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***In vitro* evaluation of thermal frontally polymerized thiol-ene composites as bone augments**

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Abstract

Because of the large number of total knee replacement (TKR) surgeries conducted per year, and with projections of increased demand to almost a million primary TKR surgeries per year by 2030 in the United States alone, there is a need to discover more efficient working materials as alternatives to current bone cements. There is a need for surgeons and hospitals to become more efficient and better control over the operative environment. One area of inefficiency is the cement steps during TKR. Currently the surgeon has very little control over cement polymerization. This leads to an increase in time, waste, and procedural inefficiencies. There is a clear need to create an extended working time, moldable, osteoconductive, and osteoinductive bone augment as a substitution for the current clinically used bone cement where the surgeon has better control over the polymerization process. This study explored several compositions of pentaerythritol-*co*-trimethylolpropane tris-(3-mercaptopropionate) hydroxyapatite composite materials prepared via benzoyl peroxide-initiated thermal frontal polymerization. The 4:1 acrylate to thiol ratio containing augment material shows promise with a maximal propagation temperature of 160°C $\pm$ 10°C, with mechanical strength of 3.65 MPa, and 111% cytocompatibility, relative to the positive control. This frontally polymerized material may have application as an augment with controlled polymerization supporting cemented implants.

Keywords

bone cement; composite/hard tissue; calcium phosphate; thiol-ene; frontal polymerization

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Additional Supporting Information may be found in the online version of this article.

Introduction

During knee replacement surgery, an orthopedic surgeon commonly uses a synthetic material, poly(methyl methacrylate), referred to as bone cement, to aid in stress distribution and adhesion of the metal prosthetic to the patients knee.^{1,2} It is projected that over a million primary total knee replacements (TKR) will occur by 2030 in the United States.³ While bone cement has been shown to last up to 15 years within a patient, it lacks in osteoconduction and osteoinduction, thus cell ingrowth and bone regeneration does not occur.⁴ Many state-of-the-art biomaterials are shifting to a composite augment utilizing calcium phosphate additives to increase the osteogenic properties of the biomaterial.⁵ Another major constraint of bone cement is the working time of the material, which greatly constrains clinicians during tissue preparation. According to Lidgren et al. for Palacos® bone cement that was thoroughly mixed by hand at 21°C, it took about 4.27 min to harden enough to mold, 5.19 min of moldable or handling time, and 10.18 min to reach a fully set polymer.⁶ Unfortunately, if the physician and operating room staff are unable to prepare the bone cement correctly or if the mold does not fit properly, the entire process must be restarted creating waste, inefficiency, and compromises quality.

A potential solution to this working lifetime issue might be found by using thermal frontal polymerization (TFP) to form a bone augment. TFP proceeds via a thermally-induced free radical polymerization mechanism. The polymerization is initiated by the decomposition and radical formation of benzoyl peroxide and follows the mechanism in Scheme 1. The thiyl radical only forms in the reactions with trimethylolpropane tris-(3-mercaptopropionate) (TMPTMP). The pores within the augment form as a result from the release of CO₂ as a by-product from using the peroxide initiator. This reaction has been demonstrated in water and other organic solvents.^{7,8} It has also been shown that any gases or water dissolved within the monomers can be volatilized during the reaction and lead to bubble formation.⁷

A study by Garber et al. on a novel bone construct displayed high biocompatibility using pentaerythritol-*co*-trimethylolpropane tris-(3-mercaptopropionate) formed by a copolymerization with an amine-catalyzed Michael addition mechanism.⁹ This study utilized a foaming mechanism to generate a porous scaffold and conducted an *in vivo* model to test the biocompatibility and osteogenic properties.¹⁰ The osteogenic results have led to the potential of utilizing these acrylate and thiol monomers, however due to their lack of cure-on demand these will be used under the free-radical frontal polymerization mechanism, to result in a biocompatible scaffold. Thermal frontal polymerization utilizes a local free-radical reaction that propagates in a wave like pattern. This wave travels due to the coupling of the thermal diffusion and the Arrhenious dependence of the reaction rate of an exothermic polymerization. The wave, or front, travels at a front velocity dependent on the initiator concentration, temperature, pressure, and the type of monomer.¹¹

An extract study was done to test cytotoxicity, and then front velocity measurements and temperature profiles were made. Finally, a pot life study, mechanical property analysis, mass loss analysis, and imaging of the augments were done. Utilizing these monomers via a TFP mechanism instead of the Michael addition mechanism, bone augment synthesis can be

conducted with an extended working time and at a rapid reaction rate. This cure-on demand capability empowers the physician to fully shape and mold the monomer form before setting the polymer. This reaction occurs in a short time period and leads to a biologically friendly augment that supports and promotes cell growth. This study uses NIH 3T3 cells for an *in vitro* cytotoxic evaluation.¹² One application of this material is could be as a substitution for currently used bone cement in total knee replacement.

