PEG-Poly(fumaric-sebacic acids)-PEG 삼중 블록 공중합체로 수용액에서 만들어진 폴리머솜의 분석과 방출특성

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Characterization and Release Behavior of Polymersomes of PEG-Poly(fumaric-sebacic acids)-PEG Triblock Copolymer in Aqueous Solution

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Abstract: Polymersomes made of biodegradable triblock copolymers based on poly(fumaric acid-co-sebacoyl chloride)/PEG (PEG-co-P(FA/SC)-co-PEG) were prepared and studied in aqueous solutions. TEM confirmed the formation of vesicles in aqueous media. Aggregation behavior of the copolymers was studied by fluorescence spectroscopy of 8-anilino-1-naphthalenesulfonic acid, and the critical aggregation concentration (c.a.c.) of the copolymer was found to be ~26.2 µM indicating desirable stability of the vesicles. Dynamic light scattering revealed that the size of the vesicles was distributed within the range of 170-270 nm. Turbidity measurements confirmed the relative short-term stability of the polymersomes. Carboxyfluorescein, a hydrophilic compound, was simply encapsulated in the vesicles during polymersome preparation. The release of encapsulant from the polymersomes at 25 and 37 °C lasted about 3 weeks, and the rate of release followed a first-order kinetics. The release is speculated to be primarily carried out through diffusion. These results confirm that these polymersomes are promising as controlled-release carriers of various drugs.

Keywords: block copolymer, dispersion, particle size distribution, critical aggregation concentration.

Introduction

Amphiphilic block copolymers self-assemble, in suitable solvents, to form various supramolecular structures making them proper candidates for various research and technology applications.1,3 Due to their potential applications, their association behaviors have been extensively studied in the last few decades.4 It has been shown that the morphology and size of the supramolecular assemblies depend on both the molecular characteristics of the starting copolymer (type (di-, tri-, multi-block copolymers), chain architecture, block lengths, and composition of the blocks) and the properties of the solution such as solvent, concentration, pH, and temperature.1,3,5,9 Amphiphilic diblock copolymers, for instance, tend to form spherical and/or highly flexible worm-like micellar1,6-13 or vesicular7,9,12,14 structures. However, the self-assembly of triblock copolymers are more complicated. Some are able to undergo core-shell

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micelle\textsuperscript{5,6,15,16} or vesicle\textsuperscript{7,18} formation, whereas others generate more complex structures such as flowerlike micelles,\textsuperscript{4,4} linked micelles,\textsuperscript{5,6} and branched or gel-like network structures.\textsuperscript{5,6,19} Some triblock copolymers even form microspheres featuring sol-gel transition characteristics and are able to form polymeric hydrogels.\textsuperscript{20-24}

In this regard, polymersomes have attracted a great deal of attention since they represent a suitable model for most of biological membrane processes like insertion of integral membrane proteins/ channel proteins docked with viruses to facilitate the transfer of viral DNA into the membranes. Such polymersomes have been effectively applied for vectorisation that could be useful in gene delivery into eukaryotic cells.\textsuperscript{38,39} Polymersomes have also been used for drug delivery purposes because they can deliver both the hydrophilic and hydrophobic therapeutic agents.\textsuperscript{7,14,27,28} Studies on clearance mechanism from the blood circulation have demonstrated that polymersomes have far more extended circulation times \textit{in vivo} than their lipid counterparts (stealth liposomes).\textsuperscript{29}

Biodegradable polymers are capable of being cleaved into biocompatible byproducts through chemical or enzyme-catalyzed hydrolysis.\textsuperscript{30,32} Therefore, they lack the serious drawbacks associated with non-biodegradable polymers, like accumulation in the body and the need for surgical removal after their implantation.\textsuperscript{22,31,33} They are, thus, considered as preferred vehicles for delivery of drugs\textsuperscript{20-22,25,27,34} and other therapeutic agents.\textsuperscript{35,37}

We have previously reported the synthesis of a novel biodegradable triblock copolymer made of PEG and poly (fumaric acid-co-sebacoyl chloride) (PEG-co-P(FA/SC)-co-PEG) triblock copolymer (MW of ca. 9500 Da; PEG4000; FA/SC molar ratio, 0.9; PDI=1.21) was prepared and characterized at Biomaterials Research Center of the University of Tehran following the method previously reported.\textsuperscript{39} The structure of the copolymer is shown in Scheme 1. 8-Anilino-1-naphthalenesulfonic acid (ANS), 5(6)-carboxyfluorescein (CF, 99%), tris(hydroxymethyl)aminomethane, and Sephadex G-50 were from Sigma (St. Louis, MO). Phosphotungstic acid was purchased from Pelco (Tustin, CA). All other chemicals were analytical grade obtained from Merck (Darmstadt, Germany).

