Functionalizing natural polymers with alkoxyisilane coupling agents: Reacting 3-glycidoxypropyl trimethoxysilane with poly(γ-glutamic acid) and gelatin

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Hybrid materials, with co-networks of organic and inorganic components, are increasing in popularity due to their tailorable degradation rates and mechanical properties. To increase mechanical stability, particularly in water, covalent bonding must occur between the components. This can be introduced using crosslinking agents such as 3-glycidoxypropyl trimethoxysilane (GPTMS). Attachment of GPTMS to polymers in aqueous conditions is hypothesized to occur by opening of the epoxide ring by nucleophiles on the polymer chain. Despite side reactions that occur between the epoxide ring of GPTMS and water, a range of NMR techniques showed that the carboxylic acid group of poly(γ-glutamic acid) reacted with GPTMS. This result was used to identify the amino acids in gelatin that reacted most rapidly with the GPTMS epoxide ring, confirming that covalent bonding occurred in gelatin-silica hybrid materials.
Introduction

The potential of creating new advanced materials that have a combination of high toughness, high strength, and tailorable degradation rates has led to significant interest in hybrid materials that have co-networks of organic and inorganic components. They have been applied to a wide range of applications including protective surfaces\(^1\),\(^2\), optical coatings\(^3\), separation membranes\(^4\),\(^5\), drug delivery\(^6\), wound healing\(^7\), and degradable tissue engineering scaffolds\(^8\),\(^9\),\(^10\).

In contrast to conventional organic and inorganic composites, the components in hybrid materials interact at the nanoscale\(^11\),\(^12\). One strategy for their synthesis is to introduce a polymer into the silica sol-gel process. However, if covalent bonds are not formed between organic and inorganic components, the hybrid may become unstable in water. Introducing covalent bonding between the components and adjusting the degree of bonding enables the tailoring of properties such as degradation rate, stiffness, hardness and mechanical strength\(^10\),\(^11\), widening the potential applications of hybrids. However, characterization of the bonding reaction is difficult, preventing fine control and design of hybrid synthesis\(^16\). Thus an aim here was to develop a methodology for identifying the reactions between coupling agents and the polymer. Bifunctional alkoxysilane coupling agents including (3-glycidoxypropyl)trimethoxysilane (GPTMS)\(^10\),\(^12\),\(^17\)-\(^21\), (3-aminoethyl)trimethoxysilane (APTES)\(^22\),\(^23\), and (3-isocyanatopropyl)trimethoxysilyl (ICPTES)\(^24\) have been used to introduce covalent bonding between organic polymers that have complementary functional groups and inorganic silica networks or particles. These popular functional alkoxysilanes contain reactive functional groups, such as epoxide (GPTMS), that can react with complementary functionalities on polymers (e.g. –COOH) and alkox groups, that undergo hydrolysis and subsequent condensation with sol-gel silicate network\(^16\),\(^25\),\(^26\). The silanol groups may originate exclusively from the functional alkoxysilane itself or an additional silica precursor may be introduced, such as hydrolysed tetraethyl orthosilicate (TEOS)\(^10\),\(^11\),\(^17\),\(^18\).

The reactive functional group in APTES is a primary amine which shows reactivity towards epoxides, activated carboxylic acids and isocyanate groups. In the case of carboxylic acids, synthesis is complicated by the fact that carbodiimide chemistry is required to activate the carboxylic group, involving by-products formation and subsequent purification steps\(^27\). In the presence of water, the basic nature of the amine in APTES catalyses the condensation of the silanol groups, tending towards particle formation and phase separation, particularly when TEOS is used as an additional silica precursor.\(^28\)

