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Bioactivity in silica/poly(γ-glutamic acid) sol–gel hybrids through calcium chelation

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ABSTRACT

Bioactive glasses and inorganic/organic hybrids have great potential as biomedical implant materials. Sol–gel hybrids with interpenetrating networks of silica and biodegradable polymers can combine the bioactive properties of a glass with the toughness of a polymer. However, traditional calcium sources such as calcium nitrate and calcium chloride are unsuitable for hybrids. In this study calcium was incorporated by chelation to the polymer component. The calcium salt form of poly(γ-glutamic acid) (γCaPGA) was synthesized for use as both a calcium source and as the biodegradable toughening component of the hybrids. Hybrids of 40 wt.% γCaPGA were successfully formed and had fine scale integration of Ca and Si ions, according to secondary ion mass spectrometry imaging, indicating a homogeneous distribution of organic and inorganic components. ^{29}Si magic angle spinning nuclear magnetic resonance data demonstrated that the network connectivity was unaltered with changing polymer molecular weight, as there was no perturbation to the overall Si speciation and silica network formation. Upon immersion in simulated body fluid a hydroxycarbonate apatite surface layer formed on the hybrids within 1 week. The polymer molecular weight (M_w 30–120 kDa) affected the mechanical properties of the resulting hybrids, but all hybrids had large strains to failure, >26%, and compressive strengths, in excess of 300 MPa. The large strain to failure values showed that γCaPGA hybrids exhibited non-brittle behaviour whilst also incorporating calcium. Thus calcium incorporation by chelation to the polymer component is justified as a novel approach in hybrids for biomedical materials.

1. Introduction

Bone regeneration therapies are needed that reduce the reliance on autograft procedures, in which harvesting of the bone can cause severe pain and complications at the donor site [1]. Acellular devices, such as synthetic scaffolds that can act as temporary templates for bone regeneration, have potential, but development of a material that can fulfil all the criteria for a regenerative scaffold is a challenge [2–4]. Synthetic bioactive ceramic bone graft substitutes that can bond with bone and stimulate osteogenesis are currently used in orthopaedic surgery. Examples are synthetic calcium phosphate-based ceramics, including tricalcium phosphate, e.g. Vitoss (Orthovita, Malvern, PA), synthetic hydroxyapatite and bioactive glass powders, e.g. NovaBone® (NovaBone Products, Jacksonville, FL) and BonAlive® (BonAlive, Turku, Finland) [5–7]. However, due to their brittle nature they are limited to non-load-bearing applications [8]. One of the greatest challenges is developing a bioactive material that has the required mechanical properties and also degrades in a controlled and congruent manner.

One strategy for creating a tough, bioactive material is to combine bioactive particles into a polymer matrix to form a composite [9–11]. However, osteoblasts should attach preferentially to the bioactive phase [12], which may be masked by the polymer matrix as it is difficult to control how many bioactive particles are exposed at the surface. Also, it is difficult to match the degradation rate of the polymer matrix with that of the bioactive component, which may lead to non-congruent degradation of the composite.

Integrating organic and inorganic components at a finer scale may allow an improved cellular response, mechanical properties and closely matched degradation rates [13,14]. One technique has been to use organo-silica polymers such as polydimethylsiloxane (PDMS) [15], or another option is to form a hybrid from separate organic and inorganic starting materials. Hybrids have the potential to achieve fine scale interactions and improved properties through interpenetrating networks (IPNs) that allow the inorganic and organic species to interact at the molecular level [16]. Through the sol–gel process hybrids can be formed by incorporating a polymer into the sol, allowing a silica network to form around the polymer

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chains. Additional bonding between the components can be provided by covalent coupling, creating a Class II hybrid material [13].

Class II hybrids have been formed with organic polymers and silica. Chitosan and gelatin have been successfully reacted with 3-glycidoxypropyltrimethoxysilane (GPTMS) to form hybrid materials [17,18] and porous structures [19,20]. Poly(γ-glutamic acid) has also been functionalized with 3-aminopropyltriethoxysilane (APTES) [21]. The concentration of APTES or GPTMS dictated both the degree of covalent coupling and the silica content. To control the degree of covalent coupling between the inorganic and organic components independently of the organic/inorganic ratio tetraethyl orthosilicate (TEOS) was added as the silica precursor. This allowed GPTMS to act solely as a coupling agent in gelatin systems [16].

While poly(γ-glutamic acid) has been shown to nucleate apatite in simulated body fluid (SBF) [22], the release of calcium ions from a hybrid should enhance the rate of bone bonding. Calcium ions and soluble silica should be released from hybrids to stimulate new bone growth [8]. However, it is not trivial to effectively incorporate calcium into a hybrid [23]. The traditional method for incorporation of calcium into the sol–gel process is to use calcium nitrate ($Ca(NO_3)_2$) as the calcium precursor [24]. The nitrate by-products are toxic to the body so sol–gel glasses are heated to a minimum stabilization temperature of 600 °C to remove the nitrates [23,25]. This temperature will destroy polymer-containing materials, thus calcium nitrate is not a viable option for hybrid materials.

Calcium chloride has been used as a calcium source in several hybrids, including chitosan/GPTMS/silica [26], polyvinyl alcohol/GPTMS/silica [27], γPGA/GPTMS/silica [28] and γPGA/APTES [21]. However, the calcium was not incorporated into the silica network and recrystallized on the surface during drying of the hybrids [23,28]. The calcium was therefore rapidly released from the hybrid materials upon immersion [28]. In other hybrids rapid calcium release led to reduced cell attachment [26], probably due to high dose exposure of calcium causing a pH burst. Calcium alkoxides are another calcium source for the sol–gel process [23]. This has been introduced into a poly(L-lactic acid)/silica system, but leads to very short gelling times [29].

γPGA is a polypeptide which has a secondary amide in the polymer backbone and is degradable by enzyme action [30]. A pendant carboxylic acid group in the repeating unit is available for functionalization. γPGA is a naturally occurring biopolymer that is best known as the health food Natto in Japan [31]. It is also produced by a fermentation reaction [32–34] and the polymer has been classified as "generally regarded as safe" by the US Food and Drug Administration [35]. Previous work by Poologasundarampillai et al. used the free acid form of poly(γ-glutamic acid), which is only soluble in the organic solvent dimethylsulfoxide (DMSO) [28], however, the salt forms (e.g. Ca, Na and K salt forms) of the polymer are soluble in water [28].

