Supporting Information

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Effect of comonomers on physical properties and cell attachment to silica-methacrylate/acrylate hybrids for bone substitution

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Materials and methods

Materials

Methyl methacrylate (MMA, 99%), *n*-butyl methacrylate (BMA, 99%), methyl acrylate (MA, 99%), 3-(trimethoxysilyl)propyl methacrylate (TMSPMA, 98%), 3-(trimethoxysilyl)propyl acrylate (TMSPA, 92%), 2-phenyl-2-propyl benzodithioate (RAFT agent, 99%), methyl 2-(dodecylthiocarbonothioylthio)-2-methylpropionate (RAFT agent, 97%), 2,2′-azobis(2-methylpropionitrile) (AIBN, initiator, 98%), 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH, free radical inhibitor, 99%), calcium hydride (CaH_2, 95%), silica gel (60 Å, 70-230 mesh), basic alumina (Al_2O_3, 95%), *n*-hexane (95%), methanol (99.5%), toluene (polymerisation solvent, 99%), tetrahydrofuran (THF, analytical and HPLC grade, 99.9%), deuterated chloroform ($CDCl_3$, 99.8%), tetraethyl orthosilicate (TEOS, 98%), and hydrochloric acid solution (1M HCl) were purchased from Sigma-Aldrich (UK). Prior to polymerisation, MMA, BMA, and MA were passed through basic alumina columns to remove inhibitors and acidic impurities (TMSPMA and TMSPA was passed through neutral alumina due to its alkoxysilane group hydrolysing). Then, they were stirred over CaH_2 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^{o}C for 4 h prior to the use. All the glassware were dried overnight at 120^{o}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 pre-osteoblast cell line (ATCC, UK) was culture expanded in monolayer cultures in basal α-MEM supplemented with 10% (v/v) FCS (foetal calf serum), 100 unit/ml penicillin and 100 μg/ml streptomycin. Cultures were maintained in humidified atmosphere at 37°C, 5% CO_2 and 21% O_2. Cells were passaged upon confluence using 500 μg/ml trypsin-EDTA (ethylene diamine tetra-acetic acid).

Copolymer synthesis

All three copolymers were synthesised by one-pot RAFT polymerisation technique. They were designed to have MMs of 15 kg/mol; p(MMA_{120}-*co*-$TMSPMA_{12}$), p(BMA_{90}-*co*-$TMSPMA_{9}$), and p(MA_{137}-*co*-$TMSPA_{13.7}$), with the cross-linking agent molar ratio of 10 fold lower than the main polymer backbone. Schlenk tubes were loaded with:

1) AIBN (0.13 mmol, 0.02 g), 2-phenyl-2-propyl benzodithioate (0.25 mmol, 0.06 ml), MMA (0.06 mol, 6.40 ml), TMSPMA (6.00 mmol, 1.43 ml),

2) AIBN (0.07 mmol, 0.01 g), 2-phenyl-2-propyl benzodithioate (0.13 mmol, 0.03 ml), BMA (0.02 mol, 3.71 ml), TMSPMA (2.33 mmol, 0.55 ml),

3) AIBN (0.13 mmol, 0.02 g), methyl 2-(dodecylthiocarbonothioylthio)-2-methylpropionate (0.25 mmol, 0.09 ml), MA (0.07 mol, 6.17 ml), TMSPA (6.85 mmol, 1.52 ml).

Toluene was used as a polymerisation solvent and added to the Schlenk tubes by the 4 fold higher amount (w/w) of the total chemicals placed in the tubes. The components of the Schlenk tubes were subsequently degassed three times by freeze-vacuum-thaw cycle under argon atmosphere, and were heated in an oil bath to 70^{o}C to initiate polymerisation. Then, p(MMA-*co*-TMSPMA) and p(MA-*co*-TMSPA) were precipitated in *n*-hexane, while p(BMA-*co*-TMSPMA) was precipitated in cold methanol.

