**Functional micelles formed by branched polymeric surfactants:
Synthesis, characteristics, and application as nanoreactors and carriers**

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Abstract Branched polymeric surfactants composed of grafted non-ionic polyethylene glycol (PEG) and anionic polyelectrolyte chains were synthesized via radical polymerization initiated by the comb-like PEG-containing polyperoxide. Above definite concentration in solution, these surfactants form micelle-like structures (MLS). The MLS formed by branched polymeric molecules are of larger size in comparison with size of MLS formed by the initial PEG-containing polyperoxide that is caused mainly by different mechanism of their self-organization and morphology of formed MLS. The availability of grafted polyelectrolyte chains in the MLS provides a possibility of their use as the containers for immobilization of bio-active substances and nucleation of the inorganic nanoparticles, as well as formation of their stable colloidal systems in water in a wide pH range. Noticeable compaction and narrowed size distribution of the MLS were revealed after immobilization of doxorubicin (Dox) molecules or Fe₂O₃ nanocrystals. MLS-based systems were used for delivery of Dox and maghemite particles at treatment of tumor cells. Both MLS-based formulations of Dox and Fe₂O₃ were efficiently engulfed by rat glioma C6 cells. A significant (10 times) decrease in the effective therapeutic dose of Dox was found when this drug was delivered by a MLS-based formulation of Dox. That effect might be explained by a specific structure and functionality of the novel carrier used for immobilization of drug that should be delivered to target cells.

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Introduction

In spite of successful synthesis of a variety of functional polymers, the creation of new polymeric surfactants and derived magnetic nanoparticles is still a topical task for the development of novel non-toxic organ targeted drug carriers and delivery systems for application in medicine and biotechnology. Thus, tailored syntheses of micelle-forming polymeric surfactants, as well as their use as carriers for drug delivery and for the nucleation of functionalized nanoparticles, are of high interest for researchers engaged in the creation and study of the formulations for chemo- and hyperthermia therapies used for tumor treatment.

During last years, numerous functional polymeric surfactants of linear, block or branched type and other desired architecture aimed at creation of micelle-like drug carriers and synthesis and functionalization of the nanoparticles of biomedical applications were synthesized and studied [1-3]. Particularly, the comb-like and branched water-soluble surface-active copolymers and derived micelles possessing high solubilization ability were developed [4].

Recently, several publications devoted to tailored synthesis of polymeric surfactants, as well as their application as carriers of bioactive substances [5-10] and soft templates for nucleation and functionalization of micro- and nanoparticles, appeared. These are amphiphilic polymers of linear, block, comb-like, star-like and dendritic structures that are capable of immobilizing drugs, particularly water insoluble ones [11-19], and can be used in biology and medicine.

Three main approaches for synthesis comb-like copolymers were used [20-23]: 1) direct grafting side polymeric branches to a backbone via polymer analogous transformation; 2) copolymerization of the macromers with conventional functional monomers; 3) “grafting from” via radical polymerization initiated by the multi-site polymeric initiators containing peroxide or azo- side groups disposed along the backbone.

To obtain the comb-like copolymers via the method of «grafting from», backbone polymers containing reactive side sites along a chain are synthesized first [23-25Error! Bookmark not defined.]. At present, the ATRP technique based on using the polymeric macroinitiators for graft-copolymerization is more often applied [26,27].

The methods of obtaining amphiphilic comb-like copolymers based on water-soluble backbone and grafted side hydrophobic polymeric chains and vice versa the synthesis of similar polymeric surfactants consisting of water insoluble backbone and grafted hydrophilic chains were developed [17,28,29]. Grafted chains can be constructed from the identical [30] or distinct [31] monomer links, and they can also contain grafted polysaccharide molecules [32]. To increase water solubility of the comb-like polymeric surfactants, hydrophilic, for instance, quaternary ammonium salt [33] or polyethylene glycol (PEG) [34] fragments are included into the grafted side chains. Such amphiphilic polymers form in water solution micelle-like structures

(MLS) [28]-or vesicles [35]. The architecture and functionality of grafted side branches, as well as the grafting degree cause a direct influence on the parameters and morphology of self-organized colloidal structures formed by the molecules of functional comb-like polymeric surfactants in solution [17,28,32,36,37].

New surface-active functional polyelectrolytes containing side peroxide fragments were successfully used for synthesis of the polymeric surfactants of branched structure. Many functional polymeric and inorganic nano- and microparticles were synthesized and tested for their potential biomedical application [25,38-42]. Previously, we have developed synthesis of comb-like PEG-containing polymers with side peroxide groups and tested them *in vitro* and *in vivo* for tumor chemotherapy [43,44]. Branched polymeric surfactants with the anionic polyelectrolyte chains and PEG were studied as thermo- and pH-sensitive drug carriers that can deliver drugs to the target organ and release them under the influence of the microenvironment conditions.

The main goal of this study was developing tailored synthesis of branched polymeric surfactants combining the non-ionic PEGylated and the anionic polyelectrolyte branches, and colloidal-chemical characterization of the obtained novel micelle-like structures. Big advantage of these structures, comparing with other similar created structures, is based on their universal capability to serve both as carriers for the organic molecules (ex. drugs) and containers for the nanoscale particles (ex. Fe₂O₃ nanocrystals). Other potential applications of these bifunctional micelle-like structures are discussed. An existence of stable colloidal systems and their low toxicity for the mammalian cells are additional features that are of great value at the biomedical use of the created materials.

Experimental

Synthesis.

