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Hydrogen peroxide generation and biocompatibility of hydrogel-bound mussel adhesive moiety

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ABSTRACT

To decouple the extracellular oxidative toxicity of catechol adhesive moiety from its intracellular non-oxidative toxicity, dopamine was chemically bound to a non-degradable polyacrylamide hydrogel through photo-initiated polymerization of dopamine methacrylamide (DMA) with acrylamide monomers. Network-bound dopamine released cytotoxic levels of H_2O_2 when its catechol side chain oxidized to quinone. Introduction of catalase at a concentration as low as 7.5 U/mL counteracted the cytotoxic effect of H_2O_2 and enhanced the viability and proliferation rate of fibroblasts. These results indicated that H_2O_2 generation is one of the main contributors to the cytotoxicity of dopamine in culture. Additionally, catalase is a potentially useful supplement to suppress the elevated oxidative stress found in typical culture conditions and can more accurately evaluate the biocompatibility of mussel-mimetic biomaterials. The release of H_2O_2 also induced a higher foreign body reaction to catechol-modified hydrogel when it was implanted subcutaneously in rat. Given that H_2O_2 has a multitude of biological effects, both beneficiary and deleterious, regulation of H_2O_2 production from catechol-containing biomaterials is necessary to optimize the performance of these materials for a desired application.

1. Introduction

Marine mussels secrete remarkable underwater adhesives that allow these animals to anchor to surfaces in turbulent intertidal zones [1]. These proteins contain a large abundance of a catecholic amino acid, 3,4-dihydroxyphenylalanine (DOPA), which functions both as a crosslinking precursor and an adhesive moiety [2]. The catechol side chain of DOPA is a unique and versatile adhesive molecule capable of binding to both organic and inorganic surfaces through either covalent attachment or strong reversible bonds. Inert, synthetic polymers modified with DOPA and other catechol derivatives (i.e., dopamine) have demonstrated strong, water-resistant adhesive properties to various biological, metallic and polymeric substrates [3–5]. The simplicity and versatility of catechol chemistry have been exploited in designing functional biomaterials for a wide range of applications including tissue adhesive and sealant [6–8], antimicrobial polymer [9], drug carrier [10], surface coating [11,12], and soft actuator [13,14].

To further advance this biomimetic technology toward clinical applications, biocompatibility of these biomimic materials needs to be carefully evaluated. Both dopamine and DOPA are naturally found in our body [15]. However, autoxidation of these catechol species generates a considerable amount of reactive oxygen species (ROS) [16]. Hydrogen peroxide (H_2O_2) is a major composition of ROS that has been detected in cell culture media containing DOPA or dopamine [17]. Routine cultures are exposed to elevated oxygen pressure (150 mmHg compared to 1–10 mmHg *in vivo*) [18,19] and are deficient of antioxidants (i.e., ascorbic acid, tocopherol, catalase) that counteract elevated oxidative stress [20], both of which promote ROS generation from these catechol species [17]. Incubation of polyphenol extracts in an nitrogen-rich atmosphere inhibited H_2O_2 generation, which indicated the importance of oxygen concentration in the production of H_2O_2 from catechol moieties [21]. Similarly, polyphenols that are well-known for their antioxidant properties have also been shown to exhibit pro-oxidant activities under certain culture conditions [20].

ROS has been found to have both beneficiary and deleterious effects [22]. Inflammatory cells produce ROS during the early stages of wound healing process [23] and a suppressed ROS production increases the chance for infection [24] and delayed wound healing [25,26]. Exogenously supplied H_2O_2 at a relatively low concentration promoted skin [25] and corneal [27] healing as well as axon regeneration [28]. Similarly, biomaterials supplemented with glucose oxidase, which increased the production of H_2O_2 at the wound site, also promoted wound healing [29]. On the other hand,

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excessive ROS concentrations, especially H_2O_2, can destroy healthy tissue, resulting in the formation of chronic wounds and promote tumor initiation [22]. Antioxidant nanoparticles (e.g. cerium oxide) have been found to accelerate topical wound healing in mice skin [30] and antioxidant (superoxide dismutase, SOD) incorporation reduced the foreign body response and increased the biocompatibility of injectable hydrogels [31]. Therefore, ROS concentration needs to be rigorously regulated depending on the application.