Hybrid synthesis

After the copolymers were purified, toluene and other precipitating solvents were removed using a rotary evaporator under vacuum pressure. Then, the polymers were dissolved in THF. In a separate beaker, inorganic sol was prepared by hydrolysing TEOS with the molar ratio of TEOS:water:HCl of 1:3.7:0.01. The amount of TEOS added such that the overall wt% of the hybrid would be 70 wt% organic and 30 wt% inorganic. When TEOS was fully hydrolysed (full hydrolysis was visually confirmed from cloudy to clear solution), polymer was poured into the beaker and the mixture was stirred for 1 h at room temperature. The mixture was poured into a Teflon mould and then placed in 40^{o}C oven to gel/age for 3 weeks. The mould was then opened to dry in a 60^{o}C oven for 10 days. Four hybrid monolith samples of each composition were synthesised with dimension of height 12.47 ± 3.30 mm, and diameter 10.23 ± 0.73 mm.

Polymer characterisation

The conversion rate of monomers to polymer and polymer composition were determined using proton Nuclear Magnetic Resonance (^{1}H-NMR) spectroscopy. This was performed in deuterated $CDCl_3$ using a 400-MHz Avance Bruker NMR spectrometer. For the % conversion calculations, trioxane was loaded to the Schlenk tube prior to the polymerisation and was used as an internal standard to determine the % conversion of the monomer to the polymer. Specifically, trioxane peak at 5.1 ppm was compared to the unreacted monomer peak at 5.5 ppm to confirm the conversion rate.

The average molar mass (MM) and dispersities ($Đ$) for all the polymers and their precursors were determined by Gel Permeation Chromatography (GPC). An Agilent, SECurity GPC system, with a Polymer Standard Service (PSS) SDV analytical linear M column (SDA083005LIM) was used. All the polymers were dissolved in THF and were filtered through 0.45 μm polytetrafuloroethylene (PTFE) syringe filters. The GPC eluent was THF, which was pumped with a flow rate of 1 ml/min by a '1260 Iso' isocratic pump. An 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 kg/mol.

Hybrid characterisation

The functional groups of the hybrids and copolymers were analysed by Fourier Transform Infrared spectroscopy (FTIR, 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. Compression testing was performed using Zwick 1474 instrument with a 100 kN load cell at a consistent speed of 0.1 mm/minute.

Thermogravimetry (TGA) analysis was used to measure the organic/inorganic % weight of the hybrid samples. TGA was performed with Netzsch sta 449c in air. The hybrid samples were placed in a platinum crucible and heated to 800ºC at 10ºC/ min.

Cytotoxicity test

The biocompatibility evaluation of the hybrid material was performed based on the guidance of ISO 10993-5[1] and ISO 10993-12[2]. Test dissolution products of the materials (0.2 g/ml in α-MEM) were prepared at 37°C over a 72 h period on a rotor. Medical grade polyethylene (PE) and polyurethane (PU) containing 0.1% (w/w) zinc diethyldithiocarbamate (ZDEC) were used as non-cytotoxic negative control and cytotoxic positive control respectively. Filter sterilised dissolution products were diluted at 25%, 50%, 75% and 100% and supplemented with 10% (v/v) FCS for *in vitro* 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) metabolic activity assays.

MC3T3-E1 cells were seeded on 96-well plates at 1×10^4 cells per well. Following 24 h of culture, cells were subjected to test dissolution products or controls for a further 24 h. The media were replaced with serum-free MTT solution (1 mg/ml in α-MEM) and incubated for 2 h. The

yellow tetrazolium MTT was reduced by metabolically active cells during the incubation period. The MTT solution was then removed and each well was filled with 100 µl dimethyl sulfoxide (DMSO) and shaken briefly to dissolve the purple formazan derivatives. The optical density was measured spectrophotometrically at 570 nm using a microplate reader (SpectraMax M5).

