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Fluorinated Methacrylamide Chitosan Sequesters Reactive Oxygen Species to Relieve Oxidative Stress while Delivering Oxygen

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Abstract

Antioxidants play an important role in regulating overabundant reactive oxygen species (ROS) in wound healing to reduce oxidative stress and inflammation. In this work we demonstrate for the first time that functionalization of methacrylamide chitosan (MAC) with aliphatic pentadecafluoro chains, to synthesize pentadecafluoro-octanoyl methacrylamide chitosan (MACF), enhances the antioxidant capacity of the MAC base hydrogel material, while being able to deliver oxygen for enhanced wound healing. As such MACF was shown to sequester more nitric oxide ($p < 0.01$) and hydroxyl ($p < 0.0001$) radicals as compared to MAC and controls even when delivering additional oxygen. MACF's beneficial antioxidant capacity was further confirmed in *in vitro* cell culture experiments using human dermal fibroblasts stressed with 2,2'-Azobis(2-methylpropionamidine) dihydrochloride (AAPH).

Keywords

chitosan; perfluorocarbons; reactive oxygen species; oxidative stress

Introduction

Wound healing is classically divided into four phases: (1) hemostasis, (2) inflammation, (3) proliferation, and (4) maturation and remodeling. Clinical and experimental evidence have clearly demonstrated that wound healing is delayed or becomes mired in a chronic state of prolonged inflammation as well as hypoxia.^{1,2} During the inflammatory phase of wound healing there is a significant increase in reactive oxygen species (ROS), which are essential to fending off infection as the skin's barrier function is reestablished.^{3,4} In chronic wounds suffering through hypoxia ROS such as HO_2^- , HO^- , O_2^- are released by neutrophils and macrophages.^{5,6} ROS in high amounts cause damage to extracellular structure proteins, lipids and DNA. Moreover, they also activate complex signal transduction pathways, leading to an enhanced expression of MMPs, serine proteases and inflammatory cytokines.⁵ ROS toxicity was shown by the severe endothelial damage in wounds of mice lacking the ROS-detoxifying enzyme peroxiredoxin-6.⁷ Overall disruption of redox balance (ROS versus antioxidant levels) has severe physiological and pathological implications such as impaired

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wound healing, cancer and general tissue necrosis.^{4,8} The majority of effective native antioxidant mechanisms in chronic wounds are oxygen dependent. These include nitric oxide (NO) formed through NO synthase, lymphocyte that activate oxidoreductase thioredoxin and macrophages expressing heme oxygenase and cysteine transporter.^{9,10} These biological mechanisms exist to combat deleterious effects of ROS and maintain redox homeostasis, but they are often deficient in chronic wound healing. Thus, controlling ROS levels during chronic impaired wound healing is advantageous for improving wound healing therapies.

A variety of biomaterials, including chitosan, have been studied for their antioxidant properties to reduce oxidative stress in biological systems.^{11–14} Chitosan is a natural polysaccharide of β -(1,4) linked D-glucosamine, which acts as an antimicrobial agent against fungi, bacteria, and viruses and as an elicitor of plant defense mechanisms.^{15–17} The antimicrobial properties of chitosan and its derivatives, along with their biodegradable nature, are valuable for biomedical applications, including but not limited to wound healing applications such as hemostasis, drug delivery, and regeneration.^{18–21}

