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Block Ionomer Complex Micelles with Cross-Linked Cores for Drug Delivery,^{1,2}

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Abstract

Soft polymeric nanomaterials were synthesized by template-assisted method involving condensation of the poly(ethylene oxide)-b-polycarboxylate anions by metal ions into core-shell block ionomer complex micelles followed by chemical cross-linking of the polyion chains in the micelle cores. The resulting materials represent nanogels and are capable of swelling in a pH-dependent manner. The structural determinants that guide the self-assembly of the initial micelle templates and the swelling behavior of the cross-linked micelles include the block ionomer structure, the chemical nature of metal ions, the structure of the cross-links and the degree of cross-linking. The application of these materials for loading and release of a drug, cisplatin, is evaluated. These cross-linked block ionomer micelles have promise for delivery of pharmaceutical agents.

INTRODUCTION

Nanoscale polymeric particles are emerging as novel drug delivery systems in biomedical applications. In particular, self-assembled block copolymer micelles have been utilized in pharmaceuticals for development of novel therapeutic [1] and diagnostic modalities [2]. Advantages of the polymer micelles include their small size, long circulation in the bloodstream, ability to circumvent renal excretion and extravasation at sites of enhanced vascular permeability. Recently nanofabrication of polymer micelles was significantly advanced by employing charge driven self-assembly of block copolymers containing ionic and nonionic blocks ("block ionomers"). The idea of using the block ionomers for design of new nanocomposite materials was stimulated by discussions that took place in 1994 between Viktor Kabanov (Moscow State University), Adi Eisenberg (McGill University), and the one of the authors of this paper, Alexander Kabanov. This was inspired by the merge of the knowledge and expertise in structural organization and solution behavior of interpolyelectrolyte complexes [3], self-assembly phenomena in the solutions of ionic block copolymers [4], and the potential application of such materials for drug and gene delivery [1]. During the last fourteen years diverse materials were synthesized by reacting the block ionomers with oppositely charged molecules such as synthetic linear polyelectrolytes [5,6] or block ionomers [7,8], surfactants/lipids [9–15], proteins [16–18], or DNA [19,20]. The

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driving force for such binding is the release of low molecular mass counterions, originally associated with the components of the complexes, into the external media, which is accompanied by a substantial entropy gain. These complexes belong to the special classes of nanostructured materials combining the properties of cooperative polyelectrolyte complexes [3] and amphiphilic block copolymers [21,22]. These materials are called “block ionomer complexes (BIC)” [5] or “polyion complex micelles” [7]. BIC containing nonionic water-soluble block, such as poly(ethylene oxide), PEO, form stable aqueous dispersions of ~10 to 100 nm diameter particles with core-shell architecture even upon complete neutralization of charges (Fig. 1). The core can comprise polyion/polyion (a), polyion/surfactant (b), or polyion/metal (c) complexes. The core of such BIC can also incorporate a variety of compounds or even particles including biologically active molecules through a combination of electrostatic, hydrophobic, and hydrogen bonding interactions. Such materials are uniquely suited for the delivery of biomacromolecules [23–26], as well as ionic and non-ionic drugs [27–31]. Ionic block lengths, charge density, and ionic strength of the solution affect the formation of stable BIC and, therefore, control the amount of the drug that can be incorporated within the micelles. BIC display transitions induced by changes in pH, salt concentration, chemical nature of low molecular mass counterions as well as temperature, and can be fine-tuned to respond to environmental changes occurring in a very wide range of conditions that could realize during delivery of biological and imaging agents.

Unique features of BIC are also relevant for their use as nanoreactors in the diverse fields of medical and biological engineering. Recently, we proposed to use BIC as micellar templates to synthesize novel polymer micelles with cross-linked ionic cores [32,33]. Indeed, the cores of the BIC formed between PEO-*b*-polymethacrylate anions (PEO-*b*-PMA) and divalent metal cations (Fig. 1c) were utilized as nanoreactors for cross-linking reactions. Resulting particles were entirely hydrophilic nanospheres, which combine several key structural features that make these systems very beneficial for effective drug delivery. These are: a cross-linked ionic core; a hydrophilic PEO shell; and nanoscale size. They are, in essence, nanoscale single molecules that are stable upon dilution and can with-stand environmental challenges such as changes in pH, ionic strength, solvent composition, and shear forces without structural deterioration. These favorable characteristics of the polymer micelles with cross-linked ionic cores motivated our ongoing efforts to elucidate their potential as efficient carriers for the delivery of anticancer drugs. Here we would like to report on the further progress in the use of BIC as templates for preparation of cross-linked polymeric micelles. Specifically, the objective of this work is to ascertain the structural determinants that guide the self-assembly of BIC templates and physicochemical characteristics of the resulting cross-linked micelles. Indeed, it is important to understand whether the properties of such particles can be easily tuned by varying the composition of the “soft” core (i.e., structure of block ionomer, cross-linker and number of cross-links) and/or by altering the length of PEO chains in the outer corona of the micelles. This, in turn, may affect drug loading efficiency, swelling, interaction and attachment to surfaces, diffusion and drug release properties.

EXPERIMENTAL

Block copolymers of PEO and methacrylic acid or acrylic acid (further designated as PEO-*b*-PMA and PEO-*b*-PAA) were purchased from Polymer Source Inc., Canada. The sodium salts of these block copolymers were used for preparation of BIC. The list of block copolymers used in this work and their molecular characteristics are presented in Table 1. Diblock copolymer samples are denoted as PEO(*x*)-*b*-PMA(or PAA)(*y*), where *x* and *y* represent the degree of polymerization of the PEO segment and PMA/PAA segment, respectively. For example, PEO(170)-*b*-PMA(180) represents a diblock copolymer containing 170 ethylene oxide repeat units and 180 sodium methacrylate units. The concentration of carboxylate groups in the copolymer samples was estimated by

potentiometric titration. Calcium chloride, barium chloride and gadolinium chloride, various cross-linker molecules (1,2-ethylenediamine, 1,5-diaminopentane, *N*-(3-aminopropyl)-1,3-propanediamine, cystamine dihydrochloride, 2,2'-(ethylenedioxy)bis(ethylamine)), *cis*-dichlorodiamminoplatinum (II) (cisplatin), and other chemicals were purchased from Sigma–Aldrich (St Louis, MO) and were used as received.