Cell adhesion

For cell adhesion tests, disks of each hybrid of approximately 5×5×1 (h) mm (n=3 per material) were produced. Following sterilisation in 70% ethanol, disks were washed with PBS and placed in serum-free α-MEM for 30 minutes prior to cell seeding. Trypsinised cells were resuspended at 1×10^6 cells/ml in basal α-MEM. 10 µl of cell suspension was seeded onto each hybrid disk and incubated for 2 h in standard conditions. Each cell seeded disk was then submerged in fresh basal α-MEM and cultured for further 72 h.

Immunohistochemistry staining

Following fixation in 4% (w/v) paraformaldehyde (PFA), cell-seeded disks were washed in PBS and permeabilised using buffered 0.5% (v/v) Triton X-100 in PBS (300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$, 20 mM HEPES at pH 7.2). Nonspecific epitopes were blocked with 10 mg/ml BSA in PBS. The samples were then 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 anti-rabbit Alexa Fluor® 488-conjugated secondary antibody (1:1000 dilution in 10 mg/ml BSA in PBS, Abcam, Cambridge, UK). Samples stained without the addition of a primary antibody served as negative controls. F-actin was labelled using Alexa Fluor® 568-conjugated phalloidin (1:1000 dilution in 10 mg/ml BSA in PBS, Abcam, Cambridge, UK) simultaneously with the secondary antibody during the incubation period. All samples were counter-stained with DAPI nucleus stain (0.1µg/ml in

PBS). Stained samples were imaged under confocal microscopy (Leica SP5 MP laser scanning confocal microscope and software, Leica Microsystems, Wetzlar, Germany). Representative images were shown in the results section.

Contact angle measurement

Contact angles of the hybrid samples were measured on DataPhysics OCA-20 (Filderstadt, Germany) contact angle goniometer by the sessile drop method. A drop (20µl) of deionised water was placed on the hybrid surface, and contact angles on each side were averaged. The sessile drop measurement was repeated three times per sample then the values were averaged.

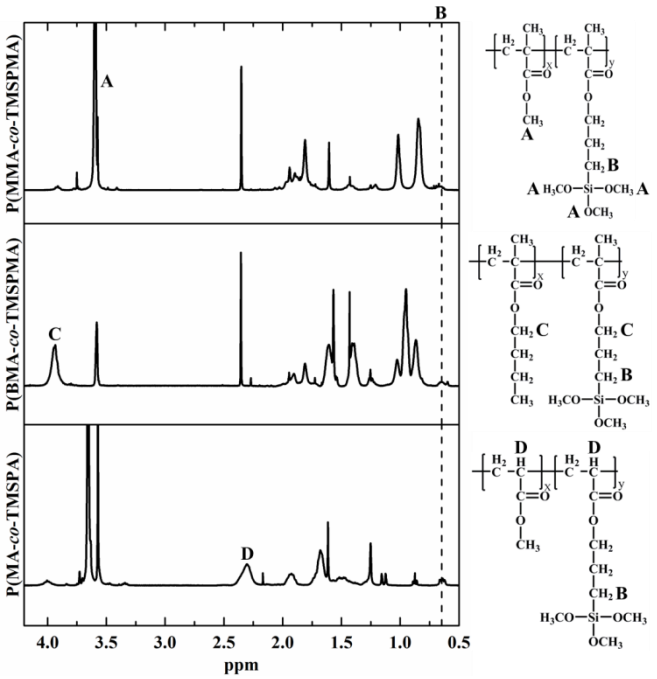

Figure S1: ^{1}H-NMR spectra of p(MMA-*co*-TMSPMA), p(BMA-*co*-TMSPMA), and p(MA-*co*-TMSPA) copolymers in $CDCl_3$. The cross-linking agent peaks for the copolymers were found in the same region at 0.67 ppm, labelled as B. B peaks were then compared with the methoxy group (3.6 ppm) of p(MMA-*co*-TMSPMA), the methylene group (3.9 ppm) of p(BMA-*co*-TMSPMA), and carbon backbone (2.3 ppm) of p(MA-*co*-TMSPA).