Materials and Methods

Materials

Materials for augment synthesis were used as obtained from Sigma Aldrich: trimethylolpropane tris(3-mercaptopropionate) (TMPTMP), luperox A98—benzoyl peroxide (BPO), hydroxyapatite (HA), and from Alfa Aesar: pentaerythritol triacrylate (PETA).

Augment synthesis

Four different composites were synthesized at different ratios of acrylate to thiol. Chen et al.'s procedure to find the lowest concentration of benzoyl peroxide was conducted, and all are synthesized with 0.5% (w/w) BPO (data not shown).¹³ Per Garber et al.'s display of bioactivity, all augments were made with 20% (w/w) HA.⁹ The ratio of acrylate to thiol is as follows in Table I.

Ratios of PETA to TMPTMP with greater amounts of TMPTMP were tested, but were unable to polymerize via thermal frontal polymerization. From henceforth all composites will be referred to by their ratio of PETA to TMPTMP.

Monomers of PETA and BPO were mixed for 24 h to addition of TMPTMP and HA. All augments containing the TMPTMP and HA were mixed in the same order and duration using a rotor for 1 min for each monomer. Fronts were initiated with a commercial soldering iron or a hot plate depending on the mold (see Supporting Information).

Cell culture

NIH 3T3 mouse fibroblast from passages 30 to 55 were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum in 25 cm² tissue culture flasks. All cells were incubated at 37°C and 5% CO₂. The media was changed in each flask every 3 days and passaged at 80% confluence.

Extract cytotoxicity

A 96 well tissue culture plate was seeded with 1×10^5 3T3 mouse fibroblast cells and incubated at 37°C and 5% CO₂ for 24 h. Three replicates of 200 mg $\pm$ 2.3 mg augment structures were soaked in 5 mL of DMEM with 10% fetal bovine serum and placed on a rocker while being incubated at 37°C and 5% CO₂ for 7 days. The extract solution was then filtered through a 0.22- μ m pore size filter, and 150 μ L per well was placed into a 96 well tissue culture plate that was seeded with 3T3 mouse fibroblast cells. Samples were incubated at 37°C and 5% CO₂ for 24 h and cellular viability was analyzed using the Alamar Blue assay. The dead control was conducted by adding 100% ethanol to wells with cells for 4 h

prior to staining. This assay called for 10 μ L of Alamar Blue reagent to be added to 100 μ L of culture medium per well and incubated at 37°C and 5% CO₂ for 4 h. The fluorescence was measured at an excitation wavelength of 535 nm and an emission wavelength of 595 nm using a plate reader.

Front velocity and temperature profile

Monomers of each sample were placed in a metal mold, measuring 0.7 cm $\times$ 1.3 cm $\times$ 10.2 cm. A commercial soldering iron was used to onset thermal frontal polymerization. The visual movement of the front, or front velocity, was calculated by capturing the reaction via video and determining the time required for the reaction front to travel 1.27 cm in three different portions of the total length of the mold. The temperature profile was captured using a type T thermocouple and collected by a USB TC-08 Thermocouple Data Logger.⁸ Polymerization was performed in triplicate and deemed successful if a self-sustaining front propagated, but not if only local polymerization occurred at the heat source.¹⁴

Pot life study

Monomers of each augment type were mixed and left at room temperature. The time until bulk polymerization occurred was determined in triplicate.

Mechanical testing

All samples were compressed at 2.5 mm per minute using an Instron (Instron Model 5696, Canton, MA) until a yield point or 10% deformation was reached. The samples were cylindrical with 6 mm in diameter and 12 mm in height. Sample testing was a modified version of Kim et al.'s protocol.¹⁵

Mass loss test

Three replicates of each augment structure were soaked in DMEM with 10% fetal bovine serum and placed on a rocker while being incubated at 37°C and 5% CO₂ for 7 days. Composite samples were trypsinized (0.25% trypsin, 1 $\times$ EDTA) for 10 min then rinsed three times in phosphate buffered saline (-Ca, -Mg) and freeze dried. Each sample was normalized to its' initial mass prior to media exposure.⁹

Scanning electron microscope analysis

The cross-sectional view was observed by scanning electron microscopy. The cylindrical augment synthesized for mechanical testing was cut to a 3 mm height. A conductive platinum coating was applied using EMS550X sputter coater for two 2-min coatings.