We have previously modified methacrylamide chitosan (MAC) with the aliphatic perfluorocarbon (PFC) pentadecafluorooctanoyl chloride to synthesize pentadecafluorooctanoyl methacrylamide chitosan (MACF) (Figure 1A). MACF hydrogels have previously been shown to uptake and release therapeutic levels of oxygen, and the effectiveness of this strategy were recently demonstrated for accelerating healing in an excisional rodent wound model.²² The high electronegativity of fluorine present in PFCs imparts reduced polarization to atoms and low intermolecular attractive forces lead to a non-polar nature.²³ These unique physical and chemical properties allow PFCs to readily dissolve respiratory gases, such as oxygen and carbon dioxide. Further, PFCs dissolve biologically relevant oxygen containing species such as nitric oxide (NO) and carbon monoxide (CO).²⁴ Unfortunately, PFCs possess poor solubility in aqueous systems, and thus are typically used as short lived colloidal suspensions. Combining PFCs with methacrylated chitosan allays solubility concerns, while providing the potentially combined benefits of oxygen delivery and antioxidant behavior (Figure 1B). It is worth mentioning that PFCs are an environmentally sensitive issue due to chemical industry pollution and widespread application of free long chain PFCs in consumer products.²⁵ However, the utility of these compounds is valuable to the field of biomedical science when properly applied and monitored.²⁶ The problem of bioaccumulation can be overcome or reduced drastically by immobilizing it with biodegradable hydrophilic polymers like methacrylated chitosan. Further, studies have reported that PFCs can be eliminated from body with short half-lives through respiration, and topical application limits the chance of bioaccumulation.²⁷ Chitosan is known to enzymatically degrade through β -glycosidic linkage and thus chitosan polysaccharides still retain side chain modifications such as methacrylation and fluorination. Because of hydrophilicity of degradation products chance of bioaccumulation is reduced. An encouraging study utilizing plant cells cultured in oxygenated perfluorodecalin (C₁₀F₁₈) liquid as a media showed improved growth and ROS mitigation,²⁸ hinting that similar dual benefits could be possible in mammalian systems. Thus, for this study we hypothesized that our MACF hydrogels could sequester ROS and relieve oxidative stress in mammalian cells *in vitro*, even while delivering supplemental oxygen.

In this study or primary goal was to study the ability of MACF hydrogels to provide both supplemental molecular oxygen while also negating potentially deleterious ROS, as such, it is important to show that localized oxygen delivery through MACF hydrogels do not contribute to elevated ROS, but instead, provide a mitigating effect on ROS and oxidative stress. Thus, this study started with in vitro testing of MACF hydrogels against individual ROS and then as a treatment to reduce oxidative stress in cells. Understanding this effect will allow refinement of oxygenating biomaterials design in the future.

Materials and Methods

Fluorinated and non-fluorinated methacrylamide chitosan hydrogels, MACF and MAC, respectively, were created and characterized as previously described.²⁹ To characterize fluorine functional modification on MAC, both MAC and MACF materials were dissolved at 2 wt% in ultrapure water (18 MΩ resistance, Millipore, Billerica, MA, USA). Polymers films were cast on KBr windows by air drying the polymer solutions on the windows. FTIR transmission spectroscopy was performed on these windows using Nicolet IS50R FTIR spectrometer (Thermo Scientific, Waltham, MA).

Griess assay for NO radicals

MAC and MACF hydrogels (200 µl) and NaNO₂ standard of 200 µM, 100 µM, 50 µM, 25 µM, 12.5 µM, 6.25 µM and 3.125 µM were incubated with 240 mM of 2,2'-Azobis(2-methylpropionamidine) dihydrochloride (AAPH) (Sigma-Aldrich) in 0.01 M phosphate buffered saline (PBS) (Sigma-Aldrich), in triplicate, for 1 h. PBS with AAPH alone was used as a control. After incubation, solution from each sample was combined 1:1 with Griess reagent (Sigma-Aldrich) and incubated at RT for 5–10 minutes before absorbance measurements were taken with Infinite M200 spectrophotometer (Tecan, Maennedorf, Switzerland) at 550 nm wavelength.

