of C/P mass ratio on the colloidal stability of the systems and the formation of PEO-b-PnBA micelles, respectively. Two different regions can be defined. Below C/P ratio 2, the polymeric micelles are not able to solubilize the whole amount of propolis. Although, all copolymers studied tended to form larger micelles in the presence of propolis as compared to the blank micelles, the coexistence of particles composed of pure propolis should be considered. The later are not sterically stabilized and, therefore, aggregate and

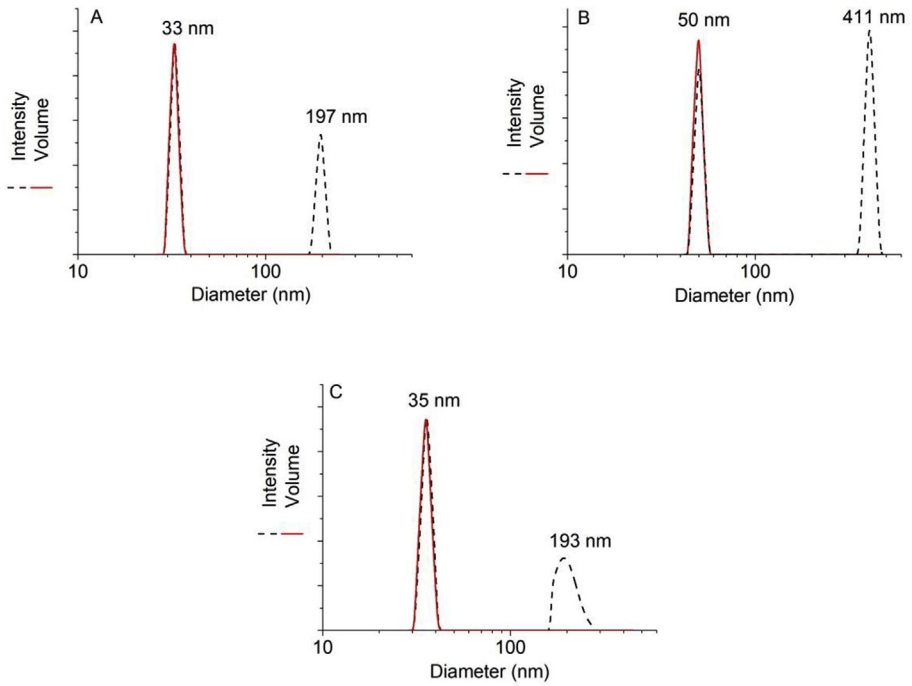


Fig. 4. Hydrodynamic diameter distribution of blank PEO_{113}-b-$PnBA_{53}$ micelles (A) and propolis-loaded PEO_{113}-b-$PnBA_{53}$ micelles formed at copolymer/propolis mass ratio 1 (B) and 2 (C).

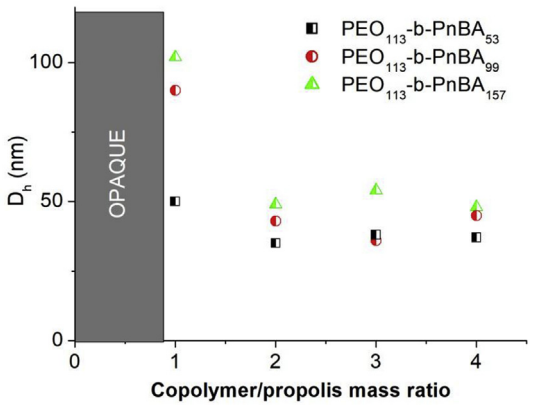


Fig. 5. Effect of copolymer/propolis mass ratio on the hydrodynamic diameter of micelles.

precipitate with time. At twofold copolymer excess the colloid systems became stable and predominantly small-size micelles were detected. Since the size of these particles is practically equal to the size of blank micelles, one may assume that propolis is completely solubilized by the micellar cores. In addition, zeta potential of both propolis-loaded micelles (- 7.56 ± 2.17 mV) and blank micelles (- 5.94 ± 2.50 mV) was identical, while the zeta potential of propolis particles without polymer was - 27.95 ± 3.12 mV. Consequently, one may conclude that PEO-b-PnBA carriers can load all biologically active lipophilic propolis constituents at C/P mass ratio 2, emphasizing a high loading capacity (*ca.* 33 wt% content of active species) of the formulations developed in this work.

