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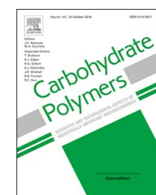

Fabrication of photo-crosslinkable glycol chitosan hydrogel as a tissue adhesive

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A B S T R A C T

In this work, an *in situ* gelling system composed of glycol chitosan (GC) was fabricated and evaluated regarding its tissue-adhesive, anti-bacterial and hemostatic properties. GC conjugated with 3-(4-hydroxyphenyl) propionic acid gelled immediately after illumination with blue light in the presence of ruthenium complex. The phenolic GC hydrogel was investigated regarding its mechanical property, hydration, degradation rate, cytotoxicity, tissue adhesiveness, and hemostatic ability. The hydrogel was shown to glue two pieces of tissues tightly in an egg-membrane model. The antibiotic-incorporated hydrogel killed bacteria effectively. When the hydrogel was applied to a wound in a mouse liver model, bleeding was reduced quickly and greatly. All the promising results show that the photo-chemically crosslinkable GC hydrogel could be used as a tissue adhesive, controlled drug release, and a hemostat.

1. Introduction

Chitosan, the deacetylation derivative of chitin, which is a naturally occurring polysaccharide from the exoskeleton of crustaceans, has been received significant attention in biomedical applications such as drug delivery, tissue engineering and wound healing owing to chitosan's excellent properties (Kumar et al., 2012; Ravi Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Dom, 2004). Chitosan is renowned for its mucoadhesive property, low cytotoxicity, biodegradability, antibacterial ability, and hemostatic ability (Alsarra, 2009; Muzzarelli, 2009). Chitosan promotes blood coagulation *via* its charge attraction to negatively charged red blood cells to form blood clots (Pei-Leun et al., 2011; Quan et al., 2015). These excellent properties especially make chitosan promising for the applications in wound dressing, tissue adhesive and hemostasis (Cho et al., 2016; Nie, Yuan, Zhao, Zhou, & Bao, 2013; Pei-Leun et al., 2011).

Tissue adhesives, serving as sealant, hemostatic and non-invasive wound dressing, attract much research attention (Reece, Maxey, & Kron, 2001; Wheat & Wolf, 2009). An ideal tissue adhesive should benefit wound sealing, hemostasis, and tissue reconstruction (Annabi, Yue, Tamayol, & Khademhosseini, 2015; Lauto, Mawad, & Foster, 2008; Spotnitz & Burks, 2008). Tissue adhesives clinically used in surgical procedures include fibrin glues (Meng-G Martin & Jones, 2005) and cyanoacrylates (Mizrahi, Weldon, & Kohane, 2010). However, these tissue adhesives have flaws when utilized in clinical applications. The limitations of fibrin glue include complicated preparation, poor mechanical strength, a risk of blood-borne disease transmission and allergic reactions to patients (Kober et al., 2008; Thomas & MacGillivray, 2003), while the toxicity of cyanoacrylate adhesives significantly circumscribe their applications (Leggat, Smith, & Kedjarune, 2007). Therefore, an ideal tissue adhesive is still in demand to resolve these concerns.

Hydrogel, a promising type of biomaterials for drug delivery and tissue engineering, could be used as tissue adhesives (Fan, Fu, Zhu, & Wang, 2016). Mechanisms of gelation, critical to the applicability of hydrogels, could be divided into two main categories. Physical crosslinking mechanisms include hydrogen bonding, ionic interaction, and hydrophobic interaction, while chemical crosslinking mechanisms include free radical polymerization, chemical reactions of complementary groups, enzyme-mediated reactions (Chen et al., 2016; Liu et al., 2017) and high-energy irradiation (Hennink & van Nostrum, 2012). However, many of the methods have certain disadvantages, such as time-consuming process, high intrinsic cytotoxicity, low mechanical strength, or inability of *in-situ* formation (Bae et al., 2014; Ryu, Hong, & Lee, 2015). Photocrosslinking, initiated by high-energy irradiation such as UV, has attracted much attention since the mechanism could perform fast *in-situ* gelation (Li et al., 2015). Nevertheless, excessive exposure to UV has risk of tissue damage (Ichihashi et al., 2003). The problem may be