Materials. The peroxide monomer 5-*tert*butylperoxy-5-methyl-1-hexen-3-yne (PM) [CH₂=CH-C≡C-C(CH₃)₂-O-O-C(CH₃)₃] was synthesized from the dimethylvinylethynyl carbinol (2-methylhex-5-en-3-yn-2-ol) [CH₂=CH-C≡C-C(CH₃)₂-OH] (0.23 mol, 96g) (Nairit Plant CJSC, Armenia) and *tert*-butyl hydroperoxide [HO-O-C(CH₃)₃] (0.24 mol, 118g) (70% in H₂O, AkzoNobel, Netherlands) was purified by vacuum distillation and characterized by: [O]=8.7%, d₄²⁰=0.867 g/ml, n_d²⁰= 1.4482 [45]. N-vinyl-2-pyrrolidone (NVP) (Merck) and acrylic acid (AA) (Merck) were purified by distillation under vacuum. Iron (II) chloride (FeCl₂×4H₂O) (99%, ABCR, Germany) and iron (III) chloride (FeCl₃×6H₂O) (99%, ABCR, Germany) were used as received. Doxorubicin (Dox) was purchased from Arterium (Ukraine). The solvents were used after additional purification [46]. Aqueous ammonia solution (25%) (Aldrich),

hydrogen peroxide (35 wt.% solution in water, stabilized) (Fluka) were obtained from ACROS Organics.

Synthesis of poly(PM-co-GMA)-graft-PEG macroinitiator was carried out via subsequent stages as ref. [43,44,47].

1. Preparation of the PM-GMA Copolymer was carried out as ref. [48]. 5-tert butylperoxy-5-methyl-1-hexene-3-yne (PM, 0.41 g, 0.5 mol) and glycidyl methacrylate (GMA, 7.72 g, 12.2 mol) (Aldrich) were dissolved in ethyl acetate (7.9 ml) and azoisobutyronitrile (AIBN, 0.129 g, 0.05 mol) (Merck) was added as radical initiator. Polymerization was carried out at 343K under argon atmosphere until a monomer conversion of 65% was reached. The resulting solution was concentrated and purified from residual monomers by triple precipitation into hexane. The obtained PM-GMA copolymer was dried in vacuum until a constant weight. After ~48 h the yield was 5.0 g. Elemental analyses: C%, 60.19; H%, 7.22. M_n : 25,000 g/mol. Copolymer composition (mol%): 3.9% PM, 96.1% GMA. Average length of the block: $l_{PM} = 1$, $l_{GMA} = 22$.

2. Preparation of the PEGylated Polymer Macroinitiator (PMI; Fig.1)

Poly(ethylene glycol) methyl ether (2.5 mg, 3.35 mmol) dissolved in the dioxane (15 ml) and boron trifluoride etherate (0.027 ml, 0.031 mol) were added to the solution of PM-GMA copolymer (1.0 g) in the dioxane (20 ml) at 313 K and stirred for 3 h until a conversion of 60% of the GMA units was observed. The resulting solution was concentrated and precipitated into hexane. The product was washed with toluene and subsequently dried at room temperature in vacuum until a constant weight was reached (~24 h). Yield: 2.6 g. M_n : 94,000 g/mol.

Fig. 1. The scheme of the poly(PM-co-GMA)-graft-PEG macroinitiator structure $l=4.0\%$ mol; $m=96.0\%$ mol; $M_n \sim 94,000$ g/mol, $[\eta]=0.61$ l/g

Structure of the poly(PM-co-GMA)-graft-PEG in the DMSO- d^6 was confirmed by 1H NMR. In the 1H NMR: the polymeric skeletal $\underline{CH_2}$ at 1.66 ppm, skeletal $\underline{CH}$ at 3.64 ppm (signal as a shoulder); the GMA-graft-PEG fragments units demonstrated the following signals: $\underline{CH_3}$ -C-C(O)-O- at 1.12 ppm (signal as a shoulder), $\underline{CH_3}$ -O-CH₂-CH₂- at 3.24 ppm, - $\underline{CH_2}$ - $\underline{CH_2}$ -O- at 3.35 – 3.65 ppm; -O- $\underline{CH_2}$ -CH(OH)- at 4.67 ppm; the PM fragments units demonstrated the following signals: ($\underline{CH_3}$)₃-C-O-O(CH₃)₂-C- at 1.18 ppm, (CH₃)₃-C-O-O($\underline{CH_3}$)₂-C- at 1.36 ppm.

Synthesis of the poly(PM-co-GMA)-graft-PEG-graft-poly(NVP-co-AA) branched polymeric surfactant (BPS) was carried out by solution copolymerization of the NVP and AA initiated by the poly(PM-co-GMA)-graft-PEG in accordance with the scheme presented on the Fig. 2.

Definite amounts of mixture NVP and AA (Table 1) were added to the solution of poly(PM-co-GMA)-graft-PEG in the dioxane. The mixture was stirred at 353K for 10 h under argon atmosphere, the resulting poly(PM-co-GMA)-graft-PEG-graft-poly(NVP-co-AA) was triply precipitated from the solution in acetone into hexane and dried for 8 h under vacuum at 313K until the constant weight.

Preparation of the Doxorubicin Delivery System. Doxorubicin (Dox) delivery systems were prepared as described earlier [44]. Briefly, solution of 100 mg/ml poly(PM-co-GMA)-graft-PEG-graft-poly(NVP-co-AA) was prepared in DMSO. Then 3.0 mg of Dox were dissolved in 0.5 ml DMSO. The resulting solutions of Dox and polymer were mixed and added drop by drop to 8.5 ml water containing 0.9% NaCl. Finally, the solution was stirred for 1 hour and then sonicated for 20 seconds. The 30-fold excess of the polymer was used to ensure the maximum loading of Dox in the MLS.

