Electronic Supplementary Material (ESI) for Biomaterials Science.
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Supporting Information

Serum Albumin Hydrogels in Broad pH and Temperature Ranges: Characterization of Their Self-Assembled Structures, Nanoscopic and Macroscopic Properties

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Rheological Characterization:

The influence of the frequency of oscillation (ω) on the gelation kinetics:

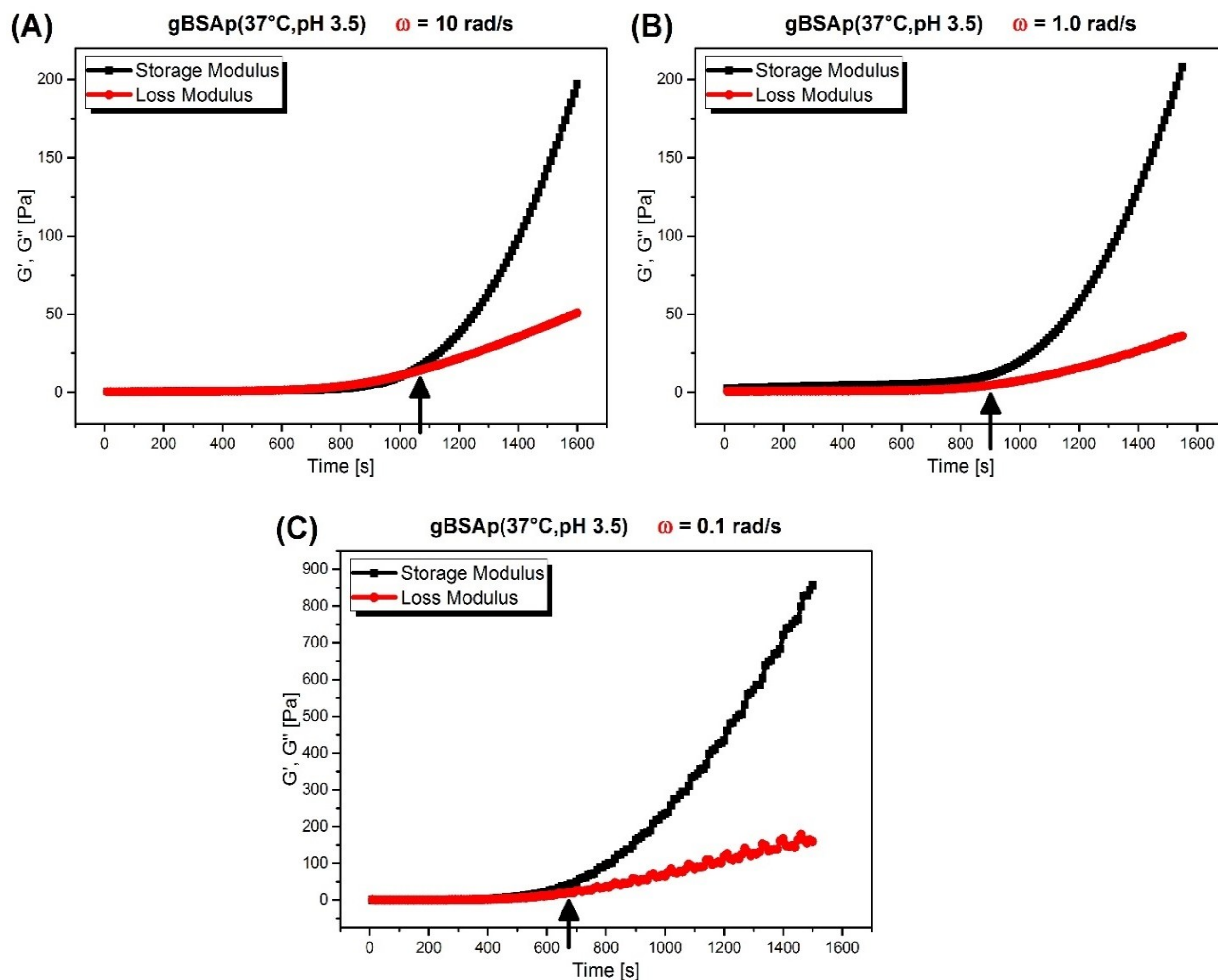


Figure S1. The dependence of gelation kinetics on the frequency of oscillation for (A) $\omega = 10$ rad/s (B) $\omega = 1.0$ rad/s (C) $\omega = 0.1$ rad/s in time-dependent storage G' and loss G" moduli of 20 wt. % BSA precursor solution at 37°C, pH 3.5 (2 M HCl used as acid) forming *g*BSA_p(37,3.5,t).

These experiments indicate that mixing would prolong hydrogel formation. These results are consistent with the actual observation, as the vials in the thermomixer are set to higher mixing rate the longer incubation time is needed for hydrogel formation. No mixing during gel preparation result in the highest gelation kinetics.