This work examines the calcium salt form of poly(γ-glutamic acid) (γCaPGA) as the organic component of the hybrid and as a source for calcium incorporation into the hybrid at a relatively low temperature whilst keeping it available for bioactivity. At low temperatures, rather than trying to incorporate the calcium directly in the silica network itself the calcium is coordinated to the polymer. This method of calcium incorporation may also provide further strengthening of the material by forming ionic crosslinking between the polymer chains.

2. Materials and methods

2.1. Materials

Poly(γ-glutamic acid) in the free acid form (γHPGA) was purchased from Natto Biosciences (cosmetic grade). All other chemicals were purchased from Sigma–Aldrich Co. Ltd. (Gillingham, Dorset, UK).

2.2. Preparation of γCaPGA

The first step was to synthesize the calcium salt form of γPGA from the free acid (γHPGA, M_w 120 kDa). γHPGA was mixed with deionized water to make a 20 wt.% slurry. $Ca(OH)_2$ was added to 45 mol.% and mixed for 1 h. γCaPGA was then ready to be incorporated into the sol–gel process and hybrid synthesis.

2.3. Polymer chain scission

γHPGA was purchased from Natto Biosciences, but it was not available in a variety of M_w. A chain scission reaction was carried out to obtain γCaPGA with a range of molecular weights that were used as the starting organic component of the hybrid materials. An alkaline pH and elevated temperatures were used to reduce the molecular weight of γCaPGA. A slurry was made of 50 g of γHPGA in 140 ml deionized water at 90 °C, to which calcium hydroxide was added to 45 mol.%. After mixing for 1 h at 90 °C, 1.86 g of sodium hydroxide was dissolved in 10 ml of deionized water containing 0.05 wt.% sodium azide (bacteriocide). At 16, 20, 24, 42 and 48 h a portion of the solution was removed and the pH was immediately reduced to pH 4.8 with 50 vol.% HNO_3. The unreacted polymer and chain scission products were submitted for M_w analysis by conventional gel permeation chromatography (GPC) by Smithers Rapra Technology Ltd (Shawbury, UK). Molecular weight distributions are shown in Fig. 2a. The M_w of the polymers decreased with reaction time (Fig. 2b) and were found to be 120, 100, 80, 60, 40 and 30 kDa. These γCaPGA solutions were used directly in the hybrid synthesis process (Fig. 1), to which GPTMS was added.

2.4. Synthesis of γCaPGA/silica hybrids

Class II hybrids using the calcium salt form of γPGA and silica (40 wt.% γPGA, 60 wt.% SiO_2) were synthesized with varying M_w of γCaPGA (Fig. 1). The final calcium content of the hybrids was 5 wt.% for a final composition of 44 wt.% γCaPGA and 56 wt.% SiO_2. GPTMS was added so that every tenth repeating unit of γPGA was functionalized and the solution mixed for 4 h. This degree of covalent coupling was chosen to give the best properties from initial testing (see Appendix B), as the amount of GPTMS was found to affect both the dissolution and mechanical properties of the hybrids. Meanwhile, the silica sol was prepared. TEOS was hydrolysed with an R ratio (TEOS:water) of 26 in 0.016 M nitric acid for 30 min

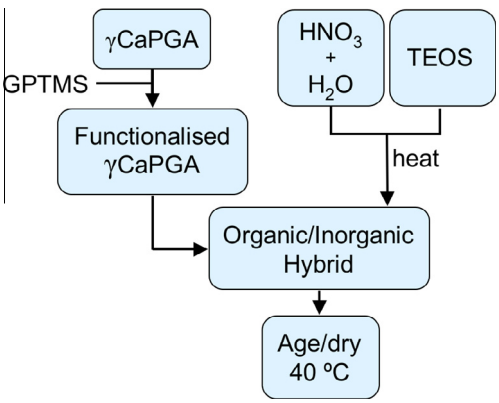

Fig. 1. Schematic for the formation of Class II hybrids of γCaPGA and silica through the sol–gel method.

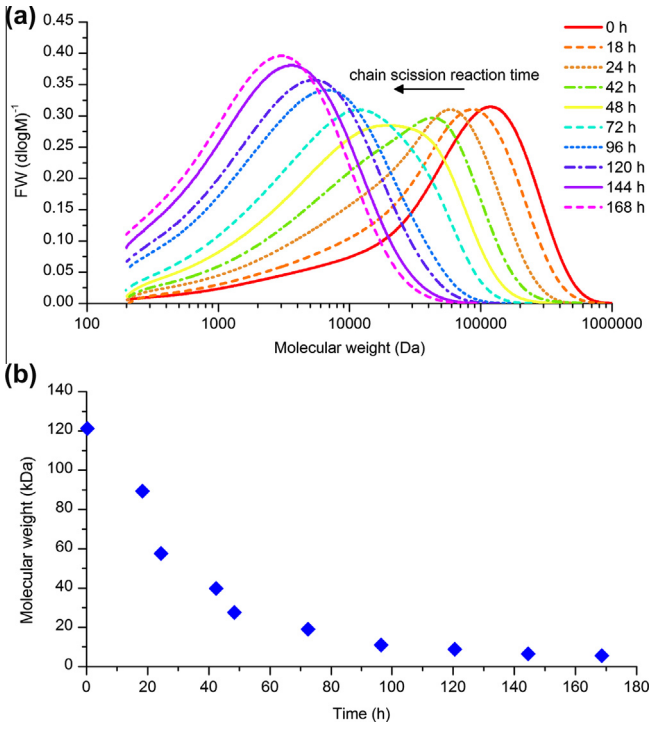

Fig. 2. (a) Molecular weight distributions of chain scission reaction products as determined by conventional GPC and (b) average M_w as a function of reaction time, courtesy of Smithers Rapra Technology Ltd. (Shawbury, UK).

until clear. The small quantities of nitric acid were used to catalyse the hydrolysis reaction. γCaPGA was not soluble in hydrolysed TEOS solution as γPGA is very insoluble in ethanol. Since ethanol was produced during the hydrolysis reaction of TEOS this ethanol had to be removed in order to combine the polymer with the inorganic sol. As ethanol was the component with the lowest boiling point it was removed from the hydrolysed TEOS by evaporation. The sol was heated at 70 °C (boiling point of ethanol) for approximately 1 h until 40% of the original aqueous volume had evaporated. The lost volume was replaced with deionized water to prevent premature gelation and to maintain the *R* ratio. The silica solution was cooled to room temperature and added to the functionalized polymer solution. The hybrid sol was placed in sealed polystyrene moulds and aged for 3 days at 40 °C, followed by drying open to the atmosphere at 40 °C.