The reactive group in ICPTES is an isocyanate which makes it amenable to reaction with alcohols and amines but it also reacts undesirably with water, requiring anhydrous reaction conditions. ICPTES is a very efficient crosslinking agent, even reacting with hydroxyl groups\(^29\). However, this may mean that polysaccharides with multiple hydroxyl and other nucleophilic species will become highly crosslinked, or react with multiple functional groups of the monomer, due to poor selectivity of the coupling agent\(^30\). For biological applications, it should also be noted that isocyanate functional groups are highly toxic, fatal if inhaled, and damage DNA\(^31\). Therefore, very stringent purification steps must follow synthesis to ensure no unreacted ICPTES remains in the material. In the case of GPTMS, one terminal end of the molecule contains an epoxide ring, prone to nucleophilic attack and ring opening, strictly dependent on reaction pH\(^32\). Polymer functionalisation with GPTMS can be carried out in a single step as the epoxide ring is activated under the mildly acid conditions typically required to solubilise natural polymers, with ring opening increasing in rate as pH reduces. For these reasons, GPTMS has found widespread use in natural polymer-based hybrid synthesis, particularly in materials for biomedical applications. Here, the focus will be on the use of GPTMS as a coupling agent in hybrid synthesis.

In order to synthesise hybrid materials using natural polymers, the polymer, e.g. alginate, chitosan, gelatin, or poly(glutamic acid) (γ-PGA), is typically dissolved in a mildly acidic (pH 4 or 5) aqueous solution and reacted with GPTMS for up to 24 h\(^10\),\(^12\),\(^17\),\(^18\),\(^31\). The functionalised polymer is subsequently introduced into the silica sol-gel process, e.g. a solution of hydrolysed TEOS, and fabricated into three dimensional (3-D) porous scaffolds using techniques such as electrospinning\(^34\), foaming or freeze-drying\(^9\),\(^11\),\(^12\),\(^35\),\(^36\). Hybrid scaffolds can be tailored to exhibit mechanical and degradation properties suitable for various applications\(^10\),\(^11\) and they have shown promising results \textit{in vitro} with regards to attachment and proliferation of mesenchymal stem cells\(^10\), osteoblast-like\(^39\) and osteosarcoma cells\(^18\),\(^19\). \textit{In vivo}, chitosan-silica hybrid scaffolds showed good cytocompatibility when implanted subcutaneously and improved tissue regeneration of crushed spinal nerve in a rat model\(^7\). Highlighting the importance of understanding hybrid systems, a gelatin-silica hybrid material showed dissolution profiles that could be tailored by altering the amount of GPTMS coupling agent\(^10\),\(^11\), yet poly(γ-glutamic acid) (γ-PGA)-silica hybrids did not\(^10\),\(^41\), despite both polymers comprising amino acids with nucleophilic carboxylic groups.
The reactions are difficult to follow as it is a challenge to adequately characterise and quantify the different species involved. Ren et al. hypothesised that both amine and carboxylic acid groups of gelatin would act as nucleophiles to ring open the protonated and activated epoxide ring in GPTMS. To study the reaction of GPTMS with gelatin, Ren et al. hydrolysed the gelatin chains before and after functionalisation in 6 M HCl at 110 °C for 22 h and used amino acid analysis to establish which amino acid residues reduced in concentration and, hence, the fraction of each amino acid that was bonded to GPTMS. Unfortunately, the hydrolysis step leads to erroneous quantification of the amino acids since some of the residues are partially destroyed by decomposition in acid (e.g. threonine and serine) or converted to other amino acids (asparagine and glutamine form aspartic acid and glutamic acid respectively) and certain bonds may not be broken (isoleucine-valine). In addition, and perhaps of more concern from a biocompatibility point of view, the amount of unreacted GPTMS, or diol species formed is unknown. The diol could also chelate, producing a range of species, such as R-Si(OH)₃, R-Si(OH)₂(OR), or R-Si(OH)OR). Despite this, a trend was observed that indicated that amino acids containing nucleophilic R groups could be reacted with GPTMS.