Micro-computerized tomography analysis

Each cylindrical structure from mechanical testing was placed into the micro-CT and imaged with a SkyScan Model 1074 microtomography scanner, the X-ray source voltage was 40 kV, the source current was 1000 microamps (uA), and the exposure time ranged from $\sim$ 300 to 600 ms. The scanner had a detector with 37 μ m pixel size, the X-ray source was 3 μ m, and the effective (reconstructed) spatial resolution was about 80 μ m full-width-at-half-maximum

(FWHM). Scaffold porosity was calculated using the Image Processing toolbox in MATLAB v7.12 (MathWorks, Natick, MA).

Statistical analysis

All results were expressed as a mean $\pm$ percent error. Data were analyzed with a two way t test. For all comparisons, a p values < 0.05 was determined significant.

Results

Effect of acrylate and thiol concentrations on cytocompatibility

Although the reaction properties mostly trended from acrylate only to the 2:1 acrylate to thiol augment, the biological properties displayed a trend toward the augments with the middle ratios of acrylate to thiol, as shown in Figure 1. High biological compatibility from the extracts of the augments was only displayed by the 9:1 and 4:1 acrylate to thiol augments. These augments displayed above 100%, normalized to the live control, of cell viability in the extract of these augments.

Effect of acrylate and thiol concentrations

In comparing the reaction properties of the different ratios of acrylate to thiol in Table I, as the amount of thiol was increased, the front velocity decreased from 0.45 cm s^{-1} for the acrylate only down to 0.12 cm s^{-1} for the 2:1 acrylate to thiol augment, as seen in Figure 2. While the front was retarded, the maximum temperature inversely correlated with thiol content with temperatures reducing from 200 to 124°C . The temperatures for the acrylate only augment was $200^\circ\text{C} \pm 10^\circ\text{C}$, 9:1 acrylate to thiol augment was $180^\circ\text{C} \pm 10^\circ\text{C}$, the 4:1 acrylate to thiol augment was $160^\circ\text{C} \pm 10^\circ\text{C}$, and the 2:1 acrylate to thiol augment was $124^\circ\text{C} \pm 4^\circ\text{C}$. The front velocity of the acrylate only augment takes $6.83 \pm 0.33 \text{ min}$ to react and cool to 40°C , while the 9:1 acrylate to thiol reached 40°C in $8.10 \pm 0.38 \text{ min}$, as seen in Table II. Similar to the decreasing trend of maximum temperature, the total time decreased from the 9:1 to the 2:1 acrylate to thiol augment with the 4:1 acrylate to thiol augment at $6.38 \pm 0.88 \text{ min}$ and the 2:1 augment at $5.17 \pm 0.53 \text{ min}$, shown in Figure 3.

Pot life study

In Figure 4, as the amount of thiol was increased there was a decrease in the stability of the system at room temperature before bulk polymerization occurred. The acrylate only augment lasted $>24 \text{ h}$, while even the 9:1 augment only lasted 2 h. The 4:1 augment and the 2:1 acrylate to thiol augment had 1 and 0.75 h pot lives, respectively.

Mechanical strength

With the addition of thiol, the ultimate compressive strength was observed to increase as shown in Figure 5 from as low as 0.87 MPa for the Acrylate only to 3.65 MPa for the 4:1 acrylate to thiol augment. The mechanical strength plateaued at the 4:1 acrylate to thiol augment, thus the 2:1 acrylate to thiol augment withstood the same amount of compressive forces.

The Young's modulus displays a similar trend as the ultimate compressive strength with the Acrylate only being the stiffest at 6.25 MPa and leveling off at 30.72 MPa for the 4:1 acrylate to thiol augment in Figure 6.

Mass retention study

Each formulation experienced a small decrease in mass as illustrated in Figure 7, except for the 4:1 acrylate to thiol augment. The mass retained was within a range of 1.1% for the acrylate only, 9:1, and 2:1 acrylate to thiol formulation. The 4:1 formulation had a larger range, and lower mass retention at 93.6% retained.

Porosity and imaging

Total porosity inversely correlated with increasing thiol concentration, except for the 4:1 acrylate to thiol composition. All samples showed <23% porosity with the 2:1 acrylate to thiol formulation having the lowest porosity at 5.9%. The 4:1 acrylate to thiol augment did not hold this trend; with higher porosity than the acrylate only augment at 22.9%, as shown in Figure 8.

