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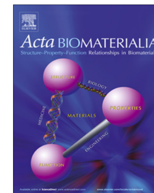

Full length article

Model polymer system for investigating the generation of hydrogen peroxide and its biological responses during the crosslinking of mussel adhesive moiety

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ABSTRACT

Mussel adhesive moiety, catechol, has been utilized to design a wide variety of biomaterials. However, the biocompatibility and biological responses associated with the byproducts generated during the curing process of catechol has never been characterized. An *in situ* curable polymer model system, 4-armed polyethylene glycol polymer end-capped with dopamine (PEG-D4), was used to characterize the production of hydrogen peroxide (H_2O_2) during the oxidative crosslinking of catechol. Although PEG-D4 cured rapidly (under 30 s), catechol continues to polymerize over several hours to form a more densely crosslinked network over time. PEG-D4 hydrogels were examined at two different time points; 5 min and 16 h after initiation of crosslinking. Catechol in the 5 min-cured PEG-D4 retained the ability to continue to crosslink and generated an order of magnitude higher H_2O_2 (40 μM) over 6 h when compared to 16 h-cured samples that ceased to crosslink. H_2O_2 generated during catechol crosslinking exhibited localized cytotoxicity in culture and upregulated the expression of an antioxidant enzyme, peroxiredoxin 2, in primary dermal and tendon fibroblasts. Subcutaneous implantation study indicated that H_2O_2 released during oxidative crosslinking of PEG-D4 hydrogel promoted superoxide generation, macrophage recruitment, and M2 macrophage polarization in tissues surrounding the implant. Given the multitude of biological responses associated with H_2O_2, it is important to monitor and tailor the production of H_2O_2 generated from catechol-containing biomaterials for a given application.

Statement of Significance

Remarkable underwater adhesion strategy employed by mussels has been utilized to design a wide variety of biomaterials ranging from tissue adhesives to drug carrier and tissue engineering scaffolds. Catechol is the main adhesive moiety that is widely incorporated to create an injectable biomaterials and bioadhesives. However, the biocompatibility and biological responses associated with the byproducts generated during the curing process of catechol has never been characterized. In this manuscript, we design a model system to systemically characterize the release of hydrogen peroxide (H_2O_2) during the crosslinking of catechol. Given the multitude of biological responses associated with H_2O_2 (i.e., wound healing, antimicrobial, chronic inflammation), its release from catechol-containing biomaterials need to be carefully monitored and controlled for a desired application.

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1. Introduction

Marine mussels secrete adhesive proteins that solidify *in situ* to facilitate the anchoring of these organisms to underwater surfaces (i.e., rocks, boats, other mussels, etc.) [1,2]. Mussel foot adhesive proteins contain an elevated amount (up to 28 mol%) of a unique catecholic amino acid, 3,4-dihydroxyphenylalanine (DOPA), which imparts these proteins with adhesive characteristics and the ability for crosslink formation [3]. DOPA is a versatile adhesive molecule and its catechol side chain can undergo various reversible and irreversible chemical interactions, making it highly attractive for designing a wide variety of injectable biomaterials and bioadhesives [3–5]. Biomaterials functionalized with DOPA and its derivatives (e.g., dopamine) have demonstrated promising results for suture-less wound closure [6], fetal membrane sealing [7], Achilles

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tendon repair [8], cell and tissue engineering [9,10], and localized drug delivery applications [11].

Catechol can be activated by enzymatic (i.e., tyrosinase) or chemical (i.e., periodate, IO_4^-) oxidants to form the highly reactive quinone, which is a necessary first step in both cohesive (i.e., *in situ* curing) and interfacial (i.e., adhesion to soft tissues) crosslinking formation [12,13]. Specifically, the use of IO_4^--mediated crosslinking is widely adopted for designing rapidly curable biomaterials [6,9,14]. Mussel inspired bioadhesives have exhibited favorable biocompatibility based on *in vitro* cytotoxicity and *in vivo* implantation testing [6,13,15]. However, these biocompatibility tests were conducted using fully cured adhesives and the potential toxic byproducts generated during the curing process were not examined. To our knowledge, there have been no documented reports that evaluated the biocompatibility of catechol during oxidative crosslinking. For products that polymerize *in situ*, the International Standard ISO 10993-12 necessitates the assessment of the potential toxicity of the reacting components during their curing process [16]. Therefore, to advance this biomimetic technology for future clinical applications, there is a need to systematically evaluate the biological responses associated with the curing of the catechol moiety.

Our lab recently identified the generation of hydrogen peroxide (H_2O_2) during autoxidation of catechol as the source of its cytotoxicity in culture [17]. H_2O_2 is one of the major reactive oxygen species (ROS) released during normal wound healing response and its biological functions is highly dependent on its concentration [18]. At a relatively low concentration (10^2–10^3 μM), H_2O_2 induces vascular endothelial growth factor expression and activates M2 phenotype macrophages differentiation, which promotes angiogenesis and tissue regeneration, respectively [19,20]. Complete removal of H_2O_2 from the wound site by antioxidant (i.e., catalase) impairs wound healing [21]. H_2O_2 also provides a natural defense against bacterial infection [22]. On the other hand, elevated levels of H_2O_2 ($\geqslant 10^5$ μM) destroys healthy tissues, resulting in the formation of chronic wounds and promote tumor initiation [18,21]. Besides the biological responses associated with H_2O_2, H_2O_2 is a mild oxidant which can oxidize the catechol moiety [23]. H_2O_2 has also been widely used as an electron acceptor in enzyme (e.g., horseradish peroxidase) mediated crosslinking of phenol-modified polymers and the concentration of H_2O_2 was correlated to changes in the crosslinking density of the hydrogel network [24,25]. Trapping the oxidant within a polymer network can potentially affect the mechanical and swelling properties of catechol-containing adhesive. Given the vital roles that H_2O_2 plays in wound healing and oxidative crosslinking, it is necessary to determine the production of H_2O_2 during the oxidative crosslinking process of catechol and correlate its release with its biological responses and its effect on the mechanical properties of the catechol-containing adhesive.