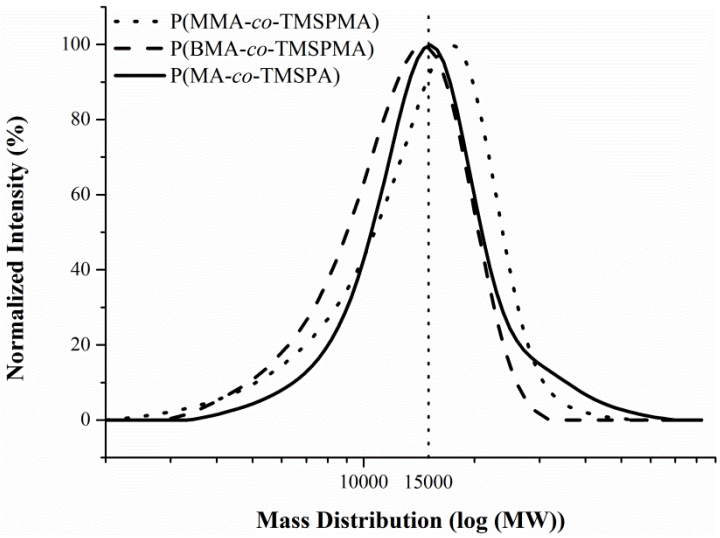

Figure S2: GPC traces of p(MMA-*co*-TMSPMA), p(BMA-*co*-TMSPMA), and p(MA-*co*-TMSPA) copolymers.

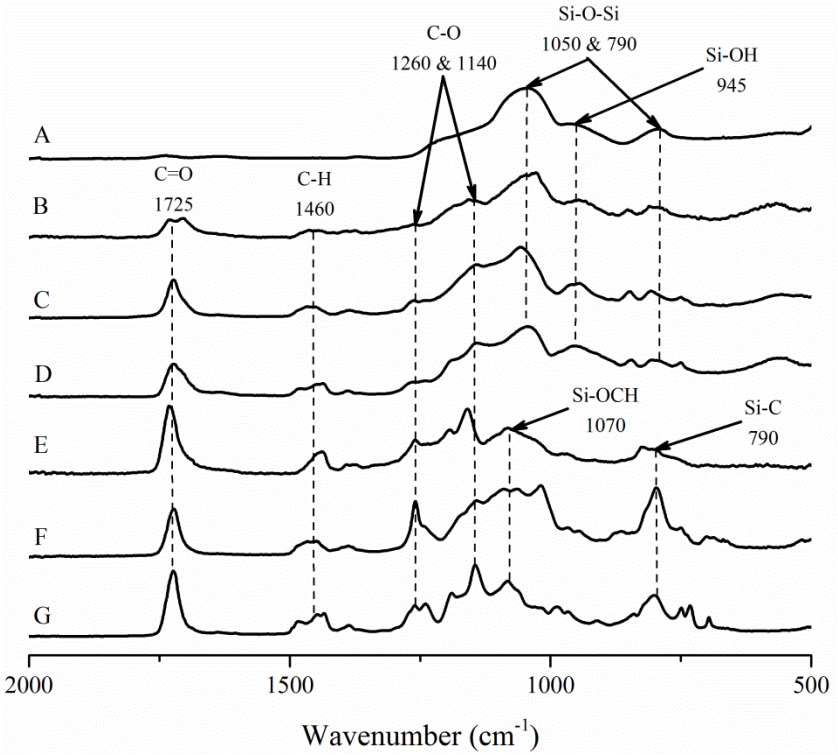

Figure S3: FTIR spectra of A) sol-gel silica glass; hybrids made with 70 wt% organic with methacrylate based copolymers: B) p(MA-*co*-TMSPA), MA(70), C) p(BMA-*co*-TMSPMA), BMA(70), D) p(MMA-*co*-TMSPMA), MMA(70), and for comparison, the copolymers prior

to hybrid synthesis: E) p(MA-*co*-TMSPA), F) p(BMA-*co*-TMSPMA), and G) p(MMA-*co*-TMSPMA).