2.5. Characterization methodology

2.5.1. Immersion in SBF solution

Apatite formation was tested by immersing the hybrids in SBF and subsequently examining the dissolution profile, along with the formation of hydroxycarbonate apatite (HCA) on the surface of the material.

SBF was formed following the method described by Kokubo and Takadama [36]. Each sample was measured in triplicate, where a constant ratio of sample mass to SBF volume was used with agitation, as defined by Jones et al. [37]: 0.15 g of hybrid was immersed in 100 ml of SBF and mixed at 120 r.p.m. in an orbital shaker held at 37 °C. Samples of 1 ml were removed at 1, 2, 4, 24, 72, 168 and 336 h and replaced by 1 ml of fresh SBF. The final solids were collected using filter paper (particle retention 5–13 μm), washed with acetone and dried overnight at 40 °C.

The solids were then examined by X-ray diffraction (XRD) and the solutions analysed by inductively coupled plasma spectroscopy (ICP).

2.5.2. Immersion in Tris buffer solution

Tris(hydroxymethyl)aminomethane (Tris) buffer solution is a physiological pH solution which was used to measure dissolution in a solution that is free of salts (including Ca^{2+}) [38]. The pH of 0.062 M Tris buffer was adjusted to pH 7.3 at 37 °C with 2 N HCl [39]. 75 mg of hybrid was immersed in 50 ml of TRIS buffer solution and mixed at 120 r.p.m. in an orbital shaker held at 37 °C for 8, 24, 72, 168, 336 and 672 h. The solutions were passed through a filter paper (particle retention 5–13 μm) and the solids washed with acetone and dried overnight at 40 °C. Ca and Si concentrations in solution were determined by ICP with optical emission spectrometry (OES) and the γPGA concentration was measured using the bicinchoninic acid (BCA) assay.

2.5.3. BCA assay

A modified protocol for the Pierce® BCA Protein Assay Kit (Thermo Scientific) was used to determine the γPGA release profile. Calibration solutions were made using γCaPGA in Tris buffer solution at concentrations of 0, 50, 100, 200, 300, 500 and 750 μg ml^{-1} γCaPGA. 150 μl of the filtered supernatant solutions from the TRIS immersion test were placed into each well of a 96-well microplate. This was followed by 150 μl of working reagent (Reagent A:Reagent B 1:20) and then mixed. The well plate was sealed and placed in a water bath at 60 °C for 30 min. It was removed, cooled to room temperature and the absorbance was immediately measured using a Molecular Devices Spectra Max M5 plate reader at 562 nm. The calibration standards were measured in triplicate and repeated for each analysis.

2.5.4. Composition analysis

Lithium metaborate fusion was carried out on the hybrid samples, with 0.1 g of finely ground sample for analysis. The hybrid sample and 0.5 g of lithium metaborate were placed in the crucible, and heated to 1400 °C for 30 min. The melt was dissolved in 100 ml of 10% analytical grade HNO_3. The solution was analysed by ICP.

2.5.5. ICP

ICP measurements were taken with a Thermo Scientific iCAP 6300 Duo ICP-OES analyser with an auto sampler. Samples eachwere diluted by a factor of 10 with analytical grade 2 M HNO_3. Mixed standards of silicon, phosphorus and calcium were prepared at 0, 2, 5 and 20 μg ml^{-1} for the calibration curve. Silicon and phosphorus were measured in the axial direction of the plasma flame, whereas calcium was measured in the radial direction.

2.5.6. Scanning electron microscopy (SEM)

SEM was performed in a Leo 1525 with Gemini column with a gun voltage of 5 keV upon hybrid fracture surfaces coated with chromium.

2.5.7. ^{29}Si magic angle spinning nuclear magnetic resonance (MAS NMR) spectroscopy

^{29}Si MAS NMR data were measured at ambient temperatures on a Varian InfinityPlus 300 MHz (7.05 T) spectrometer operating at a Larmor frequency of 59.62 MHz. All ^{29}Si MAS NMR experiments were enabled using a Bruker 7 mm MAS probe spinning at 5 kHz. A 5.0 μs (45° tip angle) pulse and 4 min recycle delay were used to facilitate quantitative study of the Si speciation by ensuring the ^{29}Si nuclear spins were fully relaxed. All ^{29}Si MAS NMR chemical shifts were referenced to kaolinite as a secondary solid reference which was located at δ −92 ppm relative to the primary IUPAC reference TMS at δ 0 ppm. The proportion of Q and T species in each sample was determined by fitting each resonance in the ^{29}Si MAS NMR data using Gaussian peaks in the DMFIT simulation program [40,41].

2.5.8. Time of flight secondary ion mass spectrometry (TOF-SIMS)

SIMS analysis was carried out with a TOF-SIMS 5 time of flight instrument (ION-TOF GMbH, Münster, Germany). Hybrid samples were prepared by grinding and polishing to 1 μm. The sample was sputtered for 1 s per scan with a $C60^+$ beam at 10 keV (1.9 nA) to form a 250 μm crater, after which an image was taken with a 25 keV Bi^+ analysis beam (1.24 pA) at a resolution of 5 μm. A 100 × 100 μm area in the crater centre was analysed to provide an image of 256 × 256 pixels for a total of 410 scans. A low energy 20 eV pulsed electron flood gun was used for charge compensation.

2.5.9. XRD

XRD was performed in a PANalytical X'Pert Pro MPD with an X'Celerator detector. The radiation used was Ni-filtered CuK_α at 40 kV, 40 mA. The diffraction was measured between $2\theta = 5°$ and 70°, with a 0.03° step size and a counting time of 25 ms for each step with a secondary graphite crystal monochromater. The sample was ground and placed on an amorphous silicon disk.

2.5.10. Compression testing

Cylindrical monoliths (diameter 7 mm, height 2 mm) were compressed using a Zwick 1474 fitted with a 100 kN load cell at 0.5 mm min^{-1}. The Young's modulus, maximum strength and strain to failure were determined. This test was repeated eight times and the mean and standard deviation were reported.

