Evaluation of the Mechanical properties and Blood compatibility of Polycarbonate Urethane and Fluorescent Self-colored Polycarbonate Urethane as Polymeric Biomaterials

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Abstract

Fluorescent polymeric biomaterials have got significant attention due to their promising applications in biomedical fields such as labeling, monitoring, diagnostics, imaging and tracking. Polycarbonate urethane (PCU) and 1,8-naphthalimide based fluorescent dyes separately have been studied and shown great biocompatibility and physical properties. Therefore, in this work we have taken advantage of excellent fluorescence properties of naphthalimide dye and biocompatibility of PCU, and covalently attached the fluorescent dye to the PCU (self-colored PCU). Covalent attachment can increase the stability of the dye in the biomedical applications especially when biomaterials are in contact with blood and can inhibit the release of the dye to surrounding media. DMTA, AFM, and contact angle measurement were used to study the mechanical and morphological properties of the self-colored PCU and results showed that incorporation of the dye to the PCU did not change the mechanical and morphological properties of the PCU. In addition, MTT assay, hemolysis assay, PT and aPTT assays as well as protein adsorption assay was used to evaluate the blood compatibility of PCU and self-colored PCU and results indicated great bio and blood compatibility of these materials. These great mechanical and blood compatibility properties of the self-colored PCU as well as their excellent fluorescent properties suggested that, these materials could be an ideal candidates to be use in biomedical applications in which non-invasive and non-destructive fluorescent based techniques are required.

Keywords: Self-colored Polycarbonate Urethane, Biocompatibility, Blood Compatibility, Fluorescent, Polycarbonate Urethane

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Introduction

Polymeric biomaterials with significant impact on medical technologies have been extensively studied in the last few decades and have been used as artificial organs, wound healing, biomedical packaging, therapeutic agents, drug delivery, and artificial kidney and hearts [1–6]. In an effort to design and synthesis of polymeric biomaterials, different synthetic polymers such as polyethylene, polypropylene, polyamide, polyurethane, polyvinyl alcohol, and poly (ethylene glycol) have been synthesized and their biocompatibility for using in biomedical application have been studied [3, 4, 7–9]. Generally, the biocompatibility (which is defined as an ability of materials to be compatible with cells, tissues and etc.)[5, 10] of these polymers is an important factor, and is governing the design and synthesis of them [11]. It has been shown that functional groups, surface properties and chemical structures of polymeric biomaterials are important factors in design and synthesis of polymeric biomaterials for desired application [1, 2, 12].

Biocompatibility of the materials includes tissue compatibility (histocompatibility) and blood compatibility (hemocompatibility) and since, biomaterials are usually in contact with blood, besides biocompatibility, their blood compatibility is key factor in design and synthesis of polymeric biomaterials for applications such as cardiovascular tissue engineering [5, 8, 9, 13–17]. Contact of blood with foreign material causes interfacial adsorption of the proteins, which can lead to formation of aggregation, platelet, coagulation, and clots [2, 5, 18]. Therefore, it is extremely needed to understand the mechanism of adsorption of proteins to minimize the formation of the clots. In an effort to design and synthesis of blood compatible polymeric materials, many researchers have tried to increase the hydrophilicity of the surfaces to reduce the formation of clots (since proteins adsorb on hydrophobic surfaces more than hydrophilic) [2, 15]. Among the synthetic polymer for biomedical applications, polyurethanes (PU) have got considerably significant attention due to their high physical properties, biocompatibility and blood compatibility [5, 8, 10].

