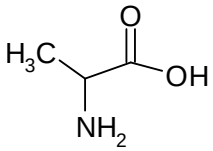
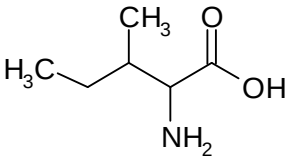
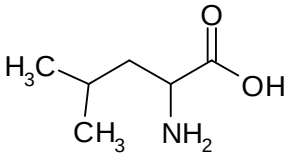
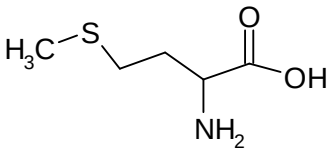
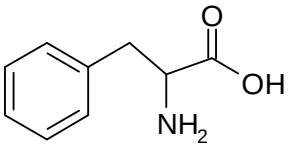
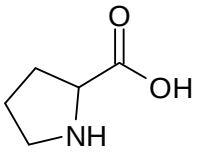
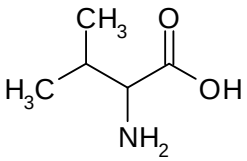
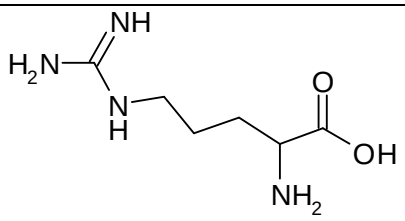
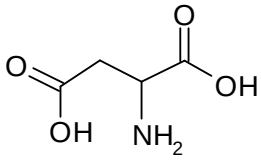
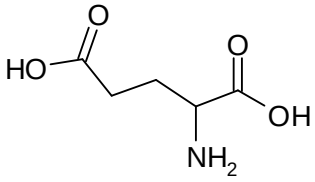
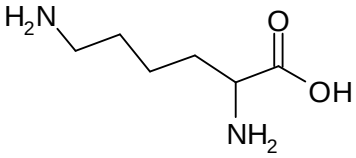
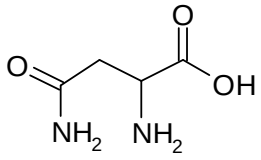
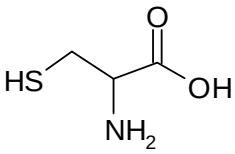
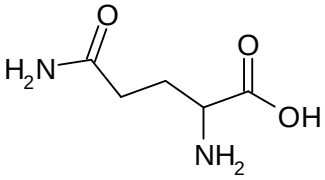
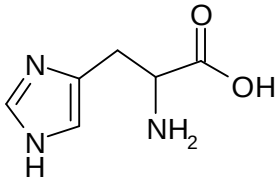
Appendix A

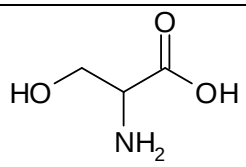
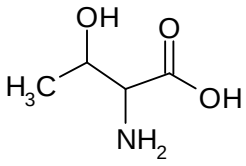
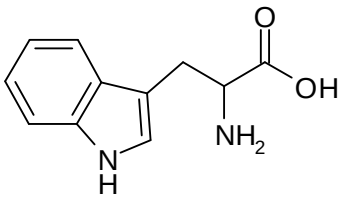
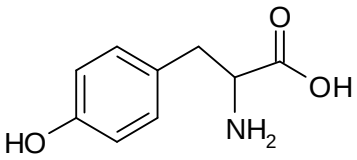
Amino acids

Amino Acid	Structure	3 Letter Abbreviation	1 Letter Abbreviation
Naturally Occurring Hydrophobic Amino Acids			
Alanine		Ala	A
Isoleucine		Ile	I
Leucine		Leu	L
Methionine		Met	M
Phenylalanine		Phe	F
Proline		Pro	P
Valine		Val	V
Naturally Occurring Charged Amino Acids			

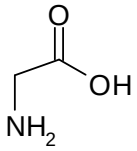
Arginine		Arg	R
Aspartic Acid		Asp	D
Glutamic Acid		Glu	E
Lysine		Lys	K

Naturally Occurring Polar Amino Acids

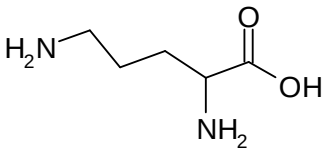
Asparagine		Asn	N
Cysteine		Cys	C
Glutamine		Gln	Q
Histidine		His	H

Serine		Ser	S
Threonine		Thr	T
Tryptophan		Trp	W
Tyrosine		Tyr	Y

Other Naturally Occurring Amino Acids

Glycine		Gly	G

Synthetic Amino Acids

Ornithine		Orn	O

Appendix B

Peptide quality control

1.1 Peptide content

Charged peptides contain bound counter-ions, due to the processing conditions used to purify them. The use of TFA in peptide synthesis and purification results in cationic peptides often being produced as trifluoroacetate salts. Any trifluoroacetate bound cannot be easily removed by freeze drying.[1] Consequently, knowledge of peptide content is important. What is labelled, for example, as a 95% HPLC pure material may contain considerably less peptide than this, due to bound counter-ions.

As a simple approximation, at a neutral pH, every positively charged residue was assumed to have an associated trifluoroacetate counter-ion, and every negatively charged residue assumed to have an associated ammonium counter-ion, due to the use of ammonium acetate buffers. The true peptide content could then be estimated by dividing the molecular weight of the peptide sequence by its effective molecular weight (peptide added to total counter ions), and multiplied by 100 to convert to a percentage.

Peptide	Calculated peptide content (%)
P_{11}-4	90.5
P_{11}-7	91.1
P_{11}-8	81.3
P_{11}-9	89.5
P_{11}-12	79.6
P_{11}-13	93.6
P_{11}-14	76.8
P_{11}-28	68.4
P_{11}-29	95.6

Table 1 - Estimated peptide content values

1.2 Individual batch data

1.2.1 P_{11}-4 (DN1 2E)

Sequence: CH3CO-Q-Q-R-F-E-W-E-F-E-Q-Q-NH2

Empirical Formula: $C_{72}H_{98}N_{20}O_{22}$

Expected Molecular weight: 1596

Net charge = -2

Actual peptide content (calculated as average from company provided data and UV analysis) = 0.944

Manufacture found MW	1595.5
HPLC (purity)	97.3 %
Manufacturer AAA	89.6 %
In house UV	99.2 %
In house elemental analysis	C 51.8%, H 6.2 %, N 16.9 %

Table 2 - Manufacturer: Polypeptide Group, Batch: CF10141A

1.2.2 P_{11}-7 (SDN1)

Sequence: CH_3CO-S-S-R-F-S-W-S-F-E-S-S-NH_2

Empirical Formula: $C_{60}H_{82}N_{16}O_{20}$

Expected molecular weight: 1347.4

Net charge = 0

Actual peptide content (calculated as average from company provided data and UV analysis) = 0.899

Manufacturer found MW	1348.2
HPLC (purity)	94.5 %
Manufacturer UV	85.8 %
In house UV	94 %
In house elemental analysis	C 48.7%, H 5.7%, N 14.4%

Table 3 – P_{11}-7 QC - Manufacturer: Neo MPS Batch: HF31 434S

1.2.3 P_{11}-8 (DN1-2O)

Sequence: CH3CO-Q-Q-R-F-O-W-O-F-E-Q-Q-NH2

Empirical Formula: $C_{68}H_{98}N_{20}O_{18}$

Expected molecular weight: 1566

Net charge = +2

Actual peptide content (calculated as average from company provided data and UV analysis) = 0.785

Manufacturer found MW	1565.7
HPLC (purity)	96.3 %
Manufacturer AAA	76.6 %
Manufacturer UV	78.7 %
In house UV	80.2 %
In house elemental analysis	C 47.1 %, H 5.6 %, N 15.3 %

Table 4 – P_{11}-8 QC - Manufacturer: Polypeptide Group, Batch: HF34148A

1.2.4 P_{11}-9 (DN1-S2E)

Sequence: CH3CO-S-S-R-F-E-W-E-F-E-S-S-NH2

Counter ions: $3NH_4^+$ and CF_3COO^-

Empirical Formula: $C_{64}H_{86}N_{16}O_{22}$

Expected molecular weight: 1432

Charge = -2

Actual peptide content (calculated as average from company provided data and UV analysis) = 0.955 (CG-01-562), 0.889 (CF08380D)

Manufacturer found MW	1431.4
HPLC (purity)	95.2%
Manufacturer AAA	90 %
In house UV	101 %
In house elemental analysis	C 51.4%, H 6.0%, N 14.7%

Table 5 – P_{11}-9 QC - Manufacturer: CPC Scientific, Batch: CG-01-562

Manufacturer found MW	1431.5
HPLC (purity)	98.8%
Manufacturer UV	91.8 %
In house UV	86 %
In house elemental analysis	C 50.2%, H 6.0%, N 14%

Table 6 – P_{11}-9 QC - Manufacturer: Polypeptide Group, Batch: CF08380D

1.2.5 P_{11}-12 (DN1-S2O)

Sequence: CH_3CO-S-S-R-F-O-W-O-F-E-S-S-NH_2

Counter ions = NH_4^+ and $3CF_3COO^-$

Empirical Formula: $C_{64}H_{92}N_{18}O_{18}$

Expected molecular weight: 1402.2

Charge = +2

Actual peptide content (calculated as average from company provided data and UV analysis) = 0.732 (AW09357a), 0.753 (AW09357b)

Manufacturer found MW	1401.2
HPLC (purity)	98.2%
Manufacturer AAA	68.4 %
In house UV	78 %
In house elemental analysis	C 46.4%, H 5.5%, N 13.8%

Table 7 – P_{11}-12 QC - Manufacturer: Neo MPS, Batch: AW09357a

Manufacturer found MW	1401.2
HPLC (purity)	96.2%
Manufacturer AAA	68.4 %
In house UV	82.1 %
In house elemental analysis	C 45.8%, H 5.5%, N 13.8%

Table 8 – P_{11}-12 QC - Manufacturer: Polypeptide Group, Batch: AW09357b

1.2.6 P_{11}-13 (DN1-6E)

Sequence: CH_3CO-E-Q-E-F-E-W-E-F-E-Q-E-HN_2

Counter ions = 6 NH_4^+

Empirical Formula:$C_{71}H_{91}N_{15}O_{26}$

Expected molecular weight: 1571

Charge = -6

Actual peptide content (calculated as average from company provided data and UV analysis) = 0.688

Manufacturer found MW	1570.3
HPLC (purity)	97.2 %
Manufacturer AAA	70.6 %
In house UV	67 %
In house elemental analysis	C 40.7 %, H 4.8%, N 12.6%

Table 9 – P_{11}-13 QC - Manufacturer: Polypeptide group, Batch: AW11279K

Manufacturer found MW	1570.5
HPLC (purity)	95.1 %
Manufacturer AAA	88 %
In house UV	94.6 %
In house elemental analysis	C 52 %, H 5.9%, N 13.4%

Table 10 – P_{11}-13 QC - Manufacturer: CPC Scientific, Batch: CG-08-00242

1.2.7 P_{11}-14 (DN1-4O)

Sequence: CH_3CO-Q-Q-O-F-O-W-O-F-O-Q-Q-NH_2

Counter ions = 4 CF_3COO^-

Empirical Formula: $C_{71}H_{109}N_{21}O_{16}$

Expected molecular weight: 1508.7

Charge = +4

Actual peptide content (calculated as average from company provided data and UV analysis) = 0.741

Manufacturer found MW	1508.1
HPLC (purity)	97.5 %
Manufacturer UV	72.6 %
In house UV	75.5%
In house elemental analysis	C 46.7 %, H 5.6%, N 14.3%

Table 11 – P_{11}-14 QC - Manufacturer: Polypeptide Group, Batch: CF08498A

1.2.8 P_{11}-28

Sequence: CH_3CO-O-Q-O-F-O-W-O-F-O-Q-O-NH_2

Counter ions = 6 CF_3COO^-

Empirical Formula: $C_{71}H_{109}N_{21}O_{14}$

Expected molecular weight: 1481.1

Charge = +6

Actual peptide content (calculated as average from company provided data and UV analysis) = 0.633

Manufacturer found MW	1480.6
HPLC (purity)	98.5 %
Manufacturer AAA	65.8 %
In house UV	60.8 %
In house elemental analysis	C 45.1%, H 5.1%, N 13%

Table 12 – P_{11}-28 QC - Manufacturer: Polypeptide Group, Batch: AK09397

1.2.9 P_{11}-29

Sequence: CH_3CO-O-Q-O-F-O-W-O-F-O-Q-O-NH_2

Counter ions = 6 CF_3COO^-

Empirical Formula: $C_{71}H_{93}N_{17}O_{24}$

Expected molecular weight: 1568.6

Charge = +6

Actual peptide content (calculated as average from company provided data and UV analysis) = 0.877

Manufacturer found MW	1568.5
HPLC (purity)	97.5 %
Manufacturer AAA	92 %
In house UV	83.3 %
In house elemental analysis	C 53.4 %, H 5.8%, N 14.8%

Table 13 – P_{11}-29 QC - Manufacturer: Polypeptide Group, Batch: AW12192D

1.3 Thermo gravimetric analysis (TGA)

A preliminary study was carried out looking into the thermo gravimetric analysis of the peptides used in this thesis. All moisture and volatiles should come off before 150^{o}C, therefore the mass lost between room temp and 150^{o}C is of interest when determining peptide content.

The runs were performed by Dr Algy Kazlauciunas (School of Chemistry, University of Leeds, UK) and were carried out on a 2050TGA V5.4A instrument at a ramp rate of 10^{o}C per minute in nitrogen gas (200ccs per min). The results of which are presented in the following figures.