3.5. Effects of copolymer composition and concertation

A pronounced effect of copolymer composition on the size of PEO-b-PnBA micelles was found. Noteworthy, in this study the molar mass of shell-forming PEO block was kept constant (5000 g mol^{-1}), while, the hydrophobic core-forming PnBA block was varied from 53 to 157 repeating units. It is evident from DLS data that D_h increased proportionally to the increase of PnBA length. The overall growth of micelles then should be attributed to enlargement of micellar cores rather than shell thickness. Considering that propolis constituents are predominantly loaded into the micellar cores, the relative loading capacity of each individual carrier was enhanced by increasing the volume of hydrophobic compartments.

One question of interest is whether propolis-loaded PEO-b-PnBA micelles can maintain their integrity upon dilution. Hence, a sample of propolis-loaded PEO_{113}-b-$PnBA_{99}$ micelles (C/P 2, copolymer concentration = 6 g L^{-1}) was diluted to 0.5 and 0.01 g L^{-1}, respectively. Fig. 6 shows plots of autocorrelation function and hydrodynamic diameter distribution measured 24 h after preparation of the two solutions. Definitely, DLS results revealed existence of polymeric micelles without any indication for notable dissociation even at concentration of 0.01 g L^{-1}. D_h values completely coincided with that of original sample, which is a proof for an extraordinary structural stability of the micelles upon rigorous dilution. Such behavior is distinctive for polymeric micelles comprising PnBA cores, which are characterized by extremely low critical micelle concentration (cmc) [18]. This phenomenon is attributed to the high interfacial tension between the hydrophobic PnBA cores and aqueous environment, determining behavior typical for frozen aggregates.

3.6. Morphology of propolis-loaded micelles

The morphology of developed formulations was visualized by atomic force microscopy. Fig. 7A shows representative height image of PEO_{113}-b-$PnBA_{99}$/propolis colloid system (C/P 4), deposited on a glass substrate by spin-coating. Uniform spherical particles with narrow size distribution are the dominant population observed on the studied specimens. The average diameter of these particles is in the range of 40 ± 6 nm, which is in a good agreement with DLS results. For comparison, a sample based on pure propolis, prepared by the same experimental procedure, was also investigated by AFM (Fig. 7B). Although the analysis was hindered by the sticky nature of propolis, one can clearly differentiate clusters composed of smaller particles of different size. The average diameter of clusters was 165 ± 15 nm. DLS analysis of aqueous suspension of propolis (sample was prepared at an initial concentration of 3 g L^{-1}, diluted to 0.1 g L^{-1} and immediately measured) revealed a dominant population of particles with similar size (D_h = 196 ± 20 nm). Obviously, the evaporation of ethanol triggered precipitation of propolis, resulting in formation of sub-micron clusters in water. Completely different situation should be considered when an amphiphilic copolymer is present in the system. In this case, the self-assembly process leads to formation of hydrophobic domains that attract the lipophilic propolis constituents. Thus, due to hydrophobic interactions, all lipophilic molecules can be accommodated within polymer carriers at specified conditions. The size of block copolymer carriers can be controlled to certain extend by playing with copolymer composition.

4. Conclusions

Amphiphilic PEO-b-PnBA diblock copolymers were successfully applied for preparing spherical propolis-loaded polymeric micelles

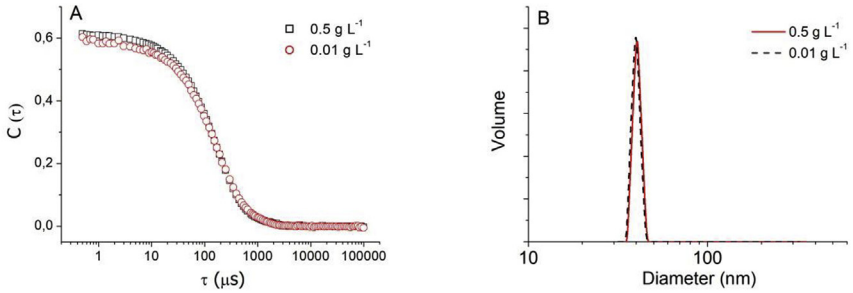


Fig. 6. Autocorrelation function plot (A) and hydrodynamic diameter distribution (B) of propolis-loaded PEO_{113}-b-$PnBA_{99}$ micelles in water at different concentrations (measurements were performed 24 h after sample preparation).