Polyurethanes can be synthesize from the polyaddition reaction between diisocyanate (hard segment) and polyl groups (soft segment) [10, 19–22]. Possibility to use different hard and soft segments for synthesizing polyurethane to achieve desired properties made them versatile and tunable polymers. For example, different kind of polyurethanes such as polycarbonate urethane, urethane acrylates, polyurethane polyols, polyester urethane, and polyether urethane have been synthesized by changing the soft segment (Polyl groups) and have been used in different applications [23–33]. Among aforementioned synthetic polyurethanes, recent studies have been shown that polycarbonate based urethanes have better biocompatibility and biostability than other kinds of polyurethanes for medical applications which was correlated to higher stability of the soft segments (polycarbonate) inside of the body [34]. Recently, Zue et al.[35] have used poly (1,6-hexanediol)carbonate diols (PCDL), 4,4′-methylenebis (cyclohexyl isocyanate) (H12MDI), 1,6-hexamethylene diisocyanate (HDI) and 1,4-butandiol (BDO) to synthesize polycarbonate urethanes and studied their biocompatibility using hemolytic and platelet adhesion tests, and they have shown that these materials have a great ability to be used as biomaterials due to their good blood compatibility. In another study, Khan et al.[36] have shown that good mechanical and chemical properties of polycarbonate urethanes make them suitable to use in biomedical applications. Furthermore, recent reports in comparison of the biocompatibility of polyether urethanes and polycarbonate urethanes showed that polycarbonate based urethanes have higher oxidative stability and biostability, which makes them good alternative to replace with polyether urethanes for biomedical applications [34, 37].
In addition, promising properties of the fluorescent polymeric biomaterials (the combination of the fluorescent compounds and polymeric biomaterials) which can be use in different biomedical applications such as biomedical labeling, monitoring, screening, diagnostics, and tracking [38], have motivated many researches to design and synthesis of biocompatible fluorescent polymeric biomaterials. Fluorescent based techniques are non-invasive, non-destructive, selective, sensitive and works based on real-time assay [39–41]. Different methods have been used to incorporate the fluorescent compounds inside of the polymeric materials. For example, fluorescent dyes have been incorporated into polymeric biomaterials with encapsulation method, in which organic dyes can be encapsulate inside of the cavity of polymeric nanoparticles [38, 42, 43]. However, increase in the size of the polymeric nanoparticles has been considered as a drawback of this system [38]. Moreover, quantum dots (QDs) have been extensively studied and used by different researches; [44–46] however, toxicity of the QDs still remained as a big concern [47]. In an effort to design of fluorescent polymeric biomaterials, covalent attachment of the dye to polymeric biomaterials have been studied by Droumaguet et al. [48]. In this research, they have synthesized copolymer with three different monomers containing rhodamine B fluorescent dye (chemical conjugation) and prepared nanoparticles and studied their ability to image the human brain endothelial cells [48]. Covalent attachment of the dye to the polymeric biomaterials can stop the release of the dye during the probing or sensing applications due to their chemical stability. However, it has been shown that dyes based on rhodamine have poor photostability [41, 49]. Fluorescent dyes based on 1,8-Naphthalimide with strong photostability, tunable photophysical properties as well as low cytotoxicity make them as an ideal alternative fluorescent dyes in biomedical application [50–53]. Therefore, the objective of this research was to use the excellent photophysical properties of naphthalimide dyes and biocompatibility of PCU simultaneously, and design and synthesis novel fluorescent polymeric biomaterials (PCU) based on naphthalimide dye and study their physical, mechanical and blood-compatibility.

In our previous work, [54] we have designed and synthesized self-colored polycarbonate urethane using 4-amino-N-propanoic acid-1,8-naphthalimide dye (Scheme 1), and after characterization of the samples, we have shown that around 99% percent of the dye reacted chemically with polycarbonate urethane (which was indicating that dye covalently attached to polymer and will not release from the sample during the washing step) and fluorescent properties was characterized. Therefore, in this work, we have studied the mechanical and morphological properties of the samples using dynamic mechanical thermal analysis (DMTA), atomic force microscopy (AFM), and contact angle measurement. In addition, we have evaluated the cytotoxicity and blood compatibility of the polymeric biomaterials to the cells and blood using MTT assay, hemolysis tests, partial thromboplastin time (APTT) and prothrombin time (PT), and protein adsorption tests. Our results showed that polycarbonate urethane has good mechanical properties and biocompatibility, in addition; incorporation of the dye to polymer did not change their mechanical and biocompatibility properties. Therefore, these materials owing to their excellent fluorescent properties and biocompatibility can be used in biomedical applications in which fluorescent properties of the biomaterials is playing important role such as imaging, diagnostics, labeling, and monitoring.

**Experimental and Methods**

**Materials**
Acenaphthene, potassium dichromate, sodium carbonate, beta-alanine and all of the organic solvents were obtained from Merck Co. of Germany. The Polycarbonate diol (Desmophen C3200 XP, (molecular weight of 2000 gr/mol)) was purchased from Covestro Co. of Germany, HDI and 1,4 butanediol were also obtained from Merck. Dibutyltin dilaurate (DBDTL) which was used as catalyst, was supplied by Sigma-Aldrich Corp. (USA).

