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Biodegradable inorganic-organic hybrids of methacrylate star polymers for bone regeneration

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Keywords
Hybrids, bioactive glass, sol-gel, star polymers, TMSPMA

Abstract
Hybrids that are molecular scale co-networks of organic and inorganic components are promising biomaterials, improving the brittleness of bioactive glass and the strength of polymers. Methacrylate polymers have high potential as the organic source for hybrids since they can be produced, through controlled polymerization, with sophisticated polymer architectures that can bond to silicate networks. Previous studies showed the mechanical properties of hybrids can be modified by polymer architecture and molar mass (MM). However, biodegradability is critical if hybrids are to be used as tissue engineering scaffolds, since the templates must be remodelled by host tissue. Degradation by-products have to either completely biodegrade or be excreted by the kidneys. Enzyme, or bio degradation is preferred to hydrolysis by water uptake as it is expected to give a more controlled degradation rate. Here, branched and star shaped poly(methyl methacrylate-co-3-(trimethoxysilyl)propyl methacrylate) (poly(MMA-co-TMSPMA)) were synthesized with disulphide based dimethacrylate (DSDMA) as a biodegradable branching agent. Biodegradability was confirmed by exposing the copolymers to the glutathione, a tripeptide which is known to cleave disulphide bonds. Cleaved parts of the star polymer from the hybrid system were detected after 2 weeks of immersion in glutathione solution, and MM was under threshold of kidney filtration. The presence of the branching agent did not reduce the mechanical properties of the hybrids and bone progenitor cells attached on the hybrids in vitro. Incorporation of the DSDMA branching agent has opened more possibilities to design biodegradable methacrylate polymer based hybrids for regenerative medicine.
1. Introduction

Porous bioactive glass scaffolds have been made as synthetic bone grafts and scaffolds for bone regeneration[1-3]. Bioactive glasses have been shown to stimulate new bone regeneration[4]. The scaffolds had interconnected pores and compressive strengths similar to that of trabecular bone but are too brittle for bone defects that are exposed to cyclic loads[1, 5, 6]. Inorganic-organic sol-gel hybrids have co-networks of inorganic and organic components that interact at the molecular level, with the hypothesis that they can reduce the brittleness of bioactive glasses but still deliver the biological benefits of the glasses, providing excellent control of degradation rates compared to conventional composites[7]. Class II hybrids have shown promising properties because the inorganic and organic components are covalently linked[8]. Hybrids of natural polymers such as poly(γ-glutamic acid)[9, 10], chitosan[11-15], and gelatin[16, 17] have been produced, using organosilane coupling agents to bond the inorganic and organic networks. The coupling agents were small molecules, such as 3-(glycidyloxypropyl)trimethoxysilane, with a functional group on one end that could bond to the polymer while the other end of the molecule was a siloxane group. Functionalisation of the polymer with the organosilane meant that the polymer contained pendant siloxane groups. When the polymer was introduced to the sol-gel process, the siloxane groups hydrolysed and polycondensation of Si-OH groups in the silicate network (formed from hydrolysed tetraethylorthosilicate, TEOS) formed Si-O-Si bonds. However, the coupling reactions were difficult to control[11, 18-20].

An alternative to coupling agents is to synthesise a polymer that already contains pendant siloxane groups, e.g. poly(3-(trimethoxysilyl)propyl methacrylate), polyTMSPMA, or poly(MMA-co-TMSPMA). Hybrids produced using polyTMSPMA showed improved ductility[21]. Further improvement was achieved using the copolymer poly(MMA-co-TMSPMA) and the effect of different methacrylate polymer architectures on mechanical properties of the hybrids was investigated[22]. The star polymer of poly(MMA-co-TMSPMA) provided higher tailorability of the mechanical properties over other architectures since the arms of the star polymer and the cross-linking core can be modified to enhance the mechanical properties. Polymethacrylates are not biodegradable due to the non-degradable C-C bond on the backbone, and a linear polymer of MM above 30 kg/mol will not
pass through pores of the glomeruli in kidney[23, 24]. Also, polymers with larger MM are required because more entanglement can improve mechanical properties. Therefore, it is important to design non-degradable fragments of polymers with precise size and architecture, through controlled polymerisation, e.g. stars. Mechanical properties can be improved by linking such fragments, but the links should be biodegradable, which could be achieved using a star polymer with a cleavable cross-linking core[25]. Hydrolytically cleavable branching agents were not considered since they can be damaged during the sol-gel process, and they would not offer controlled degradation in vivo. DSDMA, a bi-functional methacrylate branching agent with a disulphide bond in the middle, was previously used to synthesize chemically cleavable[26-29] and biologically cleavable[30, 31] polymers.