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solved by using a blue-light-mediated crosslinking approach. A ruthenium (Ru)-catalyzed photochemical crosslinking process forms covalent dityrosine bonds between phenolic groups by blue-light illumination (Elvin et al., 2009). The Ru-based method has been used for the crosslinking for hydrogels composed of fibrinogen (Elvin, Danon et al., 2010; Elvin, Vuocolo et al., 2010), keratin (Sando et al., 2010) and gelatin (Elvin, Danon et al., 2010; Elvin, Vuocolo et al., 2010; Sando et al., 2011). Besides the crosslinking between protein molecules, covalent bonds can also be formed with nearby tyrosine, thiol or amino groups on animal tissue surfaces, so this method is promising to serve as tissue adhesives.

The specific aim of this study is to develop a chitosan-based hydrogel as a tissue adhesive and hemostat. Chitosan conjugated with phenolic groups could be crosslinked *via* the Ru-catalyzed photocrosslinking mechanism (Kim, Kang, Mercado-Pagan, Maloney, & Yang, 2014). In this study, glycol chitosan was used instead of chitosan due to the improved solubility of glycol chitosan (Gohil et al., 2015), thus avoiding the use of acidic solutions. The hydrogel composed of phenolic glycol chitosan was evaluated regarding mechanical properties, hydration, degradation rate, cytotoxicity, tissue adhesiveness, and hemostatic ability. Antibiotics were further incorporated into the hydrogel to enhance its antibacterial ability. The multi-functional hydrogels potentially serve as a tissue adhesive for wound healing applications.

2. Materials and methods

2.1. Materials

Glycol-chitosan (GC, cat. no. G7753, ≧60% deacetylation), morpholinoethanesulfonic acid hydrate (MES), *N*-3-dimethylaminopropyl-*N*-ethyl carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (98%) (NHS), ruthenium trisbipyridyl chloride (RuII(bpy)$_3$Cl$_2$), sodium persulphate (SPS), lysozyme from chicken egg white, amoxicillin and gentamicin sulfate salt were purchased from Sigma Aldrich. 3-(4-Hydroxyphenyl) propionic acid (HPP, cat. no. A14567) was purchased from Alfa Aesar. Difco LB Broth, Miller (Luria-Bertani) and Agar (Granulated) were purchased from BD Biosciences (USA). CellTiter 96® AQueous One Solution Reagent was purchased from Promega (USA).

2.2. Synthesis and characterization of HPP-modified glycol chitosan

HPP-modified glycol chitosan (HPP-GC) was synthesized by grafting HPP onto GC backbones *via* EDC/NHS reaction (Gohil et al., 2015). Briefly, 50 mL of HPP solution (0.6 mg/mL in 0.1 M MES buffer, pH 5.0) was mixed with 50 mL of GC solution (4 mg/mL in 0.1 M MES buffer, pH 5.0). 0.22 g EDC and 0.12 g NHS were added into the mixture, and the reaction proceeded overnight with constant stirring. The unreacted reactants were removed by dialysis against deionized water through a seamless cellulose tube (MWCO: 12,000–14,000, Cellu Sep, USA). The product was obtained after freeze-drying.

The phenolic derivatization of HPP-GC was verified by using ^{1}H NMR (Nuclear magnetic resonance, AVIII-500 MHz FT-NMR, Bruker, USA) and FTIR (Fourier-Transform Infrared Spectrometer, Perkin Elmer Spectrum 100 in the 400–4000 cm^{-1} region). The FTIR spectrum of GC was subtracted from that of HPP-GC to identify the new peaks of phenol modification (Gohil et al., 2015). The degree of phenolic substitution (DS) of HPP-GC was quantified by the absorbance at 276 nm using UV–vis Spectrophotometer (Cary 300, Agilent, USA). Phenolic substitution was calculated from a standard curve prepared using various concentrations of HPP dissolved in ultra-pure water (the insert in Fig. A3, Supplementary material). DS is defined as the amount of conjugated HPP divided by the theoretical value of 100% conjugation of HPP.

