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Fluorinated methacrylamide chitosan hydrogel systems as adaptable oxygen carriers for wound healing

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ABSTRACT

In this study a series of novel, biocompatible hydrogels able to repeatedly takeup and deliver oxygen at beneficial levels have been developed by conjugating various perfluorocarbon (PFC) chains to methacrylamide chitosan via Schiff base nucleophilic substitution, followed by photopolymerization to form hydrogels. The synthesized fluorinated methacrylamide chitosan (MACF) hydrogels were confirmed by high resolution ^{19}F NMR. Synthesized MACF hydrogels were tested for their ability to takeup and then release oxygen for future use in dermal wound healing. Depending on the PFC substitution type maximum O_2 uptake was observed within 2–6 h, followed by complete release to the surrounding environment (5% CO_2) within 12–120 h at oxygen partial pressures of 1–25 mm Hg h^{-1}, providing outstanding system tuning for wound healing and regenerative medicine. MACFs with the most fluorines per substitution showed the greatest uptake and release of oxygen. Interestingly, adding PFC chains with a fluorinated aromatic group considerably enhanced oxygen uptake and extended release compared with a linear PFC chain with the same number of fluorine molecules. MACF hydrogels proved to be readily reloaded with oxygen once release was complete, and regeneration could be performed as long as the hydrogel was intact. Fibroblasts were cultured on MACFs and assays confirmed that materials containing more fluorines per substitution supported the most cells with the greatest metabolic activity. This result was true, even without oxygenation, suggesting PFC-facilitated oxygen diffusion from the culture medium. Finally, MACF gradient hydrogels were created, demonstrating that these materials can control oxygen levels on a spatial scale of millimeters and greatly enhance cellular proliferative and metabolic responses.

1. Introduction

Oxygen plays a significant role in wound healing, and growing evidence suggests that tissue oxygenation levels may be an important rate limiting factor in the dermal healing response [1]. Wound oxygenation level, measured as the partial pressure of oxygen (P_{O_2}), is a key determinant of healing outcome [1]. Wound P_{O_2} measurements are an important diagnostic used to guide treatment planning, even the decision to amputate. Loss of integrity or large portions of skin as a result of injury or disease may lead to major disability or even death, thus a primary goal of wound treatment is rapid wound closure with a functional and aesthetically pleasing scar [2]. Most wound healing treatments do very little to supplement the bodily response and chronic wound (defined as lasting >6 weeks) treatments often address resulting issues but rarely the root cause of a wound's inability to heal due to insufficient levels of oxygen. Clinical observations, strongly supported by experimental evidence, have shown that wound healing is delayed under hypoxia. Oxygen partial pressure (P_{O_2}) or tension in normal subcutaneous tissue is 30–40 mm Hg [3,4], as measured transcutaneously, and drops to under 30 mm Hg in an acute dermal wound [3]. In chronic diabetic ulcers $P_{O_2} < 5$ mm Hg have been observed in patients [4]. In controlled animal experiments hypoxia has been shown to delay the healing of ischemic ulcers in both young and old animals [5–7]. Poor healing wounds have delayed re-*epithelialization* by dermal fibroblasts due to inadequate oxygen levels. Thus a tailorable polymeric treatment able to precisely supply oxygen directly to a wound could overcome low oxygen levels, targeting an essential mechanism accelerating wound healing.

Oxygen regulation in cell culture is typically achieved either by controlling oxygen content inside an incubation chamber or by introducing oxygen generating species to the culture medium. Application of oxygen generating species has largely been limited to in vitro studies since they exhibit toxicity to cells within certain concentration ranges [8]. Alternatively, biomaterials have been developed integrating oxygen generators to overcome some toxicity effects [8–10]. Other approaches have focused on designing synthetic oxygen carriers to improve oxygen availability while providing better control of oxygen tension in tissue regeneration or wound healing [11–14].

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Synthetic oxygen carriers are mainly divided into two categories: modified hemoglobin solutions and perfluorocarbons (PFCs). Both artificial oxygen carriers have variable O_2 uptake, release, delivery properties. Solutions of native hemoglobin show sigmoidal O_2 dissociation behavior, which is similar to the response of whole blood [15]. PFCs show a linear relationship between P_{O_2} and concentration [13–15]. At standard temperature and pressure oxygen solubility in water is 2.2 mM. This value can be much higher, up to 44 mM, for PFCs representing a 20-fold increase over oxygen solubility in water alone [15]. The ability to dissolve high concentrations of oxygen for delivery to places with low oxygen concentrations is the primary motivation for using PFCs in most clinical applications [11–13,16]. There is considerable interest in using PFCs as oxygen carriers in a variety of biomedical and bioprocess systems and as blood substitutes. PFCs have been approved by the USA FDA for use in blood oxygenation during specific surgical procedures [17]. The downside of PFCs is that they are hydrophobic and are therefore not miscible with water. They must be emulsified or formed into colloidal suspensions to allow them to be used in aqueous conditions or to be injected intravenously. In the last decade a small subset of biomedical researchers has studied how PFCs can be harnessed to deliver oxygen to cells and tissues. Roberts et al. [11–13] have primarily studied emulsions of PFCs with alginate to improve oxygen transport, and studies with human hepatocellular carcinoma (HepG2) cells show that this approach can be used to enhance cell metabolic activity and viability. These studies used the PFCs fluorotributylamine and perfluorooctyl bromide as emulsifiers with alginate and the pluoronic stabilizer F68 to form O_2 dissolving beads. Unfortunately, toxic responses were observed when more than 1% F68 was used, limiting the stability of this system over time [13]. Recently a PFC emulsion was tested as a treatment for second degree burns and partial thickness wounds in a pig model and showed enhanced closure, however, only these data were reported and the residence time of PFCs at the wound sites was not studied [16]. The oxygen solubility of PFCs is excellent even in aqueous medium under physiological conditions [17]. It is important to note that PFCs dissolve O_2 as well as other oxygenated species, such as CO_2, CO and NO, by diffusion (driven by gradients as described by Fick's law). Thus PFCs can be utilized to not only deliver oxygen but to scavenge waste carbon dioxide gas or mitigate reactive oxygen species [14,15]. PFCs covalently immobilized to biomaterials have not been widely reported or established in clinical applications, largely due to the absence of non-toxic chemical synthesis methodologies.