Synthesis of functional maghemite/polymer composites. Maghemite particles $\gamma\text{-Fe}_2\text{O}_3$ were synthesized via mixing of solutions of $\text{FeCl}_2 \times 4\text{H}_2\text{O}$ (2.12g, 7.8 mmol) and $\text{FeCl}_3 \times 6\text{H}_2\text{O}$ (0.77g, 3.9 mmol) in the presence of BPS as a soft template in ammonium hydroxide water solution, according to the following reaction [49]:

Molar ratio of reagents was $\text{Fe}^{3+}:\text{Fe}^{2+} = 2:3$ (taking into account Fe^{2+} oxidation during solution preparation under the aerobic conditions). The amount of ammonium hydroxide (8.8 mmol, 5.9g 25% in H_2O), greater than the stoichiometric one, was applied in order to maintain solution pH > 11 [50]. Water solution of the BPS template-modifier (0.95g, concentration ~1% in the aqueous phase) was charged into the flask with iron salts and then the solution of ammonia was added. The process was carried out in a three-necked flask, equipped with a paddle stirrer and a backflow condenser at 363 K for 6 h. Then 1 M solution of HCl was added to the reaction medium to lower pH of solution to neutral, whereas the synthesized Fe_3O_4 particles were oxidized into $\gamma\text{-Fe}_2\text{O}_3$ using 10% H_2O_2 solution (0.96 g 30% in H_2O), and then washed with distilled water until the filtrate conductivity remained constant. The maghemite particles were coated with the BPS shell and kept in a form of water suspension.

Characterization

Determination of polymer composition. The content of AA in the copolymers was determined by a reverse potentiometric titration. Approximately 0.02 g of polymer was weighed accurate within 0.0002 g, dissolved in 5 ml of methanol and titrated with 0.05 N HCl solution using laboratory pH meter PH500 (Mortek Ltd, UK). The content of the NVP links was calculated from the nitrogen content estimated by the Mico–Kijedldahl's method [51].

Determination of Dox content in MLS. For determination of loaded Dox content in the **MLS (1 ml) MLS based delivery systems were dialyzed** using a dialysis membrane with a molecular weight cut-off of 2,000 against 25 ml of 0.9% NaCl buffer for 5 min at 25 °C. Then the absorbance of the buffer at $\lambda_{\text{max}}=250$ nm was measured using NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, USA). The Dox amount in buffer was calculated using calibration curve obtained by preparing Dox solutions in 0.9% NaCl with different concentrations. For calculation of the Dox content in the MLS, a kinetic method of the Dox release using UV-based measurement through dialysis membrane was used. On the basis of this analysis, the content of Dox in the micelles was $C_{\text{Dox loaded}} - C_{\text{Dox released}}$, where $C_{\text{Dox loaded}}$ is the amount of loaded Dox and $C_{\text{Dox released}}$ is the amount of Dox determined in the assay after 5 min of its release. The content of the Dox released was in the range $5 \pm 2.5\%$ from the loaded content.

Surface tension measurements. Surface tension of the polymer surfactant solutions was studied by using the method of measurement of maximum bubble pressure [52] on Sensa Dyne bubble pressure tensiometer QC3000 (Gardco, USA). The device was calibrated using bidistilled water before the measurements. Polymer solutions of different concentrations were prepared and kept for at least 24 h before the measurement. It was assumed that equilibrium was reached if the surface tension variation was lesser 0.01 mN/m during 10 minutes. Polymeric surfactants were dissolved in 0.9% solution of NaCl, solution pH value was 6.5–7.0. For the preparing solutions of enhanced pH value 2M solution of NaOH was added by drops. The measurements were carried out in the thermo stated Teflon cell at 298K. The values of polymer maximal adsorption (Γ_{∞} , mol/m²) [53] were calculated using eq. (1) and (2) [54,55] from experimental plots of isotherms of surface tension of the solutions.

$$\Gamma = -(C/RT)(d\sigma/d \lg C) \quad (1)$$

$$(C/\Gamma) = (C/\Gamma_{\infty}) + (\alpha/\Gamma_{\infty}) \quad (2)$$

in eq. (1): $-C (d\sigma/d \lg C)$ is the slope of the curve of surface tension versus the logarithm of surfactant concentration; in eq. (2): $1/\Gamma_{\infty}$ is the slope of the curve of dependence of $C/\Gamma - C$, and

α/Γ_∞ is segment on the ordinate (α - constant eq(2)); R is an ideal gas constant, T is temperature. The average minimum area per molecule ($S_0, \text{\AA}^2$) is evaluated using the equation [53]:

$$S_0 = I / (N_A \cdot \Gamma_\infty) \times 10^{20} \quad (3)$$

where N_A - Avogadro's number.

IR, ^1H , ^{13}C -NMR spectroscopy. Infrared spectra were recorded on a Tensor 27 FT-IR spectrometer (Bruker, USA) with KBr pellet. ^1H -NMR spectra were recorded using ^1H and ^{13}C Varian NMR VXR-300 (Varian/Agilent instruments, USA) with DMSO as a solvent. The ^1H chemical shifts were calibrated using tetramethyl silane (TMS).

DLS study of the micelles and particles. The sizes of polymer micelles and functionalized maghemite particles were measured by a dynamic light scattering (DLS) on DynaPro NanoStar (Wyatt Technology, Santa Barbara, USA) and Zetasizer Nano ZS (Malvern Instruments GmbH, Stuttgart, Germany) and photon correlation spectra using the NIBS (Non-Invasive Back Scatter) technology at 298K. The samples for DLS measurements were prepared by dissolution of polymeric surfactants in 0.9% solution of NaCl, pH 6.5–7.0, polymer concentration was 1×10^{-3} g/ml, next they were inserted into a pre-rinsed capillary cell for the zeta potential measurements. Minimum of three measurements were made for every sample. DLS study of the micelles containing Dox in the range 0.6×10^{-4} – 6.0×10^{-4} g/ml was performed. The solutions for DLS study were kept 24 h before the measurement.