Synthesis, Characterization and film casting

Synthesis and characterization of the naphthalimide based dye and self-colored polycarbonate urethane were done according to our previous reports [54, 55]. Briefly, acenaphthene was nitrated, oxidized, imidiated, and reduced to achieve final compound of 4-Amino-N-propanoic acid-1, 8-naphthalimide dye [55]. Then prepolymer of the polycarbonate urethane was synthesized using polycarbonate diol and Hexamethylene diisocyanate, and 0.03 wt% dye was added to prepolymer and polymerization was continued to achieve self-colored polycarbonate urethane (self-colored PCU) [54]. Polymer was casted using film applicator with thickness of 100 ± 10 µm and dried over night at oven at 100 °C.

Dynamic mechanical thermal analysis (DMTA (Tritec 2000, U.S.)) was used to study the mechanical and thermal properties of the polymeric materials and heating rate was 5 °C/min from −60 to 100 °C with frequency of 1Hz and samples were studied under nitrogen atmosphere. In addition, USPM atomic force microscopy (Ambios Technologies, U.S.) with silicon cantilever was used to study the topographical properties of the samples. Finally, the static contact angle measurements with water as probe liquid (surface tension equals to 72.8 dyne/cm at ambient temperature) was carried out using a Cam 200 optical contact angle meter (Data Physic instrument, u.s.) at room temperature according to ASTM D 7334. Ten measurements were taken and the average values were reported.

Biocompatibility Tests

Cytotoxicity of the PCU and self-colored PCU was evaluated using calorimetric test (MTT, Sigma-Aldrich, U.S.). Preparation of the lung epithelial cell and MTT assay were done according to our previous work [10]. Briefly, cells were cultured and 100 µl of MTT solution (0.5 mg/ml) was added to 96 well-plate (SPL Life Science Company, Korea) containing PCU and self-colored PCU and incubated with 5 % CO₂ for 3 h at 37 °C in shaker incubator (Heidolph, Germany). After preparation of crystal frozman, MTT solution was removed and 100 µl of dimethyl sulfoxide (DMSO, (Merck, U.S.)) was added to each wells and after dissolving crystals, ELISA reader (Finland) was used to measure the absorbance in the range of 490 nm to 570 nm. Each experiment was repeated in three replicates and following equation was used for calculating the cell viability percentage.

\[
Cell\ Viability\ (\%) = \left(\frac{Absorbance\ of\ the\ samples}{Absorbance\ of\ the\ control}\right) \times 100 \quad (Eq. 1)
\]

Hemolytic properties of PCU and self-colored PCU were studied using hemolysis test according to literature with a little modification [33, 56]. Briefly, heparinized tubes were used for collection of the blood and after centrifugation for 10 min at 700 g using Ependorf (Germany), 1 ml of the blood was splashed on the polymeric films (1cm×1cm). Then samples were incubated at 37 °C for three hours using shaker incubator. In the next step, samples were
centrifuged at 1000 g for three hours and absorbance of the supernatant was measured at 540 nm. Each experiment was done in three replicates and DI water and phosphate saline buffer (PBS, GIBCO, U.S.) were used as positive and negative controls, respectively. Finally, following equation (Eq. 2) was used for calculation of hemolysis percentage.

\[
\text{Hemolysis (\%)} = \left( \frac{\text{Absorbance of the sample at 540 nm} - \text{Absorbance of the negative control at 540 nm}}{\text{Absorbance of positive control at 540 nm} - \text{Absorbance of negative control at 540 nm}} \right) \times 100 \quad (\text{Eq. 2})
\]

Blood coagulation assay was conducted using prothrombin time (PT) and activated partial thromboplastin time (APTT) tests according to the literature [8, 33]. Briefly, blood was added to citrate (10 volume %) and plasma was obtained by centrifugation of the tubes at 2000 g for 10 min. Then, plasma was added to the samples and samples were incubated for 2 h and 48 h and coagulometer instrument was used for analyzing the samples and obtaining PT and APTT (fisher scientific, U.S.).