Turbidity measurements

The turbidity measurements were carried out at 420 nm using a Perkin-Elmer Lambda 25 UV/VIS spectrophotometer after equilibration of the system for 3 min, which was proven to be sufficient for equilibration. The data are reported as $(100 - T)/100$, where T is transmittance (%).

General procedure for the synthesis of cross-linked micelles

Cross-linked micelles were prepared by the previously described method with a slight modification [32]. In brief, PEO-*b*-polyacid/ Me^{n+} complexes were prepared by mixing an aqueous solution of corresponding PEO-*b*-polyacid with a solution of MeCl_n at a molar ratio of $[\text{Me}^{n+}]/[\text{COO}^-] = 0.3\text{--}1.3$. The 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) was added into solution of PEO-*b*-polyacid/ Me^{n+} complexes to create an active-ester intermediate with carboxylic groups of polyacid segments followed by addition of the solution of cross-linker. The extent of degree of cross-linking was controlled by the ratio of the amine functional groups to carboxylic acid groups. The reaction mixture was allowed to stir overnight at room temperature. After completion of the reaction, ethylenediaminetetraacetic acid (EDTA, 1.5 molar equivalents) was added followed by dialysis, first, against 0.5% aqueous ammonia, and then against distilled water to remove metal ions and byproducts of the cross-linking reaction.

^1H NMR analysis

^1H NMR spectra were acquired at pH 5.0 and pH 10.0 and 25°C using a Varian 500MHz spectrometer in D_2O .

Electrokinetic mobility and size measurements

Electrophoretic mobility measurements were performed using a “ZetaPlus” analyzer (Brookhaven Instrument Co.) with a 30 mW solid-state laser operating at a wavelength of 635 nm. ζ -potential of the particles was calculated from the electrophoretic mobility values using built-in software employing the Smoluchowski ζ -potential model. Effective hydrodynamic diameters (D_{eff}) of the particles were measured by photon correlation spectroscopy in a thermostatic cell at a scattering angle of 90° using the same instrument equipped with a Multi Angle Sizing Option (BI-MAS). All measurements were performed at 25°C. Software provided by the manufacturer was used to calculate the size of the particles and polydispersity indices. The diameters mean values were calculated from the measurements performed at least in triplicate.

Atomic force microscopy (AFM)

Samples for AFM imaging were prepared by depositing 5 μl of an aqueous dispersion of cross-linked micelles (ca. 0.2 mg/ml) onto positively charged 1-(3-aminopropyl)silatrane mica surface (APS-mica) for 10 minutes followed by surface washing with deionized water and drying under argon atmosphere. The AFM imaging in air was performed with regular etched silicon probes (TESP) with a spring constant of 42 N/m using a Multimode NanoScope IV system (Veeco, Santa Barbara, CA) operated in a tapping mode. The images were processed and the widths and heights of the particles were measured using Femtoscanner software (Advanced Technologies Center, Moscow, Russia).

Preparation of cisplatin-loaded micelles

The aqueous dispersions of cross-linked micelles were mixed with an aqueous solution of cisplatin (1 mg/ml) at pH 9.0 at molar ratio of cisplatin to carboxylate groups of the micelle of 0.5 followed by incubation at 37°C for 48 h. Unbound cisplatin was removed by ultrafiltration using Centricon® Plus-20 centrifugal filter units (MWCO 100 000, Millipore).

Cisplatin assay and release studies

The Pt(II) content in the micelles was determined by an ion coupled plasma-mass spectrometer, ICP-quadrupole-MS (Varian 810-MS). For the detection of platinum, two isotopes Pt194 and Pt195 were chosen because of their equal abundance (33.0% and 33.8%, respectively). Holmium was used as the internal standard for all measurements. Standard curves in the range of Pt concentrations from 2 ng/ml to 100 ng/ml were generated by using platinum atomic absorption standard. Appropriate dilutions of the test samples were prepared in 0.1 N HCl. Data were acquired and processed using the ICP-MS expert software version 2.1 b103 (Varian). The release of the Pt(II) complexes from the cross-linked polymer micelles in phosphate buffered saline (PBS, pH 7.4, 0.14 M NaCl) was evaluated by dialysis method using a membrane with 3.500 kDa cutoff. The concentration of Pt(II) species released from the micelles was determined by ICP-MS and expressed as a percentage of the total Pt(II) available and plotted as a function of time.

RESULTS AND DISCUSSION

Cross-linked micelles were synthesized using a two-step process shown schematically in Fig. 2. First, PEO-*b*-polyanion copolymers were self-assembled into BIC in the presence of divalent metal ions, such as Ca^{2+} or Ba^{2+} . Second, the inner core of the BIC was cross-linked by bifunctional agents, and the metal ions were removed by dialysis.

Specifically, PEO-*b*-PMA copolymers were reacted with CaCl_2 or BaCl_2 at pH 8.0. The solution behavior of resulting PEO-*b*-PMA complexes was strongly dependent on the length of the polymer segments of the block ionomer and the type of the metal ions. Fig. 3 presents turbidity of the PEO-*b*-PMA/ Me^{2+} systems (where Me^{2+} is Ca^{2+} or Ba^{2+} ions) as a function of the charge ratio in the mixture, Z . The latter is expressed as $Z = C_m n / C_i$ where C_m is Me^{2+} molar concentration, n is the valence of the metal ion, and C_i is the molar concentration of the carboxylate groups at a given pH. (Since turbidity measurements were performed at pH 8.0 where the ionization of the PMA chains was essentially complete, C_i equaled the base-molar concentration of the carboxylic acid units.)