**Copolymer Dispersion.** PEG-co-P(FA/SC)-co-PEG triblock copolymer was dispersed in phosphate-buffered saline (PBS), pH 7.4, (8.0 g/L of NaCl, 0.2 g/L of KCl, 1.44 g/L of NaHPO\textsubscript{4}, 0.24 g/L of KH\textsubscript{2}PO\textsubscript{4} in deionized water\textsuperscript{30} (0.1-1.5 wt%), while stirring for at least 4 h at room temperature, or 15 min at 70 °C, let cool to room temperature, and finally sonicated 3 min to obtain a homogenized dispersion. Desired concentrations were prepared by dilution of this stock solution.

**Transmission Electron Microscopy (TEM).** A drop of the dispersion in PBS, pH 7.4 (0.4 wt%) was deposited onto a 400 mesh carbon-coated Formvar filmed grid. Excess dispersion was removed by touching it with a delicate filter paper. The particles on the grid were negatively stained with phosphotungstic acid (2 wt%) solution, following the method described above. The grid was then allowed to dry at room temperature. The morphology and size of the particles were directly determined using a Zeiss 902 A transmission electron microscope operating at an accelerating voltage of 80 kV.

**Particle Size Determination.** Samples were prepared as described above. The vesicles size and size distribution were determined by photon correlation spectroscopy (90Plus, Brookhaven Instruments Co., New York, USA) with He-Ne

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**Scheme 1.** Structure of PEG-co-P(FA/SC)-co-PEG triblock copolymer.
laser (632.8 nm) at a fixed scattering angle of 90°. Measurement was performed at 25 °C in triplicate. Each analysis lasted for 2 min, with no evidence of change in size with time. The samples were filtered through a 0.65 µm Teflon filter directly into a precleaned cell. The concentration of the polymer dispersion was 0.7 wt%.

**Turbidity Measurements.** The experiments were conducted on vesicle dispersion in PBS, pH 7.4, (0.4 wt%) using a UV-vis spectrophotometer (Cary 100 Bio, Varian, Australia) at 25 and 37 °C. Turbidity was monitored by measuring the absorbance at 600 nm. The absorbance recording was started immediately after sample preparation and continued over a time period of 5 h.

**Critical Aggregation Concentration (c.a.c.).** ANS was used as a hydrophobic fluorescent probe. A given amount of the ANS stock solution in PBS, pH 7.4, (0.4 mM) was added to each of a series of the copolymer dispersions, followed by adding different amounts of buffer. The samples obtained, containing a fixed concentration (1.02×10⁻⁴ M) of ANS and varying concentrations (0.06×10⁻⁶ to 1.1×10⁻⁴ M) of the copolymer, were stirred for 2 h at room temperature to achieve equilibration. Fluorescence measurements were performed at room temperature in 1 cm path length quartz cuvettes using a fluorescence spectrophotometer (Cary Eclipse, Varian, Victoria, Australia). The excitation and emission slits were both 10.0 nm, and the scan rate was 600 nm/min. Fluorescence spectra were recorded in the range of 390-700 nm upon excitation at 370 nm. Each spectrum was scanned three times to acquire the final fluorescence emission spectra.

**Hydrophilic Encapsulant Loading.** Polymersomes (0.2 wt%) containing the water soluble fluorophore 5(6)-carboxyfluorescein (CF) were prepared in the same way as mentioned above (section: copolymer dispersion) only the PBS solution was replaced by a CF solution (50 mM) in Tris-HCl buffer (50 mM, pH 7.4). The unencapsulated CF was subsequently removed by gel filtration on a Sephadex G-50 column at room temperature, eluted with isotonic buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.4).