Here, the aim was to design biologically cleavable inorganic-organic hybrid materials for bone regeneration. Randomly branched and star copolymers of poly(MMA-co-TMSPMA) were synthesized in a similar method as the previous study[22], however, ethylene glycol dimethacrylate (EGDMA) branching agent (for randomly branched) or cross-linking core (for star) were substituted with DSDMA. The copolymers were then introduced into the sol-gel process for hybrid synthesis.

2. Experimental section

Methyl methacrylate (MMA, 99%), 3-(trimethoxysilyl)- propyl methacrylate (TMSPMA, 98%), disulphide based dimethacrylate (DSDMA), 2-phenyl-2-propyl benzodithioate (CDB, RAFT agent, 99%), 2,2’-azobis(2-methylpropionitrile) (AIBN, initiator, 98%), 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH, free radical inhibitor, 99%), calcium hydride (CaH₂, 95%), silica gel (60 Å, 70–230 mesh), basic alumina (Al₂O₃, 95%), n-hexane (95%), toluene (polymerisation solvent, 99%), tetrahydrofuran (THF, analytical and HPLC grade, 99.9%), deuterated chloroform (CDCl₃, 99.8%), tetraethyl orthosilicate (TEOS, 98%), and hydrochloric acid solution (1 M HCl) were purchased from Sigma-Aldrich. Prior to polymerisation, MMA was passed through basic alumina columns to remove inhibitors and acidic impurities (TMSPMA was passed through neutral alumina due to its alkoxy silane group hydrolysing). Then, they were stirred over CaH₂ for 1 h to neutralise traces of moisture in the presence of DPPH. Finally, all the monomers were vacuum distilled prior to the polymerisation. AIBN was recrystallized in ethanol. Toluene was dried in the presence of silica gel,
which was heated up to 250 °C for 4 h prior to use. All the glassware were dried overnight at 120 °C and assembled hot under dynamic vacuum before use. Cell culture reagents were purchased from Invitrogen and Sigma-Aldrich UK unless specified otherwise. MC3T3-E1 preosteoblast cell line (ATCC, UK) was culture expanded in monolayer cultures in basal α-MEM supplemented with 10% (v/v) FCS (fetal calf serum), 100 unit/mL penicillin, and 100 μg/mL streptomycin. Cultures were maintained in a humidified atmosphere at 37 °C, 5% CO₂, and 21% O₂. Cells were passaged upon confluence using 500 μg/mL trypsin-EDTA (ethylene diamine tetra-acetic acid).

2.1 Randomly branched copolymer synthesis

Randomly branched poly(MMA₄₈₀-co-TMSPMA₄₈) polymers were synthesised by one-pot reversible addition-fragmentation chain transfer (RAFT) polymerisation method[22]. A Schlenk tube was loaded with AIBN (0.04 mmol, 5.00 mg), CDB (0.07 mmol, 0.02 ml), MMA (0.07 mol, 7.16 ml), TMSPMA (6.72 mmol, 1.60 ml), DSDMA with molar ratio of MMA:DSDMA 100:0.7, and toluene as a solvent. The components of the Schlenk tube were subsequently degassed three times by freeze-vacuum-thaw cycle under argon atmosphere, and were heated in an oil bath to 70 °C to initiate the polymerisation. The polymerisation was terminated at 50% conversion.