Here, we use a model system to correlate the production of H_2O_2 from biomimetic catechol with the biocompatibility of the adhesive molecule both in culture and *in vivo*. Dopamine methacrylamide (DMA) was copolymerized into a non-degradable polyacrylamide (PAAm) hydrogel network. Dopamine consists of a catechol side chain that mimics the reactivity of bioadhesive moiety, DOPA. The adhesive is bound to the PAAm network so that the generation and release of the oxidation byproducts can be captured in the cell culture extract. This model system is used to distinguish the cytotoxicity effect of the oxidation byproducts (i.e., extracellular effect of ROS) from intracellular toxicity effect as a result of cellular uptake of the catechol [17]. Production of H_2O_2 from DMA containing hydrogel and its effect on cell viability and proliferation was determined. Catalase is a common enzyme found in many living organisms that are exposed to air and can catalyze H_2O_2 into water and oxygen [32]. Catalase has been found to effectively counteract the cytotoxicity induced by H_2O_2 in culture [17]. The ability for catalase to counteract the cytotoxicity effect of H_2O_2 in culture was also examined. Finally, the biocompatibility of DMA containing hydrogel was evaluated in a rat subcutaneous model.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's 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). *N,N′*-methylene-bisacrylamide (MBAA) and 2,2-dimethoxy-2-phenylacetophenone (DMPA), and dopamine hydrochloride were purchased from Acros Organics (Geel, Belgium). Acrylamide (AAm), ethanol, phosphate buffered saline (PBS, BioPerformance certified, pH 7.4), bovine liver catalase, and PolyFreeze were purchased from Sigma Aldrich (St. Louis, MO). Dimethyl sulfoxide (DMSO) and 12 M hydrochloric (HCl) acid were purchased from Fisher Scientific (Pittsburg, PA). Hydrogen peroxide (H_2O_2, 30% stock solution) was from Avantor (Center Valley, PA). Pierce Quantitative Peroxide Assay Kit with sorbitol, fetal bovine serum (FBS) and Penicillin–Streptomycin (10 U/mL) were purchased from Thermo Scientific (Rockford, IL). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide 98% (MTT) was from Alfa Aesar (Ward Hill, MA). Mouse mAb (clone MoBU-1) Alexa Fluor® 488 conjugate antibody and 4′, 6-diamidino-2-phenylindole (DAPI) were obtained from Invitrogen (Grand Island, NY). Anti-S100A4 antibody (ab27957), goat anti-rabbit IgG H&L (Alexa Fluor 488; ab150077), anti-CD68 antibody (ab125212), and goat anti-rabbit IgG H&L (Alexa Fluor 647; ab150079) were purchased from Abcam (Cambridge, MA). DMA was synthesized as previously described [33].

2.2. Hydrogel preparation

Hydrogels were prepared by photo-initiated polymerization following published protocol [34]. Briefly, precursor solutions containing 1 M AAm, 0–3 wt.% DMA, 2 mol% MBAA, and 0.1 mol% DMPA were poured into a 2-mm thick mold and photo-irradiated for 15 min using a UV crosslinking chamber (XL-1000, Spectronics Corporation, Westbury, NY) located in a N_2-filled glove box. Hydrogel samples were cut into disk-shape (diameter = 10 mm) and dialyzed in deionized water acidified to pH 3.5 using concentrated HCl for at least 3 days while changing the dialysate twice daily to remove unreacted monomers. Due to the differences in the swelling behavior of hydrogels with different concentrations of DMA (Fig. S1 and Table S1), cutting the hydrogels to a uniform size before equilibrating them in an aqueous medium ensured accurate estimation of DMA concentration in each sample.

2.3. Preparation of hydrogel extract

Hydrogels were sterilized by submersing the samples in 70% (v/v) ethanol for 45 min and washed three times with 20 mL PBS for 90 min [35,36]. The sterilized hydrogels were transferred into a 24-well plate and incubated with 1 mL of L929 cell culture medium, prepared with DMEM (addition of 10% (v/v) FBS and 0.5% (v/v) Penicillin–Streptomycin) and incubated for 1–48 h (37 °C, 5% CO_2 and 95% air). The concentrations of hydrogel-bound DMA in the extraction media were 7, 14, and 21 mM for hydrogels containing 1, 2, and 3 wt.% DMA, respectively.

2.4. Measurement of H_2O_2 generation

H_2O_2 measurement was carried out using the ferrous (Fe) ion oxidation xylenol orange (FOX) assay by Quantitative Peroxide Assay Kit [17]. 20 μL of the hydrogel extract was mixed with 200 μL of FOX reagent and incubated at room temperature for 20 min and examined using a microplate reader (SynergyTM HT, BioTek) at 595 nm. To evaluate the effect of catalase activity on H_2O_2 production, bovine liver catalase (7.8–2000 U/mL) was added to the cell culture medium prior to extraction. For comparison purposes, DMA monomer and dopamine were dissolved in DMSO (84 mM) and diluted to 14 mM (equivalent to hydrogel containing 2 wt.% DMA) using cell culture medium, and H_2O_2 generation from these solutions were determined. The H_2O_2 standard curve was prepared by preparing a stock solution (2000 μM of H_2O_2) from 30% H_2O_2 solution and serially diluting it to a concentration of 7.8–2000 μM.

2.5. Cell viability assessment

Cell viability was measured using quantitative MTT cytotoxicity assay in line with the guideline from ISO 10993-5 [37]. L929 mouse fibroblasts were suspended in cell culture medium and seeded into 96-well microculture plates with a density of 1×10^4 cells/100 μL/well and incubated for 24 h at 37 °C in a 5% CO_2 humidified incubator to obtain a monolayer of cells. Cell medium was replaced with hydrogel extracts or cell culture medium containing various controls (i.e., H_2O_2, catalase, DMA monomer, or dopamine) and further incubated for an additional 24 h. The sample solution was removed and the cells were incubated with 50 μL of 1 mg/mL of MTT in PBS for 2 h. Finally, the PBS solution was replaced with 100 μL of DMSO to dissolve formazan, and the absorbance of the DMSO solution was detected at 570 nm (reference 650 nm). The relative cell viability was calculated as the ratio between the mean absorbance value of the sample and that of cells cultured in the medium. Samples with relative cell viability less than 70% were deemed to be cytotoxic [37]. For each sample, 3 independent cultures were prepared and cytotoxicity test was repeated 3 times for each culture.

2.6. Primary cell proliferation assessment

Cell proliferation activity of rat dermal fibroblasts was obtained by BrdU assay [38]. Rat dermal fibroblasts were isolated from rat