The formation of the BIC in PEO(170)-*b*-PMA(180)/ Ca^{2+} and PEO(125)-*b*-PMA(180)/ Ca^{2+} mixtures was observed in the vicinity of $Z = 1.6$ as manifested by an increase in the turbidity of the system. In PEO(170)-*b*-PMA(180)/ Ba^{2+} and PEO(125)-*b*-PMA(180)/ Ba^{2+} mixtures the onset of the turbidity increase was shifted towards the lower values of Z of about 1.3. Such a shift may be attributed to stronger binding of Ba^{2+} ions to carboxylate groups compared to Ca^{2+} ions [34,35]. Formation of nanosized BIC particles in the resulting dispersions was detected by dynamic light scattering (DLS) at the onset of turbidity. Remarkably, no precipitation in the PEO(170)-*b*-PMA(180)/ Me^{2+} or PEO(125)-*b*-PMA(180)/ Me^{2+} mixtures was observed over the entire range of the charge ratios studied in this work. In contrast, phase-separation was observed in PMA/ Me^{2+} systems under comparable conditions (data not shown). Clearly, the block ionomer-metal complexes studied in this work can be considered as a special type of the copolymer with neutralized, hydrophobic segments from the polyion-metal complex and water-soluble PEO chains. Therefore, in the PEO-*b*-PMA/ Me^{2+} mixtures the water-soluble PEO chains prevented aggregation and macroscopic phase separation of the neutralized PMA/ Me^{2+} complexes.

The resulting complexes self-assembled into micelle-like particles of nanoscale size and formed stable aqueous dispersions [32,33,36].

Turbidimetric data also suggest that the solution properties of such complexes depended on the relative weight fraction of hydrophilic PEO blocks (f_{EO}) in the block ionomer as well as the type of the counterion (Table 1). Indeed, the BIC based on PEO(125)-*b*-PMA(180) copolymer ($f_{EO} \approx 0.26$) generally exhibited higher turbidity than those based on PEO(170)-*b*-PMA(180) copolymer with the same length of ionic block but significantly higher weight fraction of the hydrophilic PEO chains ($f_{EO} \approx 0.33$). Furthermore, the most “hydrophilic” PEO(114)-*b*-PMA(81) copolymer ($f_{EO} \approx 0.42$) did not form BIC with Ca^{2+} ions in the entire range of the charge ratios studied. However, this copolymer formed BIC with Ba^{2+} ions at relatively high $Z \approx 3$. We posit that in all cases the metal ions bind with the PMA chains of the block ionomers. However, the self-assembly of BIC is dependent on the induced amphiphilicity of the resulting complexes. The complexes containing block ionomers with low PEO fraction were most likely to aggregate. The complexes with relatively high PEO content were dependent on the counterion nature. In particular, the Ba^{2+} neutralized PMA chain was more lipophilic than Ca^{2+} neutralized PMA chain, which explained the observed differences in the aggregation behavior in these systems.

The sizes of the PEO-*b*-PMA/ Me^{2+} micelles determined by DLS are presented in Table 2. Formation of small (100 nm and less), particles with narrow particle size distribution was detected in PEO(170)-*b*-PMA(180)/ Me^{2+} and PEO(125)-*b*-PMA(180)/ Me^{2+} mixtures. Noteworthy, the complexes formed by the block ionomer with a lower content of PEO chains, PEO(125)-*b*-PMA(180), were larger than those formed by PEO(170)-*b*-PMA(180). Such trend is typical for polymeric amphiphiles forming core-corona aggregates: a decrease in the weight fraction of corona-forming block shifts aggregate structures toward smaller mean curvature and larger size [37]. These results are also in agreement with the data for similar complexes formed by hydrophilic polyhydroxyethyl-acrylate-*b*-poly(acrylic acid) copolymers and Al^{3+} ions [38]. It was demonstrated that the size of these aggregates increased as the weight fraction of the neutral block in the copolymer decreased. Also, interestingly, the PEO-*b*-PMA/ Ba^{2+} particles were smaller compared to the corresponding PEO-*b*-PMA/ Ca^{2+} particles. This may be explained by a greater lipophilicity of the Ba^{2+} neutralized PMA chain compared to the Ca^{2+} neutralized PMA chain. The PEO(114)-*b*-PMA(81)/ Ba^{2+} BIC represented relatively large aggregates with a broad size distribution.

The pronounced effect of the block ionomer structure on the self-assembly of BIC was further reinforced by examining the PEO-*b*-PAA/ Me^{2+} mixtures (see Fig. 4). The copolymer with high content of PEO block PEO(114)-*b*-PAA(93) ($f_{EO} \approx 0.43$) did not exhibit increase in turbidity in presence of Ca^{2+} ions in the entire range of the charge ratios studied. We posit that similar to the above discussed (PEO(114)-*b*-PMA(81) ($f_{EO} \approx 0.42$)) the complexes of this copolymer with Ca^{2+} ions were too hydrophilic to form self-assembled aggregates. In contrast, PEO(80)-*b*-PAA(104) copolymer with decreased weight fraction of PEO block appeared to readily form such aggregates with Ca^{2+} ions at $Z \geq 1.7$.