2.2 Star copolymer synthesis

The star polymer was synthesised by an “arm-first” approach using RAFT polymerisation technique. The arm, or the macro RAFT, of the star polymer was a linear poly(MMA₁₂₀-co-TMSPMA₁₂) with a MM of 15k g/mol. A Schlenk tube was loaded with AIBN (0.13 mmol, 0.02 g), CDB (0.25 mmol, 0.06 ml), MMA (0.06 mol, 6.50 ml), TMSPMA (6.0 mmol, 1.45 ml), and toluene as a solvent. The components of the Schlenk tube were subsequently degassed three times by freeze-vacuum-thaw cycle under argon atmosphere, and were heated in an oil bath to 70 °C to initiate the polymerisation. The polymerisation was terminated at 50% conversion. After the macro RAFT was synthesised, it was purified by precipitation in n-hexane. Then the macro RAFT was re-dissolved in a dry toluene and added in a Schlenk tube along with AIBN and the DSDMA cross-linker. The molar ratios used were Macro RAFT:DSDMA:AIBN 8:1:0.3. The contents were degassed three times by freeze-vacuum-thaw cycle under argon atmosphere, and were heated in an oil bath to 70 °C. The polymerisation was
kept for 24 h. Then, the star polymer was precipitated in n-hexane and ethanol. The precipitation was repeated 3 times in order for the unreacted arms to be removed.

2.3 Disulfide bond cleavage through glutathione

Glutathione was dissolved in deionised water in the molar ratio of glutathione:DSDMA (from the copolymers) 10:1. 0.2 g of glutathione was dissolved in 20 ml of water, and then 0.14 g of copolymer was introduced in the solution. The dissolved glutathione and copolymer mixture was stirred under argon at room temperature for 48 h. Then, cleaved copolymer was dried in the oven and dissolved in THF for gel permeation chromatography analysis which is described in 2.5 Polymer characterisation. For evaluation of hybrid’s biodegradability, 0.2 g of glutathione was dissolved in 20 ml of water, and then 0.2 g of ground hybrid sample (particle size: 357 ± 146 μm, measured by confocal microscopy images using image processing software) was introduced in the solution. The polycarbonate container was then sealed and placed in 37°C orbital shaker for 2 weeks. The ground hybrid samples were removed from the glutathione solution to dry in 1 wk, and 2 wk time points. The dried samples were immersed in THF for gel permeation chromatography analysis described in 2.5 Polymer characterisation.

2.4 Hybrid synthesis

After the copolymers were purified, toluene was removed using rotary evaporator under vacuum. It is important to point out that the TMSPMA containing polymers were not precipitated in n-hexane like most polymethacrylates because the trimethoxysilyl groups tend to cross-link. So the polymers were purified with solvent exchange (n-hexane-THF) and then were stored in THF. In a separate beaker, TEOS was hydrolysed in the molar ratio of TEOS:water:HCl of 1:3.7:0.01. The amount of TEOS was added as such so the overall wt% of the hybrid to have 70 wt% organic and 30 wt% inorganic. When TEOS was fully hydrolysed, polymer, dissolved in THF, was poured into the beaker and the mixture was stirred for 1 h at a room temperature. The mixture was then poured into a PTFE mould, and then it was sealed within another PTFE container. The container was placed in 40°C oven to gel/age for 3 weeks and then the top of the mould was unscrewed for drying at 60°C for 10 days. Four hybrid
monolith samples of each composition were synthesised with dimension of height (10.83±0.58 mm), and diameter (8.91±0.15 mm).

2.5 Polymer characterisation
Monomer to polymer conversion rate was determined using proton $^1$H-NMR spectroscopy. This was performed in deuterated CDCl$_3$ using a 400 MHz Avance Bruker NMR spectrometer. Trioxane was loaded to the Schlenk tube prior to the polymerisation and was used as an internal standard to determine the monomer to polymer % conversion. Specifically, trioxane peak at 5.1 ppm was compared to the unreacted methacrylate peak at 5.5 ppm. The polymer composition of the final copolymers was calculated using the MMA methoxy group and the TMSPMA methylene group next to the alkoxy silane group.