**In vitro Release Experiment.** The release profiles of CF from polymersomes in Tris buffer, pH 7.4, at 25 and 37 °C were determined via fluorescence spectroscopy, according to established procedures. In brief, 0.2 mL of the gel filtered polymersome dispersion was mixed with 1.1 mL of the elution buffer, as release medium, and incubated at room temperature or at 37 °C while stirring. At different time intervals, 0.1 mL aliquots of the mixture were withdrawn, diluted with 1.0 mL of Tris buffer, and then the fluorescence (Fᵢ) was measured (excitation maximum, 490 nm; emission maximum, 520 nm). To obtain the maximal fluorescence (Fᵢ), 0.05 mL of a freshly eluted fraction was disrupted with 3% (w/v) Triton X-100, followed by diluting to 3.575 mL with Tris buffer before recording the fluorescence. The extent of release (%) over time was calculated from (Fᵢ/Fᵢ)×100. It should be noted however that the time between the gel filtration of the polymersomes and the start of the experiment (30-40 min) was not considered in the release time. The SPSS statistical software was used for nonlinear curve fitting in the release profile.

The amount of encapsulated CF was determined from Fᵢ by extrapolation from the corresponding calibration curve (I=3.6717 C, R²=0.9997) and the dilution coefficient. The encapsulation efficiency (EE) was calculated from the amount of dye incorporated as a percentage of the amount initially used.

\[
EE(\%) = \frac{\text{The amount of dye incorporated}}{\text{The amount of dye initially used}} \times 100 \quad (1)
\]

**Results and Discussion**

**Characterization of Particles.** Figure 1 shows the TEM pictures of the negatively stained particles. Particles are unstained but surrounded on the support film by the stain as a result of surface tension interactions. Thus, the specimen appears lighter against a dark background (in negative contrast). At low magnification (Figure 1(a)), particles look fairly spherical with diameters in the range of 130-380 nm. Furthermore, the particles show a pronounced peripheral higher electron density compared to the interior characteristic of vesicular structures as can be observed at high magnification of TEM image (Figure 1(b)). Figure 2 shows the particle size distribution of the vesicles assessed by dynamic light scattering. DLS data show that the majority of the vesicles (96%) have diameters in the range of 170-270 nm and only a small fraction of them (4%) have diameters in the range of 28-34 nm. The smaller associates are defined as polymer aggregates containing only a few unimers. These unimer molecules may feature lower the average degrees of polymerization than others as a result of sample polydispersity. Results are summarized in Table 1, indicating that the mean diameter is 181.3±3.5 nm. Vesicles have a modal size of 207 nm. A comparison of the diameters determined by

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The two methods reveals that DLS shows a rather smaller size distribution, while it reflects the hydrodynamic diameter of particles in the swollen state. It is worth noting that there was a filtration step in sample preparation for DLS experiment, which did not exist for TEM. Thus, the filtration is considered the cause of producing particles with a size distribution of smaller as well as narrower in solution than in the dry state. Furthermore, the small associates (28-34 nm), reported by DLS, are not observed on the TEM image due to their low frequency.

Figure 1. TEM images of PEG-co-P(FA/SC)-co-PEG vesicles in PBS, pH 7.4: (a) low magnification; (b) high magnification. Scale bar: (a) 0.4 µm; (b) 0.09 µm.

Storage Stability- Short Term Aging. Physical stability of polymeric vesicles upon short-term aging can be followed by recording the turbidity as a function of time wherein an increment of the absorbance of the dispersion in the visible region is a sign of aggregation. The absorbance at 600 nm of polymersome dispersions (0.4 wt%) at 25 and 37°C remained unchanged within the chosen time interval (data not shown), implying the absence of aggregation. TEM images also show not multivesicular aggregates as well as multilamellar vesicles but only unilamellar vesicular structures (Figure 1), which could be a clear endorsement of the above-mentioned result.

The vesicular aggregates are caused due to weak repulsion between the coronal layers of the vesicles. Herein, it is presumed that the repulsion between long hydrophilic PEG blocks protruding outside of the vesicles prevents the formation of aggregated structures. Thus, the PEG-co-P(FA/SC)-co-PEG vesicles seem to be more stable than those of PEO-PPO-PEO with shorter hydrophilic blocks, which showed aggregated structures and a marked increase in the turbidity of the solution within a similar period of time.

Figure 2. Particle size distribution of polymersomes in PBS, pH 7.4, measured at 25°C.