Nash assay for OH radicals

Formaldehyde (Sigma-Aldrich) standard solutions in DI water were prepared at 200, 100, 50, 25, 12.5 and 6.25 µM. Nash reagent was prepared by adding 1.94 µM ammonium acetate (Sigma-Aldrich), 48.8 nM Acetic acid (Thermo Fisher Scientific, Waltham, MA) and 20.4 nM Acetyl acetone (Sigma-Aldrich) in DI water. Standards were added to 48 well-plates in triplicate. MACF and MAC gels (200 µl) were also added in triplicate and incubated with 240 mM of AAPH in 1X PBS for 1 h at 37°C. PBS containing AAPH without any gel was used as a control. Absorbance measurements were obtained using the Infinite M200 spectrophotometer at 444 nm wavelength. Antioxidant capacity for Griess and Nash assay was calculated as $((A_{\text{AAPH} + \text{PBS}} - A_{\text{MACF} + \text{N}_2}) / (A_{\text{AAPH} + \text{PBS}} - A_{\text{Methanol}})) \times 100\%$ in percentage of oxygenated PBS control absorbance.

Oxidative stress assay

Human dermal fibroblast cells harvested from neonatal foreskins were utilized at passage number 7, seeded in a 24 well-plate and incubated at 37°C and 5% CO₂. After 24 h, fibroblast growth media was replaced with fibroblast growth media containing Dulbecco's modified eagle medium (DMEM, Sigma-Aldrich), 10% fetal bovine serum (FBS, Sigma-

Aldrich) and 1% Penicillin/streptomycin/amphotericin (Life Technologies, Carlsbad, CA) and AAPH was added to this media at a final concentration of 30 mM. Fibroblast cells were incubated with media containing AAPH for 6 h at 37°C and 5% CO₂. CellROX green reagent (Life Technologies) was used according to the manufacturer's protocol. Briefly, CellROX reagent was added to each well to reach a final concentration of 5 μM and incubated for 30 min at 37°C. Cells were then washed with 1X PBS three times, followed by counter staining of nuclei using Hoechst 33342 trihydrochloride trihydrate (Invitrogen). Post staining, all cells were washed with 1X PBS three times and imaged using 4',6-diamidino-2-phenylindole (DAPI) and green fluorescent protein (GFP) fluorescence filters with an Olympus epifluorescence microscope (Olympus IX-81, Tokyo, Japan). Fluorescent microscopy images were obtained and analyzed to quantify the overall fraction of stressed cells. Image J (National Institute of Health, Bethesda, Maryland) software was used for counting CellROX green positive cells.

Statistical analysis

All statistical analyses were performed using JMP 11 Pro (SAS Institute, Cary, NC, USA). Two factor ANOVA with Tukey's *post hoc* analyses were performed to detect significant differences between groups using an alpha level of 0.05.

Results and Discussion

To test our main hypothesis, we first confirmed fluorine modifications in MACF by Fourier transform infrared spectroscopic (FTIR) and nuclear magnetic resonance (NMR) analyses (Figure 2). Normalized FTIR absorbance ratios were obtained then analyzed by dividing the absorbance of the N-H stretching bond associated peak (3200–3500 cm⁻¹) with the C-H stretching associated peak (2800–3000 cm⁻¹) resulting in ratio of 0.19 for MACF spectra and 0.26 for MAC, resulting in an overall reduction of 26% due to fluorine modification (Figure 2).

From FTIR, all chitosan characteristic bands and modifications were identified similar to published literature.³⁰ First, previous work has reported that C=O stretching, as associated with the amide bond (from both modification and acetylation), can be observed with FTIR between 1660 and 1630 cm⁻¹. This is observed in both groups (Figure 2A), and enhanced in MACF due to additional amine modification. Similarly, CH deformation of the β-glycosidic bonds in MAC and MACF were observed between 890 – 895 cm⁻¹ with additional bands at 1455 and 1374 cm⁻¹. Unfortunately, fluorine peaks in MACF could not be observed with FTIR as they were masked by strong C-O-C skeletal vibrations at 1100 cm⁻¹. FTIR analysis suggests that the PFC modification occurred via covalent attachment to the chitosan backbone through amine modification, thus ruling out the possibility of ester formation with the hydroxyl group on chitosan backbone. Next, fluorine modification was quantitatively measured through ¹⁹F NMR spectroscopy alongside methacrylation by ¹H NMR, using procedures described by Wijekoon et al.²⁹ It was found that 22% of MACF polymer was methacrylated based on proton modification (Figure 2B). Further, fluorine modification was determined to be 40% through quantitative comparison with CFCl₃ ¹⁹F NMR standard spectra (Figure 2B).