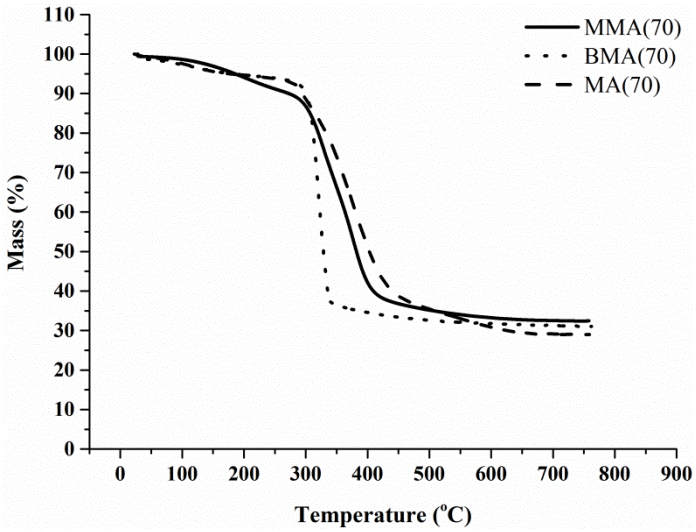

Figure S4: TGA traces from hybrids made with 70 wt% organic with methacrylate based copolymers: p(MA-*co*-TMSPA), MA(70); p(BMA-*co*-TMSPMA), BMA(70); p(MMA-*co*-TMSPMA), MMA(70).

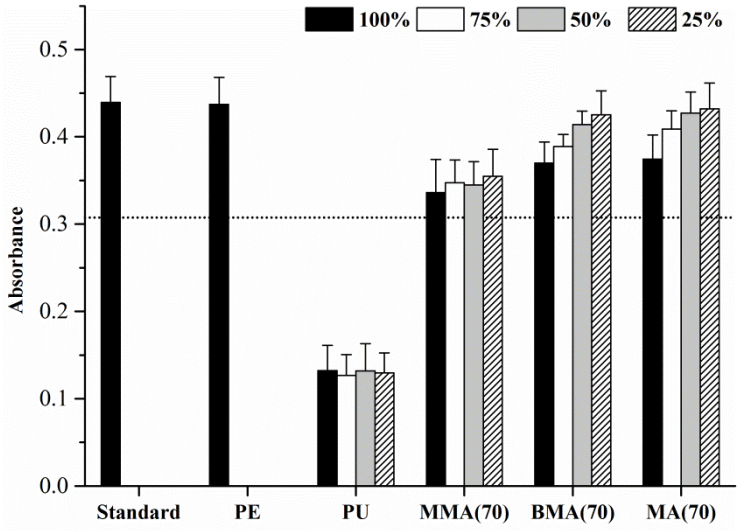

Figure S5: MTT metabolic activity assay in accordance to ISO 10993 standards[2]. The viability of MC3T3 cells in dissolution products of the hybrids were above 70% to that of the standard culture media and negative control (PE). Cell viability of the diluted dissolution products was improved. Hybrids were made with 70 wt% organic with methacrylate based

copolymers: p(MA-*co*-TMSPA), MA(70); p(BMA-*co*-TMSPMA), BMA(70); p(MMA-*co*-TMSPMA), MMA(70).

References

1. *International Standards Organization: ISO 10993-5, Biological evaluation of medical devices-Part 5: Tests for in vitro cytotoxicity, ISO* 2009, Geneva.
2. *International Standards Organization: ISO 10993-12, Biological evaluation of medical devices-Part 12: Sample preparation and reference materials, ISO* 2012, Geneva.