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

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Hybrids with a silica network covalently bonded to a polymer are promising materials for bone repair. Previous work on synthesizing methyl methacrylate (MMA) based copolymers by reversible addition-fragmentation chain transfer (RAFT) polymerization gave high tailorability of mechanical properties since sophisticated polymer structures can be designed. However, more flexible hybrids would be beneficial. Here, butyl methacrylate (BMA) and methyl acrylate (MA) based hybrids were produced. Unlike MMA, BMA and MA hybrids did not show plastic deformation, and BMA hybrid had strain to failure of 33%. Although the new hybrids were more flexible, pre-osteoblast cells did not adhere on their surfaces, due to higher hydrophobicity and lower stiffness. Comonomer choice is crucial for bone regenerative hybrids.

1. Introduction

Alternative biomaterials are required for improving current methods of bone repair. Bioactive glass scaffolds are greater osteogenic properties, than hydroxyapatite or tricalcium phosphate[1-

[a] **Supporting Information** ((bold)) is available online from the Wiley Online Library or from the author.

[4], but they are too brittle to withstand cyclic loads[5-7]. Composite versions of bioactive glass scaffolds (glass embedded in polymer matrices) have the problem of polymers masking bioactive glass from the cells and differential degradation rates during biodegradation[8, 9]. Inorganic-organic hybrids can overcome these problems, since the molecular level co-network between the flexible polymer and silica network enable cells to contact the silica and the covalent bonding between the components can give unprecedented control of mechanical properties and degradation rate[10-12]. Natural polymers, such as chitosan[13-16], gelatin[17, 18], and poly(γ-glutamic acid)[19, 20] have been functionalized with a cross-linking agent, glycidoxypropyl trimethoxysilane (GPTMS), for sol-gel silica hybrids. Despite several advantages of the naturally derived polymers, they have shortcomings for the reactions between nucleophiles of the polymers, and epoxide ring opening of the GPTMS[21].

In order to have a more versatile and reproducible organic component, biocompatible synthetic polymers are preferable. Homopolymers of 3-(trimethoxysilyl)propyl methacrylate (TMSPMA), a monomer containing alkoxysilane group, have been synthesized and fabricated to poly(TMSPMA)-SiO_2 hybrids. The hybrids have shown covalent bonding between the polymer and silica network[22], hydroxycarbonate apatite (HCA) formation was observed during simulated body fluid tests, and osteoblast precursor cell attachment was evident[23]. However, hybrids were still too brittle since every repeating monomer unit had alkoxysilane group, causing high cross-linking density. Copolymers of MMA and TMSPMA have been investigated as hybrids for bone substitute, and co-network formation was achieved without phase separation [22, 24]. The bioactivity in *in vitro*[23, 25, 26] and *in vivo*[27] have shown positive results, e.g. HCA formation, primary osteoblast adherence, and biocompatibility during subcutaneous implantation in a mouse model. The effect of polymer architecture of the copolymers on the mechanical properties of the hybrids was also investigated, and star polymer showed more tailorability[28, 29]. However, it would be more advantageous to impart higher elasticity so hybrids have higher strain to withstand cyclic loads applied in bone defect region.

BMA and MA share similar C-C backbones to MMA, while having lower glass transition temperatures (T_g), ~20ºC and ~10ºC respectively, compared to ~100ºC of MMA[30]. The T_g values are lower because the aliphatic side group in BMA limits packing of the polymer chains by increasing rotational motion[31], and absence of the methyl group in MA increases chain rotations[32]. Recently, *N*-isopropylacrylamide and TMSPMA copolymer based hybrids have been successfully investigated as thermoresponsive hydrogels[33, 34], however, swelling behavior may instigate crack formation with higher amount of silica network. Here, three different copolymers of p(MMA-*co*-TMSPMA), p(BMA-*co*-TMSPMA), and p(MA-*co*-TMSPA) were synthesized through RAFT polymerization technique. The copolymers were then fabricated to hybrids via the sol-gel process. The molecular mass (MM), cross-linking agent (TMSPMA and 3-(trimethoxysilyl)propyl acrylate (TMSPA)) molar ratio, and inorganic-organic hybrid compositions were maintained to set the uniform parameters. The aim was to evaluate the effect of softer acrylate polymers in terms of their mechanical properties of the hybrids and osteoprogenitor cell adhesion.

2. Results and Discussion

All copolymers; p(MMA-*co*-TMSPMA), p(BMA-*co*-TMSPMA), and p(MA-*co*-TMSPA), were successfully synthesized with target molar ratios of the cross-linking agents. The molar ratios were confirmed by proton nuclear magnetic resonance (**Figure S1**, supporting information), and all three copolymers contained ~9 mol% of the cross-linking agents which was close to our target copolymer structure (**Table 1**).