Interestingly, the self-assembly in PEO(80)-*b*-PAA(104)/ Me^{n+} systems can be also controlled by using metal ions with different binding affinity or valency. Indeed, the formation of PEO(80)-*b*-PAA(104)/ Gd^{3+} aggregates was observed in the vicinity of stoichiometric charge ratio, $Z = 1$. Furthermore, the PEO(80)-*b*-PAA(104)/ Ba^{2+} and PEO(80)-*b*-PAA(104)/ Gd^{3+} aggregates were much smaller (ca. 60 nm) than those formed in the presence of Ca^{2+} ions (ca. 140 nm). In contrast to PEO(114)-*b*-PAA(93)/ Ca^{2+} mixtures, which did not exhibit aggregation, the aggregates were formed in PEO(114)-*b*-PAA(93)/ Ba^{2+} or PEO(114)-*b*-PAA(93)/ Gd^{3+} mixtures at relatively high concentrations of metal ions.

For example, in PEO(114)-*b*-PAA(93)/Ba²⁺ mixture the particles with diameters of approximately 150 nm were detected only at $Z \geq 3$.

It is very important to note in our view that the onset of self-assembly in PEO(114)-*b*-PAA(93)/Ba²⁺ systems can be altered by addition of homopolyelectrolyte PAA into the mixture. Titration of an equimolar mixture of PEO(114)-*b*-PAA(93) block ionomer and PAA ($M_n = 8,000$) (based on base-molar concentrations of the carboxylic acid units) with BaCl₂ resulted in formation of BIC aggregates at $Z \approx 1$ similar to PEO(80)-*b*-PAA(104)/Ba²⁺ systems (Fig. 4). We believe that addition of PAA to the block ionomer is equivalent to a decrease in the relative weight fraction of PEO block, which favors the self-assembly behavior. Specifically, the weight fraction of PEO chains in an equimolar mixture of PEO(114)-*b*-PAA(93) and PAA corresponds to $f_{EO} \approx 0.34$, which is similar to PEO(80)-*b*-PAA(104) ($f_{EO} \approx 0.32$). It is likely that the shift in the onset of self-assembly in PEO(114)-*b*-PAA(93)/PAA/Ba²⁺ mixtures is a result of segregation of neutralized PAA chains of homopolyelectrolyte and block ionomer into joint polyion-metal cores of the BIC. Validation of this hypothesis is of considerable theoretical and practical interest and is ongoing in our laboratories. Overall, these data suggest that solution behavior and macroscopic characteristics of BIC with polyion/metal cores strongly depend on the relative ratio of nonionic and anionic blocks of the block ionomer and nature of condensing multivalent metal ions.

Block ionomer/Me²⁺ complexes were further utilized as templates for the synthesis of cross-linked polymer micelles (*c*/PEO-*b*-PMA or *c*/PEO-*b*-PAA) as presented in Fig. 2. Cross-linking of the core of the PEO-*b*-polyacid/Me²⁺ micelles was achieved via condensation reactions between the carboxylic groups of polyion chains and the amine functional groups of 1,2-ethylenediamine in the presence of a water-soluble carbodiimide, EDC. The “targeted” degree of cross-linking was defined as a ratio of amine functional groups to carboxylic acid groups. It represents the maximum theoretical amount of cross-linking that can take place, rather than the precise extent of amidation, which was shown to be lower [31]. Following the cross-linking reaction (overnight, room temperature) the size of the aggregates only slightly increased, which indicated that formation of cross-links was limited to the individual BIC particles. A strong chelating agent, EDTA was added to the reaction mixtures to bind and extract the metal ions, which cemented the ionic core. This was followed by an exhaustive dialysis, first, against 0.5% aqueous ammonia, and then against distilled water to remove the metal ions and byproducts of the cross-linking reactions. Should there be no cross-linking of the micellar core, the dissociation into individual chains would occur, since the block ionomers are soluble under these conditions. However, the particles with the net negative charge and diameters in the range of 140 nm to 180 nm were present in the aqueous dispersions (Table 3). It is important to note that after the dialysis the sizes of the formed *c*/PEO-*b*-PMA (or PAA) particles were significantly larger than the sizes of the original BIC templates. Such an expansion was consistent with the removal of the metal ions and formation of water-swollen block ionomer nanostructures. We have previously described such structures as polymer micelles with cores comprised a network of the cross-linked polyanions surrounded by a flexible hydrophilic shell composed of PEO blocks [32]. In other words such structures are believed to at least partially retain the core-shell distribution of the ionic and nonionic chains similar to the original BIC templates. Although block ionomers used to prepare BIC micellar templates had different molecular weights and compositions, there was no drastic difference in the dimensions of the resulting cross-linked micelles. The size of the particles did not change even upon 100-fold dilution, which further confirmed successful covalent cross-linking of the micelles. Notably, these cross-linked micelles had a relatively high net negative charge (Table 3). Clearly, these particles maintained significant portion of the acid functionalities although some portion of the carboxylic groups were consumed during reaction.

The hydrogel-like behavior of these micelles was observed upon a change of pH. Their size increased considerably with increasing pH, which was completely reversible (Fig. 5a). This was accompanied by an increase in the net negative charge (not shown). Evidently, the swelling was induced by ionization of the carboxylic groups at high pH. The size and shape of cross-linked block ionomer micelles at various pH values were further characterized by tapping-mode AFM. This technique allows visualization of the entire particles and yields their heights and widths. The typical images of *c*/PEO(170)-*b*-PMA(180) micelles are presented in Fig. 5. The particles were deposited at different pH and then dried at the mica. As expected, they appeared to be spherical and at pH 7.0 had a number-averaged height of 3.45 ± 0.06 nm and diameter of 111.4 ± 0.4 nm (Fig. 5b). It is known that imaging in the air usually provides lower numbers for the height of the sample as a result of the drying process, but higher numbers for the width, due to the tip convolution effect. Therefore, it is likely that *c*/PEO(170)-*b*-PMA(180) micelles collapsed on the mica surface upon drying and were visualized by AFM as flattened circular images. The high diameter versus height aspect ratio (ca. 32) was in agreement with the expected flexible, shape-adaptable character of these nanostructures imparted by the “soft” PMA cores. However, at pH 5.3 the *c*/PEO(170)-*b*-PMA(180) micelles were characterized by significantly greater height and lower diameter values. An averaged height was determined to be 18.9 ± 0.3 nm and the diameters were 82.3 ± 0.1 nm. We believed that upon acidification the PMA collapsed which resulted in the formation of more rigid structures. Such structures were more robust upon deposition on the mica, thus, were characterized by a lower dimension aspect ratios (ca. 4.4) as was determined from the analysis of the AFM images.