Transmission (TEM) and scanning (SEM) electron microscopy. The morphology of the micelles and maghemite/polymer composites was studied using a transmission electron microscope JEM-200A (JEOL, Japan) at an accelerating voltage of 200 kV. The samples were prepared via polymer dissolution in 0.9% solution of NaCl, pH value was 6.5–7.0; polymer concentration was 1.0×10^{-2} g/ml, concentration of Dox was 3.0×10^{-4} g/ml.

Samples were prepared [56] by the method of spraying tested solution on a substrate by means of the ultrasonic dispersant UZDN-1A (Ukrrospribor Ltd, Ukraine), which allows obtaining a uniform coating substrates. A thin amorphous carbon film deposited on a copper grid was used as a substrate. Dispersant options: type UZDN-1 possessing power of 50 W and frequency of 35 kHz.

The morphology of the micelles formed by the polymeric surfactants including their complexes with Dox was studied using Zeiss Supra 40/40VP scanning electron microscope (Carl Zeiss Group, Germany).

X-ray diffraction. Phase composition and size of the maghemite nanoparticles were studied by X-ray diffractometer DRON-3 (Bourestnik Inc., Russian Federation) using known additive method [57]. The size of the particles was calculated by the Scherrer formula: $d = k\lambda / (\beta \cdot \cos\theta)$, where: $k \approx 1$, constant of instrument; $\lambda = 1.7902 \text{ \AA}$ (CoK α line), the wavelength of the exposure; θ - the angle of diffraction, rad.; β - the physical width of the diffraction maximum, which was calculated by the following formulas: $\beta = (B/2) + (B(B/4 - b/3))^{0.5}$ (for $B \geq 1.5b$) and $\beta = (3b(B - b))/B$ (for $B \leq 1.5b$), where: B - half-width of sample line, rad., b - half-width of standard line, rad.

Small angle X-ray scattering (SAXS) SAXS experiments were performed at the P12 BioSAXS beamline of the European Molecular Biology Laboratory (EMBL) at the storage ring PETRA III of the Deutsche Elektronen Synchrotron (DESY, Hamburg, Germany) using a Pilatus 2M detector (1475×1679 pixels; Dectris, Switzerland) and synchrotron radiation with a wavelength $\lambda = 1 \text{ \AA}$. The sample-detector distance was 4 m, allowing for measurements in the q-range interval from 0.6 to 4 nm⁻¹. The q-range was calibrated using the diffraction patterns of silver behenate. The experimental data were normalized to the transmitted beam intensity and the background scattering of the aqueous buffer was subtracted. Samples have been placed in 1 mm glass capillaries. Temperature has been controlled by Linkam heating stage HFSX 350 (Surrey, UK) with accuracy $\pm 0.1 \text{ }^\circ\text{C}$. Twenty consecutive frames (each 0.05 s) comprising the measurement of the solvent and sample were performed. In order to verify that no artifacts as a result of radiation damage occurred, all scattering curves of a recorded dataset were compared to a reference measurement (typically the first exposure) and finally integrated by automated acquisition program given by Franke et al. [58].

Biological Experiments

Cell culture. Human T-leukemia Jurkat, murine acute L1210 leukemia, and rat glioma C6 cell lines were used in the experiments. Jurkat T-cell and L1210 leukemia cell lines were obtained from the collection of the Institute of Experimental Oncology, Pathology and Radiobiology named by R.E. Kavetsky, National Academy of Science of Ukraine (Kyiv, Ukraine). C6 line of rat glioma cells were obtained from the collection of the Institute of Molecular Biology and Genetics, National Academy of Sciences of Ukraine (Kyiv, Ukraine). Both cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma, USA) supplemented with 10% fetal bovine serum (Sigma, USA). Cells were grown in CO₂-incubator at 37 °C, 5% CO₂ and 95% humidity. The re-seeding of cells was performed at a ratio of 1:5 once in 2-3 days.

Determination of cytotoxic action of studied substances. Cells were seeded in 24-well plastic plates (Greiner bio-one, USA). The substances were added at various concentrations immediately after cell seeding without the adaptation period for Jurkat T-cells, and 24 hours after seeding of C6 cells. Counting of cell number was carried out at regular intervals in the hemocytometer chamber using Trypan Blue dye (DV-T10282, Invitrogen, Life Technologies Corporation, USA) at 0.01% final concentration 2 min after its addition to cell suspension.

Light and fluorescence microscopy. The living, apoptotic and necrotic cells were viewed and images were processed with a fluorescent Zeiss microscope (Carl Zeiss, Germany) using AxioImager A1 camera, by ~ 400 X magnifications in the relevant sections of the excitation and emission. C6 cells were seeded on glass microscopic slides in the 24-well plates (Greiner bio-one, USA). The substances were added in different concentrations 24 h after cell seeding. The material of cell nucleus was stained with DNA-binding fluorescent dye Hoechst 33342 (Sigma, USA). Cells were stained with a poly-specific Acridine orange dye (AO, Sigma, USA) [59]. These fluorochromes were added to cells at following final concentrations: AO - 0.3-1.0 $\mu\text{g/ml}$, Hoechst 33342 - 0.2-0.5 $\mu\text{g/ml}$, and cells were incubated in their presence for 20-30 min.

Statistical analysis. All experiments were repeated three times with three parallels in each variant. All data were expressed as a mean $\pm$ SD. Statistical analysis was performed using two-sided Student's t-test. P-value of <0.05 was considered as statistically significant.

Results and discussion

The combination of the backbone containing side peroxide fragments and grafted hydrophilic PEG chains regularly disposed along the backbone in the polymer molecules (see Experimental section) causes a possibility of radical grafting functional polymeric branches and provides a formation of branched water and hydrocarbon soluble surfactants combining both the non-ionic PEG and functional polyelectrolyte chains in a correspondence with scheme presented below (Fig. 1).