Given the importance of oxygen levels for tissue regeneration, the main objectives of this study were to first covalently attach three different PFC additions to photopolymerizable chitosan and determine how this would impart and vary hydrogel oxygen uptake and release properties. Then, secondly, to see how these new materials could be utilized to support fibroblast cell growth. This approach is unique, since it allows us to generate water soluble biomaterials with tunable oxygen uptake and release depending on the PFC modification. These fluorinated hydrogels are more compatible with biological systems and represent a different approach to modifying the oxygenation levels than has been previously reported. We hypothesize that more fluorines per PFC addition will allow both greater uptake and longer oxygen release from photopolymerizable chitosan hydrogels, which will support greater fibroblast growth. In response, clinically relevant oxygen rich materials were prepared via PFC substitution made through the primary amine group of chitosan. The oxygen uptake and release behavior of these materials were characterized to understand their utility in supporting cells as well as tissue regeneration. Finally, our materials were tested with dermal fibroblasts to determine cell responses to variable substrate level oxygen environments.

2. Materials and methods

Photopolymerizable methacrylamide chitosan (MAC) was synthesized as described previously [18,19] by modifying medium molecular weight chitosan (M_W 190,000–230,000 Da, Sigma-Aldrich, St. Louis, MO) with methacrylic anhydride (Sigma) to yield MAC containing 23% methacrylic groups [18]. The degree of deacetylation of chitosan was determined by ^{1}H NMR as described previously [20] and found to be 84%. Briefly, MAC was dissolved in 0.25% DCl in D_2O at 0.5% (w/v), then the ^{1}H NMR spectrum recorded (Varian 400 MHz NMR spectrometer, Palo Alto, CA). The degree of methacrylation was calculated by comparing the integrated area of the H2–H6 peaks at 2.8–4.0 p.p.m. with that of the methylene peaks at 5.6 and 6.0 p.p.m. To add PFC moietieschitosan was first modified with fluorinated ligands (pentafluoropropionic anhydride, 2,3,4,5,6-pentafluorobenzaldehyde and pentadecafluorooctanoyl chloride) (Sigma-Aldrich) followed by methacrylic anhydride to yield fluorinated methacrylamidechitosans (MACFs). The synthetic methodology (Fig. 1) was formulated to enable creation of MACF hydrogels by radical polymerization [18,19].

2.1. Preparation of pentafluoropropionic anhydride modified methacrylamide chitosan (MAC(Ali5)F)

Chitosan was first dissolved at 3% w/vin 2 vol.% acetic acid/water. A previously reported synthesis methodology [21] was altered to prepare MAC(Ali5)F. For the reaction 0.141 M pentafluoropropionic anhydride was added to the chitosan solution and stirred for 48 h at low speed (60 rpm) at room temperature (RT). The solution was then placed in a dialysis membrane (12,000–14,000 molecular weight cut-off Spectra/Por, Spectrum Labs, Rancho Dominguez, CA) and dialyzed against deionized water for 3 days with three changes per day, then lyophilized. This product was dissolved in 2 vol.% acetic acid/water, modified with methacrylic anhydride as described above, then freeze dried to yield MAC(Ali5)F.

2.2. Preparation of 2,3,4,5,6-pentafluorobenzaldehyde modified methacrylamide chitosan (MAC(Ar5)F)

First 0.04 M 2,3,4,5,6-pentafluorobenzaldehyde and 0.085 M sodium cyanoborohydride were mixed with 10 ml of 100% methanol. Then 10.58 g of 3% w/v chitosan/acetic acid solution was added and stirred at low speed (60 rpm) for 48 h at RT. This synthetic methodology was based on previously reported work [22–24]. The reaction mixture was then dialyzed against deionized water for 3 days with three changes per day, then lyophilized. The lyophilized product containing chitosan was dissolved in 2 vol.% acetic acid/water and further reacted with methacrylic anhydride to yield MAC(Ar5)F.

2.3. Preparation of pentadecafluorooctanoyl chloride modified methacrylamide chitosan (MAC(Ali15)F)

21.53 g of 3% w/v chitosan/acetic acid solution was mixed with 0.14 M pentadecafluorooctanoyl chloride. The reaction mixture was stirred at low speed (60 rpm) for 24 h at RT. The solution was then dialyzed against deionized water for 3 days with three changes per day, then lyophilized. This lyophilized fluorine-containing chitosan was further modified with methacrylic anhydride, as described above, to yield the product MAC(Ali15)F.

2.4. Preparation of hydrogels and gradient hydrogels

For hydrogel formation MAC/MACF was first dissolved in ultrapure water (MilliQ Direct 8 system at 18 MΩ resistance, Millipore,