The average MMs and dispersities ($Đ_s$) for all the polymers, macro RAFT, and cleaved polymer fragments were determined by gel permeation chromatography (GPC). An Agilent, SECurity GPC system, with a Polymer Standard Service SDV analytical linear M column (SDA083005LIM) was used. All the copolymers were dissolved in THF and were filtered through 0.45 µm polytetrafuloroethylene (PTFE) syringe filters. GPC eluent was THF, which was pumped with a flow rate of 1 ml/min by ‘1260 Iso’ isocratic pump. Agilent 1260 RID detector was used to measure the refractive index signal. The calibration curve was based on PMMA standards with MMs of 2, 4, 8, 20, 50, 100 k gmol$^{-1}$.

2.6 Hybrid characterisation
The functional groups of the copolymers and hybrids were analysed by FTIR (Fourier Transform Infrared Spectroscopy) (Nicolet iS10, Thermo Scientific) with an attenuated total reflectance module. 32 scans were averaged to yield 4 cm$^{-1}$ resolution.

The mechanical properties of the hybrids were investigated by uniaxial compression test. The hybrid samples were produced in a cylindrical monolith shapes with height/diameter >1 following ISO 640:2003 standard. The sample ends were ground with a sand paper until they were flat and parallel.
The compression testing was performed using Zwick 1474 instrument with a compression speed of 0.1 mm/min, and 10 kN load cell.

Thermogravimetric analysis (TGA) was performed with NETZSCH STA 449C. The hybrid samples were ground to a fine powder and were placed in a platinum crucible. The samples were heated up to 800°C at 10°C/min in continuously flowing air.

2.7 Cell viability test

Potential in vitro cytotoxicity effects of the hybrid material on MC3T3-E1 cells were assessed in accordance to ISO 10993-5[32] and ISO 10993-12[33]. MC3T3 cells were purchased from ATCC® LGC Standards UK (CRL-2593, MC3T3-E1 Subclone 4). Dissolution products released by the samples in powder form (0.2 g/ml in α-MEM at 37°C) over a 72 h period were prepared. Medical grade polyethylene (PE) was used as negative control (non-cytotoxic) and polyurethane (PU) containing 0.1% (w/w) zinc diethyldithiocarbamate (ZDEC) was used as positive control (reproducible cytotoxic response). The dissolution products were filter sterilized and dilution series (25%, 50%, 75% and 100%) were prepared and supplemented with 10% (v/v) FCS prior to use in cell viability assays.

Cell viability was assessed by a colorimetric cell metabolic activity assay based on the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into formazan. MC3T3-E1 cells were seeded on 96-well plates at 1×10^4 cells per well and left to grow in basal α-MEM for 24 h until a sub-confluent monolayer was formed. The culture media were removed and, MC3T3-E1 cells were then incubated with fresh basal α-MEM, the dissolution products of hybrid material or controls (100 μl/well) for a further 24 h. The culture media were removed and then MTT diluted in serum-free α-MEM at a concentration of 1 mg/ml was added (50 μl/well). Following an incubation period of 2 h, the MTT solution was removed and each well was filled with 100 μl isopropanol and shaken briefly to dissolve the formazan derivatives. The optical density was measured spectrophotometrically at 570 nm using a microplate reader (SpectraMax M5).

2.8 Cell culture on hybrids
For cell attachment studies, hybrid disks (5×5×1 (h) mm) were manufactured and sterilised with 70% ethanol for 1 min. Following washing with PBS, each sample was placed in serum-free α-MEM for 30 min prior to cell seeding. Monolayer expanded MC3T3-E1 cells were harvested and suspended in basal α-MEM at a concentration 1×10^6 cells/ml. 10 μl of cell suspension was seeded onto each hybrid disk and, incubated in humidified atmosphere at 37°C, 5% CO₂ and 21% O₂ for 2 h. Each cell seeded disk was then submerged in fresh basal α-MEM and cultured for further 72 h.