The SEM image in Figure 9, of the 4:1 acrylate to thiol augment, shows a cross-sectional image of the morphology. The average pore diameters of the macropores were 120 μm with micropores between 1 and 10 μm .

The 4:1 acrylate to thiol augment cross-sectional view was also examined via micro-CT imaging as shown in Figure 10(a) and reconstructed imaging to display the outer structure in the 3D image shown in Figure 11. Figure 10 portrays the internal porosity and some slight elongation of the pores from the bottom left to the top right of image (a) and can be compared to the 9:1 augment in image (b).

Discussion

Thermal frontal polymerization works by the feedback between the heat released by the polymerization and the Arrhenius dependence of the polymerization rate on temperature. Most of the temperature dependence is through the decomposition of the initiator. Benzoyl peroxide was chosen as an initiator due to its prevalence in bone cement and other biomaterials.^{16,17} The self-propagating front can be locally initiated and propagate from a heat source to cure the entire polymer. The initiation temperature is dependent on the size of the monomer, the materials it is in contact with, and the external environment (i.e., room temperature). These factors also affect the reaction rate, thus the temperature and reaction rate will depend on the application.¹¹ By using a fine tipped heat source the physician only needs access to a small portion of the monomer and would still be able to cure an area that is covered or not easily reachable.

This study was conducted to observe the effect of a material's structure as a function of the thiol concentration. Three ratios of acrylate to thiol were synthesized, 9:1, 4:1, and 2:1 by weight, and one composition without thiol. Ratios of 1:1 and 4:3 by weight of acrylate to thiol did not frontally polymerize due to the high concentration of thiol.¹⁸

Working with the three ratios of acrylate to thiol and the acrylate only material, the results display the high extract cell viability of the 9:1 and 4:1 augments. The cell death in the acrylate only augment is believed to have occurred due to the leeching of excess acrylate monomer that decomposes into acrylic acid.¹⁹ The build-up of excess monomer in the acrylate only augment is attributed to the low monomer conversion, especially at the surface of the augment. While the excess acrylate monomer caused cell death in the acrylate only augment, it is hypothesized that hydrolyzed thiol monomers, and/or a change in pH may have caused the cell death observed in the 2:1 ratio samples (see Supporting Information).²⁰ Thiyl radicals are known to onset the mechanism for cell damage by thiols.^{21,22} It is also possible that, due to the lower front temperature, there were unreacted thiols that diffused out of the scaffold and were able to follow a similar pathway to the thiyl radical, disrupting the cell membrane, and lysing the cells. It is presupposed that this release of thiol monomer over the 7 days occurred in all the of the augments with thiol, but the concentration released within the 2:1 acrylate to thiol augment was high enough to lead to cell lysis.

For use as a surgical augment, the temperature change caused by the reaction must be considered. The trends of the augments depict the increase in temperature with a decrease in thiol concentration. This is due to lower enthalpy of the copolymerization of a thiol with an acrylate compared to that of the homopolymerization of acrylate. With fewer acrylate radicals formed, the temperature is decreased in the 9:1, 4:1, and 2:1 augments. When comparing these augments to bone cement's temperature range, from 70°C to 120°C during polymerization the 4:1 and the 2:1 acrylate to thiol augments are the two that are closest to and within the bone cement range. The bone cement's duration at an elevated temperature is longer than that of the 4:1 acrylate to thiol augment.²³ The quick heating and rapid cooling could limit the amount of tissue damaged at the surgery site. The time for augments to react and cool to 40°C for the 4:1 augment was less than that of bone cement, for bone cements reaction time alone is greater than the duration to heat and cool the augments. Thus, this fast reaction is advantageous for the physician's total working time and the amount of time the tissue is exposed to high, potentially damaging, temperatures.

As shown by Viner et al., as the thiol concentration is increased, bulk polymerization initiated by a redox reaction between the thiol and peroxide becomes favorable. Also, the thiol acts by decreasing the oxygen inhibition. The greater the concentration of thiol the greater the decrease in bulk polymerization pot life.¹⁴ This trend of decreased pot life was shown from the 9:1 to the 2:1 acrylate to thiol augments.

The mechanical properties are shown to increase as the thiol concentration is increased, correlating indirectly with the porosity study. As the thiol concentration was increased from the 9:1 to the 2:1 acrylate to thiol formulation, the maximum temperature decreased, likely resulting in less expansion. The decrease in porosity ultimately led to an increase in the mechanical strength of the samples with lower acrylate content. Because of the porosity, the frontally polymerized augment is about one twentieth of the strength of commercially available bone cement.