Shirosaki et al. used a ninhydrin assay to quantify the degree of reaction between chitosan and GPTMS. Chitosan contains primary amine (-NH₂) groups, which were hypothesised to act as nucleophiles and open the epoxide ring of the GPTMS. The assay dye showed an absorbance in the blue region in the presence of primary amines, shifting to yellow-orange in the presence of secondary amine. However, the assay indicated that 80 % of the groups were converted to a secondary amine, even when as little as 10:1 chitosan units:GPTMS mole ratio was used. In a different study, Shirosaki et al. used GPTMS, chitosan and β-glycerophosphate and showed that even with no GPTMS, the assay indicated a degree of crosslinking of 60 %, plateauing at 80 % for 1:1 mol ratio GPTMS:chitosan. Therefore the assay does not seem suitable for this purpose. Chen et al. used a similar ninhydrin assay to determine the extent of crosslinking between GPTMS and collagen. They found that the extent of crosslinking increased from 83 % to 99 % as the weight ratio of GPTMS:collagen increased from 0.5 to 4. Unfortunately, this again does not calculate the amount of unreacted GPTMS or, in the case of collagen, the number of crosslinks that form between non-amine containing species and GPTMS, i.e. carboxylic acids groups present in amino acid side chains of glutamic and aspartic residues. The complicated structure of natural polymers, such as proteins (e.g. gelatin), which do not have a single repeating unit but up to 20 different amino acids arranged in different sequences that vary between species, individuals and tissue types, means that chemical characterization by nuclear magnetic resonance (NMR) is challenging. Researchers become reliant on using Fourier transform infrared spectroscopy (FTIR) as a characterization technique. Unfortunately, the data is not quantitative and vibration band assignment can be controversial. The carbonyl region of PGA (and also gelatin) is particularly crowded due to the presence of the two bands from amide carbonyls, plus the band from the free (unbound) COOH; it is thus difficult to find the ester band with diagnostic significance in such complex systems. In order to get a better understanding of these systems, the authors previously investigated by NMR the reactions of GPTMS in water alone under different pH conditions and with various small molecule nucleophiles, such as propanoic acid, propanol, propanthiol and propylamine as a model for larger systems. The epoxide ring opening of GPTMS was found to be catalysed by Broensted acids with an increased rate of ring opening at reduced pH. The ring opening reaction could occur either with water to result in the formation of a diol (Figure 1) or with the nucleophilic species, typically resulting in a mix of diol and nucleophile-added products. The reaction between GPTMS and the carboxylic acid group of propanoic acid led to ester formation. No reaction was observed with propanol and unfortunately, the rate of condensation of silanol groups in the presence of amine and thiol species was so rapid that the reaction with the epoxy group could not be studied by NMR.

An NMR study by Connell et al. confirmed the hypothesis of Shirosaki et al. that GPTMS reacts with the amine group of chitosan to produce a secondary amine. However, diol was formed as a side product, the ratio of diol:secondary amine products remained constant at 4:1 for pH 2, 4 and 6.

In the γ-PGA-silica system, researchers hypothesised that, analogously to gelatin, which contains glutamic acid residues, the epoxy ring of GPTMS reacts with the nucleophilic carboxylic acid species of the polymer to form ester linkages. The aim of this study was to confirm this hypothesis, characterise the covalent bonding between the species, and the (side-) reactions that occur during the reaction of GPTMS with γ-PGA and gelatin under aqueous conditions typical of hybrid synthesis. To achieve this, a series of one dimensional (1-D) and two dimensional (2-D) NMR studies were carried out. For the first time, the product of these reactions was identified and quantified, directly confirming that there is a covalent bond formed between GPTMS and the natural polymers gelatin and γ-PGA. The formation of diol was also observed, querying the efficacy of GPTMS as a suitable coupling agent to introduce covalent crosslinks under aqueous conditions.

**Experimental**

**Materials and Synthesis Methods**

Unless stated, all chemicals were analytical grade purchased from Sigma Aldrich, UK and used as received.