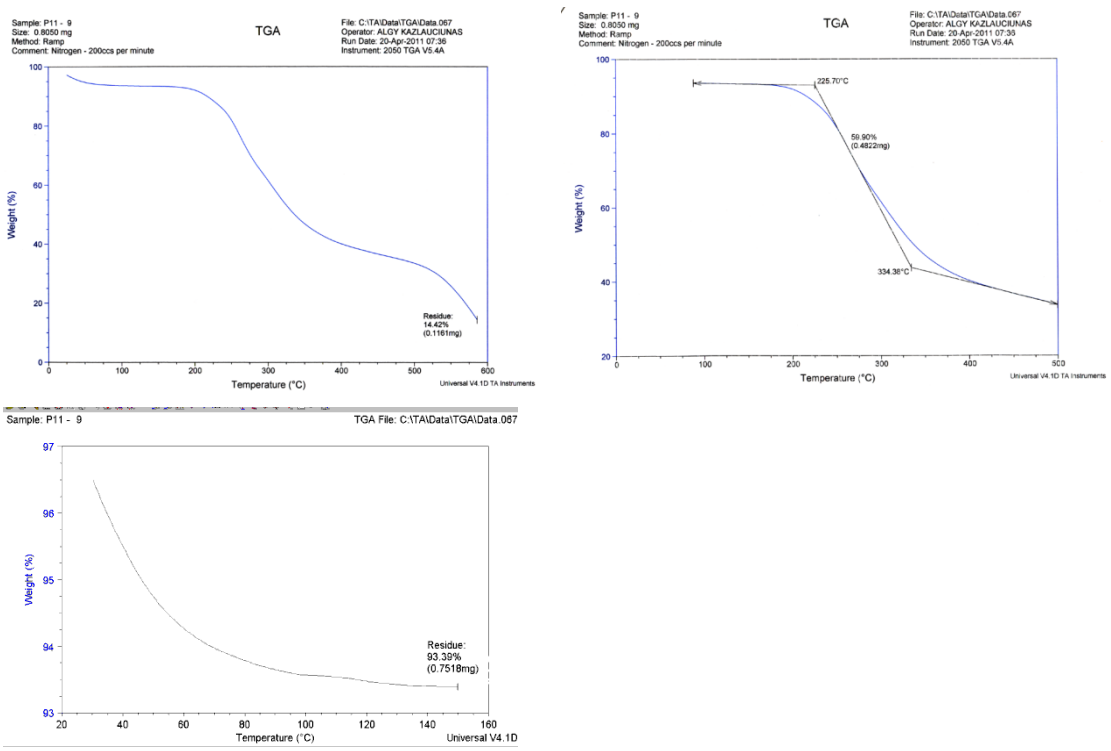


Figure 1 - P_{11}-9 TGA data

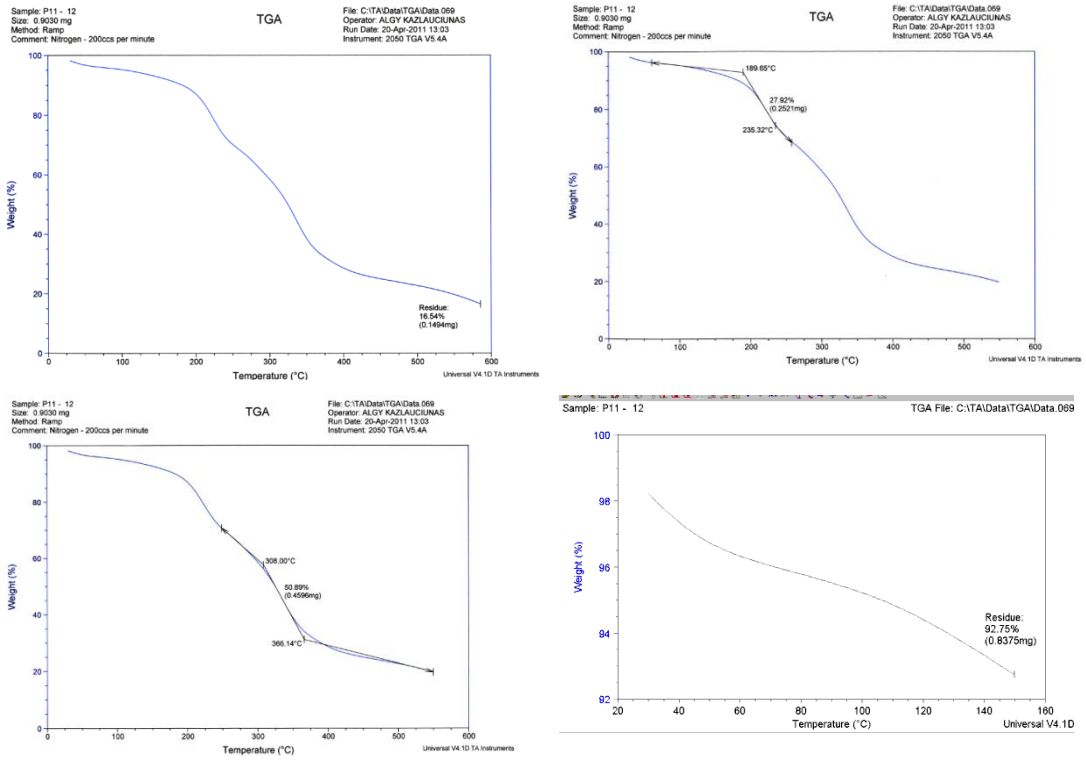


Figure 2 - P_{11}-12 TGA data

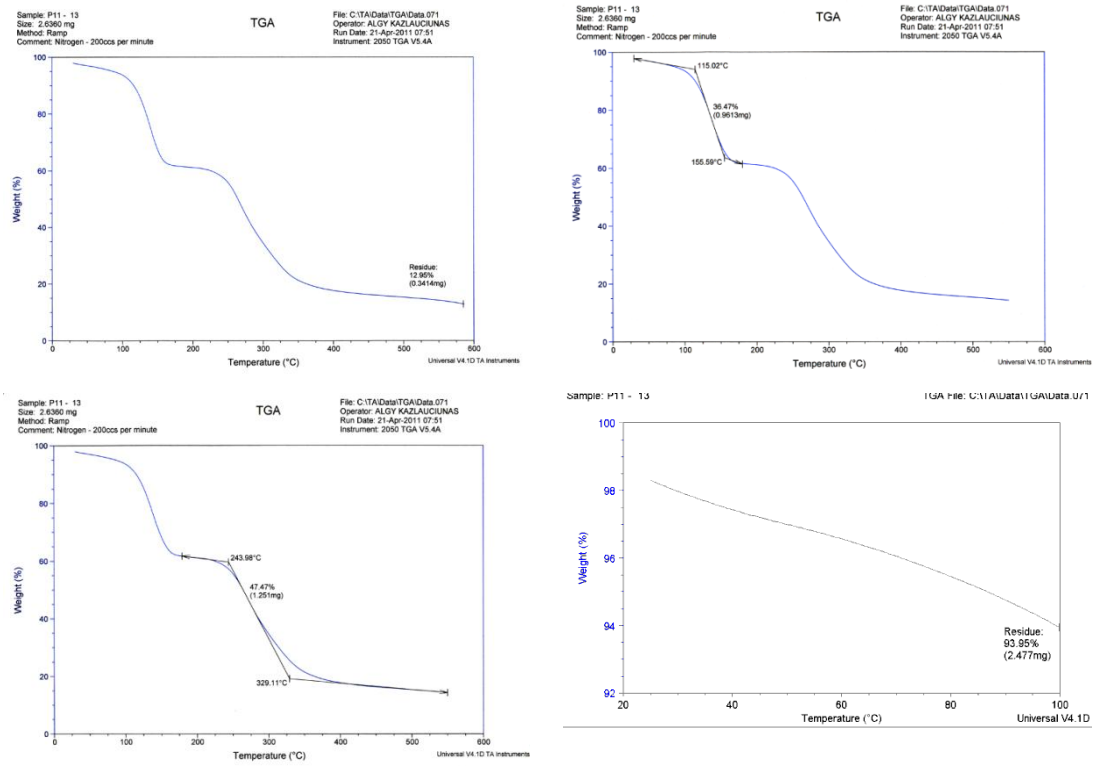


Figure 3 - P_{11}-13 TGA data

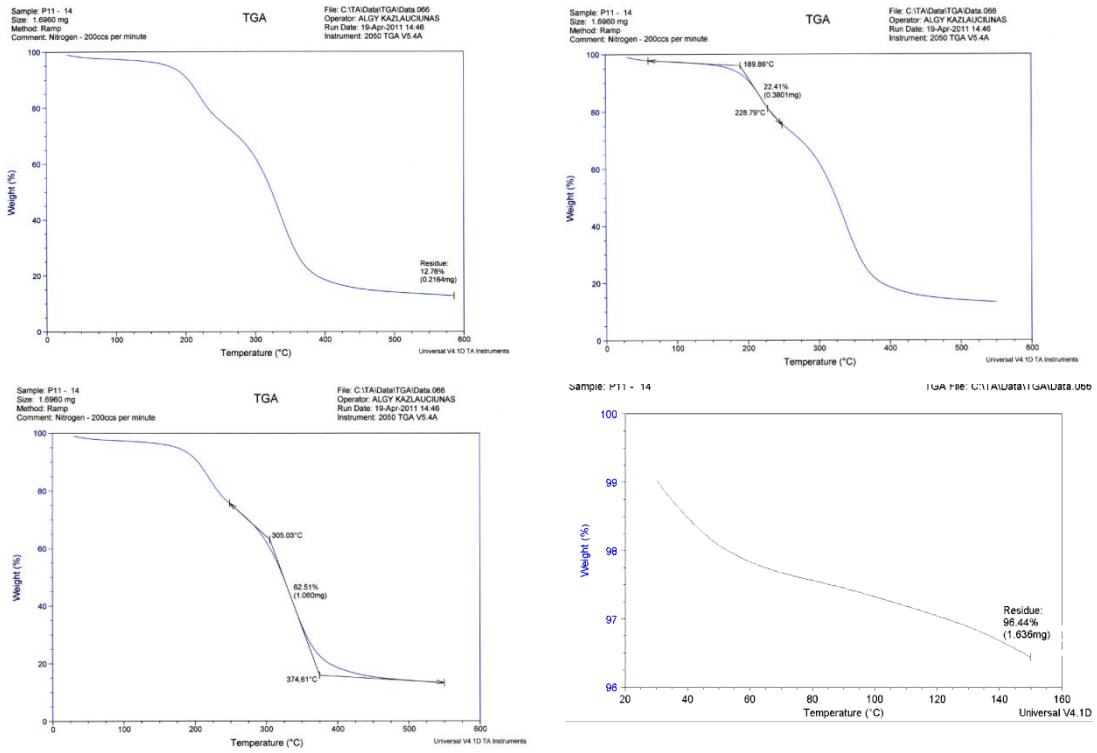


Figure 4 - P_{11}-14 TGA data

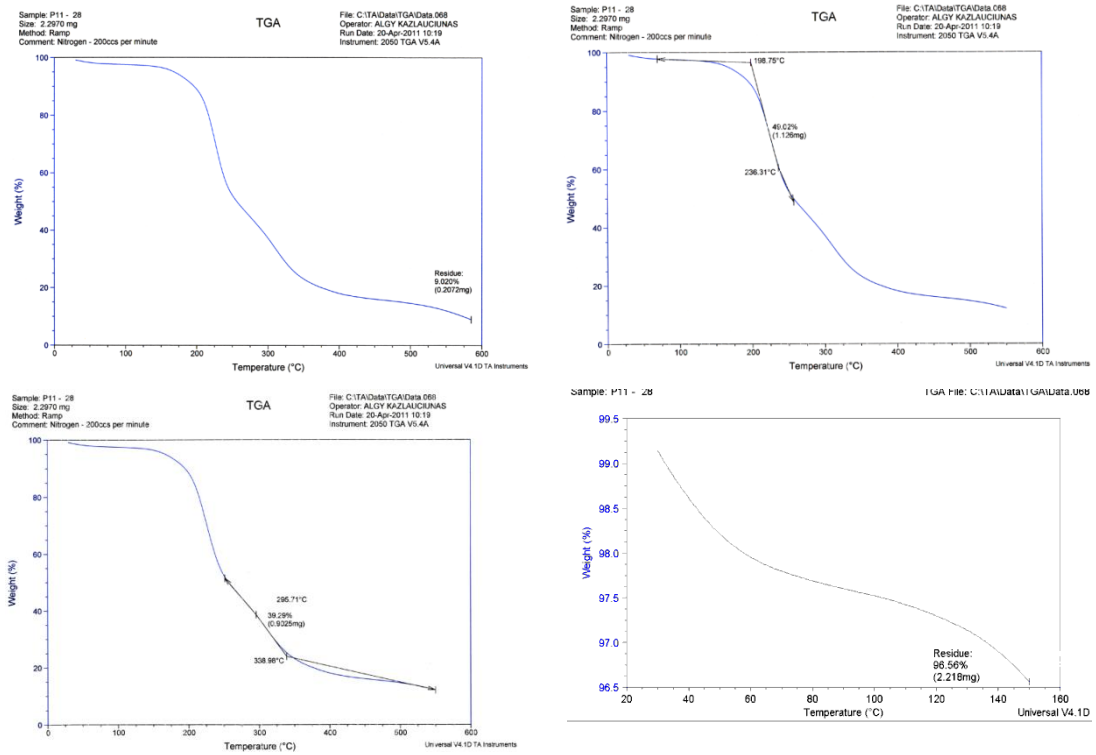


Figure 5 - P_{11}-28 TGA data

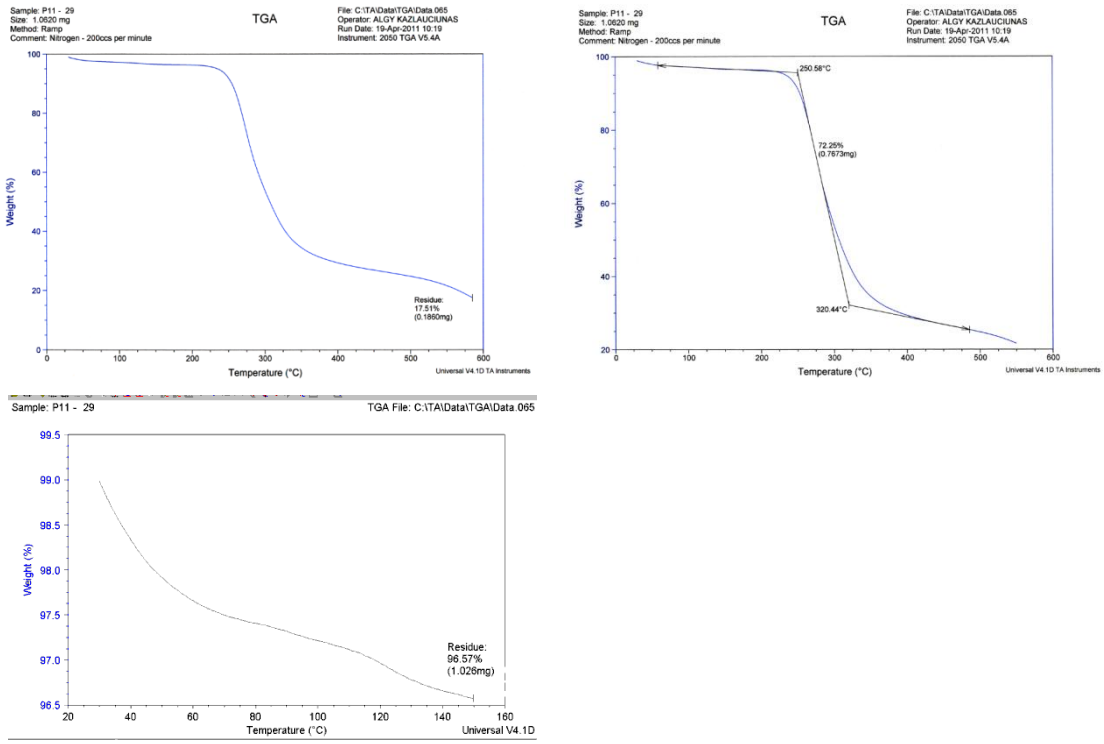


Figure 6 - P_{11}-29 TGA data

1. S. Roux, E. Zekri, B. Rousseau, M. Paternostre, J. C. Cintrat and N. Fay, *Journal of Peptide Science*, 2008, 14, 354-359.

Appendix C

Optimisation of conditions for collection of CD UV spectra in physiological like solutions

CD analysis can be used to look at the conformational change due to the self-assembly of peptides. The presence of a β-sheet band at 218 nm in the spectra will suggest that self-assembly has occurred whereas the presence of the random coil spectral bands will suggest that self-assembly has not taken place. Above a critical concentration, C* self-assembly occurs and so by obtaining the CD spectra for a range of concentrations the C* values determined from NMR studies previously carried out can be confirmed.

Initial CD analysis was carried out using a 1 mm cell with P_{11}-7, P_{11}-9 and P_{11}-12. The samples of P_{11}-7 at pH 7.4, 130mM NaCl in H_2O were not clear solutions with the presence of insoluble precipitate at concentrations higher than 100 μM and so this caused issues with the technique especially at higher concentrations. Samples with precipitate causes light scattering and this effect, affects the transmitted light giving rise to artefacts in the CD spectrum. As the self-assembly in these conditions is what is of interest it is not possible to change them to help with solubility. Therefore CD is not a suitable technique for use on peptide P_{11}-7 at a concentration higher than 100 μM but it can give valuable information at lower peptide concentrations. This will be studied further in the future.

Sodium chloride absorbs strongly over the wavelength range of interest. Blank subtraction was used to minimise this effect, however a strongly absorbing blank is not ideal.[79] The absorbance of the sample is monitored by the trace of the High Tension, HT, voltage (the voltage applied to the photomultiplier). For reliable data, this should remain within specified bounds (generally the voltage should be less than 700 V, but this value will depend on the particular instrument being used).[79]

The high salt concentration in all the peptide samples ran caused the high tension voltage to be high. In all samples the results were ignored over a HT voltage of 700

V but due to the salt effect this often resulted in a wavelength cut off before the wavelengths of interest.

From the work carried out by Kelly et al[79] on Lysozyme it can be seen that potentially changing the buffer system will result in a lower HT voltage and also possibly a better spectrum as is demonstrated by Figure 7 .

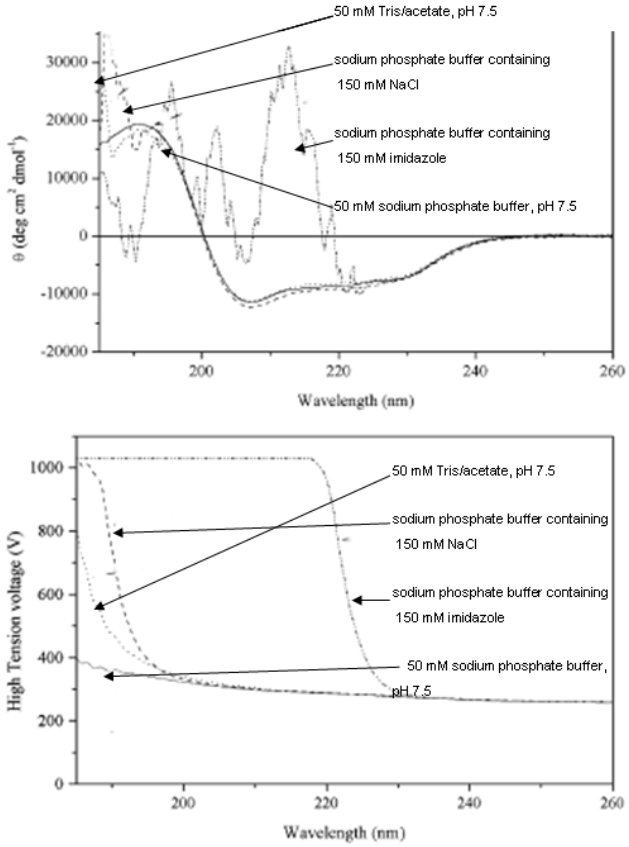


Figure 7 - The effects of buffer components on far UV CD spectra. The upper panel shows the CD spectra and the lower panel the corresponding High Tension voltage traces.[79]

In the present study three different physiological salts were chosen to determine the best salt to use to keep the high tension voltage low. The salts chosen were NaCl, NaF and Na_2HPO_4. The peptide P_{11}-9 was chosen as under the solution conditions the samples are clear solutions and so light scattering would not be a problem. A 1 mm pathlength quartz cuvette was used and the solutions conditions were 130 mM of salt, 0.02% NaN_3 (preservative to prevent bacterial growth), in H_2O at pH 7.4, with a P_{11}-9 concentration of 0.2 mM.

From the blank spectrum and high tension voltage in Figure 8 it can be seen that altering the salt does not greatly affect the HT, however the Na_2HPO_4 blank sample provides the best baseline.

a. b.

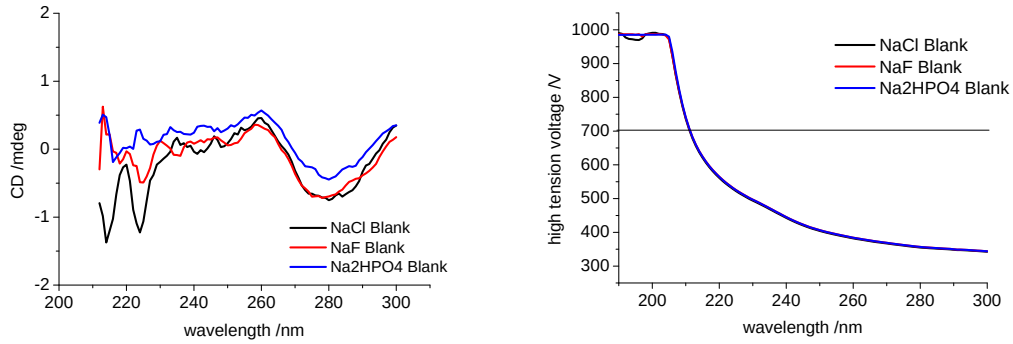


Figure 8 – a. Blank CD spectrum with a HT voltage cut-off of 700 V and b. High Tension Voltage plot for blank samples, 1mm cell, solutions 23 days old

a. b.