3. Results and discussion

Molecular weight affects many of the properties of polymers, including strength and dissolution [42]. Smaller polymers are preferred for use in implanted medical devices because molecules larger than 50 kDa should not be released into the body as they have a limited ability to pass into and out of the vascular system [43]. Non-degradable polymers with molecular weights greater than 30 kDa may restrict filtration through the kidneys [43]. Thus a range of molecular weights of γCaPGA from 30 to 120 kDa were used in hybrid synthesis, as this represents molecular weights above and within this target range.

3.1. Uniformity analysis

Hybrids of γCaPGA and silica were successfully formed through the sol–gel process using γCaPGA with M_w of 30 to 120 kDa. All hybrids were transparent (Fig. 3), amorphous and shrank in volume during drying by 85–90%. The composition was confirmed by lithium metaborate fusion, which found the calcium content to be 4.4 ± 0.8 wt.%, compared to the theoretical value of 5 wt.%.

The fine scale integration of the organic and inorganic components was confirmed by TOF-SIMS for hybrids made with 40 wt.% 120 kDa γCaPGA (Fig. 4). Both the silicon and calcium concentration maps showed fairly uniform ion distributions laterally for all the hybrids made with polymers of 30–120 kDa (data not shown). As calcium was coordinated to the polymer in the hybrids the calcium distribution also indicated the distribution of the polymer in the hybrid material. Since there are no silica- or polymer-rich domains in the hybrids these were true hybrid materials with the components present as interpenetrating networks.

From an examination of the ion concentration with depth there was a clear, defined surface layer, indicated by the initial high sodium content (Fig. 4b). Once this surface layer was removed (~130 s) the Si:Ca ratio was constant, and a strong sodium signal was also detected throughout the depth of the hybrids. This sodium contamination could be due to handling of the samples, polishing the samples to a smooth surface or preparation in the open atmosphere. Signal intensity does not convert directly to ion concentration as the detector registers a stronger response to certain ions. Sodium was an expected background contaminant in SIMS analysis due to the high sensitivity of SIMS to the sodium ion. The constant Si:Ca ratio indicates a uniform distribution and fine scale integration of polymer and silica throughout the bulk of the hybrid.

3.2. NMR of the silicate network

γCaPGA hybrids were also examined using solid state ^{29}Si MAS NMR, which indicated that M_w had no effect on the structure of the silica network and the formation of bridging oxygen bonds (Fig. 5 and Table 1). A Q^n species is defined as a Si–O tetrahedral (4 coordinate) environment with n bridging oxygen bonds to other Si tetrahedra in the glass network [44]. In contrast, T^n species have a silicon atom bonded to a carbon chain and n bridging oxygen bonds. The proportion of Q and T species in each sample was determined by fitting using a series of Gaussian peaks in the DMFIT simulation program [41]. From the intensities extracted for each species the distribution is shown to be very similar within the error (Table 1). The hybrids had a range of Q^n species (Q^4, Q^3, Q^2), which formed from condensation of the hydrolysed TEOS. The network modifier is likely to be H^+, as the other cations were coordinated to the polymer. As the network connectivity was unchanged with different polymer M_w, changes in M_w did not disrupt silica network formation. It is interesting to compare these observations with

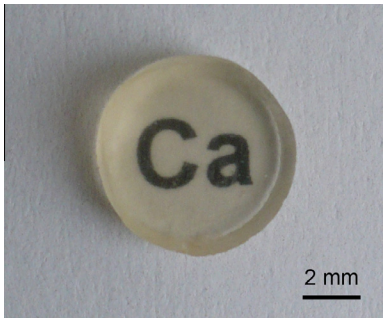

Fig. 3. Hybrid of 40 wt.% 120 kDa γCaPGA, where every tenth repeating unit of γPGA was functionalized with GPTMS.

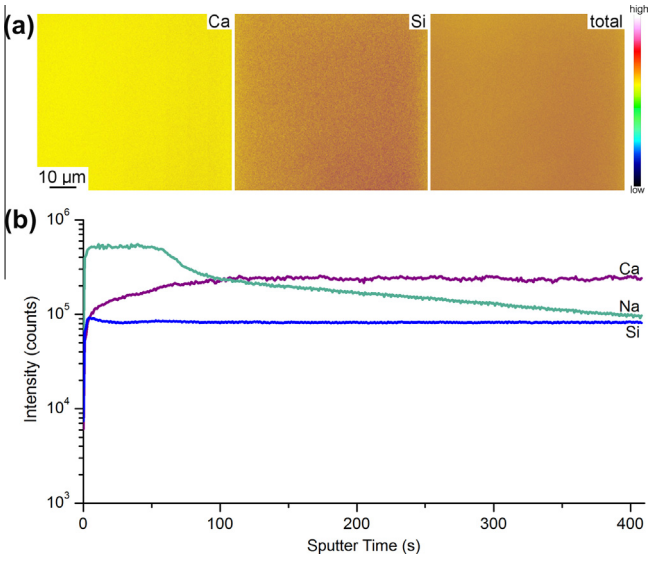

Fig. 4. (a) TOF-SIMS imaging of calcium and silicon distributions and (b) SIMS concentration depth profiles of Na, Ca and Si distributions (410 scans over a 100 × 100 μm area) for a hybrid of 40 wt.% made with 120 kDa γCaPGA.

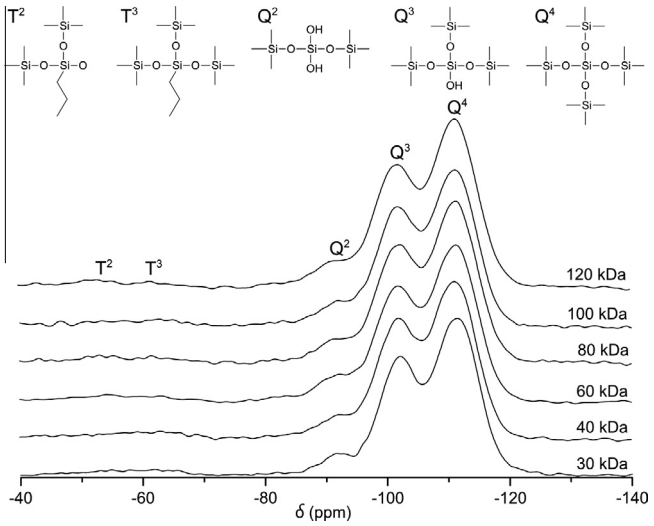

Fig. 5. ^{29}Si MAS NMR showing the silica speciation for hybrids with 40 wt.% γCaPGA of varying M_w.