Fig. 2. General scheme of synthesis of PEG- and polyelectrolyte containing BPS by using PEG-containing polyperoxide macroinitiator

As a result of copolymerization of NVP and AA initiated by PEG-containing polyperoxide as macroinitiator, branched copolymers combining both PEG and the anionic polyelectrolyte chains are formed (Table 1). It is evident that composition of the monomer mixture, predominantly the content of AA as a more active (co)monomer, defines not only kinetic parameters of the polymerization but also the amount and composition of the grafted polyelectrolyte chains. This is caused by higher reactivity of AA at copolymerization with the NVP, as referred in [60]. Both monomer conversion and polymerization rate increased with an enhancement of AA content in the initial monomer system. The enhancement of AA content in the monomer mixture leads also to an increase in content of the AA links in grafted poly(NVP-co-AA) chains. However, in spite of higher AA reactivity at copolymerization with the NVP grafted copolymer chains are depleted with their links in comparison with the NVP links. This is explained, evidently, by the inhomogeneous distribution of the monomers between the coils formed by the molecules of macroinitiator and dioxane solution that is enriched by more polar AA. A decrease in the amount of grafted copolymer with an increase in content of AA in the monomer mixture confirms this assumption.

Table 1. Characteristics of graft-copolymerization initiated by the PEGylated macroinitiator (30% per monomers) and branched copolymers (Dioxane, 353K, [Monomer]=2 mol/l)

Monomer content, % [NVP]-[AA]	Conversion, %	Polymerization rate, $\times 10^3$ %/sec	Content of AA in grafted chains, %	% grafted polymer from the initial amount of monomer	$[\eta]^*$ l/g (ethanol, 25 °C)
66:34	80.1	5.8	14.8	77.2	0.22
79:21	76.8	5.2	6.8	84.3	-
93:7	60.2	4.7	1.5	87.1	0.21

* $[\eta]$ for the initial PEG-containing macroinitiator, it is equal 0.61 l/g (ethanol, 298K)

A sharp decrease in a value of the intrinsic viscosity of final copolymers, in comparison with the initial PEGylated comb-like macroinitiator, due to more compact conformation of molecules in solution, witnesses a formation of branched copolymers as a result of grafting functional polyelectrolyte chains [61,62].

Comparative analyses of the IR- and ^1H NMR spectra confirm the formation of hybrid copolymers combining side PEG and poly(NVP-co-AA) chains (Figs.3, 4). It is obvious that the availability of the characteristic adsorption bands in the IR- and the corresponding signals in NMR-spectra attributed to grafted PEG and polyelectrolyte chains.

Fig. 3. IR-spectra of poly(PM-co-GMA)-*graft*-PEG (1) and poly(PM-co-GMA)-*graft*-PEG-*graft*-poly(NVP-co-AA) (93:7%mol) (2)

Fig. 4. ¹H NMR spectra of poly(PM-co-GMA)-*graft*-PEG-*graft*-poly(NVP-co-AA) (93:7%mol)

Surface activity of the initial non-ionic PEGylated polyperoxide surfactant does not depend on pH value of water solution (Fig. 5). However, if surface activity of the copolymer combining the non-ionic and anionic grafted chains is very close to the colloidal-chemical properties of the initial PEGylated surfactant at pH value equal 7, the availability of the carboxyl-containing links in the grafted polyelectrolyte chains causes an increase of their solubility and as a result a decrease of surface activity at pH value equal 9. It is evident that they form micelle-like structures (MLS) at the achievement of definite critical concentration in water solution.

Fig. 5. Isotherms of surface tension of water solutions of the copolymers: 1,2 - (PM-co-GMA)-*graft*-PEG; 3,4,5 - (PM-co-GMA)-*graft*-PEG-*graft*-poly(NVP-co-AA) (93:7%mol); pH=7 (1,3); pH=9 (2,4) and pH=11(5).

Taking into account the different slopes of the initial sections of the isotherms of surface tension of polymer solutions (Fig. 5) the values of maximal adsorption at the phase boundary solution/air as well as values of the area occupied by the MLS formed by the polymeric surfactants at the phase boundary were calculated. The area values occupied by surfactant molecules confirm the existence of self-organized micelle-like structures of a distinct size in the solution depending on polymer surfactant architecture. Experimental data presented in the Table 2 and on the Fig. 5 witness about the independence of surface activity of the non-ionic PEGylated polyperoxide on the pH value.

Table 2 Colloidal-chemical characteristics of the polymeric surfactants

Polymer	Solution pH value	CMC $\cdot 10^4$, mol/l	σ_{CMC} , mN/m	$\Gamma_{\infty} \cdot 10^6$, mol/m ²	S_0 , Å ²
poly(PM-co-GMA)- <i>graft</i> -PEG	7.0	1.42	30.5	0.56	270
	9.0	1.40	29.8	0.62	290
poly(PM-co-GMA)- <i>graft</i> -PEG- <i>graft</i> -poly(NVP-co-AA)	7.0	0.73	29.4	0.38	450
	9.0	0.90	37.0	0.28	600
	11.0	1.19	39.8	0.20	800

Whilst surface activity of the branched polymeric surfactant combining the non-ionic and anionic grafted chains significantly depends on pH value due to the enhancement of its solubility at elevated pH value. In spite of a decrease of their surface activity at the pH value enhancement, it is evident from surface tension isotherms that they form self-organized MLS. An increase in pH value of the branched polymer solution leads to an enhancement of the MLS size as a result of occurrence of charged carboxylate links (Table 2). Moreover, the dependence of the area occupied by branched polymer containing grafted chains with AA links at the phase boundary on solution pH value, unlike to PEGylated nonionic polyperoxide, confirms the formation of the MLS of distinct morphology.

From the results presented in Table 3 and Figs. 6,7 it is obvious that a correlation exists between the MLS sizes measured by the DLS, TEM and SEM techniques. Besides, it is evident that size and charge of the MLS formed by the branched polymeric surfactant are bigger than size and charge of the MLS formed by the PEGylated polyperoxide that is caused by different mechanism of their self-organization in the solution and distinct morphology of formed MLS.