Here, we used a model polymer, 4-armed polyethylene glycol end capped with dopamine (PEG-D4, Fig. S1), to investigate the production of H_2O_2 during IO_4^--induced crosslinking of catechol. The effect of the H_2O_2 generation on the crosslinking density of the adhesive, *in vitro* cytotoxicity, and the upregulation of the antioxidant enzyme, peroxiredoxin (Prx), in primary fibroblasts (rat dermal and tendon fibroblasts) were determined. Finally, PEG-D4 was subcutaneously implanted in rats and the generation of superoxide ($O_2^{\cdot-}$), recruitment of macrophages, and M2 macrophage polarization in the surrounding tissues were evaluated.

2. Materials and methods

2.1. Materials

$NaIO_4$ was purchased from Acros Organics (Fair Lawn, New Jersey). Polytetrafluoroethylene (PTFE) sheet was purchased from McMaster (Chicago, IL). Dulbecco's Modified Eagle Medium (DMEM; with 4.5 g/L glucose and glutamine, without sodium pyruvate) and trypsin-EDTA (0.05% Trypsin/0.53 mM EDTA in Hank's balanced salt solution) were obtained from Corning Cellgro (Manassas, VA). Phosphate buffered saline (PBS, BioPerformance certified, pH7.4), bovine liver catalase, dimethyl sulfoxide (DMSO), and PolyFreeze were purchased from Sigma Aldrich (St Louis, MO). H_2O_2 (30% stock solution) was from Avantor (Center Valley, PA). Pierce Quantitative Peroxide Assay Kit with sorbitol, 18 mm coverslips, fetal bovine serum (FBS), Penicillin-Streptomycin (10 U/mL) and dihydroethidium (DHE) were purchased from Thermo Scientific (Rockford, IL). 4′,6-Diamidino-2-phenylindole (DAPI) and calcein-AM/ethidium bromide for cell live/dead stain were purchased from Invitrogen, ThermoFisher Scientific (Rockford, IL). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide 98% (MTT) was from Alfa Aesar (WardHill, MA). TransWell® Permeable Supports (12 mm insert, 12 well plate, 3.0 μ, polycarbonate membrane) were purchased from Corning Costar (Pittston, PA). Anti-iNOS antibody (ab15323), anti-CD68 (ab125212), anti-Prx 2 antibody (ab109367) and goat anti-rabbit IgG H&L (Alexa Fluor 488, ab150077) were purchased from Abcam (Cambridge, MA). Anti-CD163 antibody (sc-58965) and goat anti-mouse IgG (sc-2781) were purchased from Santa Cruz Biotechnology (Dallas, Texas). Rat dermal fibroblasts and rat tendon fibroblasts were isolated from rat dermal tissue and tendon, respectively, and identified with anti-S100A4 antibody (ab27957) and goat anti-rabbit IgG H&L (Alexa Fluor 488) [17]. PEG-D4 was prepared following a previously published protocol [26] using 4-arm 10 kDa *N*-hydroxysuccinimide ester activated poly (ethylene glycol) purchased from JenKem U.S.A, Inc. (Allen, TX).

2.2. Hydrogel formation

Equal volume of PEG-D4 (300 mg/mL in pH 7.4 PBS) and $NaIO_4$ (54.5 mM in deionized water) were mixed in a mold consisted of 2 glass plates separated with a spacer (thickness = 0.7, 1.3, or 2.2 mm) to control the thickness of the hydrogel. Hydrogels were allowed to cure for either 5 min or 16 h. For the 16 h-cured hydrogels, the glass molds with hydrogels were sealed with parafilm and stored in a sealed zip-lock bag at room temperature to prevent liquid evaporation. To determine the effect of PEG-D4 concentration on the release of H_2O_2, hydrogels (75, 110, and 150 mg/mL PEG-D4) were prepared by mixing equal volume of PEG-D4 (150, 220, and 300 mg/mL) and $NaIO_4$ (the molar ratio of $NaIO_4$: dopamine = 0.5:1) in a glass mold with a 0.7 mm-thick spacer. For *in vitro* cell culture experiments and subcutaneous implantation in rats, the precursor solutions of PEG-D4 (300 mg/mL in pH 7.4 PBS) and $NaIO_4$ (54.5 mM in deionized water) were separately sterilized using a 0.22 μm filter prior to forming the hydrogel.

2.3. Oscillatory rheometry testing

Rheological properties of PEG-D4 hydrogels were characterized using a HR-2 rheometer (TA Instruments, New Castile, DE, USA). A frequency sweep (0.1–100 Hz at 10% strain) experiment was performed to determine the storage (G') and loss (G'') moduli of PEG-D4. Hydrogel discs (diameter = 10 mm, thickness = 0.7 mm, n = 3) were tested using a parallel plate geometry at a gap distance that is set at 90% of the individual hydrogel thickness, which was measured by a digital caliper before testing.

2.4. H_2O_2 measurement

Hydrogels were incubated with 1.5 mL of cell culture medium (DMEM with 10% (v/v) FBS and 0.5% (v/v) Penicillin-Streptomycin; pH = 7.4) for 48 h at 37 °C. H_2O_2 concentration was

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