Table 1. Properties of Polymersomes Made of the Triblock Copolymer

<table>
<thead>
<tr>
<th>Triblock copolymer</th>
<th>Diameter&lt;sup&gt;⁹&lt;/sup&gt;</th>
<th>PDI&lt;sup&gt;⁷&lt;/sup&gt;</th>
<th>c.a.c.&lt;sup&gt;⁵&lt;/sup&gt; (µM)</th>
<th>Encapsulation efficiency&lt;sup&gt;⁶&lt;/sup&gt; (%)</th>
</tr>
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<tbody>
<tr>
<td>PEG-co-P(FA/SC)-co-PEG 4.0-1.5-4.0</td>
<td>Mean&lt;sup&gt;⁰&lt;/sup&gt; (nm) 181.3±3.5</td>
<td>Mode (nm) 207</td>
<td>0.176</td>
<td>26.2</td>
</tr>
</tbody>
</table>

<sup>⁹</sup>The copolymer is denoted by PEG-polyanhydride-PEG followed by molecular weight of each block (×10<sup>-3</sup>) g/mol. <sup>⁷</sup>Determined by dynamic light scattering. <sup>⁸</sup>The value is the mean of three runs±standard error. <sup>⁵</sup>Critical aggregation concentration (c.a.c.) in PBS, pH 7.4, determined by the fluorescence probe technique using ANS at 25°C. <sup>⁶</sup>Calculated based on the encapsulation of CF as a hydrophilic fluorophore.
c.a.c. Determination. Fluorescence probe technique is a powerful tool to study association of amphiphilic block copolymers. In the present study, ANS was employed as a fluorescent probe to detect the formation of the hydrophobic parts of the vesicular structures. Changes in ANS fluorescence on vesicle formation were used to establish the critical aggregation concentration (c.a.c.), which is a measure of the stability and of the physical properties of the aggregates. Typical emission spectra of ANS (1.02×10^{-4} M) in aqueous solutions of triblock copolymer at various concentrations are shown in Figure 3(a). The two noteworthy features of these spectra are that the fluorescence intensity shows a marked enhancement with increasing copolymer concentration, reflecting an increase in the quantum yield of the fluorescence, and that there is a progressive shift in the emission maximum toward the blue, from about 529 to 516 nm. These changes are due to the transfer of ANS molecules from the aqueous environment to the hydrophobic parts of the vesicular membranes and thus provide information on the location of ANS probe in the system.

It is worth mentioning that quantum yields of a fluorophore are largely a function of the polarity of the solvating environment. However, other factors such as quenching, internal conversion, intersystem crossing, energy transfer, photochemical reactions, and complex formation in the excited state may also affect the quantum yield. In this case, these factors can be neglected. Figure 3(b) shows the fluorescence intensity at λ_{max} as a function of the copolymer concentration. It is observed that the intensity has a negligible change below a certain concentration and increases substantially thereafter. This change in intensity reflects the onset of vesicle formation and thus provides a quantitative method of c.a.c. determination. The c.a.c. is taken as the intersection of the two straight lines drawn through the points at the lower copolymer concentrations, lying on a nearly horizontal line, and the points on the rapidly rising part of the plot. This indicates a c.a.c. of 26.2 µM (Table 1), corresponding to 2.5×10^{-4} g/mL, the low value of which suggests that the triblock copolymer forms stable supramolecular structures in aqueous solutions.

Encapsulation of CF. Preparation of polymersomes in aqueous solutions containing hydrophilic compound CF resulted in the encapsulation of the dye, implying that the particles are indeed vesicles with the inner aqueous compartment. Free CF was removed by gel filtration on a Sephadex G-50 column. CF-loaded polymersomes emerged in the void volume, whereas the free dye was retarded by the gel. Isotonic Tris buffer was used as an eluant to avoid rupturing vesicles from placing in hypotonic environment of the column. The encapsulation efficiency (EE) of the polymersomes was then determined after disruption of the vesicles with Triton X-100, which had no effect on the emission maximum of fluorophore at 520 nm. The percentage of CF encapsulation was found to be 5.6% (Table 1), which is comparable with that of other kinds of polymersomes reported elsewhere.