Altogether, the cross-linked block ionomer micelles displayed volume transitions at the nanosized scale in response to changes in pH, and, therefore, can be termed nanogels. The core-shell architecture of these nanogels provides for well-defined spatial orientation of the polymer chains. Such behavior is instrumental for the design of drug carriers with controlled loading and release characteristics.

The swelling of hydrogels strongly depends on the degree of polymer cross-linking and the structure of the cross-linker. Therefore, these parameters were also expected to affect the swelling behavior of the cross-linked micelles. To elucidate the effects of the cross-linking density, the cross-linked micelles were prepared from the same stock solution of template PEO(170)-*b*-PMA(180)/Ca²⁺ micelles using different targeted degrees of cross-linking from 10% to 60%. As mentioned above, these numbers represent just the nominal extent of cross-linking that could occur while the actual degree of cross-linking is expected to be significantly less. Due to the colloidal nature of the micelles, the conventional solution-state ¹H NMR does not permit to determine the precise number of cross-links per micelle. However, at pH 10 when the cores of the micelles are completely swollen and the hindrance of the amide groups in the cores is minimal, this technique allowed us to monitor, at least qualitatively, the changes in the extent of cross-linking upon the synthesis. Indeed, the integral intensity of the signal due to the methylene protons adjacent to amide groups (resonance at $\delta \sim 3.2\text{--}3.3$ ppm) progressively increased as the targeted degree of cross-linking increased from 10% to 60%. An estimated number of cross-links per block ionomer chain in the micelles with the targeted degree of cross-linking of 10% was ca. 3. This value gradually increased and reached 9 for the micelles with the targeted cross-linking degree of 60%. It is important to note that the diamine cross-linker can also form a “loop” within a single polyanion chain instead of linking two different chains as well as attach to polyanion by only one amino group resulting in a free amine. The ¹H NMR signal at $\delta \sim 3.2\text{--}3.3$ ppm accounts for all types of amide groups including those formed due to these side reactions. Therefore, the determined numbers of the cross-links in the *c*/PEO(170)-*b*-PMA(180) micelles can be overestimated. Nevertheless, these numbers corresponded to only ca. 10 % yield of the targeted degree of cross-linking.

The hydrodynamic diameters (D_{eff}) and ζ -potentials of the *c*/PEO(170)-*b*-PMA(180) micelles with various cross-linking densities are presented in Table 4. As expected the micelles with lower degrees of core cross-linking exhibited significant swelling, whereas the highly cross-linked micelles (e.g., 40% and 60%) exhibited only a modest increase in D_{eff} compared to the precursor BIC templates micelles ($D_{\text{eff}} \approx 90$ nm). Surprisingly, the zeta-potential values of cross-linked micelles became more negative as the theoretical degree of cross-linking increased from 10% to 20%. This may be due to a decrease in the effective Stern layer of the particles with smaller diameters.

Another important factor determining the macroscopic characteristics of cross-linked micelles is a structure of the cross-linking agent. We explored the effects of the hydrophobicity of the cross-linker on the swelling behavior using several diamino cross-linkers such as 1,2-diaminepentane, 2,2'-(ethylene-dioxy)bis(ethylamine), *N*-(3-aminopropyl)-1,3-propanediamine, and cystamine (Table 5). In this study the cross-linked micelles with targeted 20% degree of cross-linking were prepared from the same stock solution of PEO(170)-*b*-PMA(180)/Ca²⁺ complexes which provided a uniform template for the reaction. Following the cross-linking and removal of the Ca²⁺ ions the pH-dependence of swelling behavior of the *c*/PEO(170)-*b*-PMA(180) micelles with various cross-linkers was studied by DLS (Fig. 6). Upon increase of pH, all types of *c*/PEO(170)-*b*-PMA(180) micelles underwent significant swelling. However, the use of more hydrophobic cross-linkers produced cross-linked micelles with smaller sizes at any given pH. The relative hydrophobicity values of the cross-linkers are presented in Table 5. Clearly, as the hydrophobicity of the cross-linker increased the size of the micelles decreased (Fig. 6). The micelles with the most hydrophilic cross-linker, 2,2'-(ethylenedioxy)bis(ethylamine) exhibited the most pronounced volume transition upon pH changes. In contrast, the micelles containing relatively hydrophobic cross-linkers such as 1,2-diaminepentane or cystamine, exhibited only a modest increase in D_{eff} . Interestingly, the ¹H NMR data suggest that the more hydrophobic the cross-linker is the higher the relative number of cross-links in the core of the micelles is achieved during the reaction (data not shown). This is understandable considering that the relatively hydrophobic core of the PEO-*b*-PMA/Ca²⁺ micelles would favor incorporation hydrophobic cross-linkers compared to the hydrophilic ones. Therefore, by adjusting the hydrophobicity of the cross-linker one can control the swelling characteristics of the cross-linked micelles.