Next, we aimed to determine the antioxidant, or ROS scavenging, capacity of MACF hydrogels under different gas equilibration conditions (Figure 3). MACF hydrogels were compared to the base material (MAC), phosphate buffered saline (PBS) controls and methanol controls under the following conditions: PBS purged to saturation with 100% O₂ gas (+O₂), 100% N₂ gas (+N₂), or left to equilibrate at atmosphere. The antioxidant capacity was probed using assays specific for the two most prevalent ROS species to wound healing: nitric oxide and hydroxyl radicals. First, the Griess assay was used to determine nitric oxide radicals sequestered from solution (Figure 3A) and revealed that amongst experimental groups MACF (+ N₂) gels reduced the most NO radicals, and PBS (+ O₂) sequestered the least (Figure 3A). MACF hydrogels sequestered significantly more hydroxyl radicals than MAC hydrogels and PBS controls ($p < 0.05$, Figure 3A), to final solution concentrations of 5.92 ± 0.26 as compared to 6.22 ± 0.24 and 6.33 ± 0.18 μM . The methanol control resulted in a final concentration of 4.87 ± 1.84 μM for hydroxyl radicals in solution.

Next the Nash assay was performed for hydroxyl radicals (Figure 3B). Interestingly both MAC and MACF hydrogels sequestered significantly more hydroxyl radicals than PBS controls from the solution ($p < 0.0001$, Figure 3B), to final concentrations of 58.08 ± 4.87 , 59.54 ± 3.93 nM as compared to 67.92 ± 1.87 nM respectively. The methanol control resulted in a final hydroxyl radical concentration of 44.00 ± 5.36 nM.

MACF hydrogels saturated with oxygen (+ O₂), initially have few free fluorine sites for oxygen and thus low affinity for ROS. We recently found that MACF hydrogels release oxygen quickly with higher flux as compare to MAC gels with no PFC modification.²² However, nitrogen equilibration (+ N₂) provides abundant available fluorine sites and improved capacity for oxidative species/oxygen containing compounds. Comparing results from both assays nitrogen equilibrated MACF gels sequestered the most ROS, but atmosphere and oxygen equilibration only slightly limited this capacity (Figure 3). This suggests that MACF hydrogels can provide oxygen when oxygenated and sequester reactive oxygen species in a variety of biologically relevant environments. Previous research has reported the scavenging ability of crab-derived chitosan at 0.1 mg/ml for hydroxyl radicals, or 62–78% capacity based on methanol controls.³¹ In another study, *B. braunii* microalga extract at 6–10 ppm exhibited 45–65% antioxidant capacity against hydroxyl radicals based on reagent blank control.³² A 20% tannic acid solution in collagen hydrogels increased antioxidant capacity for hydroxyl radicals from 56% in collagen alone to 71%.³³ This activity was further improved to 84% by glutaraldehyde crosslinking the collagen with tannic acid. For our experiments, a 300 μl (3 wt% by volume polymer) MACF (+ N₂) hydrogel demonstrated an antioxidant capacity of 67% for NO $\cdot$ and 38% for OH $\cdot$ based on methanol controls. Comparing these results to other antioxidant materials these MACF hydrogels have comparable antioxidant activity. Interestingly, MACF (+O₂) hydrogels showed a similar capacity of 57% for NO $\cdot$ and 33% for OH $\cdot$ based on methanol controls. These data suggest that MACF hydrogels saturated with oxygen could provide the dual benefit of alleviating oxidative stress while also providing supplemental oxygen to enhance and support wound healing.