Finally, to measure fibrinogen adsorption, fresh human blood was mixed with sodium citrate (3.2% w/v) at a ratio of 9:1. After centrifuging in 2000g for 10 minute, 1 ml of plasma was added to each well of 24 well plate (SPL Life Science Company, Korea) containing samples and incubated at 37 °C for 3h under gentle agitation. The samples incubated and then plasma was mixed (1 to 9 ratio) with diluent buffer (Buffer containing: antiheparin, antifibrinolytic at pH: 3.57). The incubation was continued for 2 min at 37 °C, then thrombin was added into diluted plasma and time of conversion of fibrinogen to fibrin was recorded by coagulometer. Finally, standard curved was used to convert time of clot formation to fibrinogen concentration.

**Results and Discussion**

**PCU and self-colored PCU characterization**

1,8-naphthalimide dyes owing to their excellent fluorescent properties have been extensively used in various applications such as dyeing agents for fabric, photoinduced electron transfer (PET) and internal charge transfer (ICT) based probes, ion sensors, cellular imaging, and DNA targeting [57–62]. In addition, recent reports have been shown that polycarbonate based urethanes have great blood compatibility [34–37]. Therefore, in this work, we have tried to combine the excellent fluorescent properties of the naphthalimide based dye and excellent blood compatibility of the PCU together, and evaluate the blood compatibility of covalently attached fluorescent dye to polyurethane, which can be use in different applications such as biomedical imaging, labeling, tracking, and diagnostics [38]. Chemical structures of the PCU, self-colored PCU and the naphthalimide-based dye, which were used in this research, have been showed in the scheme 1.
Scheme 1. Chemical structures of PCU, self-colored PCU, and naphthalimide based dye which have been used in this research.

Dye and polymers were synthesized and characterized according to our previous work [54, 55]. The self-colored polymer showed good fluorescence properties with maximum fluorescence intensity around 525 nm [54]. Therefore, in this work DMTA was used to study the thermal and mechanical properties of the PCU and self-colored PCU. Tan δ versus temperature and storage modulus versus temperature plots have been shown in Figure 1a and 1b, respectively. Tan δ is the ratio of loss to storage modulus and the maximum Tan δ assigned to glass transition temperature (T_g). As is shown in Figure 1a, glass transition temperature for two samples were almost similar (around -16 °C), which shows that at the ambient temperature, PCU and self-colored PCU are above their glass transition temperature and could be considered as elastomer urethanes. Self-colored PCU showed a small decrease in T_g due to the dangling of dye molecules to the PCU chain. In addition, storage modulus versus temperature plot for the PCU and self-colored PCU has been shown in Figure 1b, and the results showed that at temperature below the T_g, self-colored PCU showed higher storage modulus while in the rubbery region (after T_g) no difference was observed between PCU and self-colored PCU.
Figure 1. Storage modulus and Tan delta plots of PCU and self-colored PCU as function of temperature.

In addition, the summary of the DMTA results for PCU and self-colored PCU have been summarized in Table 1. Which is indicating that addition of dye to the PCU did not change the physical and mechanical properties of PCU, significantly.

| Polymer                | Tg (°C) | Storage modulus (at glassy region) (GPa) | Relaxation strength |
|------------------------|---------|------------------------------------------|--------------------|
| PCU                    | -14     | 0.3                                      | 0.45               |
| Self-colored PCU      | -16     | 0.43                                     | 0.43               |

Moreover, AFM imaging was used to study the morphological properties of the self-colored PCU (Figure 2). AFM images showed almost uniform surface for the self-colored PCU, and it can attributed to the low weight percent of the dye (0.03 wt%). In addition, the roughness of the sample has been calculated over whole samples and results have been shown in Table 2. As it shown in table 2, roughness values for self-colored PCU was around 8.2 nm, which was showing that samples were uniform.
Figure 2. Topographical AFM images of self-colored PCU.

Table 2. Roughness values for self-colored PCU

| Polymer          | Roughness (Ra) | Root mean square (RMS) |
|------------------|----------------|------------------------|
| Self-colored PCU | 8.20 nm        | 9.24 nm                |

It has been shown that hydrophilicity and hydrophobicity of the surfaces affects the biocompatibility of them [2], and surfaces with higher hydrophilicity have better affinity to cells and have better hemocompatibility [10, 63]. Therefore, we have used contact angle measurement to study the hydrophilicity/hydrophobicity of the PCU and self-colored PCU (Figure 3). Both PCU and self-colored PCU showed contact angle smaller than 90° (79±1° and 76±2 ° for PCU and self-colored PCU, respectively). This results were showing that both samples had hydrophilic surfaces, which can tend to adsorb lower amount of proteins and have better blood compatibility [2].