Encapsulant Release Studies. To monitor the release of aqueous contents, CF, a vital property of which is “self-quenching”, was incorporated in polymersomes. It has been observed that in solutions containing high concentrations of CF (more than 10 mM), the fluorescence emission declines rapidly because of interactions between fluorophore mole-

![Figure 3](image-url)
Thus, a dispersion of vesicles containing 50 mM of CF fluoresces slightly, while the release of the dye into the external medium, hence dilution, results in the relief of the self-quenching and in a marked increase in fluorescence intensity.\(^{41,51,52}\)

Herein the release of CF from polymersomes into Tris-HCl buffer, pH 7.4, was monitored at 25 and 37 °C and the typical release profiles are shown in Figure 4(a). The release is 38% at the beginning of the experiment (around 40 min after gel filtration), and lasts about 3 weeks at both temperatures. Within this time period, nanoparticles exhibit release of 65 and 75% of the dye at 25 and 37 °C, respectively, indicating that there is not much difference in the amount of release between the two temperatures. Furthermore, the amount of release demonstrates that there are not significant interactions between the encapsulant and the copolymer molecules.

Results also show that the release of CF from polymersomes at both temperatures of 25 and 37 °C has the identical pattern. There is an initial burst, ascribed to the release of residual dye molecules on the surface of the vesicles,\(^{12,53}\) followed by a slowly decreasing release rate which approaches zero at long time (ca. 8-9 days). This release behavior of the polymersomes seems to be studied with a “membrane-controlled reservoir device” model, as previously done by Meng et al.\(^{41}\) Provided that the polymersomes contain an unsaturated CF solution, the release rate, \(\frac{dM}{dt}\), which is proportional to the mass of the encapsulant agent and declines exponentially with time,\(^{41}\) can be described by:

\[
d\frac{M}{dt} = k(M_0 - M_t) = kM_0\exp(-kt)
\]  

(2)

where, \(M_0\) and \(M_t\) are the mass of agent in the device at time \(t = 0\) and the mass released until time “\(t\)”, respectively, and \(k\) is a first order rate constant expressed in units of time\(^{-1}\).\(^{41,54,55}\)

The exponential part of the data was redrawn as \(\ln\left(\frac{M_0}{M_0 - M_t}\right)\) versus time obtained from eq. (3):

\[
\ln(M_0/(M_0-M_t)) = kt
\]

(3)

where, \(F_t\) and \(F\) are proportional to \(M_0\) and \(M_t\), respectively. Figure 4(b) shows a linear relation at both temperatures proving that the polymersome system is a membrane-controlled reservoir device system. The slopes of the lines also show that there is no significant difference in the \(k\) values between the two temperatures, implying that the release is complete approximately at the same time.

As indicated by eq. (2), when CF is liberated, its concentration (i.e. the driving force for release) within the device \((M_0 - M_t)\) decreases resulting in a release rate which declines exponentially with time, demonstrating a first-order release kinetics.

Several mechanisms of drug release from biodegradable carriers have been proposed: Fickian diffusion through the polymer matrix, diffusion through pores in the matrix, and drug liberation through polymer erosion.\(^{11,12}\) However, it is difficult to predict a drug release profile because the profile is governed by various factors such as solubility of drug,\(^{11}\) degradation of polymer,\(^{11,20,31}\) and polymer-drug interaction.\(^{11,20}\) In this case, NMR studies have already shown that the degradation rate of the triblock copolymer is slow up to 2 weeks and thereafter it increases.\(^{39}\) Since the release is near complete within this time period, it is speculated that the release is primarily carried out through diffusion during the experimental period.
Conclusions

Vesicles of PEG-co-P(FA/SC)-co-PEG, a biodegradable triblock copolymer, are prepared by dissolving the copolymer directly in water. Dynamic light scattering shows that the particle size distribution of the vesicles is within the range of 170-270 nm, with an average size of ~181 nm. Results of turbidity measurements at 25 and 37 °C reveal the relative short-term stability of polymersome dispersion which could be the result of strong repulsions between long hydrophilic PEG blocks in the coronal layers of the vesicles. Furthermore, hydrophilic compounds can be easily encapsulated in the vesicles during polymersome preparation. The release of carboxyfluorescein (CF) from the polymersomes in Tris buffer at both temperatures of 25 and 37 °C lasts about 3 weeks and occurs in a first-order diffusion-dependent manner. Besides, changes in ANS fluorescence spectra upon increasing polymer concentration are characteristics of vesicle formation and the transfer of ANS molecules into the more hydrophobic environment. By examining the extent of the changes as a function of copolymer concentration, the critical aggregation concentration (c.a.c.) can be determined. Results show a low c.a.c. value suggesting that the polymersomes of PEG-co-P(FA/SC)-co-PEG may find promising biomedical applications as stable drug carriers.

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