those of the network connectivity previously observed by ^{29}Si MAS NMR of sol–gel produced bioactive calcium silicates and, in particular, Class II γPGA/silica hybrids in which the organic/inorganic content [28] was the same as used here. In their work Poologasundarampillai et al. reported that the T^n species detected were, as expected, directly correlated with the GPTMS content. In terms of the GPTMS content used here, one GPTMS molecule to every 10 repeating units of γCaPGA, this was expected to have a total T^n intensity of <4%, which was exactly what was observed here. The Q^n distribution was also broadly in agreement with that observed in previous studies, indicating that the silica network, although intimately mixed with the polymer here, is largely similar in its atomic scale connectivity with previously produced hybrids.

As all hybrids were found to be uniform and homogeneous over the entire range of molecular weights, they were further tested by immersion in Tris and SBF buffer solutions.

3.3. Immersion in Tris buffer solution

Hybrids were subjected to a dissolution study in Tris buffer solution to determine the effect of molecular weight on hybrid dissolution. Tris was used as it was calcium free, so the release rate of calcium ions into solution could be observed. Upon immersion in Tris buffer solution all of the hybrids (γCaPGA M_w 30–120 kDa) had similar positive slope dissolution curves, with the majority of the ion release occurring in the first 3 days (Fig. 6a). Encouragingly, calcium was released from the hybrids, indicating that the chelation was reversible. Ion release was not proportional to molecular weight, as the hybrids synthesized with mid-range molecular weight γCaPGA of 60 and 80 kDa had the highest total calcium release, at 54 and 56 μg ml^{-1} after 4 weeks. The polymer release profiles were similar for all of the hybrids up to 3 days, with approximately 35% (190 μg ml^{-1}) of the γPGA dissolving in 8 h (Fig. 6b). After 1 week the concentration of γPGA released into solution remained at 204 μg ml^{-1} for hybrids made from γPGA with M_w of 100 and 120 kDa. For all other hybrids the amount of polymer dissolved increased by ~300 μg ml^{-1}. Hybrids made with 100 and 120 kDa polymer also released soluble silica more slowly than the other hybrids (Fig. 6a). All of the other hybrids had reached their maximum silicon release of ~75 μg ml^{-1} by 3 days, whereas hybrids made with 100 and 120 kDa polymer had only released 68% and 61% of this value by this time point. Thus hybrids synthesized from 120 and 100 kDa polymers had the slowest dissolution in Tris, while hybrids made with 80 and 60 kDa polymer had the fastest.

Dissolution of the hybrid upon immersion in Tris buffer solution was also monitored by SEM (Fig. 7). After 2 weeks the appearance of the hybrid fracture surface changed, becoming rougher, so that it looked like small closely packed spheres (Fig. 7c and e). The texture resembled a pure inorganic silica/calcium sol–gel glass, which had been synthesized and dried under similar conditions to the hybrid (Fig. 7f) [45]. By 3 days this change in appearance had occurred in all hybrids, except that made with 120 kDa γCaPGA, in which not all regions had changed (Fig. 7b). This suggests that the polymer was dissolving preferentially and the exposed silica network was observed by SEM. For use in a scaffold for bone tissue engineering the components must degrade slowly and at a similar rate. The rate of dissolution of these γCaPGA hybrids must be slowed before they can be used clinically.

The hybrid made with the largest molecular weight (120 kDa) was the most stable in Tris buffer solution; it had the lowest total γPGA release, the slowest soluble silica release and one of the slowest Ca release rates. This was in contrast to the behaviour of the hybrids produced with mid-range M_w polymer of 80 and 60 kDa, which had the most γPGA release and one of the highest total Si and Ca ion releases. Thus decreasing the molecular weight did not cause a corresponding linear increase in mass loss in Tris.

3.4. Bioactivity testing in SBF solution

SBF has an ionic content similar to blood plasma as it contains salts in similar concentrations. Tris buffer solution does not contain metal ions. Immersion studies in Tris buffer allow the examination of dissolution and ion release from the material (e.g. Ca^{2+}) into a buffer solution without interference by ions in the solution. SBF is used to approximate how a solution containing physiological ion concentrations will interact with the material.

The effect of M_w on apatite deposition on the hybrids in SBF solution was examined (Fig. 8). Most of the hybrids followed similar concentration profiles with soluble silica release, phosphorus concentration decrease and an initial calcium release followed by a decrease in calcium concentration. Of particular note, the hybrid synthesized with polymer of the lowest M_w (30 kDa) had the slow-

Table 1
Silicon speciation obtained by ^{29}Si MAS NMR (relative intensity *I*) for hybrids made with 40 wt.% γCaPGA of varying M_w where average errors are ±2 ppm for the chemical shift (δ) and ±1% for *I*.

M_w (kDa)	T^2		T^3		Q^2		Q^3		Q^4	
	δ (ppm)	I (%)	δ (ppm)	I (%)	δ (ppm)	I (%)	δ (ppm)	I (%)	δ (ppm)	I (%)
120	−52.2	2	−62.0	1	−92.2	9	−101.5	33	−111.3	55
100	−55.1	2	−63.5	1	−92.4	8	−101.6	35	−111.0	54
80	−53.1	2	−62.6	1	−91.8	7	−101.4	35	−111.2	55
60	−53.3	3	−62.0	1	−92.6	11	−101.5	31	−111.1	54
40	−56.2	2	−64.1	2	−92.5	9	−101.6	33	−111.0	54
30	−55.9	2	−63.6	1	−92.0	6	−101.6	35	−111.3	56