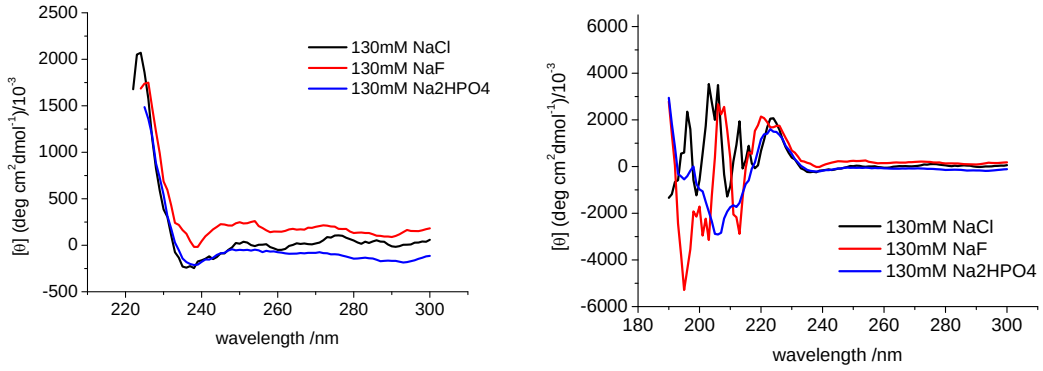


Figure 9 – a. P_{11}-9 CD spectrum with a HT voltage cut-off of 700 V and b. without a HT voltage cut off at 700V, 1mm cell, samples 23 days old

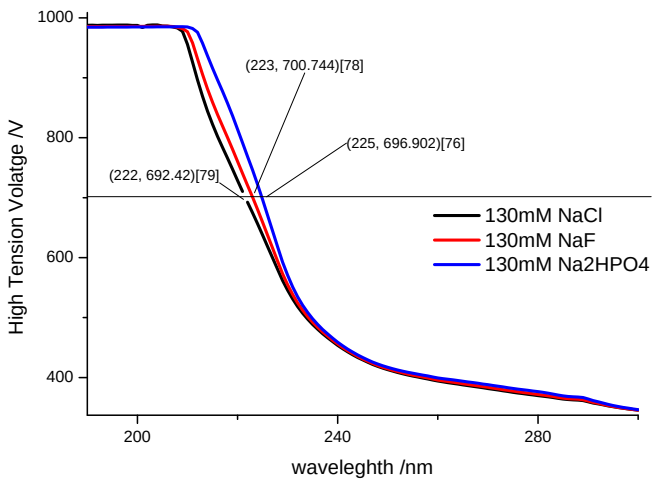


Figure 10 - High tension voltage plot for P_{11}-9, 1mm cell, samples 23 days old.

As demonstrated in Figure 10 once again with the P_{11}-9 samples altering the salt used does not greatly affect the high voltage tension of the system, and again the salt Na_2HPO_4 provides the best baseline.

The spectra collected in all three different salt for P_{11}-9 at a concentration of around 0.2 mM shows that the peptide is in a random coil conformation.

In the experiment described above the same concentration of salt was used in all three salt solutions however NaCl and NaF are 1:1 electrolytes whereas Na_2HPO_4 is a 2:1 electrolyte. This results in slightly different ionic strengths of solution. The ionic strength of a solution can be calculated using Equation 1:

$$I_c = \frac{1}{2}\sum C_B Z_B^2$$

Equation 1

Where: I_c = ionic strength of the solution, C_B = concentration of the solution / M, Z_B = charge number of the ion.

The ionic strength of NaCl and NaF in the above samples is 0.13 M and for Na_2HPO_4 the ionic strength is 0.39 M. The ionic strength of the Na_2HPO_4 solution is

much greater than that of the other two salts, this will potentially affect the self-assembly and C* concentration of the peptide but it should not affect the high tension voltage. So for this study it is not a problem, if in future it is used as the physiological salt in samples then a concentration that equals an ionic strength of 0.13 M will be used.

The effect of salt absorbance can be decreased by using a cell with a smaller pathlength. In the initial experiments run a 1 mm cell was used, however, it may be possible to look at a smaller pathlength by using a demountable cell, although a study by Miles et al has shown that the pathlength of such cells can vary by up to 50% as stated by the manufactures.[104] To investigate the effect of changing to a smaller cell the samples above of the three different salts, blank and 0.2 mM P_{11}-9 were studied using a demountable cell with a nominal pathlength of 0.1 mm. The CD data for this can only be used qualitatively rather than quantitatively as the molar ellipticity cannot be quoted as to calculate it the accurate cell pathlength is required.

a. b.

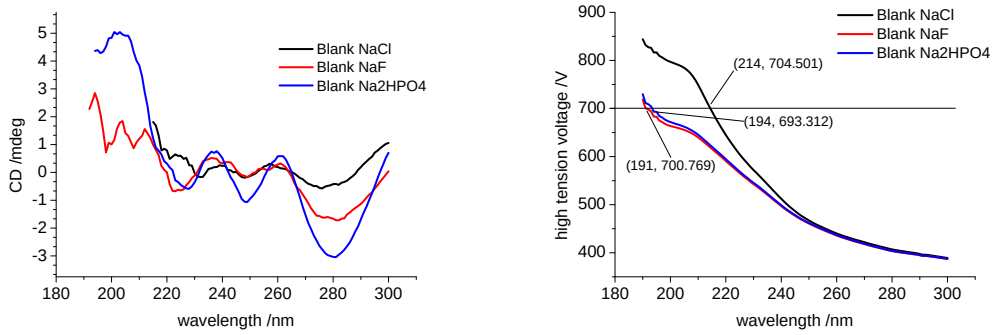
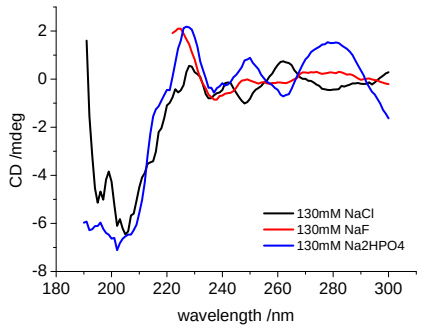


Figure 11 – a. Blank CD spectrum with a HT voltage cut-off of 700 V and b. High Tension Voltage plot for blank samples, demountable cell, solutions 23 days old,

From the blank spectrum and HT plot in Figure 11 it can be seen that by changing the salt when using the demountable cell causes a change in the HT voltage and baseline observed. For that solutions containing NaF and Na_2HPO_4 the high tension cut off point is at the lowest wavelength measured and so the entire CD spectrum can be used. With the solutions containing NaCl this is not the case the HT becomes greater than 700 V at around 215 nm and so the CD spectrum below this wavelength is ignored. The baseline for all three samples is poor.

a.



b.

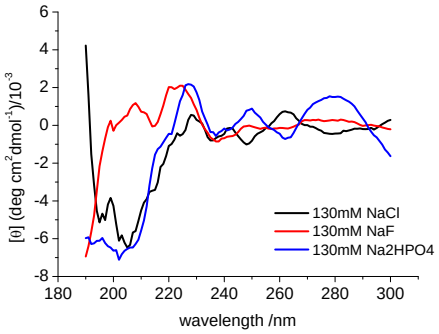


Figure 12 – a. P_{11}-9 CD spectrum with a HT voltage cut-off of 700 V and b. without a HT voltage cut off at 700V, demountable cell, samples 23 days old.

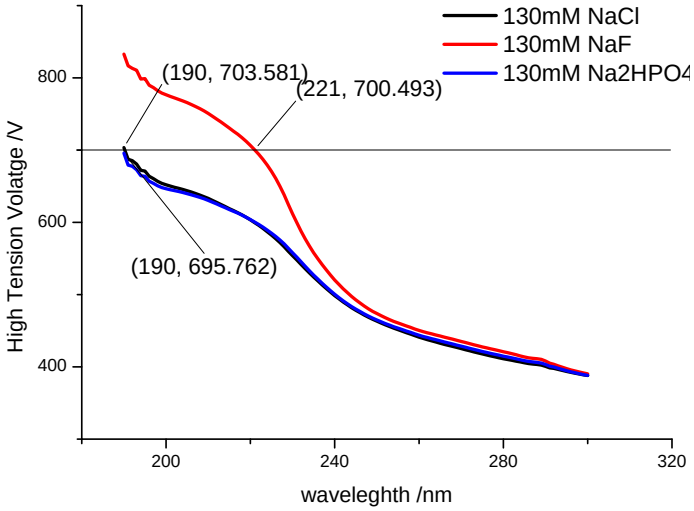


Figure 13 - High tension voltage plot for P_{11}-9, demountable cell, 23 days old

From the P_{11}-9 spectrum and HT plot in Figure 12 and Figure 13 it can be seen that by changing the salt when using the demountable cell again there is a change in the HT and baseline observed. However this time for these solutions containing NaCl and Na_2HPO_4 the high tension cut off point is at the lowest wavelength measured and so the entire CD spectrum can be used, while with the sample containing NaF this is not the case as the HT becomes greater than 700 V at around 220 nm and so the CD spectrum below this wavelength is ignored.

From looking at the blank and P_{11}-9 CD and HT plots it seems that the salt, Na_2HPO_4, would be the best to use to insure that all the wavelengths observed can

be used. However the baseline for all three salts in solution for the P_{11}-9 samples is poor, this is thought to be a result of the blank subtraction carried out. During processing the blank spectra is subtracted from the sample spectra to remove any solution absorption bands, but as the pathlength of the cell depends on how it is assembled it will not be the same for both the blank and the sample.

Even though the demountable cell offers the possibility of looking at higher concentration samples and improves the HT voltage when a high salt concentration is used it does not provide good quality spectra. It may still be possible to use the spectra to determine the presence of different conformations if the structural bands are larger than that of the noise in the baseline but this is not ideal. For the samples analysed above the spectra would suggest that for all three salt solutions P_{11}-9 at around 0.2 mM is in the random coil conformation as above with the 1 mm cell.

From the above study it can be concluded that experiments for quantitative studies in physiological like solutions carried out using CD, should be carried out using a 1 mm cell for the most accurate results and using the following solution conditions; 43 mM (0.13 M ionic strength) Na_2HPO_4 in H_2O with 0.02% NaN_3 to prevent bacterial growth at pH 7.4.

Appendix D

Supplementary rheological data on peptide gels

1.4 Equilibration time

All experiments were carried out on a sample of P_{11}-9 20 mg/ml in PBS with 0.02% NaN_3 at pH 7.4. In order to look at the effect of the equilibration time on the results, different wait times after loading prior to running the experiments was investigated. The results for the amplitude sweeps are presented in Figure 14 and for the frequency sweeps in Figure 15.

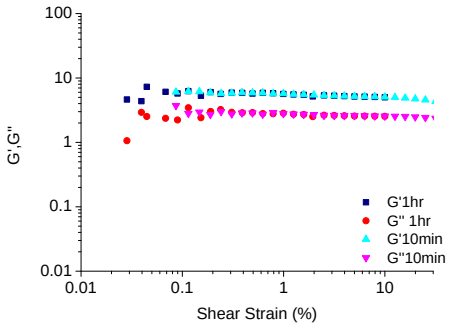


Figure 14 – P_{11}-9 comparison of 1hr wait and 10 min wait time after loading on amplitude sweeps. Strain controlled 0.01-100%, frequency 1Hz, temp 25°C

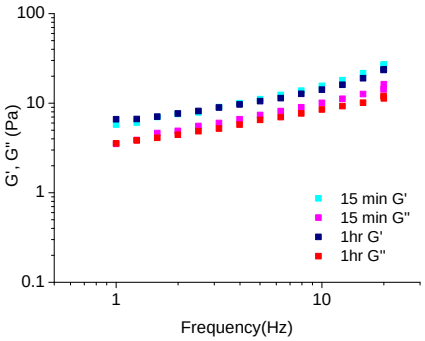


Figure 15 – P_{11}-9 comparison of 1 hour wait and 15 min wait after loading on frequency sweeps. Strain controlled 0.5%, frequency 1 - 20 Hz, temp 25°C

The two different wait times made no difference to the results and so after loading of a sample a wait time of 15 minutes was chosen. The no difference in results for the two samples also suggested good reproducibility and this was investigated further with a selection of peptides.

1.5 Reproducibility

In order to check the reproducibility of the results the same experiments were run on two different samples. The results for P_{11}-4, P_{11}-28/29 and P_{11}-12 are presented in Figure 16, Figure 17 and Figure 18 respectively.

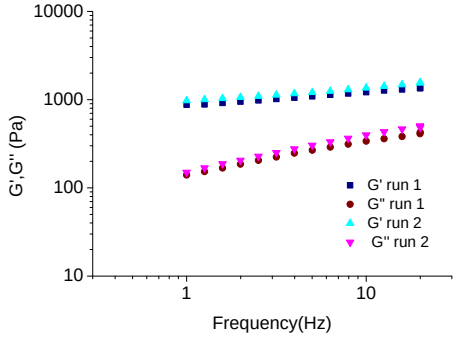


Figure 16 – P_{11}-4 frequency sweeps carried out on two separate samples. Frequency 1 - 20 Hz, strain controlled 0.15%, temperature 25°C.

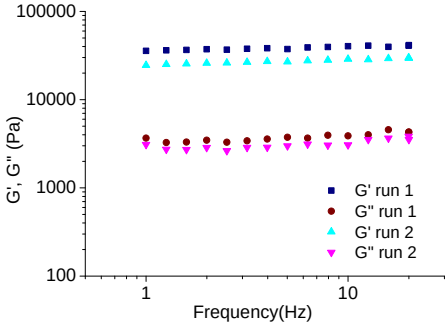


Figure 17 - P_{11}-28/29 frequency sweeps carried out on two separate samples. Frequency 1 - 20 Hz, strain controlled 0.25%, temperature 25°C.

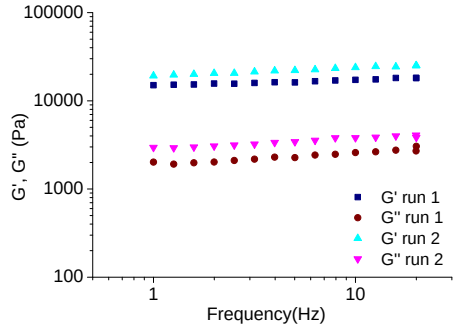


Figure 18 - P_{11}-12 frequency sweeps carried out on two separate samples. Frequency 1 - 20 Hz, strain controlled 0.25%, temperature 25°C.

1.6 LVER checks

In order to check that the frequency sweeps carried out were in the LVER region amplitude sweeps were run again in a stress controlled mode.

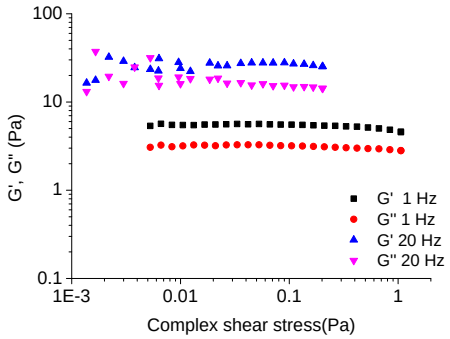


Figure 19 – P_{11}-9 amplitude sweeps carried out at 1 Hz and 20 Hz, stress controlled 0.005 - 1 Pa, temperature 25°C.

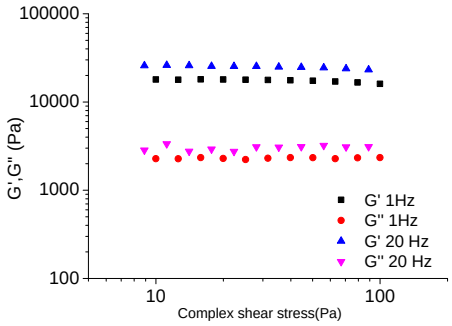


Figure 20 – P_{11}-12 amplitude sweeps carried out at 1 Hz and 20 Hz, stress controlled 10 - 100 Pa, temperature 25°C.

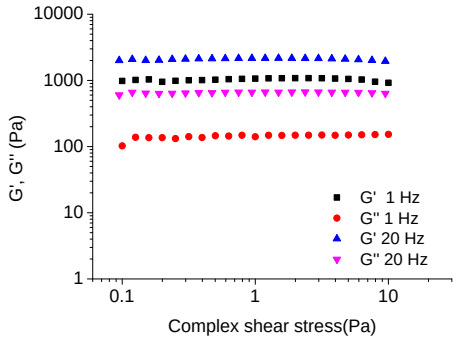


Figure 21 – P_{11}-4 amplitude sweeps carried out at 1 Hz and 20 Hz, stress controlled 0.1 - 10 Pa, temperature 25°C.

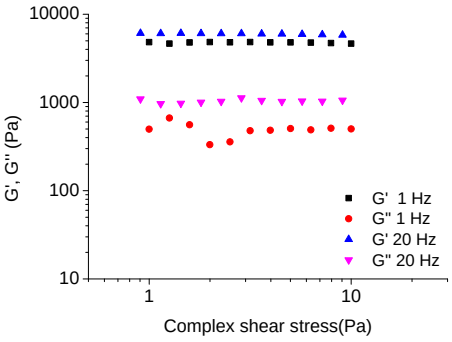


Figure 22 – P_{11}-8 amplitude sweeps carried out at 1 Hz and 20 Hz, stress controlled 1 - 10 Pa, temperature 25°C.

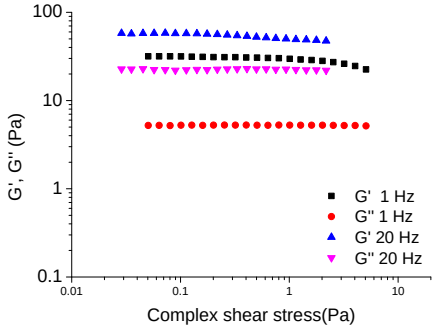


Figure 23 – P_{11}-13/14 amplitude sweeps carried out at 1 Hz and 20 Hz, stress controlled 0.05 - 5 Pa, temperature 25°C.

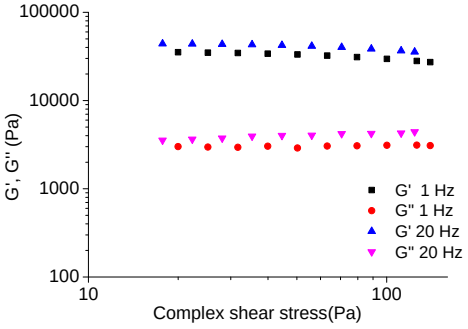


Figure 24 – P_{11}-28/29 amplitude sweeps carried out at 1 Hz and 20 Hz, stress controlled 10 - 140 Pa, temperature 25°C.