2.9 Immunohistochemistry staining

Cell-seeded disks were fixed with 4% (w/v) paraformaldehyde (PFA) and used for immunohistochemical analysis of cell attachment. Following permeabilisation with buffered 0.5% (c/v) Triton X-100 in PBS (300 mM sucrose, 50 mM NaCl, 3 mM MgCl₂, 20 mM Hepes and pH 7.2) and blocking with 10 mg/ml BSA in PBS, samples were incubated with anti-Vimentin antisera (1:500 dilution in 10 mg/ml BSA in PBS, rabbit polyclonal, IgG, Abcam, Cambridge, UK) at 4°C for 1 h. This was followed by 1 h incubation with Alexa Fluor® 488-conjugated secondary antibody. Negative controls (omission of the primary antisera) were performed in all immunohistochemistry procedures. No staining was observed in the samples used as negative controls.

F-actin was labelled using CytoPainter F-actin staining kit (Abcam, Cambridge, UK) following the manufacture’s instruction. Briefly, Alexa Fluor 568-conjugated phalloidin (1:1000 dilution in labelling buffer) was added simultaneously with the secondary antibody during the incubation period. All samples were counter-stained with DAPI (0.1μg/ml in PBS).

2.10 Confocal microscopy

The samples were imaged under confocal microscopy (Leica SP5 MP laser scanning confocal microscope and software, Leica Microsystems, Wetzlar, Germany).

3. Results & Discussion

3.1 Polymer characterisation

Randomly branched and star poly(MMA-co-TMSPMA) with DSDMA will be referred to as Rnd(D) and Str(D) respectively. Since Rnd(D) synthesis followed the same protocol as poly(MMA-co-
TMSPMA) made with EGDMA (Rnd(P)) from the previous study[22], polymerization kinetics followed a similar trend as the Rnd(P) and Macro RAFT, or arm of the star polymer (Figure 1). However, as expected, since the randomly branched polymers are not well-defined polymers, the MM and $D$ were in an unpredictable range. As Table 1 shows, Rnd(D) had a large $D$ of 2.04, while Str(D) had almost the same values of $D$ as the Str(P) synthesised previously[22]. $M_n$ of the Str(D) was lower than that of Str(P) even though the macro RAFT had a similar MM. This could be from introducing an un-distilled branching agent. DSDMA distillation was attempted prior to the polymerisation. However, during the process it was self-polymerizing due to the disulphide bond cleavage, which created additional thiol radicals.

Table 1: MMs and $D$s of the poly(MMA-co-TMSPMA) copolymers and hybrid before and after disulphide bond cleavage. Rnd= randomly branched; D= cross-linking by DSDMA; Str= star polymer; D70= wt% of polymer in hybrid. $M_n$: number average molecular weight; $M_w$: weight average molecular weight; $M_p$: maximum molecular weight in molecular weight distribution.

<table>
<thead>
<tr>
<th></th>
<th>$M_n$ (kg/mol)</th>
<th>$M_w$ (kg/mol)</th>
<th>$D$</th>
<th>$M_p$ (kg/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rnd(D)</td>
<td>15.5</td>
<td>31.6</td>
<td>2.04</td>
<td>23.8</td>
</tr>
<tr>
<td>Cleaved Rnd(D)</td>
<td>10.7</td>
<td>18.4</td>
<td>1.72</td>
<td>16.9</td>
</tr>
<tr>
<td>Macro RAFT</td>
<td>11.3</td>
<td>12.4</td>
<td>1.10</td>
<td>13.3</td>
</tr>
<tr>
<td>Str(D)</td>
<td>45.7</td>
<td>54.8</td>
<td>1.19</td>
<td>49.5</td>
</tr>
<tr>
<td>Cleaved Str(D)</td>
<td>12.7</td>
<td>18.8</td>
<td>1.48</td>
<td>15.4</td>
</tr>
<tr>
<td>Cleaved Str(D70)</td>
<td>13.7</td>
<td>23.3</td>
<td>1.7</td>
<td>14.2</td>
</tr>
</tbody>
</table>

In order to confirm biodegradation through a reduction reaction, the copolymers were introduced and mixed with glutathione. Glutathione is a tripeptide that is present in mammalian tissues. It is known to reduce cell’s oxidative state and disulphide bonds within other proteins by converting itself to an oxidised form[34-36]. Therefore, glutathione was hypothesised to cleave Rnd(D) and Str(D) down to smaller polymer fragments than the original MMs as schematically illustrated in Figure 2.

