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Citric acid-derived *in situ* crosslinkable biodegradable polymers for cell delivery

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ABSTRACT

Herein, we report a first citric acid (CA)-derived *in situ* crosslinkable biodegradable polymer, poly (ethylene glycol) maleate citrate (PEGMC). The synthesis of PEGMC could be carried out via a one-pot polycondensation reaction without using organic solvents or catalysts. PEGMC could be *in situ* crosslinked into elastomeric PPEGMC hydrogels. The performance of hydrogels in terms of swelling, degradation, and mechanical properties were highly dependent on the molar ratio of monomers, crosslinker concentration, and crosslinking mechanism used in the synthesis process. Cyclic conditioning tests showed that PPEGMC hydrogels could be compressed up to 75% strain without permanent deformation and with negligible hysteresis. Water-soluble PEGMC demonstrated excellent cytocompatibilty *in vitro*. The degradation products of PPEGMC also showed minimal cytotoxicity *in vitro*. Animal studies in rats clearly demonstrated the excellent injectability of PEGMC and degradability of the *in situ*-formed PPEGMC. PPEGMC elicited minimal inflammation in the early stages post-injection and was completely degraded within 30 days in rats. In conclusion, the development of CA-derived injectable biodegradable PEGMC presents numerous opportunities for material innovation and offers excellent candidate materials for *in situ* tissue engineering and drug delivery applications.

1. Introduction

Seeking ideal biomaterials for specific biomedical applications has been an ongoing effort in biomedical engineering. Carefully selecting monomers for biomaterial syntheses is essential for determining and controlling the functionality and biocompatibility of the biomaterials to be produced. Citric acid (CA) is a multifunctional chemical compound that is involved in Kreb cycle and used in many aspects of our lives such as in food additives, water softening, anti-coagulant, anti-viral tissues, and cleaning products. In recent years, there has been increasing attention in using citric acid as a robust multifunctional monomer for biomaterial syntheses. A key feature for CA-derived biomaterials is that CA provides valuable pendant functionality participating in the ester bond-crosslink formation, enhancing hemocompatibility, balancing the hydrophilicity of the polymer network, and providing hydrogen bonding and additional binding sites for bioconjugation to confer additional functionality such as optical properties [1,2].

The recent developments on citric acid-derived biomaterials were driven by the significant needs for biodegradable elastomers in tissue engineering. Poly(diol citrate) was the first type of CA-derived biodegradable elastomer [3,4]. CA reacted with aliphatic diols such as 1,8-octanediol to form oligomers (pre-polymers) which can be crosslinked into elastomeric polyesters, poly (diol citrates). Poly(diol citrates) have shown promise as biomaterials for hemocompatible and compliant vascular graft coatings [5], small diameter blood vessel and cartilage tissue engineering [6], and orthopedic fixation devices [7]. More recently, significant efforts in our laboratories have focused on expanding the tunability and functionality of the citric acid-derived biodegradable elastomers. By doping urethane bonds in a polyester network, crosslinked urethane-doped polyester (CUPE) was developed based on poly (diol citrate). CUPE addresses the challenges in developing soft, elastic but strong biodegradable elastomers that can serve as immediately implantable tissue engineering scaffolding materials for *in vivo* tissue engineering [2,8]. By introducing double bond-containing monomers into the poly(diol citrates) pre-polymer network, such as maleic acid and maleic anhydride, poly(alkylene maleate citrates) (PAMCs) were synthesized [9,10]. PAMCs feature a dual-crosslinking mechanism through which the polymers can be crosslinked by ester bond formation as similar to poly(diol citrates)

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and photocrosslinking polymerization due to the presence of double bonds from the maleate units in the polymer backbones. This dual-crosslinking mechanism allows fine tuning the mechanical properties and degradation rates of PAMCs to better fit the versatile needs in various soft tissue engineering. Even more exciting development made recently, the first biodegradable photoluminescent polymers (BPLPs) were developed by adding α-amino acids to poly(diol citrates) polymer backbones [11]. The side-added amino acids further reacted with the germinal −OH on the same citrate units to form fluorescent 6-membered amide-ester rings which resulted in bright fluorescence with high quantum yields (up to 79%) and tunable fluorescence emission (up to 825 nm). The fully degradable photoluminescent polymers hold great promise for tissue engineering and drug delivery where quantitatively non-invasive or minimally-invasive monitoring or tracking of scaffold degradation/tissue regeneration and drug delivery processes remain challenges.

In recent years, *in situ* crosslinkable biodegradable materials have gained much attention for potential applications in tissue engineering, drug delivery, and wound care [12–18]. For tissue engineering applications, *in situ* crosslinkable biodegradable materials can be used as injectable scaffolds for tissue regeneration through a minimally-invasive delivery method [19,20]. For drug delivery applications, injectable biomaterials can be used for the localized delivery of therapeutic agents to a diseased site avoiding dangerous and costly surgical procedures [21]. A wide variety of *in situ* crosslinkable biomaterials have been reported ranging from naturally derived extracellular matrices (ECM) such as chemically-modified glycosaminoglycans (GAGs) [22–27] to synthetic polymers such as poly (vinyl alcohol) (PVA) [28,29], poly(ethylene glycol) (PEG) [30,31], poly(propylene fumarate) (PPF) [32,33], polyphosphoester [34,35], and polylactone-based hydrogels [36–38].