The MMs of the copolymers were confirmed by gel permeation chromatography (GPC) analysis (traces shown in **Figure S2**, supporting information), and **Table 1** shows the actual MMs and dispersities ($Đ$) values. MMs of the copolymers were close to the target MM of 15 kg/mol with very narrow $Đ$s (<1.2). Particularly, p(MMA-*co*-TMSPMA) had a $Đ$ value similar to previous work[28]. P(BMA-*co*-TMSPMA) was previously synthesized to fabricate hybrids

for thin films and optics[35-38], but polymers were produced with *Đ*s greater than 2.21, which is much higher than in this study. For p(MA-*co*-TMSPA), to the best of our knowledge, this was the first time such a copolymer has been synthesized for hybrids. It is also the first time that the different chemistry of the polymers within the hybrid system has been compared.

The copolymers; p(MMA-*co*-TMSPMA), p(BMA-*co*-TMSPMA), and p(MA-*co*-TMSPA), were made into hybrids with the same process as previous study[28], and will be referred to as MMA(70), BMA(70), and MA(70) respectively, where 70 refers to the organic wt.%. The molecular structures of the copolymers and the hybrids were determined by Fourier transform infrared spectroscopy (**Figure S3**, supporting information). All the hybrids possessed bands characteristic of acrylic polymers: C-H vibration, C=O, C-C-O asymmetric, and C-O-C symmetric stretching, plus absorption bands of the condensed silica network: Si-O-Si asymmetric and Si-OH stretching. Thermogravimetric analysis confirmed the inorganic to organic compositions (**Figure S4**, supporting information). All three hybrids had similar residual mass (silica network) post thermal degradation of ~30 wt% which agreed with the nominal value set for the hybrid synthesis (**Table 2**).

Uniaxial compression tests were performed to evaluate mechanical properties of the hybrids (**Figure 1)**. As expected, the hybrids displayed different behaviors. The most noticeable difference was the absence of a yield point and plastic deformation for BMA(70) and MA(70). Both of these softer hybrids displayed elastic deformation until they reached ultimate stresses of 17-18 MPa (**Table 2**). BMA(70) had compressive modulus 2 fold lower than MA(70), while the strain to failure increased two fold. MMA(70) showed a very similar compressive deformation as seen in previous tests on a similar hybrid[28]. It first experienced elastic deformation followed by plastic deformation and failure. However, the yield stress, *E*, and ultimate stress were nearly two fold lower than those of the previous MMA based hybrid, while yield strain and ultimate strain values were very similar. This is possibly due to the MM of the organic source. Former p(MMA-*co*-TMSPMA) hybrids had copolymer MM of 60 kg/mol, here

the MM was 15 kg/mol. As MM increases, the potential for chain entanglement increases which could enhance mechanical properties of the hybrids[39]. MMA(70) had much higher ultimate stress and *E* values compared to the softer hybrids. *E* was roughly six fold higher than that of MA(70) and more than 10 fold to BMA(70). The ultimate stress was almost two fold higher than both softer hybrids. However, strain to failure of MMA(70) and MA(70) were similar at 19% and 16% respectively, while BMA(70) had a much higher strain of 33%.

Cell viability was assessed by MTT metabolic activity assay following ISO 10993 standards[40] (**Figure S5**, supporting information). Despite of all the hybrids passing the ISO standard, cells were not able to attach on the softer hybrids. **Figure 2** shows confocal microscopy of immunohistochemical stained hybrids after 72 h of MC3T3 cell culture. Expression of intermediate filament, microfilaments, and nucleus was present for MMA(70) as expected. However, attachment of MC3T3 cells were not evident on surfaces of BMA(70) and MA(70) samples due to the negative stain for cell nuclei and spreading cytoskeleton proteins.

Cell adhesion, a key parameter of cell-biomaterial interaction, is a fundamental process that directly affects cell growth, migration, differentiation and the long-term success of an implanted medical device. Cellular response to a biomaterial is dictated by a number of factors that affect adsorption of cell-adhesive proteins onto the surface, which occurs immediately after implantation. Among them, surface properties such as wettability can be of crucial importance. In general, optimal cell adhesion can be achieved on a moderately hydrophilic surface [41, 42]. Extremely hydrophilic materials fail to support cell adhesion as adsorbed water layers block protein adsorption[43]. Cellular behavior on highly hydrophobic surfaces is more complex as surface structure and chemistry will affect whether proteins adsorb without changing the protein's behavior. Repellence of culture medium prevent cells from being in contact with the material surface and the inability to form focal adhesion plaques in turn results in poor cell adhesion[44, 45]. The cell adhesion on very hydrophobic surfaces could be, however, potentially improved by other chemical and/or physical modifications[46, 47].