Appendix E

Peptide:GAG mixing study

1. Visual observations

1.1 P_{11}-9

The following figures show how the physical appearance of the gels changes over time:

Figure 25 – samples 1 day old. From left to right P11-9:GAG 1:1, 1:0.5, 1:0.2, 1:0.1 and P11-9 control

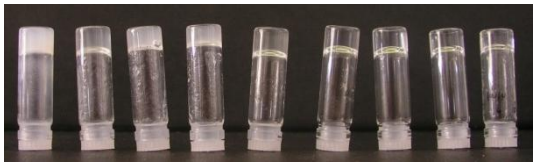


Figure 26 - samples 3 days old. From left to right P11-9:GAG 1:10, 1:4, 1:3, 1:2, 1:1, 1:0.5, 1:0.2, 1:0.1 and P11-9 control

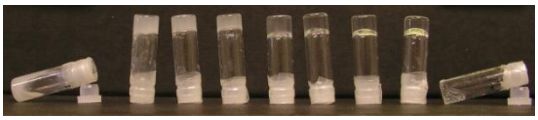


Figure 27 - samples 3 months old. From left to right P11-9:GAG 1:10, 1:4, 1:3, 1:2, 1:1, 1:0.5, 1:0.2, 1:0.1 and P11-9 control

Figure 28 - samples 7.5 months old. From left to right P11-9:GAG 1:4, 1:3, 1:1, 1:0.5, 1:0.2, 1:0.1

Figure 29 - samples 1 year and 4 months old. From left to right P11-9:GAG 1:10, 1:4, 1:3, 1:2, 1:1, 1:0.5, 1:0.2, 1:0.1 and P11-9 control

P_{11}-9 : GAG	Time taken for gel to form	Appearance	Other observations
1:0.1	minutes	clear self-supporting gel same after 3 months same after 222 days 1yr 4mths clear self-supporting gel	forms a viscous liquid upon shearing birefringent
1:0.2	minutes	clear self-supporting gel same after 3 months same after 222 days 1yr 4mths clear liquid with white precipitate	forms a viscous liquid upon shearing birefringent
1:0.5	minutes	clear self-supporting gel with a very small amount of white precipitate after 3 months - clear self-supporting gel after 222 days clear viscous liquid with white precipitate 1yr 4mths clear liquid with white precipitate	forms a viscous liquid upon shearing birefringent
1:1	minutes	slightly cloudy self-supporting gel same after 3 months slightly cloudy self supporting gel 1yr 4mths cloudy gel	forms a viscous liquid upon heavy shearing birefringent
1:2	seconds when still warm	cloudy self supporting gel with a lot of gel like precipitate on the vial walls same after 3 months 1yr 4mths clear liquid some cloudy self-supporting gel	a lot of heating and vortexing needed to mix and dissolve gag forms a cloudy solution on heating and quickly reforms previous gel with slight cooling
1:3	seconds when	cloudy self supporting gel with a lot of	a lot of heating

	still warm	gel like precipitate on the vial walls same after 3 months after 222 days self supporting gel but top part cloudy and bottom part clear 1yr 4mths some clear liquid some white viscous gel	and vortexing needed to mix and dissolve gag forms a cloudy solution on heating and quickly reforms previous gel with slight cooling
1:4	seconds when still warm	less cloudy self-supporting gel than 1:2 and 1:3 with some gel like precipitate on the vial walls same after 3 months	a lot of heating and vortexing needed to mix and dissolve gag forms a cloudy solution on heating and quickly reforms previous gel with slight cooling
1:10	seconds when still warm	very cloudy self-supporting gel with no precipitate on the vial walls after 3 months- cloudy viscous liquid 1yr 4mths cloudy self-supporting gel	a lot of heating and vortexing needed to mix and dissolve gag forms a cloudy solution on heating and quickly reforms previous gel with slight cooling (same after 3 months)
Peptide control	minutes	clear self-supporting gel same after 3 months 1yr 4mths clear self-supporting gel	forms a viscous liquid upon light shearing (same after 3 months) birefringent

Table 14 – P_{11}-9:GAG mixing study observations

1.2 P_{11}-12

The following figures show how the physical appearance of the gels changes over time:

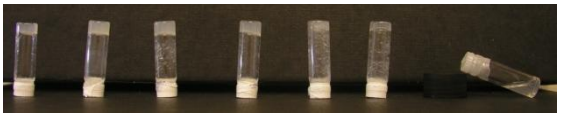


Figure 30 - samples 1 week old. From left to right P11-12 control 1:10, 1:4, 1:3 1:2, 1:1 and GAG control

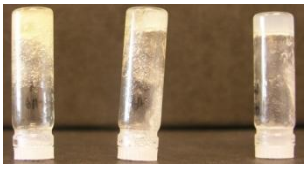


Figure 31 – samples 1 day old. From left to right 1:100, 1:50, 1:20

Figure 32 - samples 5 days old. From left to right 1:100, 1:50, 1:20

Figure 33 – from left to right GAG control, 1:10, 1:4, 1:3, 1:2, 1:1, 1:0.5, 1:0.2, 1:0.1, P11-12 control. P11-12 control, 1:1, 1:2, 1:3, 1:4, 1:10, Gag control samples all 1 month old and 1:0.1, 1:0.2, 1:0.5 samples 2 weeks old.

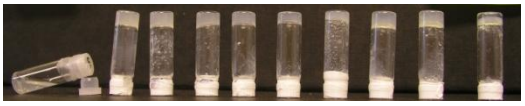


Figure 34 – Samples 4 months old. From left to right GAG control, 1:10, 1:4, 1:3, 1:2, 1:1, 1:0.5, 1:0.2, 1:0.1, P11-12 control. (1:0.1, 1:0.2, 1:1, 1:2 samples all reheated after 1 month)

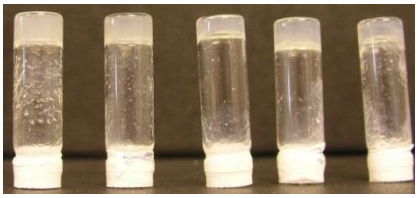


Figure 35 – samples 10 months old. From left to right 1:4, 1:3, 1:1, 1:0.2, 1:0.1

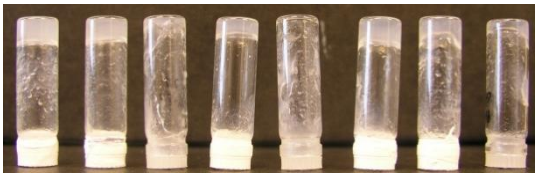


Figure 36 – samples 1 year and 5 months old. From left to right 1:4, 1:3, 1:2, 1:1, 1:0.5, 1:0.2, 1:0.1, P11-12 control

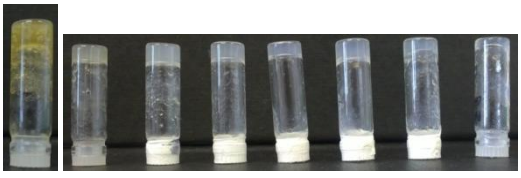


Figure 37 –From left to right 1:100, 1:50, 1:20, 1:4, 1:3, 1:1, 1:0.2, 1:0.1, P11-12 control. Samples 1:100, 1:50 and 1:20 6.5 months old rest of samples 1 year and 11months old.

P_{11}-12 : GAG	Time taken for gel to form	Appearance	Other observations	Picture	
1:0.1	2 hrs no gel	after 72 hours cloudy solution with gel like precipitate on the walls same after 1 month same after 4 months after 8 months cloudy self-supporting gels with bits of gel on sides of the vial walls 1yr 5 mth – slightly cloudy self-supporting gel	after 2 weeks sample was reheated and upon cooling within seconds a cloudy self supporting gel formed	 initial solution with gel like precipitate on the walls	 after reheating after 1 month

1:0.2	2 hrs no gel	after 72 hours cloudy solution with gel like precipitate on the walls of the sample vial same after 1 month same after 4 months after 8 months cloudy self-supporting gels with bits of gel on sides of the vial walls 1yr 5 mth – slightly cloudy self-supporting gel	after 2 weeks sample was reheated and upon cooling within seconds a cloudy self supporting gel formed	 initial solution with gel like precipitate or the walls	 gel after reheating after 1 month
1:0.5	minutes	cloudy gel with precipitate same after 4 months 1yr 5 mth – slightly cloudy self-supporting gel	upon reheating = cloudy solution		

1:1	seconds	cloudy gel with precipitate after 4 months (with reheating after 1 month) cloudy self-supporting gel after 8 months cloudy self-supporting gels with bits of gel on sides of the vial walls 1yr 5 mth – slightly cloudy self-supporting gel	upon reheating = cloudy solution after 2 weeks gel stops being self-supporting after 1 month sample was reheated and upon cooling within seconds a cloudy self supporting gel formed	   initial gel formed 2 weeks old 1 month after reheating
1:2	seconds	cloudy gel with precipitate 1yr 5 mth – slightly cloudy self-supporting gel	upon reheating = cloudy solution after 2 weeks gel stops being self-supporting after 1 month sample was reheated and upon cooling within seconds a cloudy self supporting gel formed	   initial gel formed 2 weeks old 1 month after reheating

-

1:3	seconds	cloudy gel with precipitate same after 1 ½ months same after 4 months after 8 months cloudy self-supporting gels with bits of gel on sides of the vial walls 1yr 5 mth – slightly cloudy self-supporting gel	upon reheating = cloudy solution	
1:4	seconds	cloudy gel with precipitate same after 1 ½ months same after 4 months after 8 months cloudy self-supporting gels with bits of gel on sides of the vial walls 1yr 5 mth – slightly cloudy self-supporting gel	upon reheating = cloudy solution	

1:10	seconds	cloudy gel same after 1 ½ months same after 4 months	upon reheating = cloudy solution	
1:20	spontaneous	Cloudy gel	5 days - Cloudy self supporting gel with gel on walls of vial 6.5mth - Cloudy self supporting gel with gel on walls of vial	
1:50	spontaneous	Opaque thick gel	Took a long time for all the gag to mix/dissolve 5 days - Cloudy self supporting gel with gel on walls of vial, slightly yellow in colour 6.5 mths - Cloudy self supporting gel with gel	

			on walls of vial		
1:100	spontaneous	Opaque thick gel	Took a long time for all the gag to mix/dissolve 5 days - Cloudy self supporting gel with gel on walls of vial, yellow in colour, lots of bubbles 6.5 mth yellow gel	 Day of prep	 6.5 mth

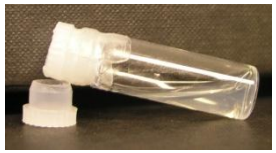
Peptide control	minutes	cloudy gel same after 1 ½ months after 4 months self-supporting gel with two layers one layer is clear and the other cloudy.	upon reheating = clear solution		 self-supporting gel with 2 layers 1=clear 1=cloudy
Gag Control	no gel formed	cloudy viscous liquid same after 1 ½ months same after 4 months	upon reheating = cloudy solution		

Table 15 – P_{11}-12: GAG mixing study observations

1.3 P_{11}-4

The following figures show how the physical appearances of the gels change over time:

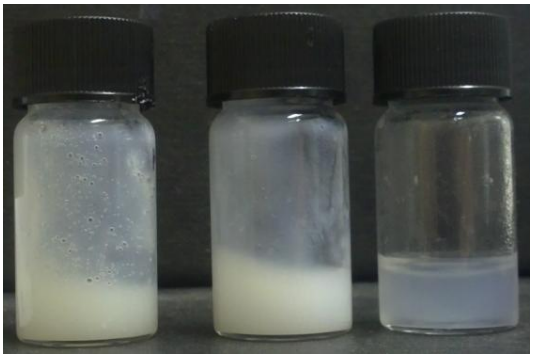


Figure 38 - samples on day of prep after heating to remove bubbles. From left to right, P_{11}-4:GAG 1:10, 1:2 and P_{11}-4 control.

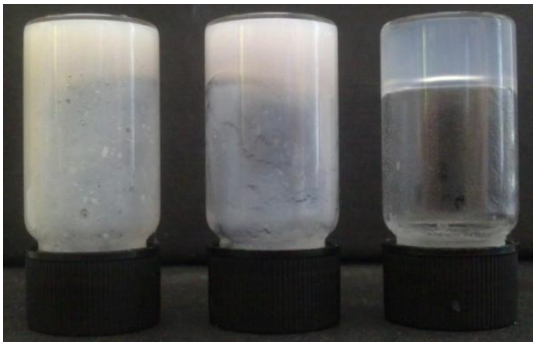


Figure 39 - samples 2 days old. From left to right From left to right, P_{11}-4:GAG 1:10, 1:2 and P_{11}-4 control.

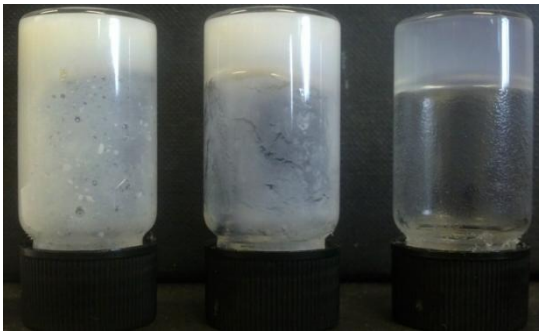


Figure 40 - samples 10 days old. From left to right From left to right, P11-4:GAG 1:10, 1:2 and P11-4 control.

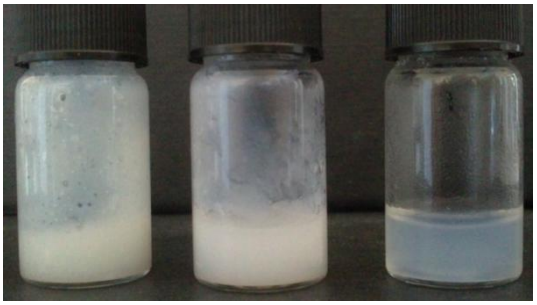


Figure 41 - samples 20 days old. From left to right From left to right, P_{11}-4:GAG 1:10, 1:2 and P_{11}-4 control. Still self-supporting gels.

P11-4: GAG	Time taken for gel to form	Appearance	Other observations
1:2	Secs-mins	Cloudy self-supporting gel	Cloudy viscous liquid when warm Thicker than that of control Few bubbles upon mixing
1:10	Secs	Cloudy/white self-supporting gel	Took a while to get gag to dissolve Thicker than that of 1:2 Lots of bubbles took lots of heating to remove bubbles
control	Mins - hours	Slightly cloudy self-supporting gel	

Table 16 – P_{11}-4:GAG mixing study observations

1.4 P_{11}-8

The following figures show how the physical appearances of the gels change over time:

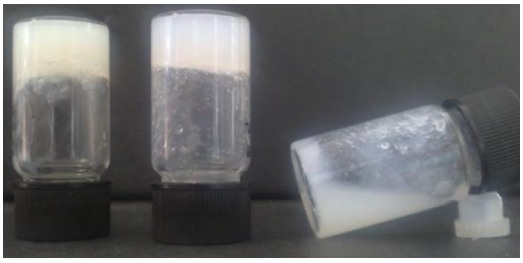


Figure 42 – Day of preparation. From left to right P_{11}-8:GAG 1:10, 1:2 and P_{11}-8 control

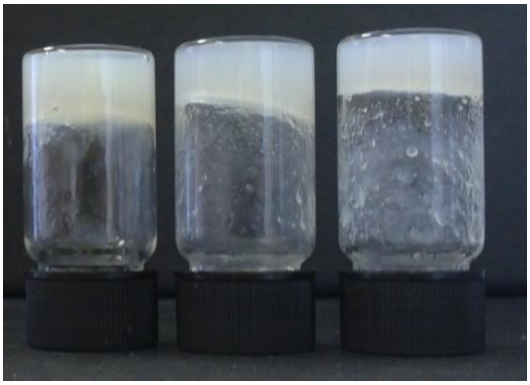


Figure 43 – Samples 5 days old. From left to right P_{11}-8:GAG 1:10, 1:2 and P_{11}-8 control

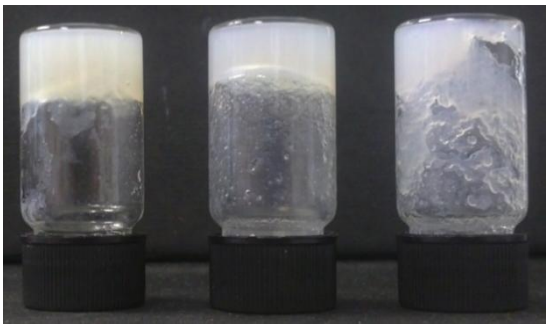


Figure 44 -Samples 13 days old. From left to right P_{11}-8:GAG 1:10, 1:2 and P_{11}-8 control

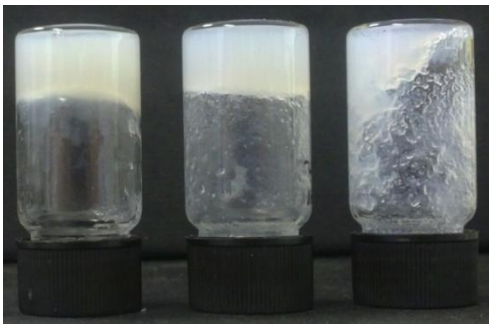


Figure 45 - Samples 40 days old. From left to right P_{11}-8:GAG 1:10, 1:2 and P_{11}-8 control

P11-8 : GAG	Time taken for gel to form	Appearance	Other observations
1:2	Secs	Cloudy slightly bitty self-supporting gel	13 days cloudy self supporting gel with bitty gel visible on walls 40 days cloudy bitty self-supporting gel that's less bitty than control 90 days cloudy self-supporting gel
1:10	Secs	Cloudy self-supporting gel	13 days cloudy self supporting gel 40 days cloudy "smooth" self-supporting gel 90 days cloudy self-supporting gel
control	days	Cloudy liquid that forms a weak self supporting gel after a few days	13 days bitty cloudy self-supporting gel with liquid phase 40 days cloudy very bitty self-supporting gel with liquid phase 90 days cloudy self-supporting gel with some liquid phase

Table 17 – P_{11}-8:GAG mixing study observation

1.5 P_{11}-13:GAG + P_{11}-14

The following figures show how the physical appearances of the gels change over time:

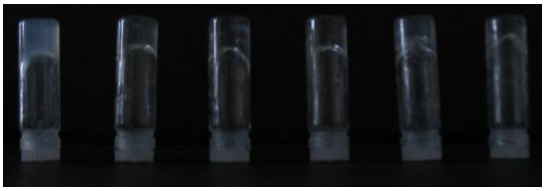


Figure 46 – 3 days old. From left to right P_{11}-13/14:GAG 1:10, 1:5, 1:2 1:1, 1:0.1 and P_{11}-13/14 control

Figure 47 - 19 days old. From left to right P_{11}-13/14:GAG 1:10, 1:5, 1:2, 1:1, 1:0.1 and P_{11}-13/14 control

Figure 48 - 47 days old. From left to right P_{11}-13/14:GAG 1:20, 1:10, 1:5, 1:2, 1:1, 1:0.1 and P_{11}-13/14 control

Figure 49 – 6 months old. From left to right P_{11}-13/14:GAG 1:20, 1:10, 1:5, 1:2, 1:1, 1:0.1 and P_{11}-13/14 control.