**FUNCTIONALIZATION OF POLY(γ-GLUTAMIC ACID) WITH (3-GLYCIDYLOXYPROPYL)TRIMETHOXYSILANE IN DIMETHYL SULFOXIDE.** Based on a previous protocol, poly(γ-glutamic acid) (γ-PGA, 120kDa (~930 repeating units), Natto Biosciences) was dissolved in deuterated dimethyl sulfoxide (DMSO-d₆, 99.99% deuteration) at 70 °C to give a polymer concentration of 50 mg.mL⁻¹. (3-glycidyloxypropyl) trimethoxysilane (GPTMS) was added to the solution to give a molar ratio of γ-PGA monomer units to GPTMS (GC) of 10. The solution was mixed for 3 h at 70 °C before an aliquot of 0.7 mL was transferred to a 5 mm nuclear magnetic resonance (NMR) tube. The tube was sealed and ¹H and ¹³C NMR spectra were recorded over 1 h.
**Functionalization of Na-γ-PGA with GPTMS in D$_2$O.** γ-PGA (Natto Biosciences) was added to D$_2$O (99.99% deuteration) at 40 °C to give a polymer concentration of 50 mg.mL$^{-1}$. NaOH (90 mol % relative to γ-PGA monomer unit) was added and the solution stirred vigorously until the polymer had completely dissolved, producing the sodium salt form of γ-PGA (Na-γ-PGA). The solution was adjusted to pH 6 by adding DCI (2M in D$_2$O, 99.99% deuteration) dropwise. pH 6 was chosen because previous work on GPTMS behaviour in aqueous conditions showed that below pH 5 the epoxide ring opened without interaction with the polymer, and above pH 7 gelation of the silicate network was promoted$^{38}$. GPTMS (GC of 10) was added to the Na-γ-PGA solution and the reaction was allowed to proceed for up to 96 h at 40 °C. Aliquots of 0.7 mL were removed at 14 h, 24 h, 40 h and 96 h, placed into 5 mm NMR tubes, sealed, and $^1$H, $^{13}$C one dimensional (1-D), $^1$H-$^1$C heteronuclear single quantum coherence spectroscopy (HSQC) and $^1$H-$^{13}$C heteronuclear multiple-bond correlation spectroscopy (HMBC) spectra were recorded.

**Functionalization of gelatin with GPTMS in D$_2$O.** Gelatin was dissolved in D$_2$O (99.99% deuteration) at 40 °C to give a polymer concentration of 50 mg.mL$^{-1}$ and adjusted to pH 5 using DCI (2M in D$_2$O, 99.99% deuteration). GPTMS was added to give a GC of 10 and the solutions were mixed at 40 °C for 48 h to allow reaction to progress. 0.7 mL solution was extracted, placed into a 5 mm NMR tube and sealed to obtain $^1$H NMR spectra.

**Sample Characterisation**

**Preparation of Samples for Reference Spectra.** Samples for reference spectra were obtained for the unreacemed Na-γ-PGA and gelatin dissolved in D$_2$O (99.99% deuteration) at 40 °C and pH 6 by following the relevant protocol above but taking an aliquot for NMR analysis prior to addition of GPTMS. Samples for reference spectra of unreacemed GPTMS was prepared by adding 0.2 µL GPTMS to 0.7 mL CDC13 (99.9% deuteration) in a 5 mm NMR tube. GPTMS with partial epoxy ring opening to form diol in the absence of other nucleophiles was prepared by adding 0.2 µL GPTMS to 1.5 mL D$_2$O (99.99% deuteration) adjusted to pH 6 with DCl (2 M in D$_2$O, 99.99% deuteration) dropwise. The reaction was carried out for up to 48 h at 40 °C. Aliquots of solution were removed at various time points and placed into 5 mm NMR tubes to obtain $^1$H, $^{13}$C and $^1$H-$^{13}$C HSQC NMR spectra.

**Solution State Nuclear Magnetic Resonance Spectroscopy.** One dimensional (1-D) spectra were acquired either at 295.8 K using a Bruker Avance III 400 HD spectrometer operating at 400 MHz $^1$H frequency and equipped with a bbof/5 mm probe running TopSpin 3.2 software or Varian 400 MHz Mercury instrument, operating at a proton frequency equal to 400 MHz. Chemical shifts were referenced to methanol at 3.40 ppm. The two dimensional (2-D) $^1$H-$^{13}$C HSQC experiments were run at 295 K on a Bruker 400MHz AV II spectrometer running TopSpin 2 and equipped with a z-gradient bbo/5mm tuneable probe and a GAB gradient unit providing a maximum gradient output of 53.5 G/cm. The $^1$H-$^{13}$C HSQC spectrum was acquired using the Bruker pulse sequence hsqetgpsi2. The directly detected proton (400.2 MHz) dimension used a spectral width of 4480 Hz (centred on 5 ppm) and 1024 data points giving an acquisition time of 0.11 s. The indirectly detected carbon (100.63 MHz) dimension used a spectral width of 24154 Hz (centred on 110 ppm) and 240 experiments were collected each with 2 transients. A relaxation delay of 1 s was employed and sine shaped gradients used. The indirect dimension was linearly predicted to 1024 points and the data was processed using 1024 data points for the direct dimension and a cosine squared window function in both dimensions.