As Table 1 shows, the MMs decreased significantly after 48 h of mixing the copolymers with glutathione. $M_n$ and $M_w$ values of both Rnd(D) and Str(D) were decreased, particularly Str(D) values were decreased by more than 2 fold. Also, the MMs of the cleaved polymers were lower than 30
kg/mol, which was the target MM for kidney filtration. GPC traces of the copolymers and their cleaved fragments are shown in Figure 3. Both Rnd(D) and Str(D) GPC traces show shifts towards longer elution time. The Str(D) trace, as expected, had an unreacted linear macro RAFT trace at higher elution time which was also seen in star polymer synthesized with EGDMA cross-linking core[22]. The cleaved Str(D) trace displayed a significant shift of peak molecular mass ($M_p$) towards that of macro RAFT, from 49.5 to 15.4 kg/mol. However, there was an unexpected trace in the lower elution time region. This was possibly due to the condensed alkoxysilane groups from TMSPMA, since the polymer was exposed to water for 48 h. The other possibility is that, two arms, or macro RAFTs, are linked together on the same side of the crosslinker (attached on the same double bond) thus forming a linear copolymers with double the MM of the initial arm, as it has been observed before in other studies on degradable core star polymers by Group Transfer Polymerization[37-39].

### 3.2 Hybrid characterisation

Sol-gel silica hybrids containing Rnd(D), and Str(D) will be referred to as Rnd(D70), and Str(D70) respectively. FTIR was used to confirm molecular structure of the hybrids and Str(D). The Str(D) spectrum was very similar to that of the methacrylate based polymers previously synthesised[22], with bands for C=O stretching, C-C-O asymmetric stretching, and C-O-C symmetric stretching of the ester group from the methacrylate moieties (Figure 4). As expected for the hybrids, stronger absorption bands of the condensed silica network were present: Si-O-Si asymmetric stretching and Si-OH stretching, with the presence of the methacrylate absorption bands. FTIR confirmed that the organic and inorganic sources were both present in the hybrid system.

Crack-free poly(MMA-co-TMSPMA)-SiO$_2$ class II hybrids were produced and shown in Figure 5. They were transparent from the front (Figure 5 A) and the top view (Figure 5 B). Hybrids had inherent yellow colour from the thermally degraded thiol groups from the RAFT agent. The organic-inorganic ratio was confirmed by TGA (Figure 6A), with both hybrids showing thermal decomposition starting at ~340°C, which was similar to poly(MMA-co-TMSPMA)[40] and previous poly(MMA-co-TMSPMA)-SiO$_2$ hybrids[22, 41]. The final residual mass was 30.9% and 31.2% for Rnd(D70) and Str(D70) respectively, which was close to the target inorganic content. The differential
thermogravimetry (DTG) curves shown in Figure 6B indicated trivial thermal degradation related to water and trace of organic solvent evaporation. However, there were no major peaks below 300°C, which would be attributed to weak linkages of PMMA[42], implicating that both samples were true class II hybrids.

Uniaxial compression tests were conducted to evaluate the mechanical properties of the hybrids. Mechanical properties of the hybrids followed the same trends as previously synthesised methacrylate based hybrids. As shown in Table 2, all the values were similar to that of the Rnd(70) and Str(70) values from the previous work[22]. This indicated that substitution of DSDMA in the place of EGDMA did not affect mechanical properties. The representative stress/strain curve is shown in Figure 7. As expected, hybrids first experienced elastic deformation until they reached yield stress. Then, plastic deformation and self-hardening occurred up to the ultimate failure which are similar to PMMA modes of failure[43]. The differences in mechanical properties between different polymer architectures could be due to the hydrodynamic radius of each structure. Star architecture offers higher strain to failure, which is possibly from having elastic cross-linking cores. The branched architecture can be viewed as having multiple lengths of short linear polymers connected to each other in an uncontrolled manner which could lead to more stiff mechanical properties compared to that of star architecture.