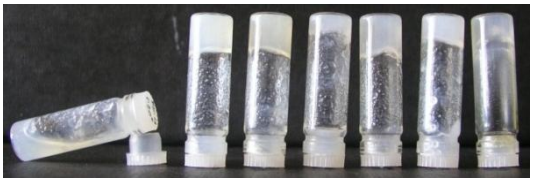


Figure 50 - 10 months old. From left to right P_{11}-13/14:GAG 1:20, 1:10, 1:5, 1:2, 1:1, 1:0.1 and P_{11}-13/14 control. All samples heated, vortexed and then cooled after 6 months.

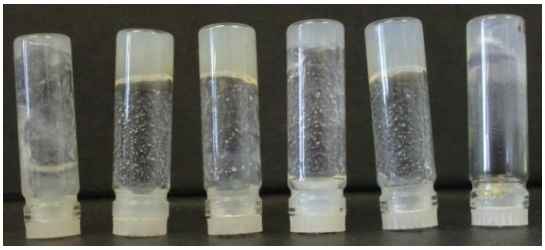


Figure 51 - 1 year 5 months old. From left to right P_{11}-13/14:GAG 1:20, 1:10, 1:5, 1:2, 1:1 and P_{11}-13/14 control.

-

P11-13: GAG+14	Time taken for gel to form	Appearance	Other observations
1:0.1	instantaneous	clear self-supporting gel at 19 days = cloudy self supporting gel	when left overnight as just P11-13 and gag formed clear gel that upon light shearing became a viscous liquid 19 days - turnt into cloudy liquid upon vortexing after heating and then cooling still cloudy liquid 37 days – cloudy bitty gel in clear liquid with gel up edges of the vial wall 6 mth bitty gel on vial walls Sample heated and vortexed→cloudy bitty liquid 10 mth - cloudy self-supporting gel +gel on walls
1:1	instantaneous	clear self-supporting gel at 19 days = cloudy self supporting gel	when left overnight as just P11-13 and gag = clear liquid 19 days - turnt into cloudy liquid upon vortexing after heating and then cooling still cloudy liquid 37 days – cloudy bitty gel in clear liquid with gel up edges of the vial wall

			6 mth bitty gel on vial walls some liquid phase Sample heated and vortexed→cloudy bitty liquid 10 mth - cloudy self-supporting gel +gel on walls
1:2	instantaneous	clear self-supporting gel at 19 days = cloudy self supporting gel → cloudy liquid upon light shearing	when left overnight as just P11-13 and gag = clear liquid 19 days after heating and then cooling still cloudy liquid 37 days – cloudy bitty gel in clear liquid with gel up edges of the vial wall 6 mth bitty gel on vial walls some liquid phase Sample heated and vortexed→cloudy bitty liquid 10 mth - cloudy self-supporting gel +gel on walls

1:5	instantaneous	clear self-supporting gel at 19 days = clear self supporting gel	when left overnight as just P11-13 and gag = clear liquid 19 days - upon vortexing after heating and then cooling still clear self supporting gel not birefringent 37 – days clear self-supporting gel not birefringent 6^{th} mth cloudy self supporting gel upon shearing becomes viscous Sample heated and vortexed→cloudy bitty liquid 10mth cloudy self-supporting gel +gel on walls with slight yellow colour
1:10	instantaneous	cloudy self-supporting gel at 19 days = cloudy liquid with precipitate on walls	when left overnight as just P11-13 and gag = clear liquid 19 days - upon vortexing after heating and then cooling still cloudy gel 37 days– cloudy bitty gel in clear liquid with gel up edges of the vial wall 6 mth – small amount of cloudy slef-supporting gel plus gel on vial walls Sample heated and vortexed→cloudy bitty liquid 10 mth cloudy self-supporting gel +gel on walls with slight yellow colour

1:20	seconds	cloudy self- supporting gel	13 days- cloudy self supporting gel 6^{th} mth cloudy self supporting gel upon shearing becomes viscous Sample heated and vortexed→cloudy bitty liquid 10 mth viscous liguid/gel
control	instantaneous	clear self-supporting gel at 19 days = clear self supporting gel	19 days - upon vortexing after heating and then cooling still clear self supporting gel not birefringent 37 days – cloudy self-supporting gel 10 mth cloudy self-supporting gel

Table 18 - P_{11}-13:GAG+P_{11}-14 mixing study observations

1.6 P_{11}-14:GAG+P_{11}-13

The following figures show how the physical appearances of the gels change over time:

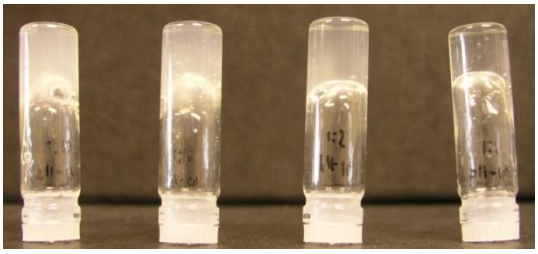


Figure 52 – Day of preparation. From left to right P_{11}-14:GAG+P_{11}-13 1:20, 1:10, 1:5 and 1:1

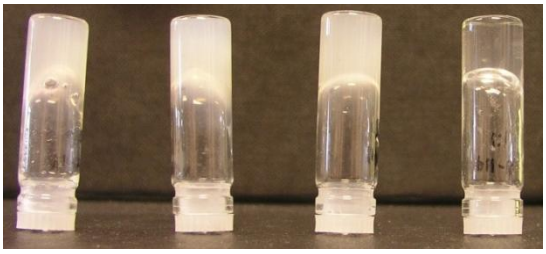


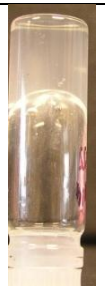
Figure 53 – 13 days old. From left to right P_{11}-14:GAG+P_{11}-13 1:20, 1:10, 1:5 and 1:1

Figure 54 – 5 months old. From left to right P_{11}-14:GAG+P_{11}-13 1:20, 1:10, 1:5, 1:1 and P_{11}-13/14 control

Figure 55 - 9 months old. From left to right P_{11}-14:GAG+P_{11}-13 1:20, 1:10, 1:5, 1:1 and P_{11}-13/14 control

Figure 56 – 1 year and 3.5 months old. From left to right P_{11}-14:GAG+P_{11}-13 1:20, 1:10, 1:5, 1:1 and P_{11}-13/14 control

P_{11}-14:GAG+13	Time taken for gel to form	Appearance	Other observations	Picture	
1:1	seconds	clear gel	addition of gag to P_{11}-14 → cloudy liquid formed gel upon vortexing, upon light shearing gel becomes viscous not self-supporting 13 days clear self supporting birefringent gel doesn't break upon vortexing 5mths – clear self-supporting gel Heated ~80C and vortexed → stil clear self-supporting gel 9mths clear self-supporting gel + very small amount of clear liquid 1yr 3.5mths slightly cloudy self-supporting gel	 on day of prep	 13 days old

1:5	seconds	slightly cloudy gel	addition of gag to P_{11}-14 → clear liquid formed gel upon vortexing, upon light shearing gel becomes viscous not self-supporting 13 days – cloudy self supporting birefringent gel doesn't break upon vortexing 5mths – cloudy self-supporting gel Heated ~80C and vortexed → stil cloudy self-supporting gel 9mths cloudy self-supporting gel 1yr 3.5mths liquid phase with some bitty gel on walls	 on day of prep	 13 days old
1:10	seconds	cloudy gel	addition of gag to P_{11}-14 → spontaneous gel formed with gag powder sitting on top upon vortexing becomes viscous liquid gel formed before vortexing and upon	 on day of prep	 13 days old

1:10	shearing will form a viscous liquid 13 days – cloudy self supporting birefringent gel upon vortexing gel no longer self-supporting 5mths – cloudy self-supporting gel with gel on walls Heated ~80C and vortexed → cloudy bitty viscous liquid 9mths cloudy viscous liquid full of gel precipitate 1yr 3.5mths cloudy self-supporting gel with some liquid phase

1:20	seconds	cloudy gel	addition of gag to P_{11}-14 → spontaneous gel formed with gag powder sitting on top upon vortexing becomes viscous liquid gel formed before vortexing and upon shearing will form a viscous liquid 13 days – cloudy self supporting birefringent gel upon vortexing gel no longer self-supporting 5mths – bitty gel on walls and some liquid phase Heated ~80C and vortexed → cloudy bitty viscous liquid 9mths cloudy self-supporting gel hint of yellow colour and a small amount of liquid 1yr 3.5mths cloudy self-supporting gel	 P_{11}-14:Gag only	 on day of prep	 13 days old

Table 19 – P_{11}-14:GAG+P_{11}-13 mixing study observation

1.7 P_{11}-28:GAG+ P_{11}-29

The following figures show how the physical appearances of the gels change over time:

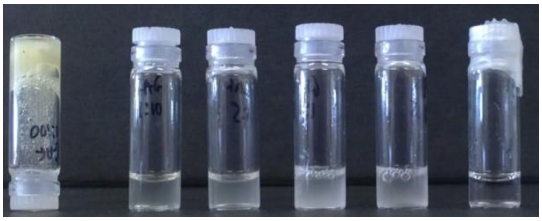


Figure 57 – P_{11}-28:GAG on day of preparation. From left to right 1:100, 1:10, 1:5, 1:2, 1:0.5, control

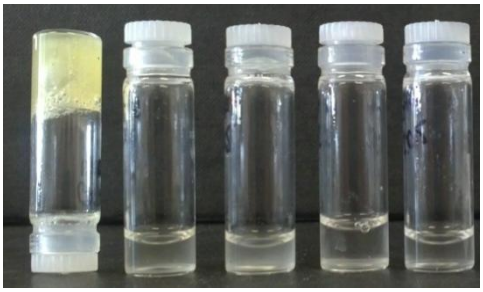


Figure 58 - P_{11}-28:GAG 1 day old. From left to right 1:100, 1:10, 1:5, 1:2, 1:0.5, control

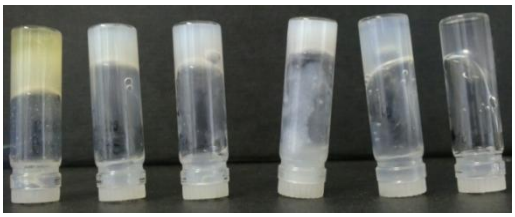


Figure 59 - P_{11}-28:GAG + P_{11}-29 on day of preparation. From left to right 1:100, 1:10, 1:5, 1:2, 1:0.5, control

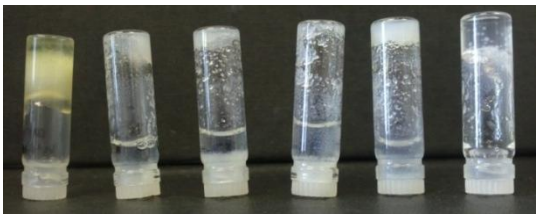


Figure 60 - P_{11}-28:GAG + P_{11}-29 20 days old. From left to right 1:100, 1:10, 1:5, 1:2, 1:0.5, control

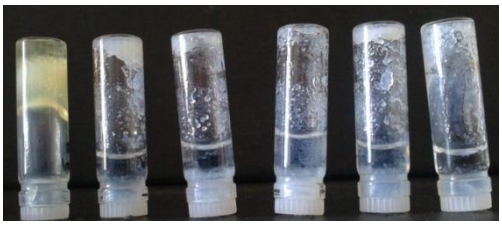


Figure 61 - P_{11}-28:GAG + P_{11}-29 40 days old. From left to right 1:100, 1:10, 1:5, 1:2, 1:0.5, control

Figure 62 - P_{11}-28:GAG + P_{11}-29 40 days old. From left to right 1:100, 1:10, 1:5, 1:2, 1:0.5, control. All samples vortexed

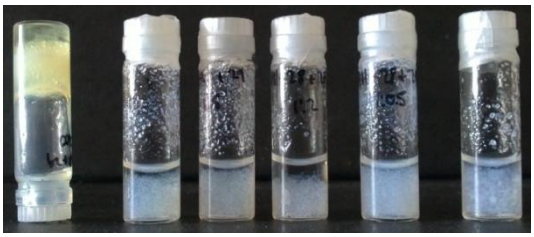


Figure 63 - P_{11}-28:GAG + P_{11}-29 40 days old. From left to right 1:100, 1:10, 1:5, 1:2, 1:0.5, control. All samples vortexed and heated

Figure 64 - P_{11}-28:GAG + P_{11}-29 1:100 81 days old

P_{11}-28 +GAG: 29	**Time taken for gel to form**	**Appearance**	**Other observations**
1:0.5	spontaneous	cloudy gel	addition of gag to P_{11}-28 → cloudy liquid becoming clear overnight spontaneous self-supporting gel formation on addition on P11-29 to P11-28:GAG 20 days – 2 phases clear liquid with white gel precipitate and self-supporting cloudy gel 40 days – as 20 days After vortexing and reheating sample still the same precipitate didn't solubilise
1:2	mins	cloudy gel	addition of gag to P_{11}-28 → cloudy liquid becoming clear overnight self-supporting gel formed after a few mins post addition of P11-29 to P11-28:GAG 20 days –clear liquid with white gel precipitate some gel on walls of vial 40 days – as 20 days After vortexing and reheating sample still the same precipitate didn't solubilise

1:5	secs	cloudy gel	addition of gag to P_{11}-28 → cloudy liquid self-supporting gel formed within seconds after addition of P11-29 to P11-28:GAG 20 days –clear liquid with white gel precipitate some gel on walls of vial 40 days – as 20 days After vortexing and reheating sample still the same precipitate didn't solubilise
1:10	secs	cloudy gel	addition of gag to P_{11}-28 → cloudy liquid self-supporting gel formed within seconds after addition of P11-29 to P11-28:GAG (quicker than 1:5) 20 days – 2 phases clear liquid with white gel precipitate and self-supporting cloudy ge 40 days – as 20 days After vortexing and reheating sample still the same precipitate didn't solubilise
1:100	spontaneous	cloudy yellow gel	addition of gag to P_{11}-28 → very hard to dissolve even after lots of vortexing and heating still some GAG not dissolved. left over night → yellow self-supporting gel that doesn't become viscous on vortexing took a lot of vortexing to get P11-29 to mix with P11-28:GAG gel 20 days old yellow self-supporting gel that doesn't become viscous on vortexing

			40 days – as 20 days 81 days same as above! 99 days same as above 4 mths (131 days) same as above
control	spontaneous	cloudy	20 days old precipitate forming 40 days old liquid with gel precipitate After vortexing and reheating sample still the same precipitate didn't solubilise

Table 20 –P_{11}-28:GAG +P_{11}-29 mixing study observations

1.8 P_{11}-29:GAG + P_{11}-28

The following figures show how the physical appearances of the gels change over time:

Figure 65 - P_{11}-29:GAG day of preparation. From left to right 1:50, 1:10, 1:2 and control

Figure 66 - P_{11}-29:GAG day after preparation. From left to right 1:50, 1:10, 1:2 and control

Figure 67 - P_{11}-29:GAG+P_{11}-28 day of preparation. From left to right 1:50, 1:10, 1:2 and control

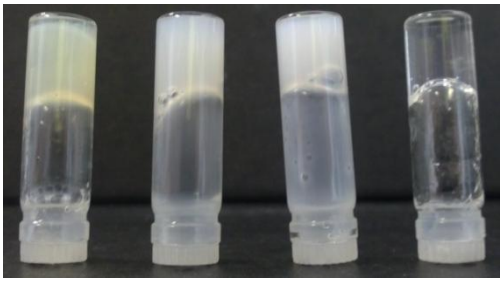


Figure 68 - P_{11}-29:GAG+P_{11}-28 6 days old. From left to right 1:50, 1:10, 1:2 and control

Figure 69 - P_{11}-29:GAG+P_{11}-28 15 days old. From left to right 1:50, 1:10, 1:2 and control

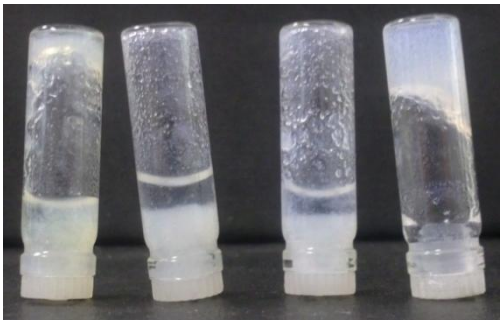


Figure 70 - P_{11}-29:GAG+P_{11}-28 41 days old. From left to right 1:50, 1:10, 1:2 and control

P_{11}-29 +GAG :28	Time taken for gel to form	Appearance	Other observations
1:2	secs after vortexing	milk white self-supporting gel 15 days old liquid with cloudy precipitate 41 days clear liquid with some cloudy gel precipitate 59 days liquid with gel precipitate 91 days grainy gel on walls and liquid phase	turnt white upon addition of P11-28
1:10	secs-1 min slightly longer than 1:2 after vortexing	milk white self-supporting gel 15 days cloudy self-supporting gel v small amount of clear liquid 41 days clear liquid with some cloudy gel precipitate 59 days liquid with gel precipitate 91 days grainy gel on walls and liquid phase	P11-29 +GAG left overnight = slightly cloudy liquid turnt white upon addition of P11-28

1:50	spontaneous with vortexing	cloudy slightly yellow gel 15 days slightly yellow cloudy self-supporting gel 41 days slightly yellow viscous liquid with cloudy gel precipitate 59 days some yellow gel phase v small amount is self-supporting 91 days Some self-supporting grainy gel and liquid phase plus gel on walls	self-supporting gel formed within hrs of GAG addition to P11-29 clear liquid P11-28 sat on top of gel P11-29 until vortexing when spontaneous gelation was observed.
control	spontaneous with vortexing	Clear self-supporting gel Same at 15 days 41 days cloudy self-supporting gel 91 days grainy cloudy self-supporting gel	

Table 21 - P_{11}-29:GAG +P_{11}-28 mixing study observation

2. Rheology

2.1 LVER checks

Following the frequency sweeps amplitude sweeps were performed again at 1 Hz and 20 Hz in a stress controlled mode to confirm that the frequency sweeps were carried out in the LVER. The following plots are the LVER checks carried out for all the peptide:GAG ratios studied.