The HMBC experiment was measured at 298 K on a Bruker 500MHz AV II spectrometer running TopSpin 2 and equipped with a z-gradient bbo/5 mm tuneable probe and a GAB gradient unit providing a maximum gradient output of 53.5 G/cm. The spectrum was acquired using the Bruker pulse sequence hmbctgp3ind. The directly detected proton (500.13 MHz) dimension used a spectral width of 5000 Hz (centred on 4 ppm) and 1024 data points giving an acquisition time of 0.1 s. The indirectly detected carbon (125.76 MHz) dimension used a spectral width of 27670 Hz (centred on 110 ppm) and 220 experiments collected each with 64 transients. The minimum and maximum $^1$JCH couplings were set to 110 Hz and 180 Hz respectively and a long range coupling of 8.3 Hz used. A relaxation delay of 1 s was employed along with sine shaped gradients. The indirect dimension was linearly predicted to 256 points and zero-filled to 512 points, then the data processed using 2048 data points for the direct dimension and a cosine squared window function in both dimensions.

**Results and discussion**

**Poly(γ-glutamic acid)**

Due to the complicated chemical structure of the gelatin chain, γ-PGA was first investigated, which has a single amino acid as its repeating unit. γ-PGA is a good candidate for many biomaterial applications as it is biodegradable, generally regarded as safe by the FDA, and is naturally occurring via fermentation.$^{50}$ However, the free acid form of the polymer is not water soluble, requiring dimethyl sulfoxide (DMSO) to be used as a solvent. Following an adapted protocol,$^{18}$ no reaction
between γ-PGA and GPTMS in DMSO was observed by NMR (Figure S20 in the Supporting information). Presumably, there is no Brønsted acid in the solvent to catalyse the epoxide ring opening and hence, functionalization will not occur. Similarly, no hydrolysis of the methoxysilane groups was observed.

Sodium and calcium salt forms of the polymer (Na-γ-PGA and Ca-γ-PGA respectively) can be used instead, which are soluble in mildly acidic conditions, and hence, will catalyse the epoxide ring opening. However, chelation of the carboxylic acid groups was required to render the polymer soluble and stoichiometric addition of sodium salt would interfere with the functionalization reaction.

So, for this work, Na-γ-PGA was produced from the free acid form of the polymer, using 90 mol % NaOH.

After reaction of Na-γ-PGA with GPTMS for 48 h at pH 6 and 40 °C, 1-D 1H NMR (Figure 2, and Figures S10-S12 in Supporting Information) showed a reduction in the relative area of peaks f1 and f2 at 2.7 ppm and 2.8 ppm in GPTMS/D2O and Na-γ-PGA/GPTMS, which means that the epoxy ring opened under the mildly acidic conditions, in line with previous observations. The disappearance of the sharp peak at 3.17 ppm (GPTMS/D2O pH 7) and the appearance of the large peak at 3.34 ppm (GPTMS/D2O pH 2 and Na-γ-PGA/GPTMS) was due to the hydrolysis of the methoxysilane groups to silanol and methanol. Both the epoxy and methoxysilane functional groups reacted simultaneously. In addition, a number of new peaks were observed in the GPTMS/D2O and Na-γ-PGA/GPTMS samples between 3.25-4.00 ppm but considerable overlap prevented identification and quantification of the different species. From previous studies where GPTMS is reacted with model small molecules and in water at different pH values, where there is no overlap in the NMR signals, these new peaks are due to the formation of diol species as a consequence of the epoxy ring reaction with water. In the Na-γ-PGA/GPTMS solution, further new peaks were observed between 4.00 ppm and 4.33 ppm.