<table>
<thead>
<tr>
<th>Table 2: Mechanical properties of silica/poly(MMA-co-TMSPMA) biodegradable hybrids synthesised with poly(MMA-co-TMSPMA) cross-linked by DSDMA in the form of a star, Str(D70), or randomly branched, Rnd(D70), with 70 wt% organic polymer.</th>
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<tr>
<td><strong>Yield Stress</strong> (MPa)</td>
</tr>
<tr>
<td>-------------------------</td>
</tr>
<tr>
<td>Rnd(D70)</td>
</tr>
<tr>
<td>Str(D70)</td>
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The MTT assay was performed in accordance to ISO 10993 standards[32]. As shown Figure 8, both Rnd(D70) and Str(D70) were biocompatible according to ISO 10993 standards, where the cell
viability of the neat dissolution products (100%) of the material under test should be greater than 70% of the positive control. The dissolution products of Rnd(D70) hybrid did not appear to have adverse effects on cell viability. The reduction in cell viability when exposed to Str(D70) dissolution products was likely due to residue monomers remained in the samples. The viability of MC3T3-E1 culture dissolution products of the Str(D70) hybrids remained above the 70% threshold in comparison to that of the non-toxic controls despite a noticeable reduction. Dilution series (25%, 50%, and 75%) of the extracts using the extract vehicle as diluent (α-MEM in the present study) were also examined. The 50% dilution of the test sample had at least the same or a higher viability in comparison to the neat dissolution products, therefore the cytotoxicity test presented here was deemed valid under the guidance of ISO standard.

Cell attachment on the hybrids was examined by immunohistochemistry and confocal microscopy. Following 72 h of cell culture, the expression of Vimentin (green) and F-actin (red) were evident in MC3T3-E1 regardless of the polymer architectures (Figure 9).

As a proof of principle that the degradation of the new polymer translates to degradation of the hybrid, Str(D70) hybrid was immersed in glutathione solution under the same procedure (3.1 Polymer characterization). The hybrid sample was ground to a particulate (357 ± 146 μm), since the surface area of the monolith was too low for glutathione to react within the test time frame. For the ground particulate, no cleaved copolymer or star polymer was detected using GPC after the first week of immersion. However, after 2 weeks, cleaved star polymer was detected by GPC. As shown in Table 1, the measured MM ($M_n$: 13.7 kg/mol; $M_w$: 23.3 kg/mol; $M_p$: 14.2 kg/mol) was similar to that of the macro RAFT, or arm of the star polymer, which proved that controlled degradation occurred. Similar to cleaved Str(D) values, $MM$ and $D$ values were higher than macro RAFT values, because the cleaved fragments are possibility containing parts of DSDMA and condensed alkoxy silane from TMSPMA. However, the MM values were under kidney filtration threshold.

4. Conclusion
Poly(MMA-co-TMSPMA) of randomly branched and star architectures were successfully synthesized with a biodegradable branching agent, DSDMA. Then the polymers were introduced to class II hybrids via sol-gel process. The biodegradability of the polymers and hybrid were confirmed by cleaving the disulphide bonds with a tripeptide (glutathione). The MM of the cleaved polymers reduced to below the threshold values which can be removed by the kidney filtration. The hybrids had similar mechanical and cell attachment characteristics to the non-biodegradable equivalent hybrids synthesized with EGDMA; previously synthesized Rnd(70) and Str(70)[22]. The hybrids passed the cytotoxicity test, and MC3T3-E1 preosteoblast cells were able to attach on the surface within 72 h. Star shaped poly(MMA-co-TMSPMA) with DSDMA as a cross-linking agent shows a great potential as an organic source for biodegradable hybrids.