2.1.1 P_{11}-9

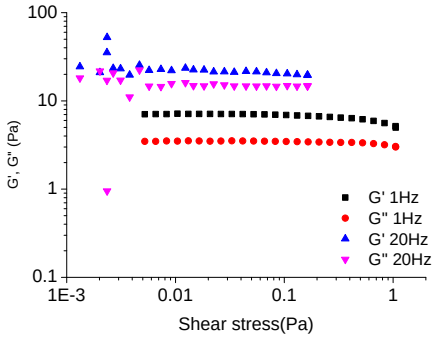


Figure 71 – 1:0.5, frequency = 1 Hz and 20 Hz, temperature = 25°C, stress controlled 0.005-1 Pa

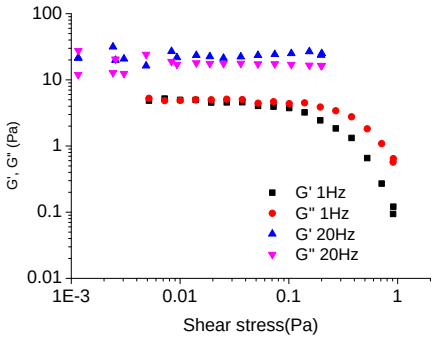


Figure 72 – 1:2, frequency = 1 Hz and 20 Hz, temperature = 25°C, stress controlled 0.005-1 Pa

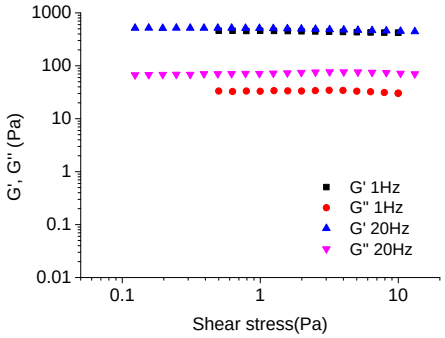


Figure 73 – 1:10, frequency = 1 Hz, stress controlled 0.5-10 Pa and 20Hz stress controlled 0.1-10 Pa, temperature = 25°C

2.1.2 P_{11}-12

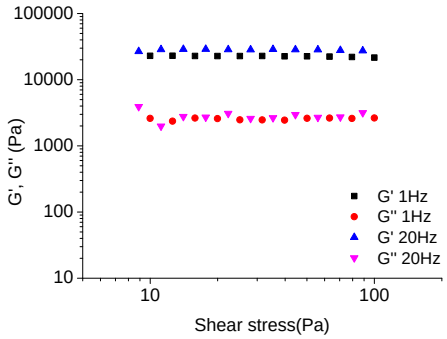


Figure 74 – 1:0.5, frequency = 1 Hz and 20 Hz, temperature = 25°C, stress controlled 10-100 Pa

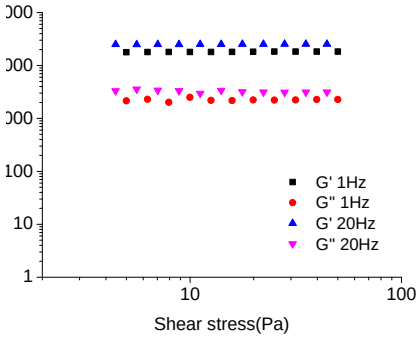


Figure 75 - 1:2, frequency = 1 Hz and 20 Hz, temperature = 25°C, stress controlled 5 - 50 Pa

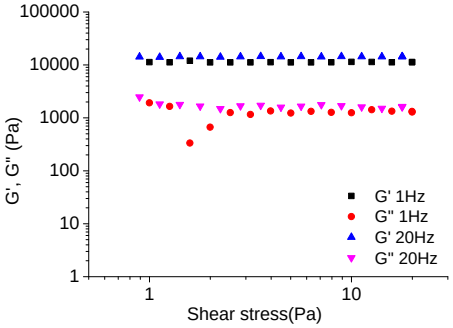


Figure 76 - 1:10, frequency = 1 Hz and 20 Hz, temperature = 25°C, stress controlled 1 -20 Pa

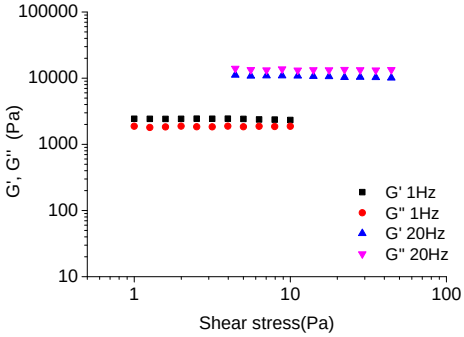


Figure 77 - 1:100, frequency = 1 Hz, stress controlled 1 -10 Pa, and frequency = 20 Hz, stress controlled 5 - 50 Pa, both at temperature = 25°C,

2.1.3 P_{11}-4

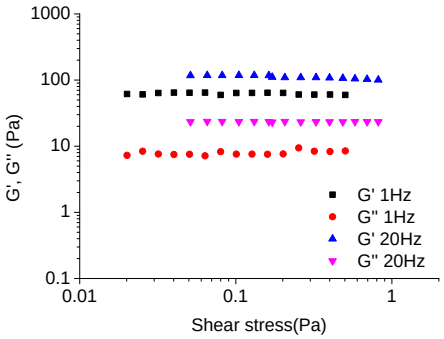


Figure 78 - 1:2, frequency = 1 Hz and 20 Hz, temperature = 25°C, stress controlled 0.02 - 0.5 Pa

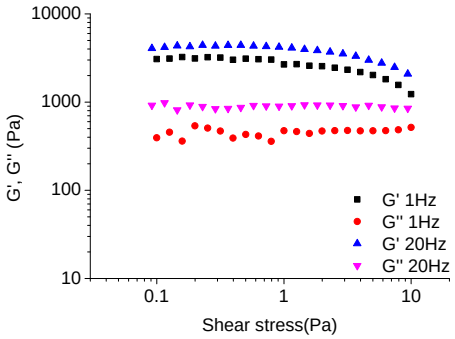


Figure 79 - 1:10, frequency = 1 Hz and 20 Hz, temperature = 25°C, stress controlled 0.1 - 10 Pa

2.1.4 P_{11}-8

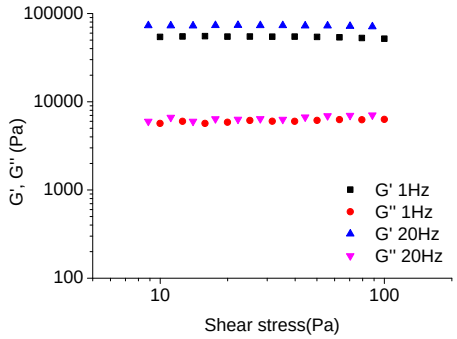


Figure 80 - 1:2, frequency = 1 Hz and 20 Hz, temperature = 25°C, stress controlled 10 - 100 Pa

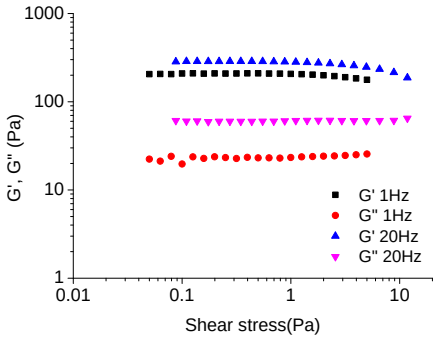


Figure 81 - 1:10, frequency = 1 Hz and 20 Hz, temperature = 25°C, stress controlled 0.05 - 5 Pa

2.1.5 P_{11}-13:GAG+P_{11}-14

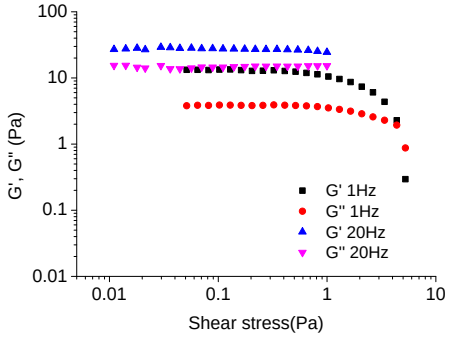


Figure 82 - 1:2, frequency = 1 Hz and 20 Hz, temperature = 25°C, stress controlled 0.05 - 5 Pa

2.1.6 P_{11}-14:GAG+P_{11}-13

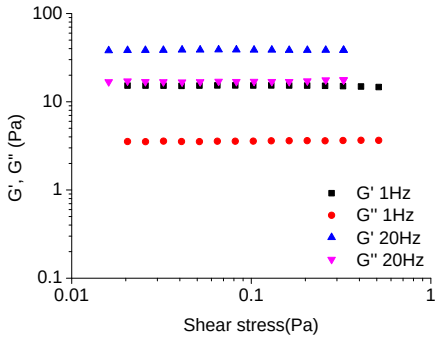


Figure 83 - 1:2, frequency = 1 Hz , stress controlled 0.02 – 0.5 Pa, and 20 Hz stress controlled 0.05 – 1 Pa, temperature = 25°C

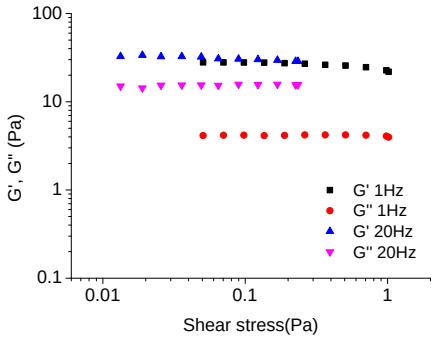


Figure 84 - 1:10, frequency = 1 Hz, stress controlled 0.01 – 1 Pa and frequency = 20 Hz, stress controlled 0.05 - 1 Pa, both at at 25°C,

2.1.7 P_{11}-28:GAG + P_{11}-29

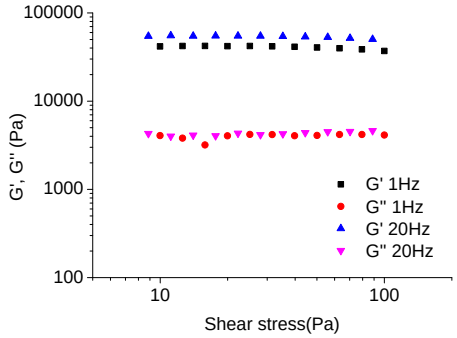


Figure 85 - 1:2, frequency = 1 Hz and 20 Hz, temperature = 25°C, stress controlled 10 – 100 Pa

2.1.8 P_{11}-29:GAG + P_{11}-28

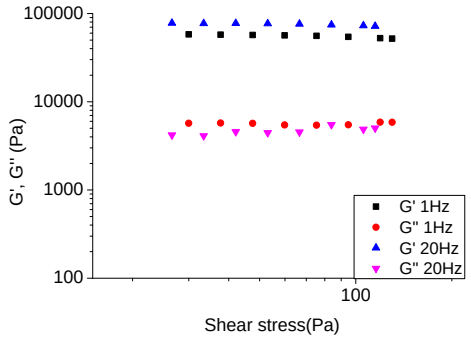


Figure 86 - 1:2, frequency = 1 Hz and 20 Hz, temperature = 25°C, stress controlled 30 – 130 Pa

2.1.9 P_{11}-28+29:GAG

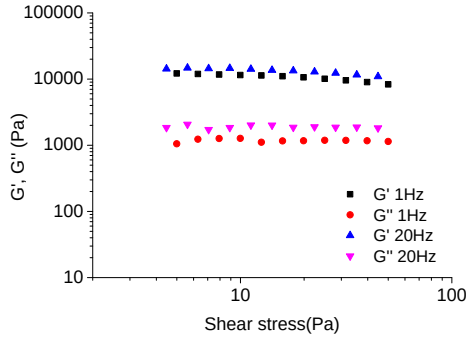


Figure 87 - 1:10, frequency = 1 Hz and 20 Hz, temperature = 25°C, stress controlled 5 – 50 Pa

2.2 Reproducibility

For a selection of the peptide:GAG samples the same frequency sweep was carried out on two separate samples in order to check the reproducibility of the results.

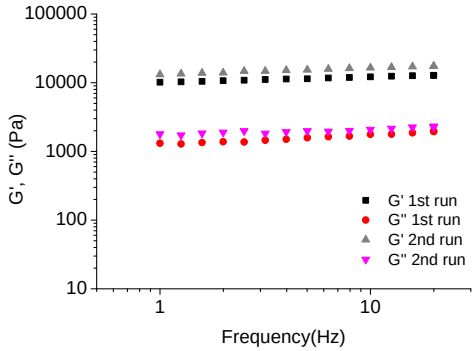


Figure 88 – P_{11}-12 1:2 frequency sweeps, frequency 1 – 20 Hz, strain controlled 0.1%, temperature = 25°C

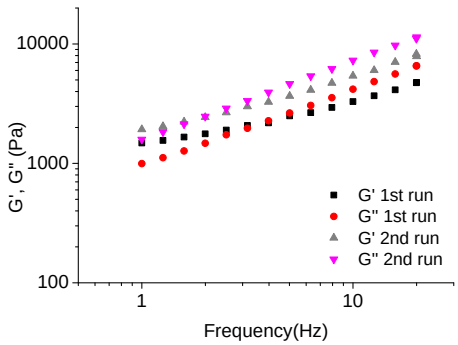


Figure 89 - P_{11}-12 1:100 frequency sweeps, frequency 1 – 20 Hz, strain controlled 0.15%, temperature = 25°C

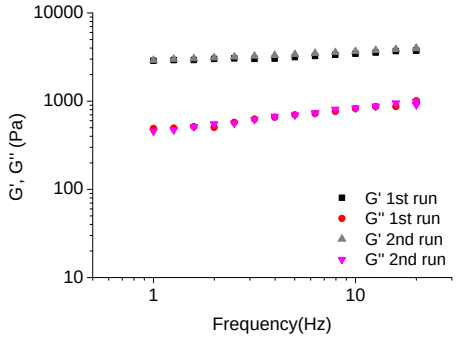


Figure 90 – P_{11}-4 1:10 frequency sweeps, frequency 1 – 20 Hz, strain controlled 0.04%, temperature = 25°C

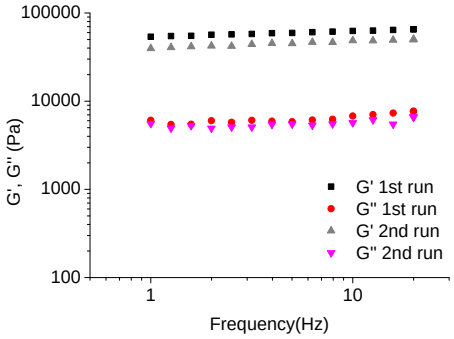


Figure 91 – P_{11}-8 1:2 frequency sweeps, frequency 1 – 20 Hz, strain controlled 0.1%, temperature = 25°C

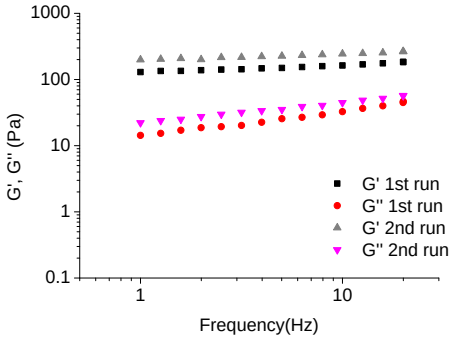


Figure 92 – P_{11}-8 1:10 frequency sweeps, frequency 1 – 20 Hz, strain controlled 0.1%, temperature = 25°C

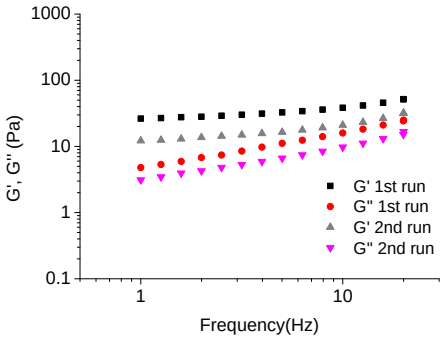


Figure 93 - P_{11}-14:GAG+P_{11}-13 1:2 frequency sweeps, frequency 1 – 20 Hz, strain controlled 0.6%, temperature = 25°C

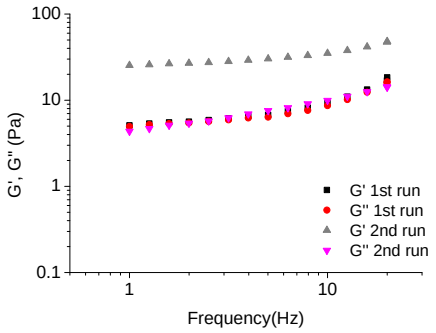


Figure 94 - P_{11}-14:GAG+P_{11}-13 1:10 frequency sweeps, frequency 1 – 20 Hz, strain controlled 0.7%, temperature = 25°C