Finally, we proceeded to assessing the potential antioxidant benefits of MACF hydrogels in *in vitro* cell tissue culture. Human dermal fibroblasts were plated then oxidatively stressed

using 2,2'-Azobis(2-methylpropionamidine) dihydrochloride (AAPH), a free radical-generating azo compound. Human dermal fibroblasts were selected because they are a major and essential component of dermal tissue. The same treatments and controls used in antioxidant assays (Figure 3) were applied during cellular AAPH exposure experiments, and outcomes were measured after 6 h using the CellROX oxidative stress fluorescent assay (Figure 4, Figure S1). Two-factor ANOVA with Tukey's *post hoc* analysis revealed that MAC and MACF groups were significantly lower than the PBS only control group ($p < 0.0001$). For treatment equilibration conditions, statistical analysis revealed that nitrogen equilibration resulted in significantly less stressed cells as compared to oxygenated and atmospheric hydrogel treated cells ($p < 0.01$). Oxygen saturation did not elevate oxidative stress as confirmed by a similar fraction of stressed cells in both oxygenated and atmospheric gel treated groups ($p > 0.01$). Published confirmation with other PFC systems *in vitro* is limited, however, previous *in vivo* work has shown that Perflubron (C_8BrF_{17}) partial liquid ventilation reduces ROS production in the lungs by 32%.³⁴ One future direction to take this work is to study chronic hypoxic environments in culture, where the effects of oxygenated MACF gels would be more pronounced as it would activate several local antioxidant mechanisms to relieve oxidative stress burdens.

Conclusions

This work demonstrates that PFC modified methacrylamide chitosan (MACF) can serve as a potent antioxidant to negate ROS that are known to impact the wound healing process, even while delivering supplemental oxygen. This ability is improved when oxygen containing species are limited in MACF gels via saturation with nitrogen. Along with oxygen delivery, the combined ability of MACF hydrogels to deliver oxygen while providing valuable antioxidant capabilities provides significant future utility for wound healing, as well as in tissue engineering and regenerative medicine applications where oxygen supply and control of ROS is important to successful clinical outcomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A) Synthesis of MACF by successive methacrylation and fluorine functionalization of chitosan. Substitution percentages from NMR (1H or ^{19}F) provided in green. B) Proposed mechanism of wound healing related ROS generation and elimination by MACF hydrogels.

Figure 2.

A) Normalized FTIR absorbance spectra for MACF, MAC and the subtracted spectra (MACF - MAC). The subtracted spectrum demonstrates a reduction in amino group normalized intensity. B) Left spectrum shows ¹H NMR results for MACF with integration of methacryl group protons and sugar ring protons. Right spectrum shows ¹⁹F NMR results of MACF with integration of fluorine peaks.

Figure 3.

A) Griess assay for ability to sequester $\text{NO}\cdot$ from solution (lower concentration is best). * denotes significance by two-factor ANOVA with Tukey's *post hoc* analysis ($p < 0.01$). Mean $\pm$ SD, $n = 3$ B) Nash assay for ability to sequester $\cdot\text{OH}$ radicals from solution (lower concentration is best). *** denotes significance by two-factor ANOVA with Tukey's *post hoc* analysis ($p < 0.0001$). Mean $\pm$ SD, $n = 3$.

Figure 4.

CellROX assay for cell oxidative stress in response to AAPH (30 mM) and MACF hydrogel treatments as compared to 'PBS' controls (lower is better). A) % of CellROX positive cells expressing oxidative stress. * denotes significance by two factor ANOVA with Tukey's *post hoc* analysis ($p < 0.01$) and *** denotes significance by two factor ANOVA with Tukey's *post hoc* analysis (control highest mean, $p < 0.0001$). Mean $\pm$ SD, $n = 3$. B) Representative fluorescent images of oxidatively stressed cells (green) under MACF nitrogen saturated, MACF oxygen saturated and PBS control treatments. Hoechst 33342 staining (blue) is used to visualize nuclei. Scale bars in all images are 100 μ m.