13C spectra (Figure 3 and S19 in Supporting Information) show that when GPTMS was reacted with only water at pH 2 for 24 h, new signals appeared at 62, 69 and 71 ppm as a new diol species forms, in agreement with Gabrielli et al. The chemical shift of e’ and f’ in the new species increased due to the epoxide ring opening and addition of a second electronegative atom. When Na-γ-PGA was reacted with GPTMS for 48 h, two new signals were observed at 70 and 63 ppm (labelled e” and f” in Figure 3) in addition to the untreated GPTMS peaks (a-f), the diol peaks (d’-f’) and the polymer peaks (1, 2, α-γ). Broadening of d occurred around 72 ppm and may be attributed to the emergence of d” species. Broadening of the α peak of the polymer at 55 ppm indicated that the reaction between GPTMS and Na-γ-PGA occurred close to this carbon. A very small signal was detected at 172.7 ppm, (see inset of Figure 3 and S1) indicating that the reaction product lead to the formation of a new type of carbonyl carbon, namely...
an ester carbonyl. Quaternary carbon atoms which are not bonded to hydrogen atoms such as in an ester or carboxylic acid group, give rise to low intensity NMR signals. Hence, the low intensity of the peak compared to $e''$ and $f''$ is due to its quaternary nature.

2-D NMR of $^1$H,$^13$C HSQC shows correlations between atomic nuclei. Signals are detected where a proton is directly bonded to a carbon atom. The coordinates on the plot give the chemical shift of the nuclei and hence enable identification. This is particularly useful when overlapping signals occur in 1-D spectra and can give an indication of the structure of the species. The $^1$H,$^13$C HSQC spectrum of Na-$\gamma$-PGA/GPTMS (Figure 4 and Figures S13-S16 in the Supporting Information) clearly shows the unreacted GPTMS ($a$-$f$) and the diol side product ($d'$-$f'$) which forms when the water hydrolyses the epoxide ring. In agreement with literature, new GPTMS signals $e''$ and $f''$ were observed at ($\delta^1$H 4.00 ppm, $\delta^{13}$C 67.7) and ($\delta^1$H 4.12-4.16 ppm, $\delta^{13}$C 66.50 ppm) respectively. The new signal $d''$ appeared at ($\delta^1$H 3.25 ppm, $\delta^{13}$C 71.00 ppm), very close to the $d'$ signal at ($\delta^1$H 3.49 ppm, $\delta^{13}$C 70.72 ppm), when the epoxide ring reacted either with the water to form a diol or with the Na-$\gamma$-PGA. The opening of the epoxide ring caused the simultaneous coalescence of the signals ($d$ and $f$) as conformational constraints are relaxed.

No signal was detected in the carbonyl region as expected since there were no directly bonded protons to the carbon. A prominent signal was observed at ($\delta^1$H 4.33 ppm, $\delta^{13}$C 52.53 ppm) which indicates that the monomers of the Na-$\gamma$-PGA chain were modified and corresponds to the $\alpha$' carbon of the polymer chain. These signals are in close agreement with the propanoic-GPTMS study of Gabrielli et al.\textsuperscript{2}, which confirmed that carboxylic acid at pH 6 can react with the epoxide ring of GPTMS to form an ester (Figure S16, Supplementary Information). In the Na-$\gamma$-PGA, which is a polypeptide with carboxylic acid pendant groups, the reaction occurred slowly with 93 mol % epoxide ring remaining after 24 h and 24 mol % after 96 h, determined from $^1$H NMR by comparing the integral of $f_1$ and $f_2$ with that of a and b. At early time points, the amount of $\alpha''$ formed was below the detection limits of the machine. But at 24 h, 1 mol% of $\alpha''$ signal was detected (with respect to unreacted $a$) and increased to 2% at 96 h. Considering that a mol ratio of 12.8 Na-$\gamma$-PGA monomer units:GPTMS was used, this corresponds to 13% and 26% of the added GPTMS reacting with Na-$\gamma$-PGA within 24 h and 96 h respectively. Calculating the remainder, we determined that 7% and 22% epoxide rings opened to form diols during side reactions at 24 h and 96 h respectively. Unfortunately, this cannot be quantified directly from the 1-D $^1$H NMR spectra due to considerable overlap of the peaks.