5. Acknowledgement

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6. References

7. Figure legends

[Figure 1] ln(M₀/Mₜ) and conversion as a function of time for poly(MMA-co-TMSPMA) synthesised by: RAFT polymerisation with EGDMA cross-linking (Rnd(P)); with DSDMA cross-linking (Rnd(D)); linear polymer as arm of the star polymer (Macro RAFT).

[Figure 2] Theoretical disulphide bond cleavage of poly(MMA-co-TMSPMA) cross-linked by DSDMA through introduction of glutathione, and biodegradation of the copolymer architecture (randomly branched and star architectures).

[Figure 3] GPC traces of poly(MMA-co-TMSPMA) cross-linked by DSDMA: A) Rnd(D), and B) Str(D) before and after disulphide bond cleavage through introducing glutathione. Macro RAFT is the poly(MMA-co-TMSPMA) arms of the star polymer prior to making the star.

[Figure 4] FTIR spectra of A) silica sol-gel hybrids synthesised with 70 wt% organic using poly(MMA-co-TMSPMA) cross-linked by DSDMA in the form of a star, Str(D70); B) silica sol-gel hybrids synthesised with 70 wt% organic using poly(MMA-co-TMSPMA) randomly branched with DSDMA, Rnd(D70); and C) poly(MMA-co-TMSPMA) cross-linked by DSDMA in the form of a star, Str(D), for comparison.

[Figure 5] A) Front and B) top view photos of silica/poly(MMA-co-TMSPMA) hybrids made with poly(MMA-co-TMSPMA) cross-linked by DSDMA in randomly branched (Rnd(D70)), and star (Str(D70)) polymer structure.

[Figure 6] Thermal analysis of silica/poly(MMA-co-TMSPMA) hybrids cross-linked by DSDMA, using randomly branched polymer, Rnd(D70), and star polymer, Str(D70): A) TGA and B) DTG.

[Figure 7] Representative compression test curve of 70S30C bioactive glass monolith[10], silica/poly(MMA-co-TMSPMA) hybrids synthesized with poly(MMA-co-TMSPMA) cross-linked by
EGDMA in the form of randomly branched, Rnd(70), and star, Str(70) from the previous study[22], and hybrids with poly(MMA-co-TMSPMA) cross-linked with DSDMA in the form of randomly branched, Rnd(D70), and star, Str(D70).

[Figure 8] Viability of MC3T3 cells cultured in dissolution products of silica/poly(MMA-co-TMSPMA) hybrids Rnd(D70) and Str(D70), by MTT metabolic activity assay, relative to controls of standard culture media and PE (negative non-toxic controls) and positive (cytotoxic) control (PU), after 72 h of culture, for progressive dilutions in media, following ISO 10993 standards[32]. The 70% limit of the standard control is represented as dotted line. Under the guidance of ISO standard, both neat dissolution products (100%) and dilution series (25%, 50%, and 75%) prepared using the extract vehicle as diluent (α-MEM) were also examined. * p < 0.05 when compared to cytotoxic PU control.

[Figure 9] Immunohistochemical staining of the MC3T3 cytoskeleton on the silica/poly(MMA-co-TMSPMA) hybrids A) Rnd(D70), and B) Str(D70). Vimentin immunostain (green), F-actin labelling (red), and DAPI nuclear counter stain (blue) produced via confocal microscopy.
Glutathione reduced
Bioactive glasses can regenerate bone but are brittle. Hybrids can overcome this problem as intimate interactions between glass and polymer creates synergetic properties. Implants have previously been made with synthetic polymers that degrade by water, however, they degrade catastrophically, causing rapid loss of strength. Polymers that degrade by biological agents may degrade at a more controlled rate, which should give time for tissue repair and transfer of load. Previously, hybrids made with star shaped poly(methyl methacrylate-co-3-(trimethoxysilyl)propyl methacrylate) (p(MMA-co-TMSPMA)) showed enhanced properties. However, methacrylates are not bio-degradable. Here, star shaped p(MMA-co-TMSPMA) was synthesised with a core that can be cleaved by glutathione, a tripeptide. On exposure to glutathione, the hybrid degraded, producing products with molecular weights below the kidney filtration threshold.