Here, MC3T3 cells attached only on MMA(70) which had moderate contact angle of 93^{o}, while the other hybrids had contact angles of 99^{o} or higher (values in **Table 2**, and representative photos in **Figure 2**). It is difficult to conclude the exact contact angle cut-off point for cell attachment as the interactions between cells and hybrids are a much more complex issue. Previous studies have suggested that differences in cell behaviors, indirectly related to different hydrophilicity/hydrophobicity, could be mediated by not only the quantity but also the composition and conformation of the adsorbed protein[48, 49]. The exact mechanism involved between cells and hybrids developed in this study, however, require further analysis in future studies. Other surface properties that can contribute to cell response to a material include surface charge and topography. All three copolymers synthesized in this study contained neutral $–CH_3$ group. There are previous evidences of robust cell attachment on regions of methyl-modified surfaces[50, 51]. The poor cell attachment on BMA(70) and MA(70), however, was also likely to be due to low stiffness (**Table 2**). Studies have reported that cell attachment, proliferation and subsequent function are all dependent on the stiffness of the substrate, in relation to the stiffness of the native tissue where the cells derived from[52, 53]. All disks used for the cell adhesion study were polished in a similar way, therefore it was unlikely that surface topography influenced the differences in cell attachment seen in **Figure 2**. The poor cell attachment can be improved by pre-conditioning softer hybrids, as this is an established method that has been previously used to enhance hydrophilicity of poly-ε-caprolactone, FDA approved polymer, for cell attachment[54].

3. Conclusions

Hybrids of silica and different methacrylate based copolymers were synthesized to compare physical properties for bone substitutes. Hybrids made with p(BMA-*co*-TMSPMA) and p(MA-*co*-TMSPA) copolymers had lower stiffness and fracture stress compared to the p(MMA-*co*-TMSPMA) hybrid (all with MM of 15 kg/mol). However, combination of the low stiffness and

increase in hydrophobicity inhibited osteoprogenitor cell attachment. MMA based hybrids appear to be the more promising material for bone substitute compared to BMA and MA based hybrids in terms of physical and biological properties. Designing more sophisticated copolymers of MMA and BMA could enhance hydrophobicity and mechanical properties for bone substitute materials.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author

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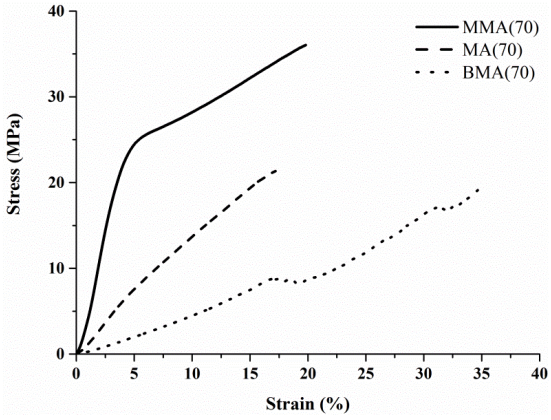

Figure 1: Representative uniaxial compression deformation trend for hybrids made with 70 wt.% organic with methacrylate based copolymers: p(MA-*co*-TMSPA), MA(70); p(BMA-*co*-TMSPMA), BMA(70); p(MMA-*co*-TMSPMA), MMA(70).