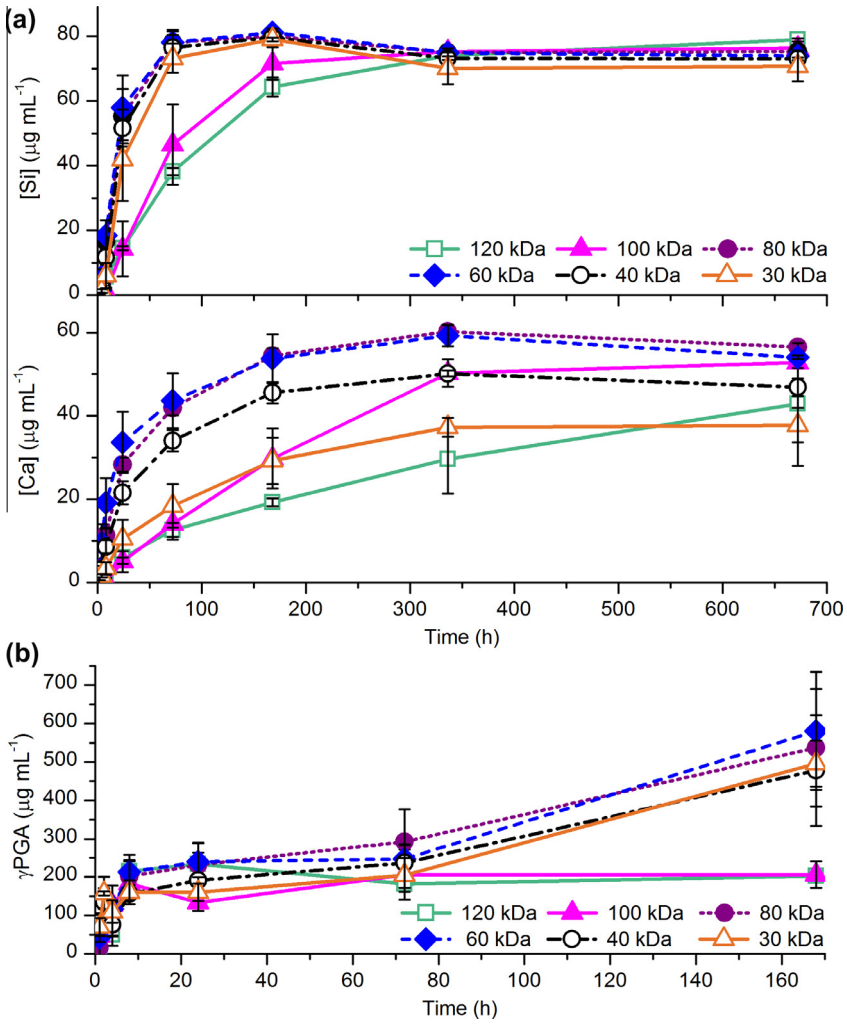

Fig. 6. (a) Ca and Si profile in Tris buffer solution for up to 4 weeks and (b) polymer release profile for up to 1 week for hybrids of 40 wt.% γCaPGA of varying M_w.

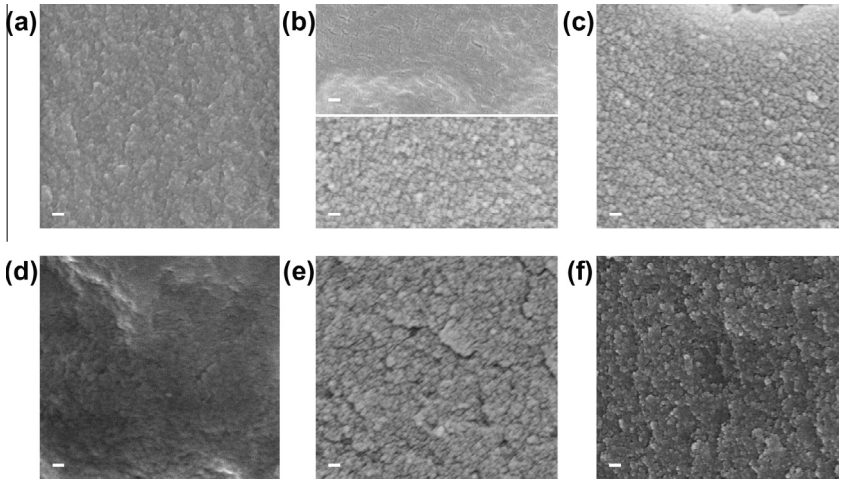

Fig. 7. SEM images of 40 wt.% 120 kDa γCaPGA hybrid (a) before immersion, (b) after 3 days (2 regions) and (c) 2 weeks immersion in Tris buffer solution. SEM images of 40 wt.% 60 kDa γCaPGA hybrid (d) before immersion and (e) after 2 weeks immersion in Tris buffer solution. (f) 70S30C glass dried at 40 °C. Scale bar 100 nm.

est kinetics of silicon release, whereas the hybrid formed from the polymer with the highest M_w (120 kDa) had the fastest kinetics. In the calcium profile, the hybrid containing the polymer with the lowest M_w (30 kDa) had the lowest final calcium concentration of 88 μg ml^{-1} at 336 h and the hybrid made with the highest M_w polymer (120 kDa) has the highest calcium concentration of 107 μg ml^{-1} at 336 h. This could be due to finer integration between the inorganic and organic components in the interpenetrat-