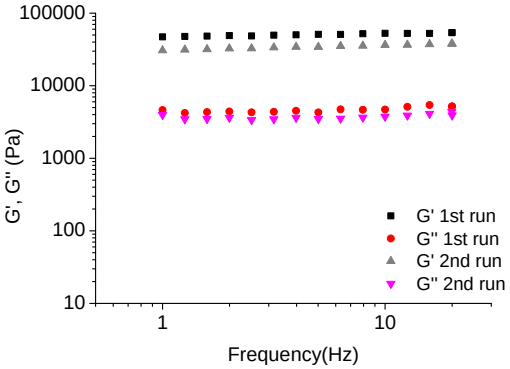


Figure 95- P_{11}-28:GAG+P_{11}-29 1:2 frequency sweeps, frequency 1 – 20 Hz, strain controlled 0.14%, temperature = 25°C

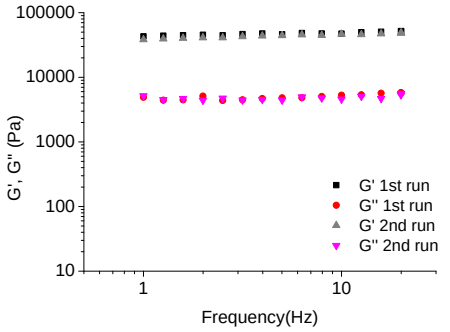


Figure 96 - P_{11}-29:GAG+P_{11}-28 1:2 frequency sweeps, frequency 1 – 20 Hz, strain controlled 0.16%, temperature = 25°C

Appendix F

Ex vivo study

1. GAG leakage study

	Sample type	Disc to use		
A	No inj	T1C1	T2C3	T1C5
B	1:2 CS	T2C1	T1C3	T2C5
C	1:10 CS	T1C2	T2C4	T1C6
E	No inj	T3C1	T4C3	T3C5
F	1:10 P11-12:GAG	T4C1	T3C3	T4C5
G	1:100 CS dry powder	T3C2	T4C4	T3C6
H	1:100 P11-12:GAG CS dry powder	T4C2	T3C4	T4C6
I	no inj	T5C1	T6C3	T5C5
J	1:100 CS premade gel	T5C2	T6C4	T5C6
K	1:100 P11-12:GAG premade gel	T6C2	T5C4	T6C6
L	NP put back in disc	T6C1	T5C3	T6C5

Table 22 - Sample types and discs used

	Tail	Disc	Injection	NP removed (g)	Notes
A	T1	C1	Nothing	0.39	
	T2	C3	Nothing	0.21	
	T1	C5	Nothing	0.24	
B	T2	C1	250 µl 0.027M CS (1:2)	0.25	
	T1	C3	250 µl 0.027M CS (1:2)	0.39	
	T2	C5	250 µl 0.027M CS (1:2)	0.17	Back pressure on inj ∴seal broke Disc removed from Perspex and restuck Reinjected however may have been some residual from last inj
C	T1	C2	250 µl 0.133M CS (1:10)	0.47	Small leakage on inj Edge resealed with super clue Some CS lost ∴inj 100 µl more of CS
	T2	C4	250 µl 0.133M CS (1:10)	0.22	Edge sealed with super glue prior to inj
	T1	C6	250 µl 0.133M CS (1:10)	0.17	Edge sealed with super glue prior to inj

Table 23 - Samples A-C amount of NP removed and injection notes

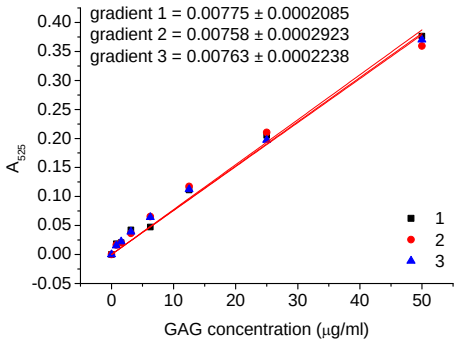


Figure 97 – DMB assay standard curve used to calculate GAG concentration of samples at 24 hours (y=mx+c. y=A_{525}, m=0.007653, c=0)

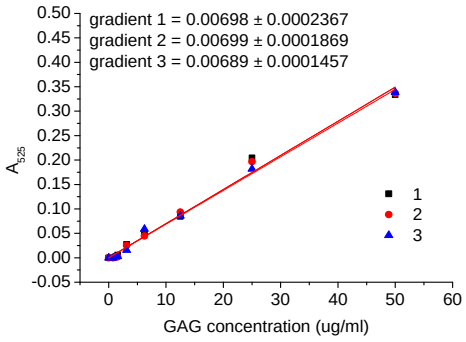


Figure 98 - DMB assay standard curve used to calculate GAG concentration of samples at 48 hours (y=mx+c. y=A_{525}, m=0.00695, c=0)

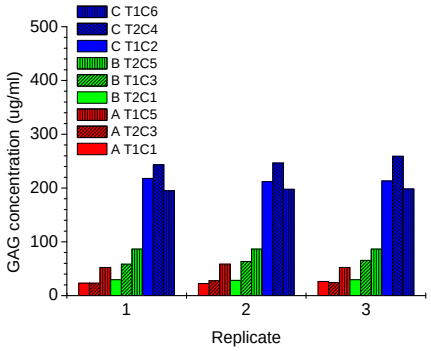


Figure 99 – GAG concentration in PBS solution at 24 hours for each assay replicate for each of the individual discs in samples A-C

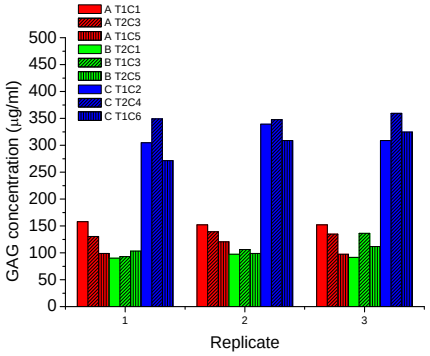


Figure 100 - GAG concentration in PBS solution at 48 hours for each assay replicate for each of the individual discs in samples A-C

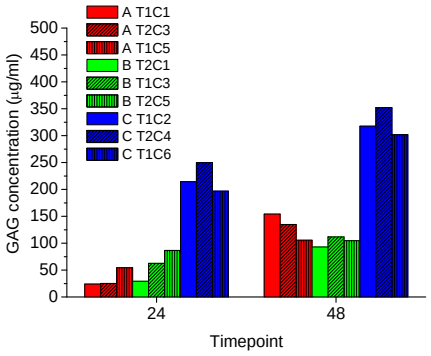
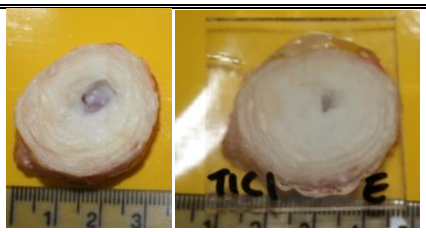
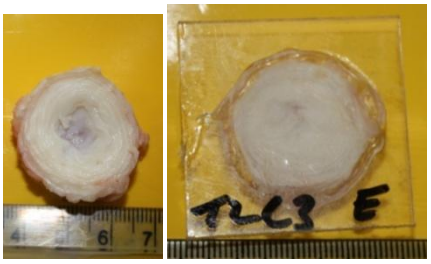
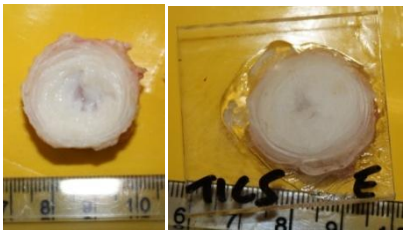
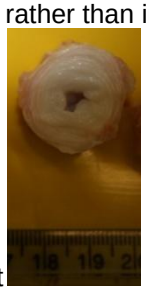
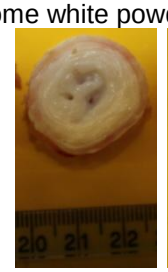
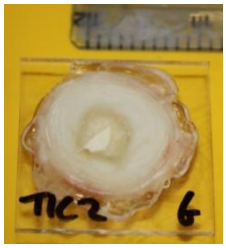
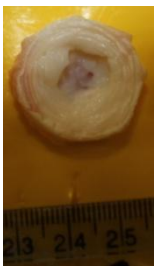
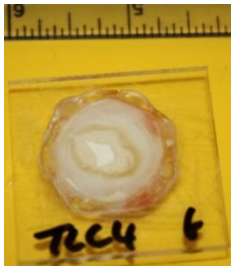
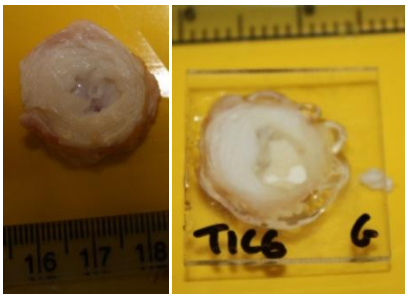
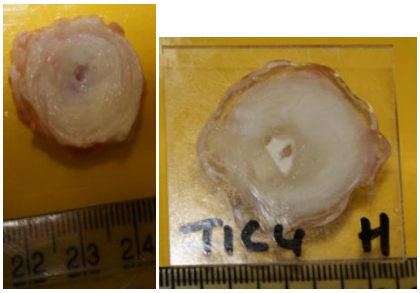


Figure 101 – Average of DMB assay replicates for each timepoint for each of the individual discs in samples A-C

	Tail	Disc	Injection	NP removed (g)	Notes
E	T3	C1	Nothing	0.59	
	T4	C3	Nothing	0.78	
	T3	C5	Nothing	0.55	
F	T4	C1	125 µl P11-12 40 mg/ml 125 µl 1:20 CS 136 mg/ml	0.46	P11-12 inj fine Injected half GAG through opposite needle then back pressure became to high so inj through same needle as P11-12 
	T3	C3	125 µl P11-12 40 mg/ml 125 µl 1:20 CS 136 mg/ml	0.25	About 20 µl of P11-12 came out of air hole on injection Injected CS through same hole as P11-12

	T4	C5	125 µl P11-12 40 mg/ml 125 µl 1:20 CS 136 mg/ml	0.39	 Inj P11-12 and CS through different holes After about 50 µl of GAG addition back pressure was felt and it came out of needle injecting through rather than into disc ~10ul of CS lost 
G	T3	C2	125 µl P11-12 40mg/ml 125 µl PBS 0.171 g CS added as dry powder	0.40	P11-12 inj fine PBS inj fine through same hole as P11-12 Some white powder still visible after inj  
	T4	C4	125 µl P11-12 40mg/ml 125 µl PBS 0.169 g CS added as dry powder	0.54	P11-12 inj fine PBS inj fine through same hole as P11-12 Some white powder still visible after inj  

	T3	C6	125 µl P11-12 40mg/ml 125 µl PBS 0.169 g CS added as dry powder	0.25	P11-12 inj fine PBS inj through same hole as P11-12 experienced back pressure and gel leakage through air hole ~ 20µl Some white powder still visible after inj 
H	T4	C2	250 µl PBS 0.170 g CS added as dry powder	0.39	PBS inj fine Some powder still visible after inj 
	T3	C4	250 µl PBS 0.171 g CS added as dry powder	0.19	PBS inj fine Some powder still visible after inj 
	T4	C6	250 µl PBS 0.169 g CS added as dry powder	0.24	PBS inj fine Some powder still visible after inj

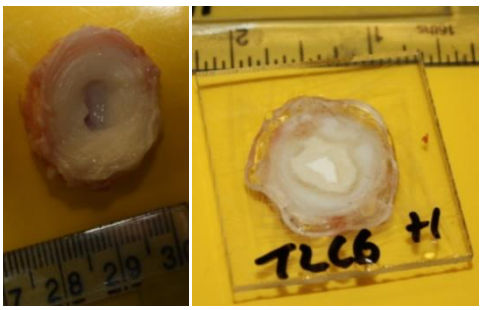


Table 24 - Samples E-H amount of NP removed and injection notes

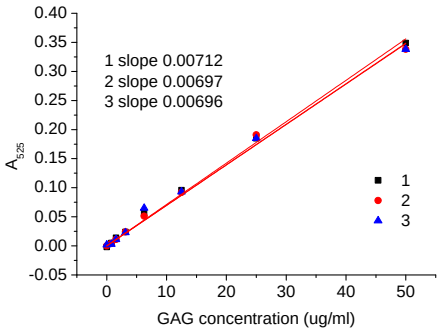


Figure 102 – DMB assay standard curve used to calculate GAG concentration of samples E-H at 24 and 48 hours (y=mx+c. $y=A_{525}$, m=0.0070167, c=0)

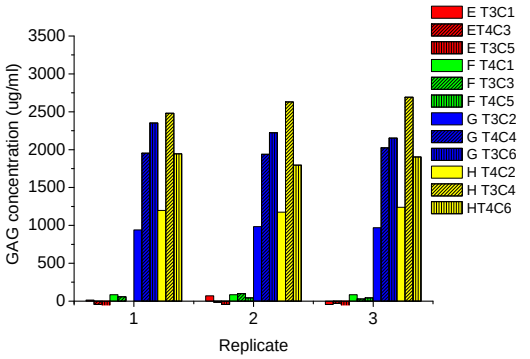


Figure 103 - GAG concentration in PBS soluition at 24 hours for each assay replicate for each of the individual discs in samples E-H

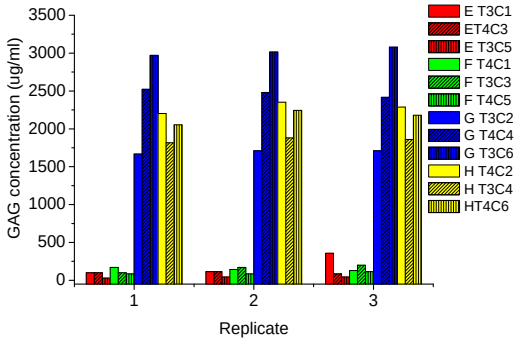


Figure 104 - GAG concentration in PBS solution at 48 hours for each assay replicate for each of the individual discs in samples E-H

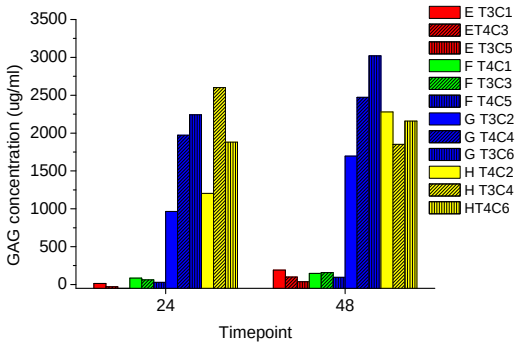


Figure 105 - Average of DMB assay replicates for each timepoint for each of the individual discs in samples E-H

	Tail	Disc	Injection	NP removed (g)	Amount of NP/Gel put back in (g)
	T5	C1	nothing	0.39	
I	T6	C3	nothing	0.25	
	T5	C5	nothing	0.18	
	T5	C2	GAG 1:100 gel spooned in	0.27	0.25
J	T6	C4	GAG 1:100 gel spooned in	0.14	0.14
	T5	C6	GAG 1:100 gel spooned in	0.17	0.15
	T6	C2	P11-12: GAG 1:100 gel spooned in	0.29	0.17
K	T5	C4	P11-12: GAG 1:100 gel spooned in	0.20	0.14
	T6	C6	P11-12: GAG 1:100 gel spooned in	0.19	0.09
	T6	C1	NP cut out and then put back in	0.32	0.28
L	T5	C3	NP cut out and then put back in	0.29	0.23
	T6	C5	NP cut out and then put back in	0.16	0.25

Table 25 - Samples I-L amount of NP removed and injection notes

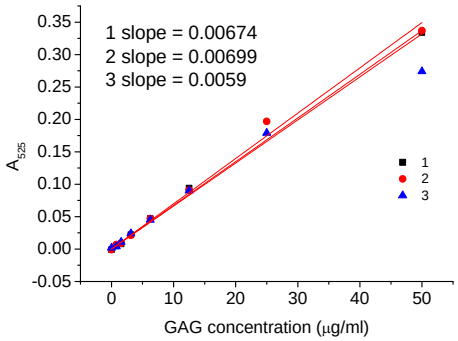


Figure 106 - DMB assay standard curve used to calculate GAG concentration of samples I and L at 24 and 48 hours (y=mx+c. y=A_{525}, m=0.00654, c=0)

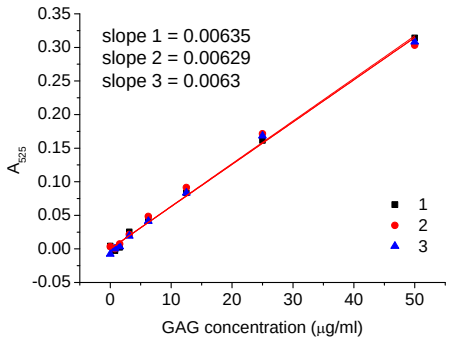


Figure 107 - DMB assay standard curve used to calculate GAG concentration of samples J and K at 24 and 48 hours (y=mx+c. y=A_{525}, m=0.006313, c=0)

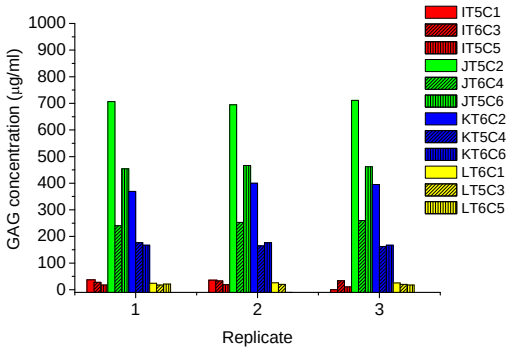


Figure 108 - GAG concentration in PBS solution at 24 hours for each assay replicate for each of the individual discs in samples I-L