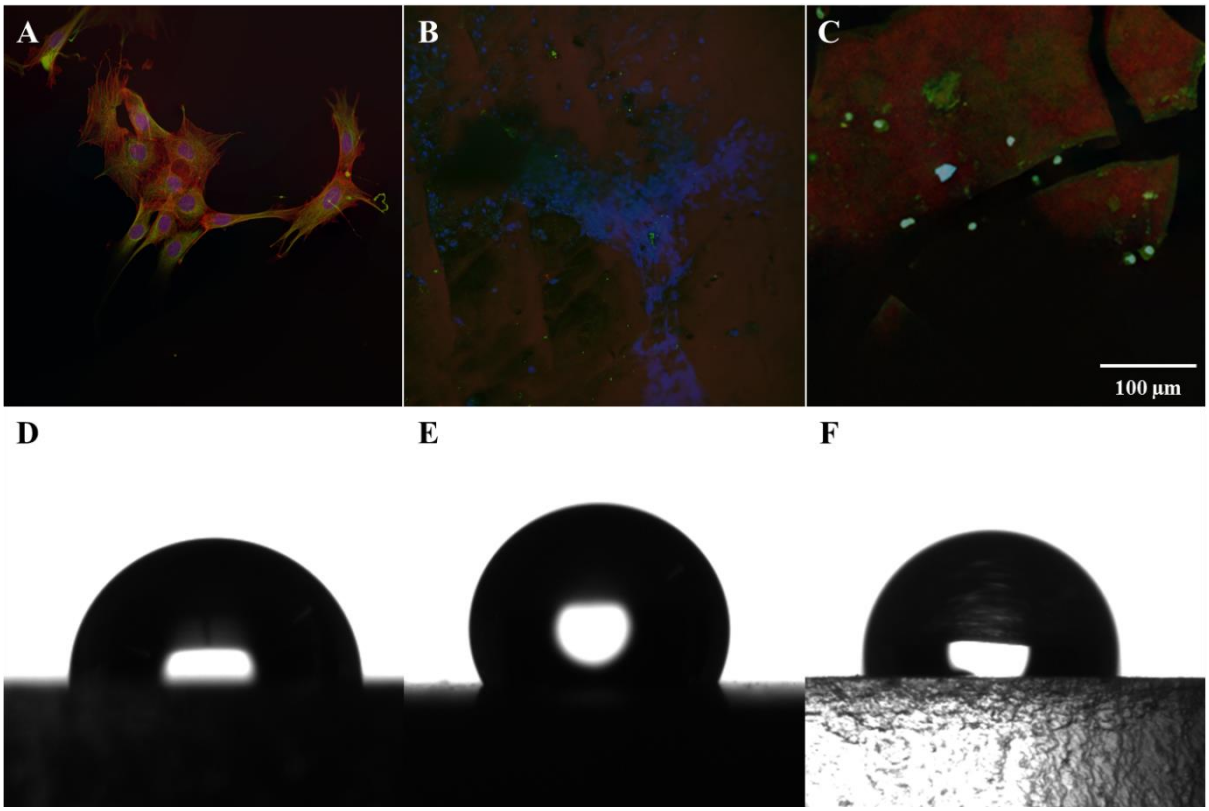

Figure 2: Confocal microscopy image of immunohistochemical stained MC3T3 cells cultured for 72 h on surfaces of hybrids made with 70 wt.% organic with methacrylate based copolymers: A) p(MMA-*co*-TMSPMA), MMA(70); B) p(BMA-*co*-TMSPMA), BMA(70); and C) p(MA-*co*-TMSPA), MA(70). The images were stacked of Vimentin immunostain (green), F-actin labelling (red), and DAPI nuclear stain (blue). Representative photo of sessile drop contact angle measurement of D) MMA(70), E) BMA(70), and F) MA(70)

Table 1: Molecular weights and dispersities (*Đ*) of the copolymers. M_n: number average molecular weight; M_p: maximum molecular weight.

	M_n[a] (kg/mol)	M_p[a] (kg/mol)	*Đ*[a]	Cross-linking agent (%mol)
P(MMA-*co*-TMSPMA)	14.5	17.7	1.12	8
P(BMA-*co*-TMSPMA)	12.8	14.6	1.11	9.3
P(MA-*co*-TMSPA)	14.1	14.9	1.10	8.7

[a)] Determined using GPC with an RI detector, which was calibrated with PMMA standards.

Table 2: Mechanical properties, residual mass, and contact angle measurement of the 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). Standard deviations are derived from the average values.

	Yield Stress (MPa)	Yield Strain (%)	*E* (GPa)	Ultimate Stress (MPa)	Ultimate Strain (%)	Residual Mass (%)	Contact Angle (deg)
MMA(70)	25 ± 0.3	4 ± 0.4	0.64 ± 0.08	33 ± 4	19 ± 2	32	93 ± 1

BMA(70)	-	-	0.06 ± 0.004	17 ± 3	33 ± 3	31	117 ± 2
MA(70)	-	-	0.12 ± 0.01	18 ± 3	16 ± 1	29	99 ± 2

Osteoblast precursor cells adhere on methyl methacrylate based hybrid, while they cannot adhere on more flexible butyl methacrylate and methyl acrylate based hybrids. Stiffness and hydrophobicity of the hybrids are critical properties for cell attachment and biomaterials design. Copolymer of methyl methacrylate and 3-(trimethoxysilyl)propyl methacrylate is the most promising polymer source of hybrids for bone substitute application.

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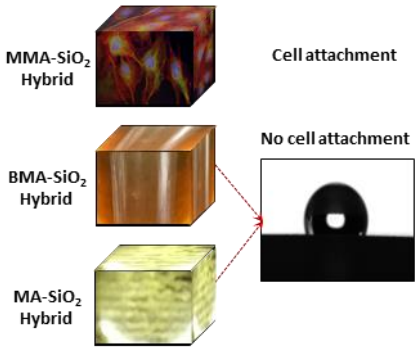
MMA-SiO2
Hybrid
Cell attachment
BMA-SiO2
Hybrid
No cell attachment
MA-SiO2
Hybrid

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