Figure 3. Water contact angle of PCU (a) and Self-colored PCU (b).

Biocompatibility of PCU and self-colored PCU.

Cytotoxicity of the polymeric biomaterials which will be use in biomedical applications is an important factor which governs the design and synthase of polymers with biomedical application [36]. Generally, cytotoxicity can be measured using fluorescent based technique (live-dead assay using flow cytometry)[64] or calorimetric based techniques (such as MTT) [10, 56, 65]. In this study, we have used MTT assay for studying the biocompatibility of PCU and self-colored PCU to cultured cells after 48 h. Cell viability was calculated according to equation 1 and results have been shown in Figure 4. The results showed that around 100 % of the cells were viable after incubation with PCU, and in agreement with previous study, PCU was not toxic to the cells [36]. In addition, the results showed that the incorporation of the dye to PCU did not affect the non-toxicity of the samples to the cells and incubated cells with
self-colored PCU were still viable after 48 h. Therefore, both samples were similar to control biocompatible and no significant change was observed due to incorporation of dye.

**Figure 4.** Cell viability (%) of lung epithelial cells after 48 incubations with PCU and self-colored PCU. Each experiment was done in three replicates.

Blood incompatible materials can rupture the red blood cells and induce release of hemoglobin or platelet; therefore, hemolytic activity of polymeric biomaterials is another serious concern in designing and synthesizing biocompatible biomaterials [66, 67]. Therefore, in the next step we studied hemolytic activity of PCU and self-colored PCU and hemolysis percentage was calculated according to equation 2 and results have been shown in table 3. The absorbance was measured at 540 nm, which was indication of hemoglobin release and hemolysis percentage less than 5 % was considered as non-toxicity of the samples (permissible value for polymeric biomaterials) [35, 66, 68]. The results showed that PCU is not causing hemolysis of the cells (hemolysis percentage is around 1%) and the results were in agreement with the previous studies for PCU and other polymeric biomaterials, in which the hemolysis value was around 1% and lower than 5% [13, 14, 33, 35]. In addition, results showed that incorporation of naphthalimide based fluorescent dye to the polycarbonate urethane with covalent bond, did not affect the hemolytic activity of PCU (self colored PCU did not cause any hemolysis and no significant change with pristine polymer). Similar hemolytic activity results have been obtained by Zhue et al.[35] for different kinds of PCUs, and the good blood compatibility of the PCUs was attributed to number of the side chains of polymer. Therefore, our results have been shown that PCU and self-colored PCU have low hemolytic activity and are in the permissible range for using in the biomaterials with in blood contact applications.

**Table 3.** Hemolysis percentage of PCU and self-colored PCU. Each experiment was done in three replicates.

| Polymer       | Hemolysis percentage (Mean ± SD) |
|---------------|----------------------------------|
| PCU           | 1.10 ± 0.15 %                    |

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APTT and PT (coagulation time) for plasma incubated with PCU and self-colored PCU after 2 h and 48 h have been shown in table 4, which are two other parameters to evaluate the blood compatibility of the polymeric biomaterials. The results showed that PT was 15.23 ± .15 sec and 18.16 ± 0.55 sec for PCU samples after 2 h and 48 h, respectively, which was almost similar to PT values obtained for control samples (14.60 ± 0.35 sec and 19.70 ±1.55 sec after incubation for 2 and 48 h, respectively). Also, APTT time for samples incubated with PCU were 28.07 ± 1.94 sec after 2 h incubation and 29.47 ± 1.19 sec after 48 h incubation, which were not significantly different from the results obtained for control samples (28.80 ± 2.08 sec and 30.8 ± 2.92 sec after 2 h and 48 h incubation, respectively). In addition, results showed that the addition of the dye to PCU did not change the coagulation time of plasma after incubation for 2 h and 48 h, in which PT values were 14.60 ± 0.4 sec and 19.03 ± 0.42 sec after 2 h and 48 h, respectively, and APTT values were 30.47 ± 2.55 sec and 31.37 ± 4.41 sec after 2 h and 48 h, respectively. Therefore, these results showed that PCU and self-colored PCU did not affect coagulation time of the plasma and the results were almost similar to control samples, which was another indicator of appropriate blood compatibility of the samples.