2.3. Characterization of the photocrosslinked HPP-GC hydrogels

HPP-GC solution (3% w/v) was prepared by the addition of HPP-GC into deionized water under constant stirring for 1 h until complete dissolution, and then RuII(bpy)$_3$Cl$_2$ (1 mM) and SPS (20 mM) was added. The HPP-GC solution gelled after irradiated with a LED lamp (8 W, 440–460 nm: PAR20, VITALUX, ROC) from a distance of 15 cm for 30 s.

2.3.1. Rheological properties

The rheological properties were evaluated by using a Modular compact rheometer (MCR-102, Anton Paar, Canada). Parallel plate geometry (20 mm diameters) was used to monitor the elastic modulus values (G') and the viscous modulus values (G") changes of the hydrogels with a constant strain 1% from 0.1 Hz to 1 Hz frequency at 37 °C.

2.3.2. Mass swelling

HPP-GC hydrogels were lyophilized and their dry weights (W_{dry}) were weighed. The dried samples were immersed in PBS solution (pH 7.4). At certain time periods (0.5, 1.5, 4 or 24 h), the wet samples were weighed. The wet weights (W_{wet}) of the samples were determined after the removal of excess surface water using Kimwipes. Mass swelling percentage of the HPP-GC hydrogels were calculated by following equation:

$$\text{Mass swelling percentage } (\%) = \frac{(W_{wet} - W_{dry})}{W_{dry}} \times 100\%$$

2.3.3. Degradation

For the degradation test, HPP-GC hydrogels were immersed in 5 mL of PBS solution (pH 7.4) containing lysozyme (1 mg/mL) at 37 °C for up to 8 weeks. The solution was replenished every 3 or 4 days. The wet weights (W_{wet}) of the samples were determined and then divided by the initial wet weight ($W_{wet,i}$) on the first day as the remaining mass percentage, which calculated by following equation:

$$\text{Remaining mass percentage } (\%) = \frac{W_{wet}}{W_{wet,i}} \times 100\%$$

2.3.4. Cytotoxicity

The cytotoxicity of the hydrogel was studied using an MTS assay. L929 cells were seeded in a 96-well plate at 2×10^4 cells per well, and cultured in 100 μL of the culture medium for 1 day. The hydrogels (200 mg) was placed in 10 mL of a serum-free medium at 37 °C for 1 day. 100 μL of the hydrogel extract was replenished with 10% fetal bovine serum and then replaced the culture medium in the cell-cultured plate. After 1 day of cell culture, 20 μL of CellTiter 96® AQueous One Solution Reagent was added in each well and then incubated for 3 h. Finally, the absorbance at 490 nm was measured using an ELISA reader (ELx800, BioTek, USA). The fresh culture medium and the culture medium containing 0.64% phenol were used as negative and positive controls, respectively.

2.4. Tissue adhesiveness

Egg membrane, which is mainly composed of fibrous proteins such as collagen, was used as a model tissue membrane. The tissue adhesiveness of the hydrogels was examined using a digital force gauge (FGP-5, NIDEC-SHIMPO, Japan) with a 50 N digital force gauge (Fig. A1 in Supplementary material). Egg membranes were separated from eggshells, rinsed with water, and cut into suitable sizes (diameter ≥ 10 mm). The egg membrane was firmly fixed onto one end of a glass cylinder (10 mm in diameter) by O-ring. 90 μL HPP-GC solution (1, 2 or 3% w/v) was dropped on the lower egg membrane/glass

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