The reaction of the carboxylic acids of Na-$\gamma$-PGA with the epoxide ring of GPTMS at pH 6 was more efficient than that of the amine in chitosan with GPTMS at pH 6 since at 24 h, 65% of the reacted GPTMS formed the intended product and 35% formed the diol species (compared to only 20% intended product and 80% diol formation in chitosan)\textsuperscript{12}. However, this effect becomes less pronounced at longer reaction times, potentially due to the reduction in available carboxylic acid groups for reaction.

An added complication of the functionalization method was that simultaneously to the reaction occurring at the epoxide end, the alkoxysilane was hydrolyzing and condensing by means of a Bronsted acid catalysed reaction. This meant that at long reaction times, the reaction solution became very viscous and eventually gelled. Gelation was accelerated at increasing temperatures meaning that a compromise had to be found between high temperatures to promote functionalization of the polymer and lower temperature to slow gelation. For this reason, 40 °C was selected for functionalization. The fact that significant diol formation occurs may actually be beneficial for the materials synthesis. The diol can act as a plasticiser, creating extra free volume in the material and slow the gelation of the hybrid. This allows the time of the functionalization step to be extended to ensure the epoxide ring becomes fully opened, which may impact on bioactivity of the material, and ultimately prevents the hybrid from becoming too brittle due to high levels of crosslinking and restricting the movement of polymer chains.

All this data points to the probable reaction of GPTMS epoxide ring with the 1 carbonyl of Na-$\gamma$-PGA but a further 2-D NMR technique was required to confirm that the reaction product was an ester. $^1$H,$^13$C HMBC (Figure 5) shows the correlations that occur between protons and carbons that are separated by two or three bonds. Despite the complexity and noise of the spectra, a correlation was detected between the new $f''$ and $l''$ signals at ($\delta^1$H 4.14 ppm, $\delta^{13}$C 172.7 ppm). While the signals are of low intensity, they are in the typical region for an ester functional group and were in the same position and were of similar intensity of those observed by Gabrielli et al.\textsuperscript{12} with the model molecule propanoic acid.\textsuperscript{12} The low intensity is due to the features of the atoms affording the signal and their low abundance in very complex systems. The low intensity the cross-peak suggests the presence of the ester bond.

This confirms that the epoxide ring of GPTMS reacts with the carboxylic acid group of polypeptides to give covalent bonding in hybrid materials even under aqueous acidic conditions. It should be noted, however, that this does not prove that only carboxylic acid reacts with the epoxide ring of GPTMS. A more complex polypeptide such as gelatin will contain other nucleophilic species such as amines that may also react. However, since the efficiency of GPTMS reaction with carboxylic acid in this system was so much greater than the reaction with amine in chitosan previously reported, one might expect the dominant crosslinking reaction to involve carboxylic acid groups rather than amines.

**Gelatin**

With compelling evidence that the epoxide ring reacts with carboxylic acid functional groups, and with this reaction dominant over the side reaction of GPTMS that forms diols, it is possible to start analysing more complicated polypeptide chains such as gelatin. A pH of 5 was used for gelatin because using pH 6 caused gelation of the gelatin. At pH 5 the ring opening of the epoxide was previously found to be very slow (just 13% of the epoxide ring results hydrolysed after three days)\textsuperscript{48}, so pH 5 is unlikely to hamper gelatin reaction with GPTMS compared to pH 6.