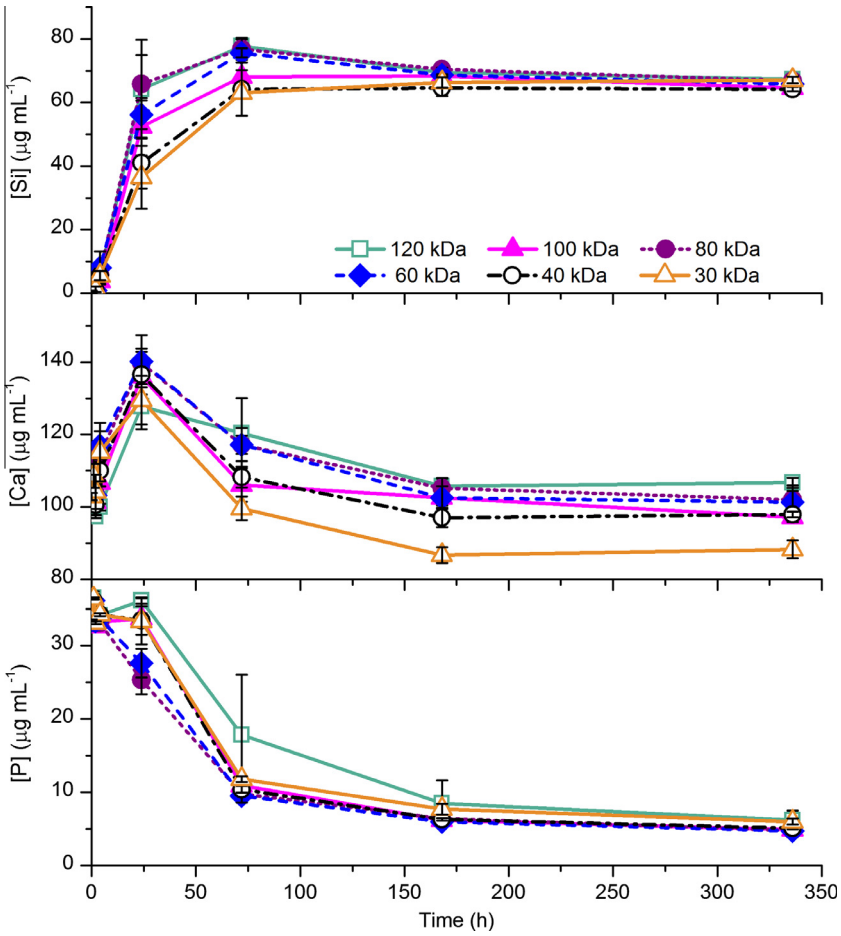

Fig. 8. Si, Ca and P concentration profiles in SBF solution for up to 2 weeks for hybrids of 40 wt.% γCaPGA of varying M_w.

ing network as M_w decreased. As the degree of coupling remained constant (every tenth repeating unit of the polymer), the total number of coupling units on each individual polymer chain was greater on the larger molecular weight polymers. This could lead to a more rigid structure that was more likely to fracture due to the stress of water absorption, and thus led to faster silicon release. However, this trend differed from the dissolution behaviour in Tris buffer solution, where the hybrids made from γPGA with molecular weights of 60 and 80 kDa had the fastest silicon release.

All hybrids produced a reduction in phosphorus concentration from 33 μg ml^{-1} in the initial SBF to ~8 μg ml^{-1} after 1 week immersion in SBF. When combined with a corresponding decrease in the calcium concentration, the decrease in phosphorus concentration indicated precipitation of calcium phosphate. HCA formation was confirmed by XRD and SEM (Fig. 9). This indicates that the hybrids might be bioactive, even though they only contained 5 wt.% calcium, which was much lower than conventional bioactive glasses (17.5 wt.% Ca in the original Bioglass®).

3.5. Compression testing

The fine scale integration of interpenetrating networks of silica and the polymer at the molecular level was expected to result in improved mechanical properties over bioactive glasses. Composite materials tend to fail at the interface between components. Thus removing this interface and having covalent coupling between the components should lead to improved mechanical properties. All stress–strain curves for the hybrids had a linear elastic region followed by slight plastic deformation before failure (Fig. 10a), reaching compressive strengths greater than 300 MPa and a strain to failure of >26% (Fig. 10c and d). This is a significant improvement over sol–gel glasses, which are quite brittle with a strain to failure of 4% and compressive strength of 66 MPa (70S30C sintered to 800 °C). When comparing the stress–strain curves of a hybrid and sol–gel glass (Fig. 10a) there was a difference in the shapes of the curves as well as the ultimate stress and strain values. The hybrid had a gradually increasing slope at low strain values and a gradual decrease in slope just before failure, whereas the sol–

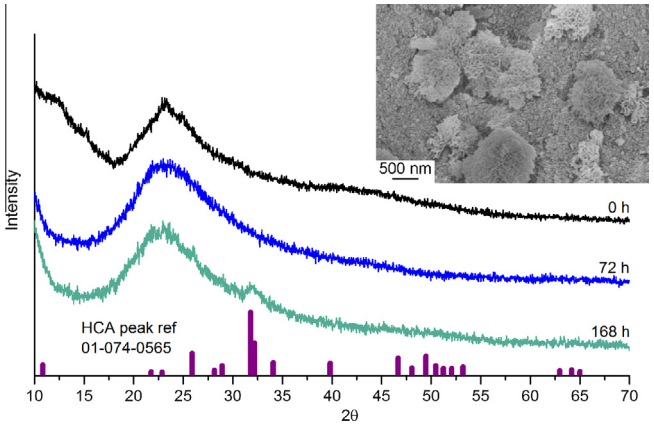

Fig. 9. XRD diffraction pattern for immersion in SBF solution for 0, 72 and 168 h. (Inset) SEM image at 50,000× magnification after 2 weeks in SBF for a hybrid of 40 wt.% 60 kDa γCaPGA.