Table 4. aPTT and PT for PCU and self-colored PCU after 2 h and 48 h. Each experiment was done in three replicates.

| Polymer         | Time (h) | PT (Mean ± SD) (s) | aPTT (Mean ± SD) (s) |
|-----------------|----------|--------------------|----------------------|
| Control         | 2 h      | 14.60 ± 0.35       | 28.80 ± 2.08         |
|                 | 48 h     | 19.70 ±1.55        | 30.8 ± 2.92          |
| PCU             | 2 h      | 15.23 ± 0.15       | 28.07 ± 1.94         |
|                 | 48 h     | 18.16 ± 0.55       | 29.47 ± 1.19         |
| Self-colored PCU| 2 h      | 14.60 ± 0.4        | 30.47 ± 2.55         |
|                 | 48 h     | 19.03 ± 0.42       | 31.37 ± 4.41         |

Albumin and fibrinogen are major proteins available in the plasma and have been used for characterization of biocompatibility of materials [18, 69]. Fibrinogen is one of the most important protein in the coagulation of blood and its adsorption causes adhesion and activation of other components of coagulation cascade and platelets. Previous studies have been shown that long aliphatic groups of polyurethane have an affinity to proteins and this is one of the reasons for their good blood compatibility [33]. Therefore for understanding, the protein adsorption by PCU and self-colored PCU, we have measured the fibrinogen adsorption. The counted fibrinogen for blood was 269 mg/dl and the blood incubated with polystyrene plate was containing 225±7 mg/dl. Our results showed that fibrinogen concentration after exposure to PCU and self-colored PCU were measured around 231±5 mg/dl and 233±4 mg/dl, respectively, and there was a little increase from the control value but not significant, which was showing that lower amount of
fibrinogen was adsorbed by samples than control samples, which could be attributed to hydrophilic properties of the PCU and self-colored PCU. In addition, the results were showing that addition of the dye to PCU did not affect the affinity of the samples to fibrinogen, which could be attributed to similar contact angle values for PCU and self-colored PCU.

Conclusion.

In this paper, we have evaluated the mechanical properties, morphological properties and biocompatibility of PCU and covalently attached fluorescent dye to PCU (self-colored PCU) using different techniques. DMTA results showed that addition of the dye to PCU did not change glass transition temperature (Tg) of the PCU (Tg was around -16 °C), also AFM imaging showed that incorporation of the dye to PCU, did not cause surface roughness. In addition, Contact angle measurement showed that both PCU and self-colored PCU had similar water contact angle (around 76 °). Furthermore, cytotoxicity of the PCU and self-colored PCU against cells was evaluated using MTT assay, which showed that almost all of the cells were viable after 48 h incubation with samples (100 % cell viability). Moreover, blood compatibility of PCU and self-colored PCU was evaluated with hemolysis assay which has been consider as a serious concern in design of polymeric biomaterials and results showed around 1.1 % and 0.97 % hemolytic activity for PCU and self-colored PCU respectively, which were in the accepted range for polymeric biomaterials. Also, plasma coagulation time induced by PCU and self-colored PCU was evaluated using PT and aPTT assays, in which PCU and self-colored PCU showed PT and aPTT value around 15-19 s and 28-30 s respectively, which were similar to the coagulation time of control sample. Finally, fibrinogen adsorption was evaluated and results showed that PCU and self-colored PCU had adsorbed 231±5 mg/dl and 233±4 mg/dl proteins respectively, which was similar to control sample. This great mechanical and blood compatibility properties of the self-colored PCU as well as their excellent fluorescent properties suggested that, these materials could be good candidates to use in biomedical applications such as diagnostics, monitoring, labeling and biomedical imaging.

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Conflict of Interest/Competing Interests

Authors declare no conflict of interest.

Availability of data and material

All of the data generated were used in this research.

Code availability

Not applicable.

Authors’ contribution

E.Z. and H.Y. designed the conception and experiments, and synthesized and characterized polymers. E.Z., H.Y., and M.Z. performed the biocompatibility test. All authors wrote and revised the manuscript.
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