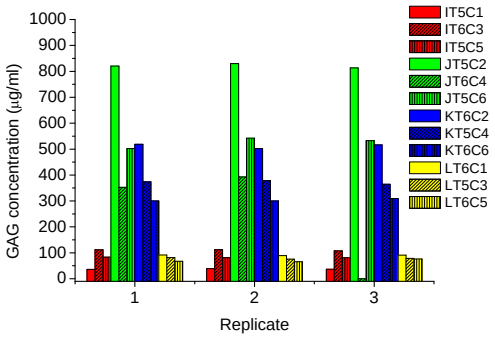


Figure 109 - GAG concentration in PBS solution at 48 hours for each assay replicate for each of the individual discs in samples I-L

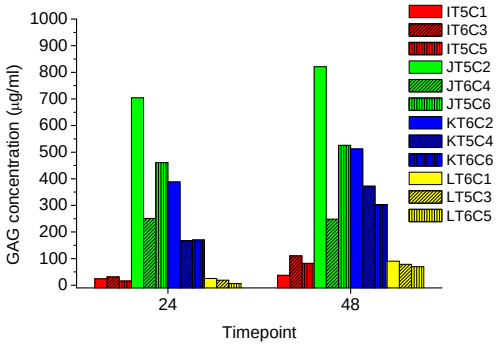


Figure 110 - Average of DMB assay replicates for each timepoint for each of the individual discs in samples I-L

	Disc	GAG added /g	if all leaked out ug/ml	measured in sample ug/ml				natural leakage subtracted ug/ml				% of added GAG leaked			
				24	SEM	48	SEM	24	±	48	±	24	±	48	±
A	No injection	-	-	34	10	131	14								
E	No injection	-	-	-24	21	109	44								
I	No injection	-	-	24	4	77	21								
	No injection average	**-**	**-**	**12**	**5**	**106**	**9**								
L	NP put back in	-	-	17	6	80	6	5	7	-26	11				
B	1:2 GAG	0.0035	116.67	59	17	103	5	48	17	-3	11	41	15	-2	9
C	1:10 GAG	0.01575	525.00	220	16	324	15	209	16	218	17	40	3	42	3
F	1:10 P11-12:GAG	0.017	566.67	59	17	133	19	47	17	27	21	8	3	5	4
H	1:100 GAG (dry powder)	0.17	5666.67	1895	403	2097	127	1884	403	1992	128	33	7	35	2
G	1:100 p11-12:gag (dry powder)	0.17	5666.67	1726	389	2397	384	1714	389	2291	385	30	7	40	7
J	1:100 GAG (gel)	0.177	5900.00	472	131	532	165	460	131	426	166	8	2	7	3
K	1:100 GAG:P11-12 (gel)	0.157	5233.33	242	73	394	62	230	73	290	62	4	1	6	1

Table 26 - GAG leakage raw data used to calculate percentage of added GAG leaked

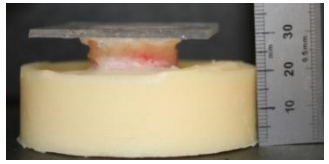
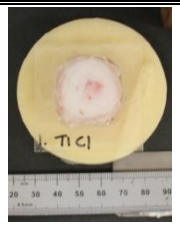
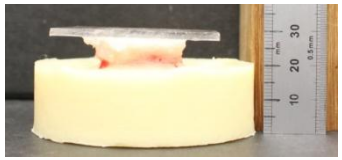
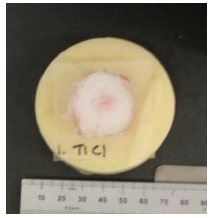
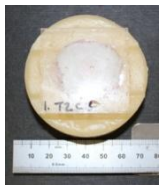
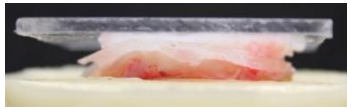
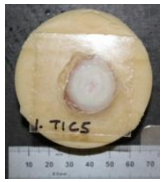
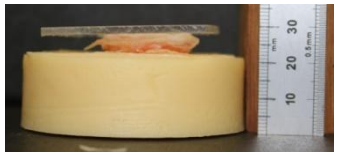
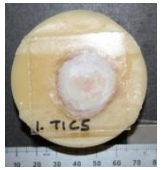
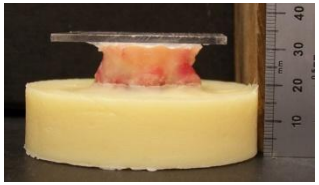
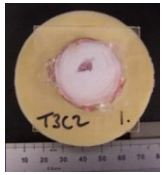
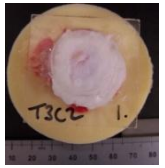
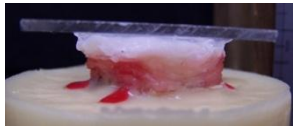
2. Compressive loading study

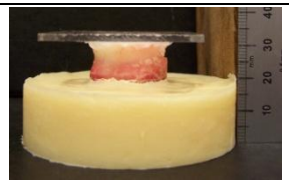
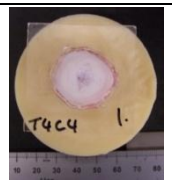
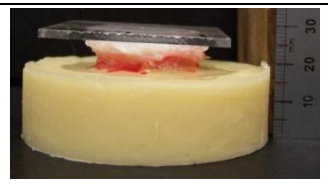
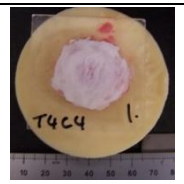
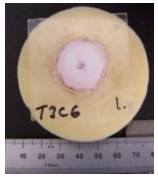
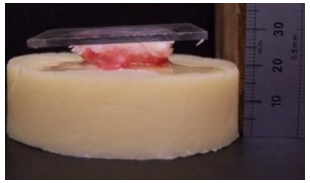
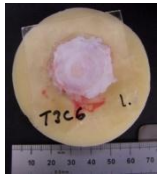
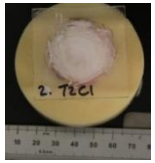
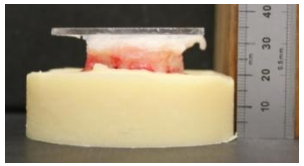
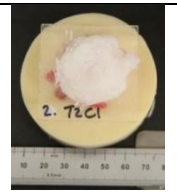
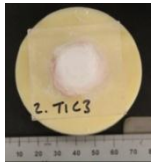
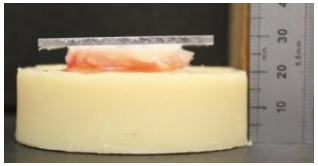
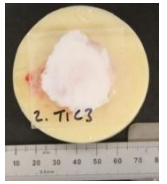
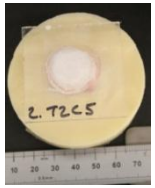
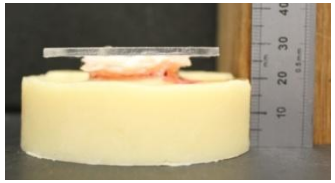
Group	Tail and Disc used		
No NP	T1C1	T2C3	T1C5
	T3C4	T4C4	T3C6
NP	T2C1	T1C3	T2C5
	T4C2	T3C4	T4C6
1:10	T1C2	T2C4	T1C6
	T3C1	T4C3	T3C5
1:2	T2C2	T1C4	T2C6
	T4C1	T3C3	T4C5

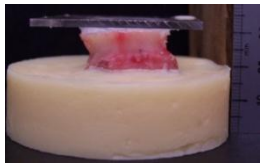
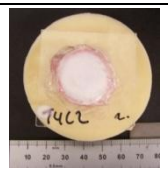
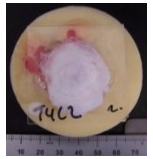
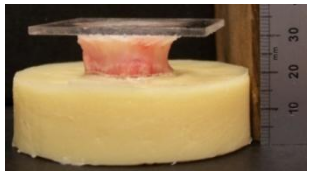
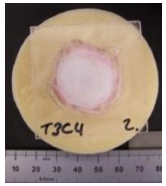
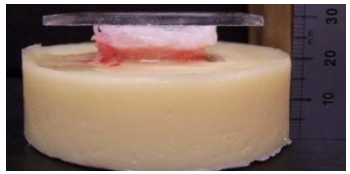
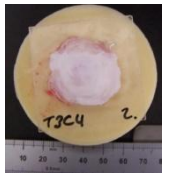
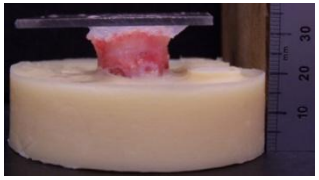
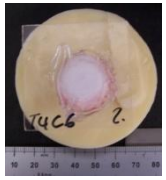
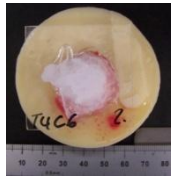
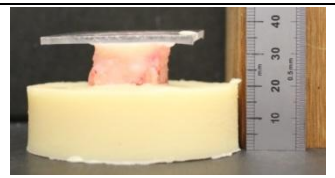
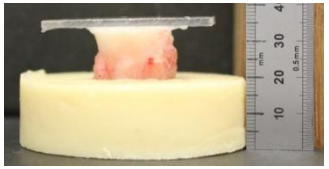
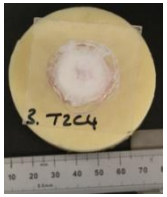
Table 27 - Discs used for each group

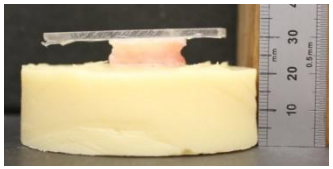
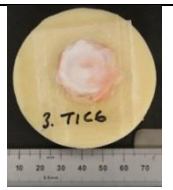
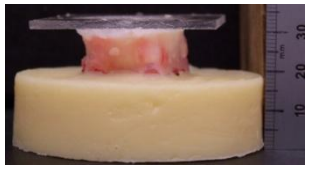
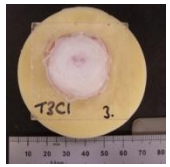
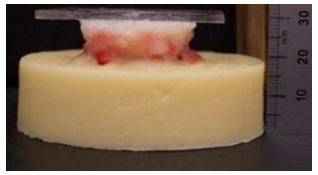
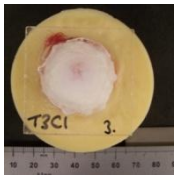
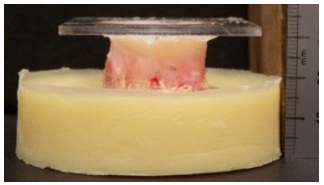
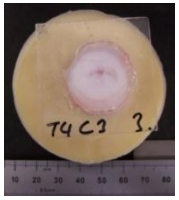
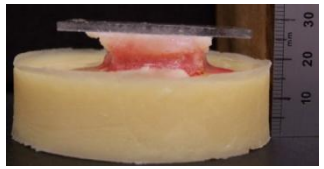
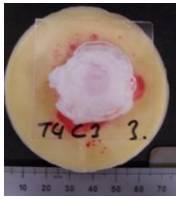
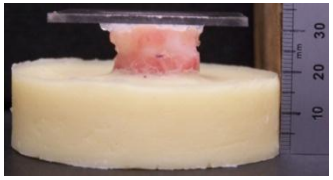
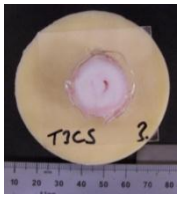
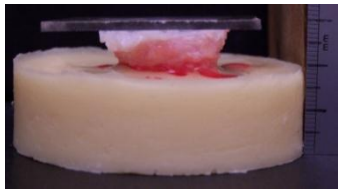
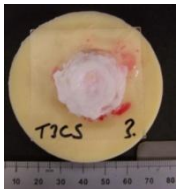
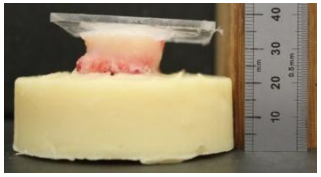
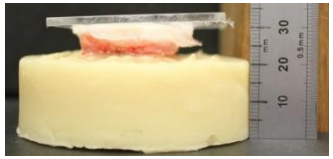
Group	Disc	Disc weight	Np weight	Disc without NP weight
Denucleated	T1C1	10.75	0.5049	10.2218
	T2C3	10.522	0.4076	9.8973
	T1C5	8.395	0.2741	7.9659
	T3C2	12.614	0.553	11.909
	T4C4	9.224	0.323	8.765
	T3C6	8.127	0.323	7.683
Intact NP	T2C1	16.761		
	T1C3	12.5381		
	T2C5	8.6744		
	T4C2	12.629		
	T3C4	8.466		
	T4C6	7.795		
1:10	T1C2	14.24	0.7169	13.3514
	T2C4	10.8911	0.3317	10.3436
	T1C6	7.552	0.1714	7.2677
	T3C1	17.208	0.419	16.558
	T4C3	12.233	0.44	11.691
	T3C5	8.523	0.354	8.082
1:2	T2C2	13.4987	0.5857	12.6557
	T1C4	7.81	0.3262	7.3923
	T2C6	6.4178	0.1501	6.1869
	T4C1	16.462	0.731	15.563
	T3C3	11.756	0.438	11.159
	T4C5	8.602	0.256	8.239
	Average	**10.904**	**0.406**	**10.274**
	SD	**3.116**	**0.164**	**2.926**

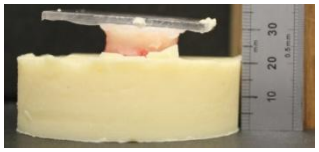
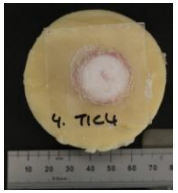
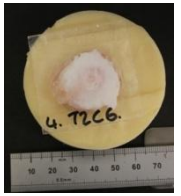
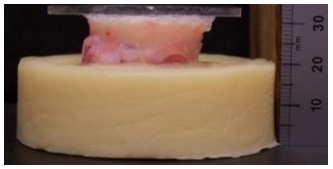
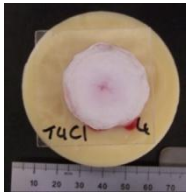
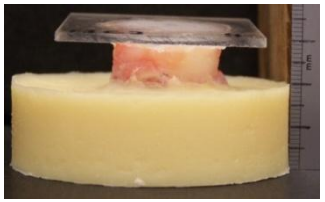
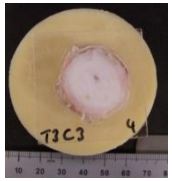
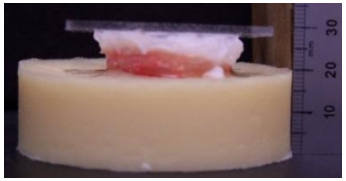
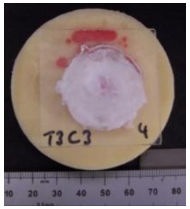
Table 28 – Disc weights pre- and post- nucleus removal

Group	Disc	Preload		Post load		Other
Denucleated	T1C1					
	T2C3					post load 
	T1C5					
	T3C2					post load 

	T4C4						
	T3C6						
NP intact	T2C1					during load	
	T1C3					during load	
	T2C5						

	T4C2						
	T3C4						
	T4C6						
1:10	T1C2					during load: 	
	T2C4						

	T1C6				
	T3C1				
	T4C3				
	T3C5				
1:2	T2C2				

T1C4					
T2C6					
T4C1					
T3C3					

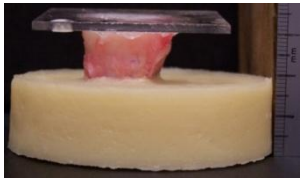
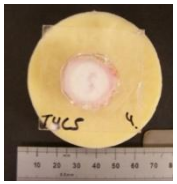
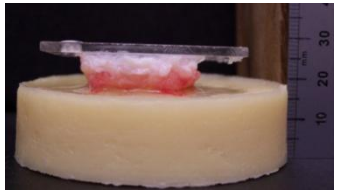
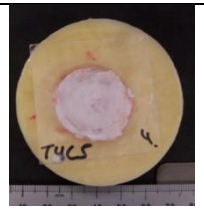
	T4C5					

Table 29 - Photographs of discs pre- and post-load

Group	Disc	Slope of linear region 200-500N	disc height	disc width a	disc width b	disc length	A	normalised stiffness				
		N/mm	mm	mm	mm	mm	mm	N/mm	AVERAGE	SD	95% conf lim	SEM
Denucleated	T1C1	2534.2	5.0	26.5	28.0	5.0	582.8	21.7	16.4	3.978568	3.183459	1.624244
	T2C3	1391.5	4.5	25.0	27.0	4.5	530.1	11.8				
	T1C5	1563.0	5.0	21.0	23.0	5.0	379.3	20.6				
	T3C2	1090.2	12	30	27.5	8	648.0	13.5				
	T4C4	1320.1	7	29	22	6	501.1	15.8				
	T3C6	973.26	6	23	22	6	397.4	14.7				
Np intact	T2C1	1310.8	8.0	30.0	30.0	8.0	706.9	14.8	11.7	2.915461	2.332812	1.190232
	T1C3	731.1	6.0	26.0	26.0	6.0	530.9	8.3				
	T2C5	1097.3	5.5	23.0	22.0	5.5	397.4	15.2				
	T4C2	827.47	6	26	27	6.5	551.3	9.8				
	T3C4	946.01	5	29	26	6	592.2	9.6				
	T4C6	971.54	8	24	25	6	471.2	12.4				
1:10	T1C2	1067.8	7.0	26.5	28.0	7.0	582.8	12.8	17.5	4.584942	3.66865	1.871795
	T2C4	1190.1	6.0	24.0	24.0	6.0	452.4	15.8				
	T1C6	1234.5	4.0	22.0	20.0	4.0	345.6	14.3				
	T3C1	1912.5	8	27	28	8	593.8	25.8				
	T4C3	1227.4	9	24	26	7.5	490.1	18.8				
	T3C5	962.99	8	22	24	7.5	414.7	17.4				
1:2	T2C2	906.2	6.0	27.0	26.0	6.0	551.3	9.9	13.9	3.97962	3.184301	1.624673
	T1C4	1335.1	5.0	25.0	25.0	5.0	490.9	13.6				
	T2C6	1464.6	4.0	23.0	23.0	4.0	415.5	14.1				
	T4C1	1657.7	8	29	31	7	706.1	16.4				

	T3C3	822.26	7	27	27	6.5	572.6	9.3
	T4C5	1176.4	8	24	22	7	414.7	19.9

Table 30 - Raw data used to calculate normalised stiffness values