Interestingly, *c*/PEO(170)-*b*-PMA(180) micelles with cross-links formed by *N*-(3-aminopropyl)-1,3-propanediamine exhibited a sharp increase of the size in the pH range from 8 to 6. This cross-linker contains an ionizable secondary amino group with pK value around 8.3 [39]. Therefore, we believe that pH-induced protonation of this group resulted in electrostatic interaction of the cross-linker with the carboxylate groups in the cores of the micelles and decrease of the volume. All together, the properties of the cross-linked micelles can be altered by varying the block ionomer structure, number of the cross-links, the hydrophobicity of the cross-linker, and the use of cross-linkers with ionizable groups. Therefore, a wide variety of cross-linked micelles with different sizes and charges can be synthesized from the same micellar template simply by using different types of linkers.

The ionic character of the core of the cross-linked micelles allowed for the encapsulation of charged therapeutic molecules. As was previously demonstrated [31] cisplatin, a potent anticancer drug, can be successfully incorporated in such micelles. It was of interest to investigate whether the cisplatin loading capacities of cross-linked micelles and release profile of the drug can also be tailored by altering the degree of cross-linking. Therefore, cisplatin was immobilized in *c*/PEO(170)-*b*-PMA(180) micelles with targeted degrees of cross-linking of 10, 20, 40, and 70%. The loading was achieved by simple mixing of the drug solutions with aqueous dispersion of the micelles at 37°C and pH 9.0. This resulted in a

decrease of the size and net negative charge of the cross-linked micelles due to progressive neutralization of the PMA segments as a result of cisplatin reaction with the carboxylate groups. Notably, the drug loaded cross-linked micelles were stable in aqueous dispersions, exhibiting no aggregation or precipitation for a prolonged period of time (weeks). The cisplatin loading capacity of *c*/PEO(170)-*b*-PMA(180) micelles with different degrees of cross-linking is shown in Fig. 7a. Interestingly, the micelles with higher degree of cross-linking exhibited lower loading. This is probably due to the differences in the total content of carboxylate groups that decreases as the cross-linking degree increases. Additionally, the distribution of cross-linkers in the micelle core can also affect the loading capacity. Upon cross-linking reaction the carboxylate groups are buried in the polyion-metal complex core and their number and accessibility for carbodiimide activation are limited. In consequence, it is possible that the cross-linking reactions are limited to some exterior layer of the core. At the higher degrees of cross-linking, the free volume of this layer should decrease, which may hinder the accessibility of the micelle core to the drug molecules. Furthermore, the micelles with higher degree of cross-linking may have relatively more hydrophobic core environment, which additionally hinder incorporation of rather hydrophilic molecules of cisplatin.

The release of the Pt(II) compounds from the micelles is essential for exhibiting the drug activities in the physiological conditions. It proceeds via an exchange reaction between chloride ions and carboxylic groups of PMA in the platinum complexes. Hence, the release profiles were examined for cisplatin-loaded polymer micelles with various degree of cross-linking in phosphate buffered saline (PBS, pH 7.4, 0.14 M NaCl) using equilibrium dialysis. All types of micelles displayed sustained release of Pt species under physiological conditions as shown in Fig. 7b and no burst release was observed. Of particular interest was the finding that the release rate was higher for the micelles with high density of cross-links. For instance, during 24 hours the micelles with 70% degree of cross-linking released $20.9 \pm 3.1\%$ of incorporated Pt(II), while the micelles with 10% degree of cross-linking released only $9.2 \pm 0.5\%$. This may be due to some spatial differences in drug localization in micelles with different density of cross-links in the cores. Micelles with a higher degree of cross-linking may have more peripheral drug localization allowing easier chloride exchange and hence faster release. Indeed, for the drugs that are chemically incorporated into the polymer carrier the mechanism of release is complex, multistep process. In our case it should involve, first, the penetration of chloride ions into the micelle core, followed by exchange between chloride ions and carboxylic groups in Pt(II) complexes, and, finally, diffusion of Pt(II) species out of the micelles. The observed decay of drug release with the increasing degree of cross-linking of the micelles may be also related to the hydrophobic nature of the core of cisplatin-loaded micelles. Since the core of the micelles with low density of cross-linking can accommodate higher amount of cisplatin, it became more hydrophobic which may hamper the in and out diffusion of water, chloride ions and Pt(II) species.

The observed release characteristics are likely to be beneficial from the standpoint of the chemotherapeutic drug delivery in the body. By preventing the premature drug release the drug-loaded micelles will be more efficiently delivered to the tumor. Importantly, the composition of the “soft” core (i.e. cross-linking density and structure of cross-linker) can be easily tuned to modulate drug loading efficiency, swelling, diffusion and drug release properties. Furthermore, since PEO-coated nanoparticles avoid renal excretion [40], we anticipate that an unwanted side effect of free cisplatin, namely, nephrotoxicity can be also suppressed.

CONCLUSIONS

Block ionomer complexes based on PEO-*b*-poly-carboxylate anions and divalent metal ions were utilized for template-assisted synthesis of a new type of functional nanosystems—hydrophilic polymer micelles with cross-linked ionic cores. Importantly, the overall synthetic approach is robust and versatile to allow for modification of the core and shell compositions that then control the macroscopic properties of the micelles. The ionic character of the core allows for the encapsulation of charged therapeutic or diagnostic molecules while the cross-linking of the core will suppress dissociation of the micelle upon dilution. The finding on the dependence of the loading and release of the cisplatin demonstrate that polymer micelles with cross-linked ionic cores are promising supramolecular carriers that may allow to control the biodistribution and pharmacokinetics of the drug to improve the therapeutic outcomes.