Given the aforementioned benefits of using citric acid for biomaterial syntheses and the fact that none of the previously developed citric acid-derived biodegradable elastomers can be made into a water-soluble form for *in situ* drug and cell delivery, herein, we reported the syntheses and characterization of the first citric acid-derived water-soluble, *in situ* crosslinkable, and biodegradable polymers, poly(ethylene glycol) maleate citrates (PEGMCs). PEGMCs were synthesized by reacting citric acid (CA) with poly (ethylene glycol) (PEG) 200 and maleic acid (MA). PEGMCs can be *in situ* crosslinked into biodegradable elastomeric polyester hydrogels (PPEGMCs). CA is a multifunctional acid and a metabolic product of the Krebs cycle [39]. PEG is the most widely-used water-soluble macro diol in biomedical applications. MA, an important component of the citric acid cycle, has been used in many synthetic biomaterial designs, and was chosen as a difunctional acid bringing vinyl functionality to the polymers [40–43].

2. Materials and methods

All chemicals, cell culture medium, and supplements were purchased from Sigma–Aldrich (St. Louis, MO), except where mentioned otherwise. All chemicals were used as received.

2.1. Synthesis and characterization of PEGMC

PEGMC is an oligomer (pre-polymer) which was synthesized as described in Fig. 1. CA, PEG, and MA were melted in a 250 ml three-necked round bottom flask fitted with an inlet adapter and outlet adapter by stirring the contents in the flask at a temperature of 160 °C under nitrogen gas flow for 20 min. Once the constituents melted, the temperature was reduced to 145 °C for 2 h. The pressure was then dropped to 50 mTorr for another 2 h. The prepared pre-polymer was dissolved in deionized water and dialyzed with a 500 Da molecular weight cut off membrane for 2 days followed by lyophilization to achieve a purified form of PEGMC (pre-PPEGMC). Different ratios of acids (MA/CA) were adjusted in the initial composition of the pre-polymer as 8/2, 6/4, and 4/6, respectively, as shown in Table 1. The overall ratio of the acids over the diol was kept at 1:1.

To analyze the functional groups present in PEGMC, a 5% (w/v) PEGMC solution in 1,4-dioxane was prepared and cast onto a potassium bromide crystal and allowed to dry overnight in a vacuum hood. Fourier Transform Infra Red (FTIR) spectroscopy measurements were recorded at room temperature using a Nicolet 6700 FTIR (Thermo Scientific, Waltham, MA) equipped with OMNIC Software using 128 scans across the wave numbers 4000–400 cm^{-1} at a resolution of 2 cm^{-1}. For proton analysis, the pre-polymers were purified twice as mentioned above and then dissolved in dimethyl sulfoxide-d_6 (DMSO-d_6) to make a 3% (w/v) pre-polymer solution and placed in a 5 mm-outer diameter tube. Proton Nuclear Magnetic Resonance (^{1}H NMR) was used for the analysis of the actual composition of PEGMCs for all ratios on 300 MHz JNM ECS 300 (JEOL, Tokyo, Japan). The chemical shifts for the ^{1}H NMR spectra were recorded in parts per million (ppm), and were referenced relative to tetramethylsilane (TMS, 0.00 ppm) as the internal reference.

2.2. Preparation and characterization of PPEGMC hydrogel

For the crosslinking of PPEGMC by photoinitiators, the purified pre-polymer was dissolved in water to make a 30% polymer by weight concentration. Acrylic acid was used as a crosslinker and 2,2′-Azobis(2-methyl propionamidine) dihydrochloride was used as a photoinitiator. In this study, the percentage of photoinitiator was fixed as 0.11 M, whereas the concentration of crosslinker was varied as 1.5–6% (w/v) to study the effect of crosslinker on the overall hydrogel performance. Next, the solution was poured into a Teflon mold and placed under a UVP 365 nm Long Wave Ultraviolet Lamp (Upland, CA) for 60 s.

For the crosslinking of PPEGMC by water-soluble redox initiators, a 30% (w/v) pre-polymer solution in water was mixed with 3% (w/v) of acrylic acid. The mixture was then added to an aqueous solution of 0.026 M ammonium persulfate (APS) and 0.11 m *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED). Next, the mixture was placed in an air tight vial and incubated at 37 °C for 60 s. The schematics of polymer syntheses are shown in Fig. 1. The resulting PPEGMC was characterized by FTIR to verify the crosslinking formation as compared to the PEGMC.

2.3. Sol content of PPEGMC hydrogel

Sol content was determined by measuring dry mass differential before and after incubation with 1,4-dioxane, which dissolves unreacted pre-polymers. Freshly made photocrosslinked hydrogels were lyophilized for 48 h to achieve dry hydrogel discs and weighed (M_i). These hydrogel discs were immersed in 1,4-dioxane for 2 days and further lyophilized and weighed (M_f). The sol gel fraction (SFG%) was calculated using Equation (1). Sol content experiments were also designed to understand the effect of MA/CA ratio in the polymer chain, amount of crosslinker, and crosslinking mechanism (photo and redox) on the sol content of the hydrogel.

$$\mathrm{sol}(\%) = \frac{M_i - M_f}{M_i} \times 100 \quad (1)$$

2.4. Swelling ratio of PPEGMC hydrogel

Sol free lyophilized PPEGMC discs were incubated in PBS, deionized water, and buffer solutions with different pHs (2.4, 3.4,