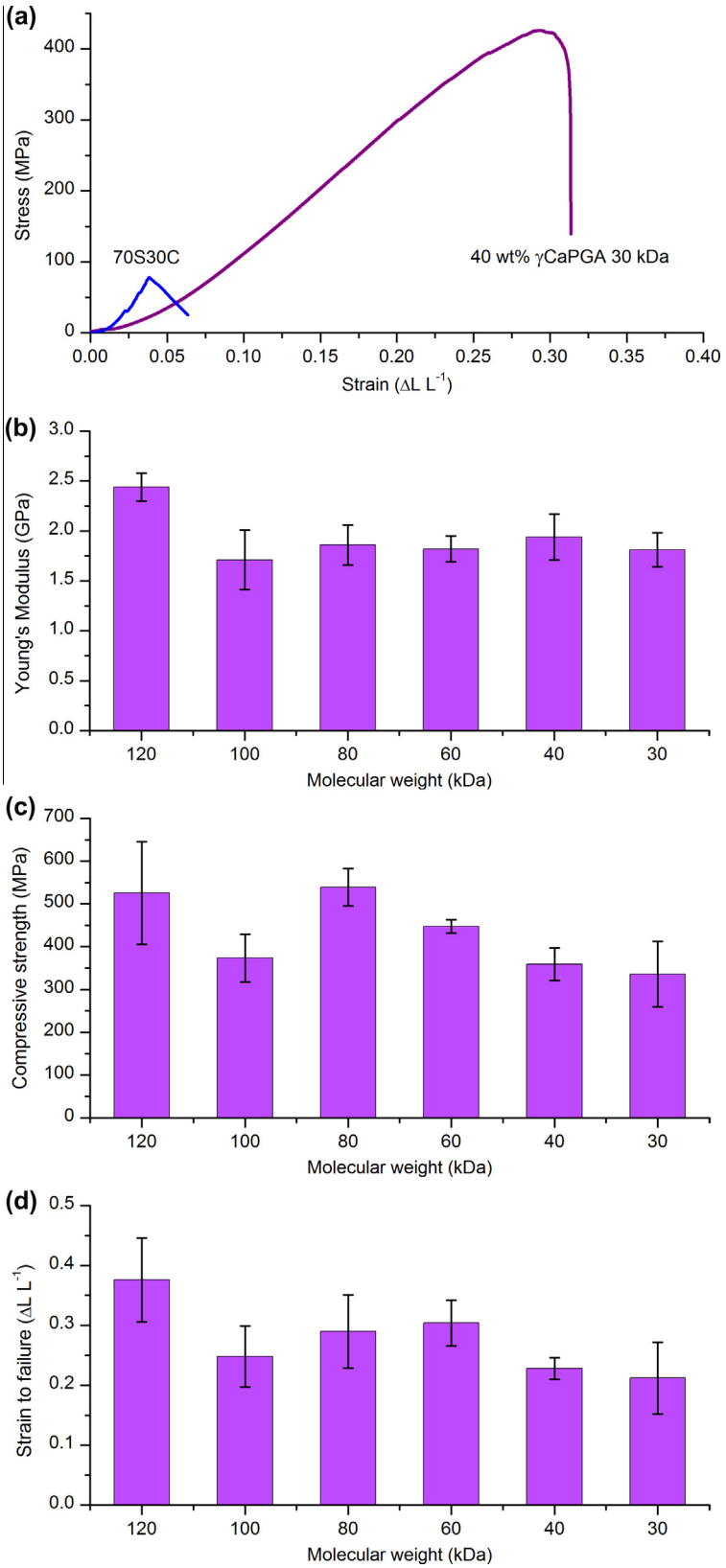

Fig. 10. Compression testing for hybrids of 40 wt.% γCaPGA of varying M_w. (a) Sample stress–strain curve for 30 kDa γPGA hybrid and a sample stress–strain curve for a 70 mol.% SiO_2, 30 mol.% CaO glass (70S30C). (b) Young's modulus, (c) maximum compressive strength and (d) strain to failure.

gel glass only underwent linear elastic behaviour before failure. This deformation of the hybrids showed that incorporation of γPGA into the sol–gel process had eliminated the brittle nature of the sol–gel glass.

The maximum compressive strength of the γCaPGA hybrids was 540 MPa for a hybrid synthesized with γCaPGA of 80 kDa. This was a greater compressive strength than that of cortical bone at 190–209 MPa, bearing in mind that cortical bone does contain some pores [46]. This same hybrid had a Young's modulus of 1.9 GPa, which was an order of magnitude lower than that of cortical bone at 17.4 GPa [47]. The compression testing in Fig. 10 was carried out on hybrid monoliths, not on porous scaffolds. However, in bone tissue engineering applications a scaffold should be porous with interconnects large enough for vascularisation. The compressive strength will decrease once the hybrid materials have been formed into a porous scaffold.

From examining the mechanical properties of hybrids made from polymers with varying molecular weights it could be seen that the strength of the hybrids generally increased as molecular weight increased (Fig. 10c). This left the hybrids made from 100 kDa γPGA as an erroneous data point. To confirm this, the hybrid of 40 wt.% γCaPGA made with the 100 kDa polymer was synthesized again and subjected to compression testing. The repeated test yielded the same mechanical properties, as they were equivalent within error (maximum compressive strength 377 ± 57 vs. 371 ± 59 MPa), which confirmed this as a true value and not simply synthesis error. The hybrid was synthesized with the 120 kDa polymer, as received from Natto Biosciences, but all of the lower molecular weight polymers (30–100 kDa) were the products of a chain scission reaction. This suggests that there was something inherent in the chain scission process that has adversely affected the mechanical properties of the resulting hybrids. Upon comparing the hybrids made with polymer from the chain scission reaction, those made with the 80 kDa polymer had the best (statistically significant at $p < 0.05$) mechanical properties. A lower M_w may encourage finer scale integration of the components in the hybrid, but this was counterbalanced by the lower strength of the polymer as the M_w decreased. However, the hybrids made with the 80 and 60 kDa polymers had the fastest dissolution in Tris and SBF. It is important to balance the polymer strength and integration.

For all hybrids the strain to failure was >26%, which is a significant improvement over sol–gel glasses and desirable for a calcium-containing material suitable for bone tissue engineering. However, the stability in solution must be increased in order for the γCaPGA hybrids to be used in bone tissue engineering. This work shows that calcium chelating polymers can be successfully incorporated into the sol–gel process as a calcium source to encourage bioactive behaviour.

4. Conclusions

Calcium salt γPGA was used as a calcium source and a glass toughening agent. Hybrids of 40 wt.% γCaPGA were successfully formed and found to be transparent and homogeneous. There was a uniform distribution of Ca and Si ions throughout the hybrids, as measured by SIMS, which indicated a uniform distribution of the organic and inorganic components. The successful incorporation of calcium aided the formation of HCA within 1 week in SBF. In Tris buffer solution γPGA was found to dissolve preferentially compared with silica for hybrids made with all M_w polymer. In compression the hybrids of γCaPGA all had large strains to failure (>26%) (γPGA M_w 30–120 kDa), with a maximum compressive strength of 540 MPa and a strain to failure at 40% for the hybrids synthesized with 80 kDa γPGA. This showed that γCaPGA sol-gel hybrids are of particular interest for bone tissue engineering as have non-brittle behaviour and encapsulate calcium.

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Appendix A. Figures with essential colour discrimination

Certain figures in this article, particularly Figs. 1–4, 6 and 8–10 are difficult to interpret in black and white. The full colour images can be found in the on-line version, at http://dx.doi.org/10.1016/j.actbio.2013.04.037.

Appendix B. Supplementary data

The amount of covalent coupling was found to affect both the dissolution and mechanical properties of the silica/γCaPGA hybrids (Supplementary figures 1-3). As the amount of covalent coupling increased, from no coupling (no GPTMS) to coupling at every tenth repeating unit of the polymer (10 EC), the rate of dissolution of silica, calcium and γPGA decreased. As covalent coupling was increased further, to bond at every other repeating unit of the polymer (2 EC), the rate of ion and polymer release increased. Thus covalent coupling at every tenth repeating unit of polymer was chosen to produce hybrids with the optimum mechanical and dissolution properties. Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actbio.2013.04.037.

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