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References

1. Batrakova, EV.; Bronich, TK.; Vetro, JA.; Kabanov, AV. Nanoparticulates as Drug Carriers. Torchilin, V., editor. London: Imperial College Press; 2006. p. 57
2. Torchilin VP. Adv. Drug Delivery Rev 2002;54:235.
3. Kabanov, V. Multilayer Thin Films. Decher, G.; Schlenoff, JB., editors. Weinheim: Wiley-VCH; 2003. p. 47
4. Moffitt M, Khougaz K, Eisenberg A. Acc. Chem. Res 1996;29:95.
5. Kabanov AV, Bronich TK, Kabanov VA, et al. Macromolecules 1996;29:6797.
6. Gohy J-F, Varshney SK, Antoun S, Jerome R. Macromolecules 2001;34:2745.
7. Harada A, Kataoka K. Macromolecules 1995;28:5294.
8. Gohy J-F, Varshney SK, Jerome R. Macromolecules 2001;34:3361.
9. Bronich TK, Kabanov AV, Kabanov VA, et al. Macromolecules 1997;30:3519.
10. Kabanov AV, Bronich TK, Kabanov VA, et al. J. Am. Chem. Soc 1998;120:9941.
11. Bronich TK, Cherry T, Vinogradov SV, et al. Langmuir 1998;14:6101.
12. Bronich TK, Popov AM, Eisenberg A, et al. Langmuir 2000;16:481.
13. Bronich TK, Solomatina SV, Yaroslavov AA, et al. Langmuir 2000;16:4877.
14. Bronich TK, Ouyang M, Kabanov VA, et al. J. Am. Chem. Soc 2002;124:11872. [PubMed: 12358530]
15. Solomatina SV, Bronich TK, Eisenberg A, et al. Langmuir 2007;23:2838. [PubMed: 17266337]
16. Harada A, Kataoka K. Macromolecules 1998;31:288.
17. Kishimura A, Koide A, Osada K, et al. Angew. Chem., Int. Ed. Engl 2007;46:6085. [PubMed: 17628476]
18. Batrakova EV, Li S, Reynolds AD, et al. Bioconjugate Chem 2007;18:1498.
19. Kabanov AV, Vinogradov SV, Suzdaltseva YG, Alakhov VY. Bioconjugate Chem 1995;6:639.
20. Kataoka K, Togawa H, Harada A, et al. Macromolecules 1996;29:8556.
21. Hillmyer MA. Science (Washington, D. C.) 2007;317:604.
22. Zhang L, Khougaz K, Moffitt M, Eisenberg A. Amphiphilic Block Copolymers 2000:87.
23. Vinogradov SV, Bronich TK, Kabanov AV. Bioconjugate Chem 1998;9:805.
24. Katayose S, Kataoka K. Bioconjugate Chem 1997;8:702.
25. Bronich TK, Nguyen H-K, Eisenberg A, Kabanov AV. J. Am. Chem. Soc 2000;122:8339.
26. Oishi M, Nagasaki Y, Itaka K, et al. J. Am. Chem. Soc 2005;127:1624. [PubMed: 15700981]

27. Bronich TK, Nehls A, Eisenberg A, et al. Colloids Surf., B 1999;16:243.
28. Nishiyama N, Yokoyama M, Aoyagi T, et al. Langmuir 1999;15:377.
29. Stapert HR, Nishiyama N, Jiang D-L, et al. Langmuir 2000;16:8182.
30. Oh KT, Bronich TK, Bromberg L, et al. J. Controlled Release 2006;115:9.
31. Bontha S, Kabanov AV, Bronich TK. J. Controlled Release 2006;114:163.
32. Bronich TK, Keifer PA, Shlyakhtenko LS, Kabanov AV. J. Am. Chem. Soc 2005;127:8236. [PubMed: 15941228]
33. Bronich TK, Bontha S, Shlyakhtenko LS, et al. J. Drug Target 2006;14:357. [PubMed: 17092836]
34. Hunt JP. Metal Ions in Aqueous Solution. 1963
35. Ogawara K, Kawazoe S, Tamura T, et al. Polymer 1997;39:437.
36. Li Y, Gong Y-K, Nakashima K, Murata Y. Langmuir 2002;18:6727.
37. Jain S, Bates FS. Science (Washington, D. C.) 2003;300:460.
38. Sanson N, Bouyer F, Gerardin C, In M. Phys. Chem. Chem. Phys 2004;6:1463.
39. Palmer BN, Powell HKJ. J. Chem. Soc., Dalton Trans 1974:2089.
40. Greish K, Fang J, Inutsuka T, et al. Clin. Pharmacokinet 2003;42:1089. [PubMed: 14531722]

Fig. 1.

BIC compositions and morphologies comprise a broad and series of complexes for incorporating non-natural and natural charged molecules including: (a) synthetic polyions, (b) ionic surfactants, (c) metal ions, (d) DNA, (e) RNA, (f) proteins/protein complexes, (g) low molecular mass peptides and (h) pharmaceutical drugs.

Fig. 2.
Scheme for the synthesis of polymer micelles with cross-linked cores.

Fig. 3. Turbidity in the PEO-*b*-PMA/Me²⁺ mixtures as a function of the charge ratio in the mixture, Z: (▲) PEO(170)-*b*-PMA(180)/Ca²⁺, (Δ) PEO(170)-*b*-PMA(180)/Ba²⁺ (■) PEO(125)-*b*-PMA(180)/Ca²⁺, (□) PEO(125)-*b*-PMA(180)/Ba²⁺, (○) PEO(114)-*b*-PMA(81)/Ca²⁺ and (●) PEO(114)-*b*-PMA(81)/Ba²⁺.

Fig. 4.

Turbidity of PEO-*b*-PAA/Me^{2+/3+} mixtures as a function of the charge ratio in the mixture, *Z*. (●) PEO(114)-*b*-PAA(93)/Ca²⁺, (■) PEO(114)-*b*-PAA(93)/Ba²⁺, (▲) PEO(114)-*b*-PAA(93)/Gd³⁺, (◆) mixture of PEO(114)-*b*-PAA(93) and PAA/Ba²⁺, (○) PEO(80)-*b*-PAA(104)/Ca²⁺, (□) PEO(80)-*b*-PAA(104)/Ba²⁺, (Δ) PEO(80)-*b*-PAA(104)/Gd³⁺.