Table 3. The results of MLS size and charge study using DLS and microscopy methods.

MLS type	DLS Z-Average hydrodynamic diameter (nm)	TEM Average diameter (nm)	SEM Average diameter (nm)	Zeta Potential, Zeta potential (mV)
poly(PM-co-GMA)- <i>graft</i> -PEG	59.0	39.0	35.0	-0.25
complex of Dox and poly(PM-co-GMA)- <i>graft</i> -PEG	40.6	30.0		2.25
poly(PM-co-GMA)- <i>graft</i> -PEG- <i>graft</i> -poly(NVP-co-AA)	340.0	300.0	350.0	-0.60
complex of Dox and poly(PM-co-GMA)- <i>graft</i> -PEG- <i>graft</i> -poly(NVP-co-AA)	250.0	-	275.0	10.00

Fig. 6. TEM images of the MLS formed by the poly(PM-co-GMA)-*graft*-PEG(1) and poly(PM-co-GMA)-*graft*-PEG-*graft*-poly(NVP-co-AA) (2) in water (x 30000).

Fig. 7. SEM images of the MLS formed by the poly(PM-co-GMA)-*graft*-PEG (1) and poly(PM-co-GMA)-*graft*-PEG-*graft*-poly(NVP-co-AA) (2) in water.

MLS formed by the molecules of branched polymeric surfactant and availability of grafted anionic polyelectrolyte chains in their structures provide a possibility of their use as nano- and microcontainers for immobilization of bioactive substances and nanoparticles, as well as formation of stable water-based colloidal systems for their targeted transportation. Study of the hydrodynamic diameter of the Dox...polymer complexes (Table 3, Fig. 8) revealed a noticeable decrease in size and charge of the MLS containing Dox in comparison with MLS formed by branched polymer molecules without Dox. Such a compaction of the MLS was observed also as a result of binding Dox by the MLS formed by the comb-like PEGylated surfactant. However, that did not lead to a significant decrease in their size comparing with the MLS formed by the branched polymer molecules. Moreover, it is obvious that branched polymer surfactant forms in preference the monodispersed MLS including the MLS containing bound Dox.

Fig. 8. Hydrodynamic diameter of MLS (by intensity (1-4) and by number (1a-4a)) formed by the poly(PM-co-GMA)-*graft*-PEG(1); complex Dox...poly(PM-co-GMA)-*graft*-PEG(2); poly(PM-co-GMA)-*graft*-PEG-*graft*-poly(NVP-co-AA) (93:7% mol) (3), complex Dox...poly(PM-co-GMA)-*graft*-PEG-*graft*-poly(NVP-co-AA) (93:7%mol) (4)

Internal structure of MLS has been studied by small angle X-ray scattering, which recognizes the difference between poly(PM-co-GMA)-*graft*-PEG, poly(PM-co-GMA)-*graft*-PEG-*graft*-poly(NVP-co-AA) and by complex of poly(PM-co-GMA)-*graft*-PEG-*graft*-poly(NVP-co-AA) with Dox (Fig. 9). At Fig. 9 we did not observed plateau at lowest q which point that mean size of aggregates is larger than observation window of SAXS experiment i.e., $>2\pi/q_{min} \sim 120$ nm. It is in agreement with SEM data. In SAXS experiments we have observed scattering from part of MLS most probably from interface between MLS and solvent. Interval of low scattering vectors was interpreted using scaling concept $I(q) \sim q^{-\alpha}$. An evidence for fractals in the submicron and nanometer scale can be conveniently derived [63,64]. The estimated limit of the length scales L for which the structure is fractal can be defined as $1/q_{max} < L < \pi/q_{min}$. One should take into

account that the observed q -interval for fractal behavior is limited by the lowest q -value of the instrument ($q_{\min}$) and the influence of residual background scattering ($q_{\max}$). Therefore, the actual cutoffs for the fractal behavior can be wider than experimentally observed.

Fig. 9. SAXS data of solutions of the poly(PM-co-GMA)-*graft*-PEG (**1**), poly(PM-co-GMA)-*graft*-PEG-*graft*-poly(NVP-co-AA) (**2**) and of poly(PM-co-GMA)-*graft*-PEG-*graft*-poly(NVP-co-AA) with Dox (**3**) (polymer – 0.001 g /L, Dox – 0.0003 g/l), T 293 K. Solid lines represent linear fit according to scaling concept

Linear fit in q range from 0.08 to 0.2 nm⁻¹ (corresponds to length scale of 5-40 nm) gives the parameter α for poly(PM-co-GMA)-*graft*-PEG-*graft*-poly(NVP-co-AA) of 4.4 ± 0.1 and for poly(PM-co-GMA)-*graft*-PEG of 2.6 ± 0.1 . Value below 3 points on formation of volume (mass fractal) aggregates in the case of poly(PM-co-GMA)-*graft*-PEG i.e., non-compact core and interface. Value of α above 4 points on aggregates of compact core and non-compact (developed interface) [63,64]. Addition of Dox to poly(PM-co-GMA)-*graft*-PEG-*graft*-poly(NVP-co-AA) changes value of α to 3.6 ± 0.1 which points on formation of compact aggregates with fractal interface and suggests possible location of Dox at interface of MLS.

From the Figs. 10,11 presenting the results of DLS study of the micelles containing different amount of the Dox it is obvious that there is a significant dependence of the MLS size on the content of Dox molecules per polymer confirming the MLS shrinkage in a result of Dox binding. This dependence is more significant for the branched polymer containing chains of the anionic and non-ionic type in comparison with the MLS formed by the non-ionic PEGylated polyperoxide.