In literature, there are numerous reports of assigned NMR spectra of different types of polypeptide and these have been used to assign the NMR spectra in Figure 6\textsuperscript{52-54}. Considering the abundance of each amino acid\textsuperscript{15} (Table 1) in the type II bovine gelatin used herein we may assume that any reaction with amino acids of less than 2 mol% abundance will be
negligible and not detectable by NMR even if 100 % reacted. This allows us to consider only the amino acids: glycine, proline, alanine, hydroxyproline, glutamic acid, arginine, aspartic acid, serine, lysine and leucine. At pH 5, amine groups are almost fully protonated so nucleophilic behaviour of amines is ruled out. Of the candidate amino acids, we

would therefore expect the most reactive (nucleophilic) repeating units in gelatin to be glutamic acid and aspartic acid. Comparison with the spectra of unreacted gelatin with GPTMS/diol, the NMR spectra of gelatin exposed to GPTMS for 48 h shows a new signal at 2.25 ppm and changes in signal at 2.33 ppm, 2.75-2.67 ppm and 4.26 ppm (Figure 7 and Figures S21 and S22 in Supporting Information). Using the assigned spectra and considering only the amino acids with abundances greater than 2 %, these changes in signal were attributed to the following amino acids: 2.33 ppm, proline and glutamic acid; 2.75-2.67 ppm, aspartic acid; 4.26 ppm, alanine, glutamic acid, lysine, and arginine. As stated previously, proline and alanine are unable to react with the epoxide of GPTMS, leaving glutamic acid and aspartic acid as likely candidates for reaction. The greatest change in signal was observed around 2.42-2.20 ppm, where the signal at 2.33 ppm disappeared and the signal at 2.25 ppm appeared. We propose that this is as a result of the glutamic acid residue becoming functionalised (since the other amino acid that may give rise to this signal is proline which is unable to react with GPTMS). The third affected signal at 4.26 ppm is as a result of the proton bonded to the C2 of glutamic acid, arginine and lysine. We have already shown that glutamic acid reacts with GPTMS, and it is possible that the change in signal comes exclusively from this amino acid. However, it is not possible to determine the extent of the reaction or completely rule out arginine and lysine’s amino groups reacting with the epoxide of the GPTMS and producing the change in signal. It should be noted that the resonance-stabilized amine of arginine may render the amino acid non-reactive and no other change in signal was observed in the regions typical of lysine and arginine. If any reaction did occur involving lysine and arginine, it was to a lesser extent than that of carboxylic acid containing amino acids, in particular glutamic acid which has been shown to be the most important amino acid when functionalizing gelatin.

Conclusions

This study has shown that the epoxide ring of GPTMS reacts with the carboxylic acid groups of Na-γ-PGA and gelatin to form covalent bond between the alkoxysilane and the polymer via ester linkages. The reaction is catalysed by Broensted acids, so may occur under weakly acidic conditions such as pH 6, but Na-γ-PGA must be in its salt form in order to be soluble. Ester formation is the dominant reaction with almost twice as much ester product in the reaction milieu than diol species.

Increased temperature speeds up the functionalization reaction but also the gelation by silanol condensation, hence a compromise must be made to balance the two. The authors would recommend a reaction temperature of 40 °C, which is particularly important for gelatin which gels at temperatures below 35 °C.

Glutamic acid and aspartic acid were identified as the two most important amino acids involved in the functionalization of gelatin based on their abundance and reactivity. Poly(glutamic acid) and poly(aspartic acid) are therefore potentially useful polymers for bio-hybrid synthesis.

A series of 1-D and 2-D NMR experiments were used to show directly for the first time that GPTMS can be used as a covalent crosslinker in hybrid materials between complex natural polymers and inorganic silica networks.

![Figure 6](image-url)  
**Figure 6** – 1H NMR spectra of gelatin in D2O at pH 5 overlaid with predicted signals from amino acids from literature. Where multiple signals are shown for the same protons they represent a possible range.

![Figure 7](image-url)  
**Figure 7** – 1H NMR spectrum of gelatin after reaction with GPTMS for 48 h at pH 5 (black line) superimposed with the spectra of unreacted gelatin and partially hydrolysed GPTMS (grey line). Differences in the spectra are indicated with *.

Table 1 – Bovine skin gelatin composition from Mahmoodani *et al.*

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<thead>
<tr>
<th>Amino Acid</th>
<th>Code</th>
<th>Rel abundance %</th>
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<tbody>
<tr>
<td>Glycine</td>
<td>Gly</td>
<td>42.71</td>
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