Fig. 5.

(a) Effective diameter (D_{eff}) of cross-linked micelles as a function of pH: ($\square$) $cl\text{PEO}(170)\text{-}b\text{-PMA}(180)$; ($\blacksquare$) $cl\text{PEO}(125)\text{-}b\text{-PMA}(180)$; ($\blacktriangle$) $cl\text{PEO}(114)\text{-}b\text{-PMA}(81)$; and (Δ) $cl\text{PEO}(114)\text{-}b\text{-PAA}(93)$. (b), (c) Tapping-mode AFM images of $cl\text{PEO}(170)\text{-}b\text{-PMA}(180)$ micelles in air at pH 7.0 (b) and pH 5.5 (c) deposited on APS-mica. Scan size is 3.0 μm .

Fig. 6.

Effective diameter (D_{eff}) of *cIPEO(170)-b-PMA(180)* micelles prepared using different cross-linkers as a function of pH: (▲) ethylenediamine, (□) 1,5-diaminopentane, (■) cystamine, (Δ) N-(3-aminopropyl)-1,3-propanediamine, (●) 2,2'-(ethylenedioxy)-bis(ethylamine). D_{eff} values were calculated from three measurements performed on each sample and are within ± 10 nm.

Fig. 7. (a) Cisplatin loading capacity of *c*/PEO(170)-*b*-PMA(180) micelles with different degrees of cross-linking at 37°C; (b) release of Pt(II) complexes from cisplatin-loaded polymer micelles with various targeted degrees of cross-linking in PBS (pH 7.4, 0.14 M NaCl) at 37°C: (■) 10%, (□) 20%, (●) 40%, (Δ) 70%.

Table 1

Physicochemical characteristics of block copolymers

Block copolymer ^a	$M_w \times 10^{-3}$	M_w/M_n	Weight fraction of PEO blocks (f_{EO}) ^a
PEO(170)- <i>b</i> -PMA(180)	23.0	1.45	0.33
PEO(125)- <i>b</i> -PMA(180)	21.0	1.16	0.26
PEO(114)- <i>b</i> -PMA(81)	12.0	1.15	0.42
PEO(114)- <i>b</i> -PAA(93)	11.7	1.20	0.43
PEO(80)- <i>b</i> -PAA(104)	11.0	1.25	0.32

^aThe average number of monomer units in PEO and polyacid blocks and content of PEO blocks in block copolymers were calculated using the average molecular weights provided by manufacturer.

Table 2Effective diameters (D_{eff}) of the PEO-*b*-PMA/ Me^{2+} complexes

Copolymer	PEO- <i>b</i> -PMA/ Ca^{2+} complexes ($Z = 1.6$)	PEO- <i>b</i> -PMA/ Ba^{2+} complexes ($Z = 1.4$)
	D_{eff}^a , nm	
PEO(170)- <i>b</i> -PMA(180)	89	71
PEO(125)- <i>b</i> -PMA(180)	102	76
PEO(114)- <i>b</i> -PMA(81)	n/a	410 ^b

^a Effective diameter denote averaged value calculated from three measurements performed on each sample. D_{eff} values were within ± 10 nm.

^b Size measurements were performed at the onset of turbidity increase at $Z = 3$.

Table 3Physicochemical characteristics of cross-linked micelles at pH 7.0^a

BIC template	Theoretical (“targeted”) cross-linking degree (%)	Z	D_{eff} , b nm	ζ , ξ mV
PEO(170)- <i>b</i> -PMA(180)/Ca ²⁺	20	1.6	156	−30
PEO(125)- <i>b</i> -PMA(180)/Ca ²⁺	20	1.6	176	−25
PEO(114)- <i>b</i> -PMA(81)/Gd ³⁺	20	1.2	141	−23
PEO(114)- <i>b</i> -PAA(93)/Ba ²⁺	40	3.2	169	−32

^a Cross-linked micelles were prepared using 1,2-ethylenediamine as cross-linking agent.^b Effective diameter denote averaged value calculated from three measurements performed on each sample. D_{eff} values were within ± 5 nm.^c ζ are potential values were calculated from three measurements performed on each sample and were within ± 5 mV.

Table 4

Physicochemical characteristics of *c*/PEO(170)-*b*-PMA(180) micelles at pH 7.0

Targeted degree of cross-linking	10%	20%	30%	40%	60%
D_{eff} (nm)	324.7 ± 5.9	214.6 ± 3.1	172.9 ± 1.8	134.8 ± 1.7	127.2 ± 2.3
ζ -potential	-25.8 ± 3.4	-31.3 ± 3.7	-29.4 ± 1.4	-35.2 ± 1.4	-35.3 ± 1.4

Table 5

Characteristics of the cross-linkers used in the study

Cross-linker	Structure	M. w. ^a	xlogP ^b
1,2-ethylenediamine	NH ₂ CH ₂ CH ₂ NH ₂	60.1	-1.3
1,2-diaminepentane	NH ₂ (CH ₂) ₅ NH ₂	102.2	-0.3
<i>N</i> -(3-aminopropyl-1,3-propanediamine)	NH ₂ (CH ₂) ₃ NH(CH ₂) ₃ NH ₂	131.1	-1.0
2,2'-(ethylenedioxy)bis(ethylamine)	NH ₂ (CH ₂) ₂ O(CH ₂) ₂ O(CH ₂) ₂ NH ₂	148.2	-1.7
Cystamine	NH ₂ CH ₂ CH ₂ SSCH ₂ CH ₂ NH ₂	152.2	-0.1

^aData are presented for free bases.^bThe values of xlogP are from PubChem website and represent the predicted values of the octanol/water partition coefficients (logP) of organic compounds.