Fig. 10. The dependences of the MLS size on content of Dox in the system: a) complex Dox...poly(PM-co-GMA)-*graft*-PEG; b) complex Dox...poly(PM-co-GMA)-*graft*-PEG-*graft*-poly(NVP-co-AA) ([polymer]= $1.0 \cdot 10^{-2}$ g/ml, pH=6.7; 0.9% NaCl)

Fig. 11. Hydrodynamic diameter of the MLS (measured by intensity) formed by the complex Dox...poly(PM-co-GMA)-*graft*-PEG (a); and complex Dox...poly(PM-co-GMA)-*graft*-PEG-*graft*-poly(NVP-co-AA) (93:7%mol) (b): [Dox]= 0 g/ml (1,6); $1.0 \cdot 10^{-4}$ g/ml (2,7); $3.0 \cdot 10^{-4}$ g/ml (3,8); $5.0 \cdot 10^{-4}$ g/ml (4) and $9.0 \cdot 10^{-4}$ g/ml (5,9).

The change of the MLS charge from negative to positive as a result of Dox binding can be explained by the formation of the Dox...polymer complexes through different mechanisms for PEGylated polyperoxide and polymer containing $-\text{COOH}$ groups. Our calculations witness that at the same content of the Dox per formulations one micelle formed by branched polymer contains more Dox molecules than a micelle formed by the PEGylated polyperoxide due to a difference in their size and amount in the system. Moreover, a significantly larger positive charge of the MLS...Dox formed by the branched polymeric surfactant can be caused also by the formation of the complex of Dox with both PEG and carboxyl-containing grafted polymeric

chains. We have shown that the COOH-containing branched surfactant forms a complex of salt type as a result of Dox interaction with predominantly the carboxylic groups, while the micelles formed by the PEGylated polyperoxide bind Dox molecules via a formation of the hydrogen bonds with the PEG chains. As it can be concluded from spectrum (Fig. 12), formation of the microscale complexes of branched surfactant and Dox is, evidently, caused by the interaction of Dox amino group with carboxyls of grafted polyelectrolyte chains. A shift in the adsorption band attributed to $n \rightarrow \pi^*$ transfer of carboxyl C=O electrons confirms the formation of complexes of a salt type between the poly(PM-co-GMA)-*graft*-PEG-*graft*-poly(NVP-co-AA) and Dox amino group and witnesses about a change of its solvate surroundings in comparison with the UV-spectrum of water solution of branched copolymer.

Fig. 12. UV-spectra of water solutions of poly(PM-co-GMA)-*graft*-PEG-*graft*-poly(NVP-co-AA) (1), complex of Dox and poly(PM-co-GMA)-*graft*-PEG-*graft*-poly(NVP-co-AA) (2), Dox (3)

One can conclude from the results of comparison of the hydrodynamic diameters of the microscale MLS measured **by the number and by the intensity** (Fig. 8) that they do not form secondary structures, as a result of aggregation due to, evidently, high stabilizing ability of the hydrophilic exterior shell of the MLS, in contrary to the MLS formed by the PEGylated comb-like surfactant.

Fig. 13. The hydrodynamic diameter of the MLS (measured by intensity) formed by the complex Dox...poly(PM-co-GMA)-*graft*-PEG (a); and the complex Dox...poly(PM-co-GMA)-*graft*-PEG-*graft*-poly(NVP-co-AA)(b) (one week after the preparation of the conjugates (1) and conjugates of the same after 3 months of storage (2).

Moreover, it is obvious from the results of DLS study of the MLS (Fig. 13) that their size did not change significantly even after three months of storage.

Specific architecture and colloidal-chemical properties of the branched polymeric surfactant and derived MLS cause their ability to form the hydrated complexes with Fe^{+2} and Fe^{+3} cations via their interaction with the carboxylic groups of grafted polyelectrolyte chains, corresponding to a suggested scheme (Fig. 14). The MLS containing $\text{Fe}^{+2/+3}$ cations are spatially restricted templates wherein the super-paramagnetic $\gamma\text{-Fe}_2\text{O}_3$ nanoparticles are nucleated. That provides both the control of size, narrowed size distribution of $\gamma\text{-Fe}_2\text{O}_3$ nanocrystals, and formation of the micro scale composite coated with functional polymeric shell.

Fig. 14. The scheme of the maghemite nanoparticle nucleation and functionalization.

X-ray diffraction and TEM images of the maghemite nanoparticles suggest that particles' size is in the range of 6.6 ± 0.7 nm (TEM) (Fig. 15) or 8.9 ± 0.4 nm (X-ray diffraction) (Fig. 16), and a tendency to narrowed particle size distribution takes place.

Fig. 15. TEM image of the maghemite nanoparticles functionalized with the poly(PM-co GMA)-*graft*-PEG-*graft*-poly(NVP-co-AA) (a) and the graph of particle size distribution according to TEM (b)

Fig. 16. X-ray Diffractions of Fe₂O₃ particles obtained in the presence of of oleic acid (1), poly(PM-co GMA)-*graft*-PEG(2) , poly(PM-co GMA)-*graft*-PEG-*graft*-poly(NVP-co-AA) (3)

However, the hydrodynamic diameter of the functionalized maghemite particles measured by the DLS technique is much bigger. This discrepancy could be explained by nucleation of the nanoparticles in carboxyl-containing sites of the grafted polyelectrolyte chains of one or several polymeric molecules and following formation of functionalized γ -Fe₂O₃-polymer micro-composites (Fig. 17).

Fig. 17. Hydrodynamic diameter of the maghemite micro composites obtained in the presence of poly(PM-co-GMA)-*graft*-PEG-*graft*-poly(NVP-co-AA) (1) (Curves recorded at intervals of 5 seconds (a,b,c)), MLS of complex Dox ... poly(PM-co-GMA)-*graft*-PEG-*graft*-poly(NVP-co-AA) (93:7%mol) (2) and MLS formed by poly(PM-co-GMA)-*graft*-PEG-*graft*-poly(NVP-co-AA) (93:7% mol) (3).