The large distribution in mass retention and porosity for the 4:1 sample is hypothesized to be a result of the carbon dioxide released from the scaffold. In future studies, adjusting the

porosity can be achieved by a change in the concentration of BPO, initiator.^{11,24} This could decrease the amount of gas released, providing a narrower distribution of mass retention and porosity, and result in consistent porosity similar to what is observed within the 9:1 and 2:1 acrylate to thiol augments.⁷

Analysis of the scanning electron microscopy

Cordell et al. has shown the importance of micropores for initial cell adhesion and the importance of macropores between 100 and 150 μm pores for cell growth and mineral formation.²⁵ With these augments having both micropores and macropores; adequate structure for adhesion, nutrient and waste flow, could lead to cell ingrowth post implantation.

In conclusion, utilizing thermal frontal polymerization provides an opportunity for physicians to utilize an alternative to traditional bone cement with an extended working time but a “cure-on demand” capability. This study explored the effect of different ratios of frontally polymerized acrylate to thiol and concluded that the 4:1 ratio of acrylate to thiol augment displayed the optimal cytocompatibility, mechanical strength, and porosity when compared to the acrylate only, 9:1 and 2:1 acrylate to thiol augments. Based in these results the 4:1 augment may prove useful as a substitution for bone cement in orthopedic repair procedures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Relative metabolic activity of 3T3 mouse fibroblast cells in acrylate or thiol-acrylate extractives as quantified via an Alamar Blue fluorescent assay. Asterisk indicates sample is significantly different from the dead control.

Figure 2.

Front velocity versus time for all acrylate to thiol ratios with 0.5% (w/w) benzoyl peroxide and 20% (w/w) hydroxyapatite.

Figure 3. Temperature profile of acrylate only, 9 to 1, 4 to 1, and 2:1 ratio of acrylate to thiol with 0.5% (w/w) benzoyl peroxide and 20% (w/w) hydroxyapatite. Thermocouple located at the center of a 1.3 cm × 0.7 cm × 10.3 cm mold.

Figure 4. Pot life study of lifetime of the reaction once all monomers are mixed. **All samples were significant from each other.

Figure 5.

Compressive strength tested at a rate of 2.5 mm min^{-1} until the yield point was reached or at 10% deformation. *Indicates significance of $p < 0.05$.

Figure 6.

Young's modulus tested using an Instron at a rate of 2.5 mm min^{-1} until the yield point was reached or at 10% deformation. *Indicates significance of $p < 0.05$.

Figure 7. Mass retained after 7 days in tissue culture media at 37°C. *Indicates significance of p 0.05.

Figure 8. Porosity of augments with varying ratios of acrylate to thiol. All ratios have 0.5% (w/w) benzoyl peroxide and 20% (w/w) hydroxyapatite. **All samples were significant from each other.

Figure 9. Scanning electron microscopy of: (a) acrylate only, (b) 9:1 ratio of acrylate to thiol, (c) 4:1 ratio of acrylate to thiol, (d) 2 to 1 ratio of acrylate to thiol displaying consistent porosity.

Figure 10. micro-CT cross-sectional slice of: (a) acrylate only, (b) 9:1 ratio of acrylate to thiol, (c) 4:1 ratio of acrylate to thiol, (d) 2 to 1 ratio of acrylate to thiol. Analyzed via MATLAB. Augment cylinders are 6 mm in diameter and 12 mm in height. Scale bar is 6 mm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 11. micro-CT 3D image of: (a) Acrylate only, (b) 9:1 ratio of acrylate to thiol, (c) 4:1 ratio of acrylate to thiol, (d) 2 to 1 ratio of acrylate to thiol. Augment cylinder is 6 mm in diameter and 12 mm in height. Scale bar is 6 mm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Scheme 1.

Thiol-ene free-radical chain growth polymerization mechanism.

Table I
Ratios of PETA to TMPTMP Per Construct by Mass

Sample Name	Percent PETA by Mass	Percent TMPTMP by Mass
Acrylate only	100	0
9 to 1	90	10
4 to 1	80	20
2 to 1	66	33

Table II
Front Velocity, Maximum Temperature, and Reaction Duration Per Ratio of Acrylate to Thiol by Mass

Sample Name	Front Velocity (cm s ⁻¹)	Maximum Temperature (°C)	Duration of Reaction and Cooling (From 25°C to 40°C in min)
Acrylate Only	0.45±0.01	200±10	6.33±0.33
9 to 1	0.28±0.01	180±13	8.02±0.84
4 to 1	0.22±0.03	160±10	6.18±0.88
2 to 1	0.12±0.03	124±4	5.17±0.93