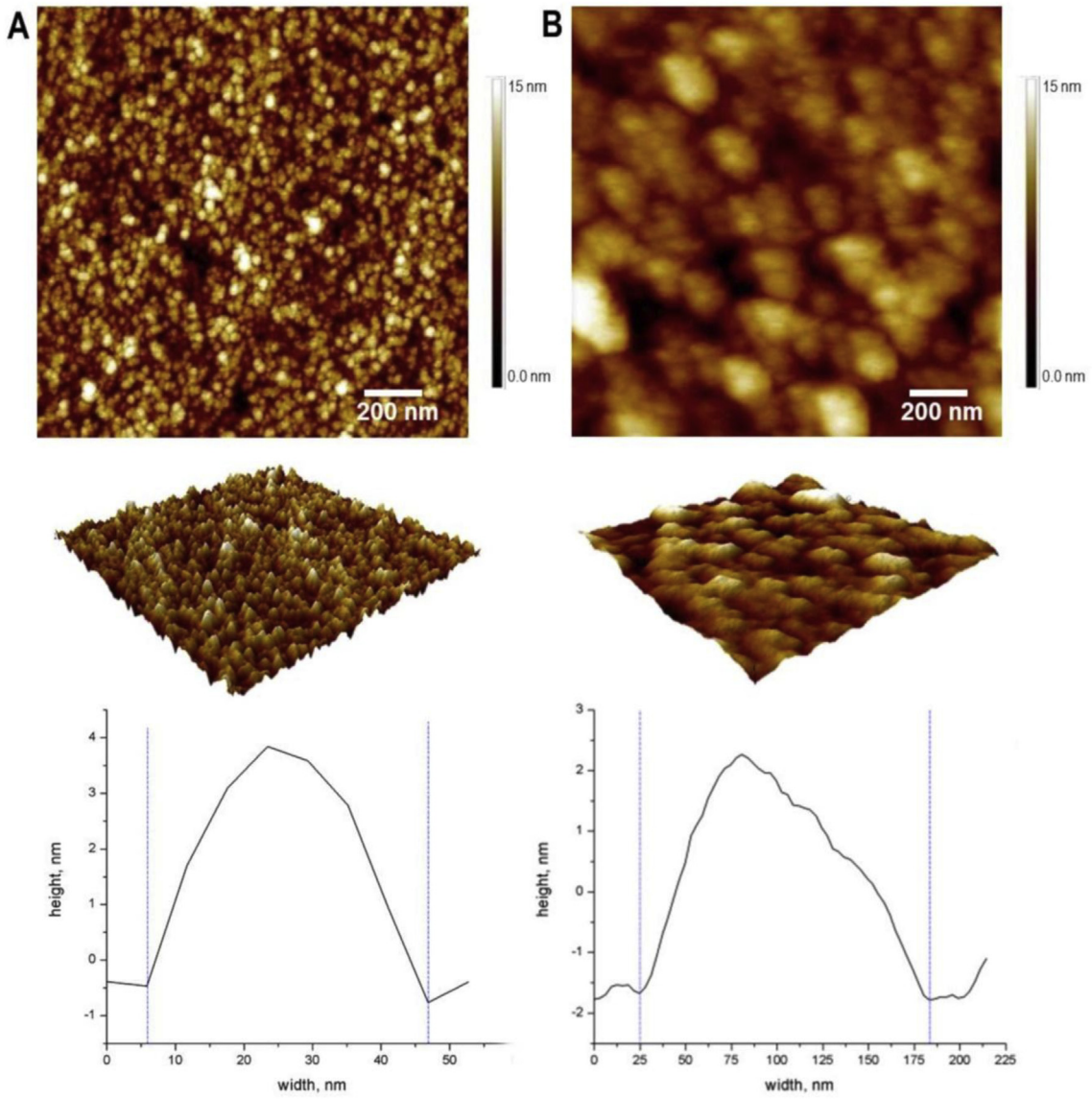


Fig. 7. Tapping mode 2D and 3D AFM height images of propolis-loaded PEO_{113}-b-PBA_{99} micelles (A) and propolis particles (B) deposited on a glass substrate from 0.5 g L^{-1} aqueous solution by spin-coating; Bottom – cross-section through one of the micelles (A) and clusters (B).

in aqueous media. Stable, slightly opalescent colloid solutions were obtained at a copolymer/propolis mass ratio $\geq$2 for the three copolymer compositions studied. The very similar D_h of blank and propolis-loaded micelles and the absence of any precipitate in solutions indicated for a complete loading of all biologically active lipophilic propolis constituents into the micellar cores. The relatively large amount of encapsulated propolis with respect to copolymer revealed a high loading capacity of the carriers. The size of PEO-b-PnBA micelles was dependent on the copolymer composition. D_h increased proportionally to the increase of PnBA chain length from 53 to 157 monomer units, at a constant PEO length of 113 units. Propolis-loaded micelles exhibited a superior structural stability upon rigorous dilution and maintained their morphology at a concentration as low as 0.01 g L^{-1}. Consequently, PEO-b-PnBA micelles, due to numerous favorable characteristics like nanoscopic size, good colloid stability, high loading capacity and resistance to fast dissociation at very low concentrations, have great potential for application as long-circulating carriers of propolis in biomedicine via parenteral administration.

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