One can see from data in the Table 4 that only using of the branched polymeric surfactant provides obtaining the smallest nanoparticles coated with an elevated amount of the polymer molecules preventing their aggregation and providing an improved stability. A dependence of the transmission density of the dispersions of the nanoparticles on time (Fig. 18) confirms high sedimentation stability of the maghemite nanoparticles modified with the branched polymeric surfactant.

Table 4.Characterization of γ -Fe₂O₃ nanoparticles

γ -Fe ₂ O ₃ nanoparticles	Amount of the polymer on surface of synthesized particles, %	Number-average particle diameter, nm	
		X-ray	TEM
Fe ₂ O ₃ without a polymeric shell (synthesis in presence of the oleic acid)	0.89	15.3±1.3	14.0
Fe ₂ O ₃ with a poly(PM-co GMA)- <i>graft</i> -PEG shell	1.01	13.5± 0.9	12.5
Fe ₂ O ₃ with poly(PM-co GMA)- <i>graft</i> -PEG- <i>graft</i> -poly(NVP-co-AA) shell	19.5	8.9±0.4	6.3

These composites are of narrow size distribution and have attached functional polymeric shell that provides an improved stability of the composite particles (Figs. 17, 18). As one can see in Fig. 18, a dependence of the intensity of light scattering on the hydrodynamic diameter of the magnetic particles measured at intervals of 5 sec does not change noticeably owing to their high stability and prevention of composite aggregation in water dispersion. Due to formation of several durable bonds of the polymer-grafted chains with the particle surface and compaction of

the MLS-based composite, the particle size is decreased in a comparison with size of the initial MLS and size of the same MLS containing the immobilized Dox (Fig. 17).

Fig. 18. Time dependence of the transmission density of the dispersions of $\gamma\text{-Fe}_2\text{O}_3$ synthesized in presence of the oleic acid (1); poly(PM-co-GMA)-*graft*-PEG (2) and the branched poly(PM-co-GMA)-*graft*-PEG-*graft*-poly(NVP-co-AA) (3,4) at pH=7.1 (1-3) and pH =8.5 (4) ($[\text{Fe}_2\text{O}_3\text{-particles}] = 0.02\text{g}/100\text{ml}$, H_2O).

Fig. 19. Change in number of murine acute L1210 leukemia cells under their treatment with free Dox and with complex Dox...poly(PM-co-GMA)-*graft*-PEG-*graft*-poly(NVP-co-AA) (48 h incubation). a) complex of Dox...poly(PM-co-GMA)-*graft*-PEG-*graft*-poly(NVP-co-AA); b) complex Dox...poly(PM-co-GMA)-*graft*-PEG-*graft*-poly(NVP-co-AA) containing inbuilt phosphatidyl choline (PC)

Therefore, a decrease in size of the MLS formed by branched polymeric surfactants due to the compaction occurring as a result of formation of bonds with the immobilized substances is a characteristic property of the branched polymeric surfactant studied in present work.

An enhanced stability of dispersions of the MLS formed by the complex of Dox with the poly(PM-co-GMA)-*graft*-PEG-*graft*-poly(NVP-co-AA) and prevention of their aggregation

provide durable circulation of the MLS at transportation and a possibility of its use for chemotherapy of murine L1210 leukemia cells. The results of such testing witness 10-times decrease in the effective therapeutic dose that is necessary for the achievement of the same treatment (cytotoxic) effect as at using of free (un-bound) Dox after 48 h incubation (Fig. 19).

The results of DIC and fluorescent microscopy of rat C6 glioblastoma cells in control and after cell treatment (72 h) with the synthesized magnetic particles are presented in Fig. 20. One can see that the particles are of approximately 150-200 nm size, and they are concentrated in the perinuclear region of the cytoplasm. Such a specific localization of the particles inside the treated cells is of great interest and it was not described earlier for other nanoparticles. It can be suggested that the engulfment of the magnetic composites is promoted by the availability of specific exterior functional shell formed by the hybrid branched polymer. The reasons and potential consequences of the appearance of this phenomenon are presently under investigation.

Fig. 20. DIC (A and B) and DIC-fluorescent microscopy (C) of rat glioblastoma C6 cells in control (A) and after treatment (72 h) with magnetic particles M12 (B). (C) – treated cells stained with fluorescent dyes: Hoechst-33342 (blue color of stained nuclear chromatin) and Acridin orange (green color of the nucleic acids and reddish – of the lysosomes).

Fig. 21. Change in number of human T-leukemia Jurkat cells under their treatment (24, 48, 72 hours) with uncoated maghemite particles (Fe_2O_3) and such particles with the poly(PM-co-GMA)-*graft*-PEG-*graft*-poly(NVP-co-AA) shell.

It should be noted that the treated cells do not die after the engulfment of the applied particles (Fig. 20), however their proliferation is significantly retarded (Fig. 21). An increase in number of the polyploid (undivided) cells (noted by arrows on Fig. 20) was observed, and this phenomenon could be one of probable mechanisms responsible for the revealed cytostatic action of the studied particles. One can speculate that the perinuclear localization of the particles blocks separation of nucleus between the daughter cells, and, thus, cell proliferation stops.

Conclusion

A developed approach in synthesis provides obtaining of branched functional polymeric surfactants combining grafted PEG and polyelectrolyte chains. Novel hybrid copolymers form micelle-like structures of the narrowed size distribution in the microscale range, in contrary to the initial PEGylated surfactants possessing different micelle architecture. Such micelles form highly stable water dispersions and can be used as functional containers and carriers capable of compaction of the micelles providing their size decrease and narrowing of size distribution. Owing to availability of functional polymeric shell, the MLS bearing Dox or maghemite nanoparticles penetrate through plasma membrane of tumor cells leading to a cytotoxic or cytostatic effect, respectively.

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