The influence of the fatty acid presence on the hydrogel formation:

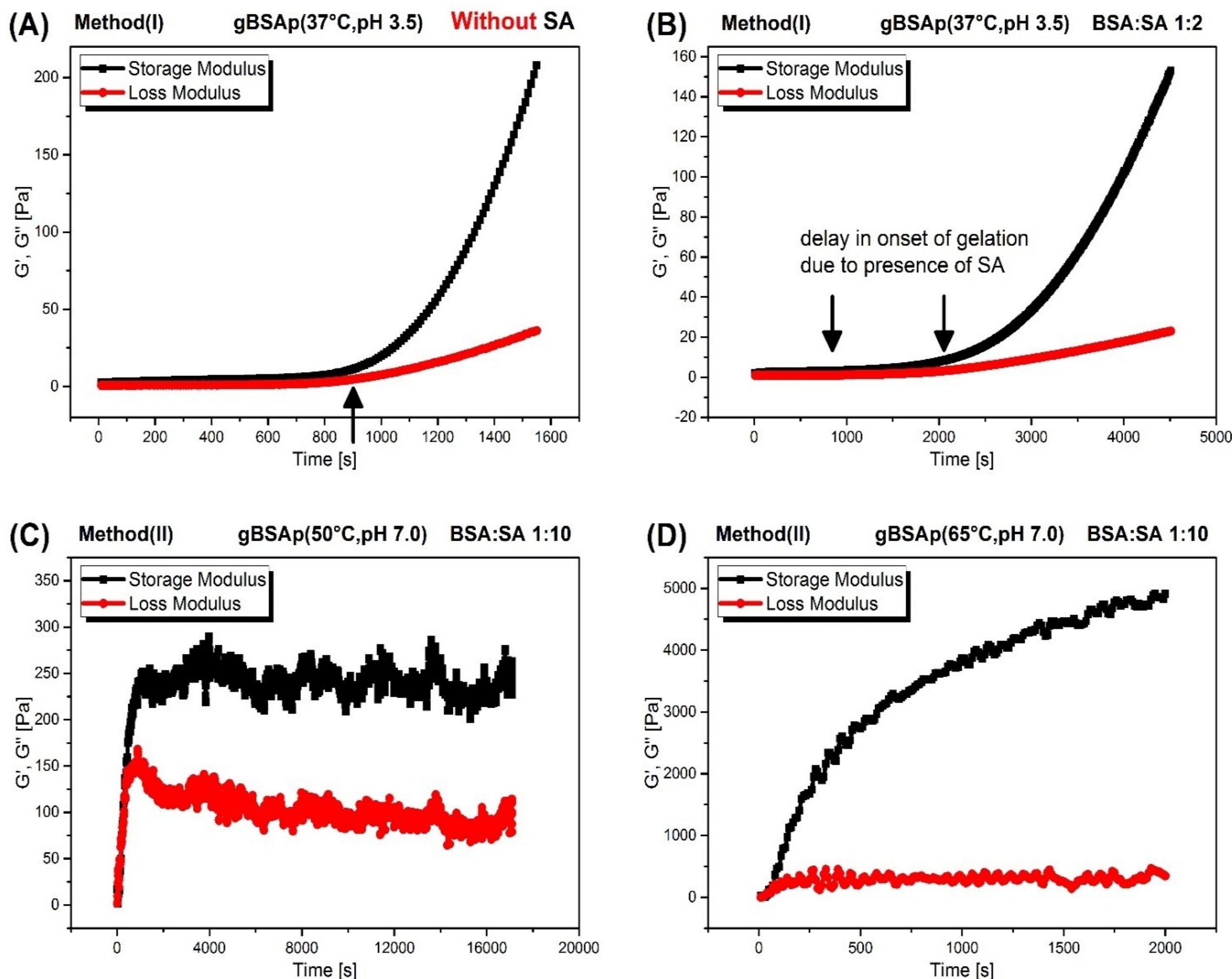


Figure S2. The influence of fatty acid on the hydrogel formation. Time-dependent storage (G') and loss (G") moduli of 20 wt. % (A) BSA precursor solution at 37°C, pH 3.5 (2 M HCl used as acid) forming gBSAp(37,3.5,t) (B) BSA precursor solution at 37°C wi

To analyze the potential effects of stearic acid on the mechanical properties of the hydrogels, we repeated the experiments explained in the rheological characterization part for gBSAp and gBSAT. In Figure S2. (A) the gBSAp precursor solution is prepared without SA and the storage and loss modulus are recorded till obtaining gBSAp whereas in (B) SAs are introduced to the precursor solution with method (I), see the Loading FAs into the hydrogels part, and the rest was the same. We see that presence of SA delays the gel formation. Figure S2 (C) shows gBSAT at 50°C and (D) gBSAT at 65°C formation with a molar ratio of SA:BSA 10:1 in the precursor solution which prepared by method (II). These results show that there was no hydrogel formation at 50°C in the presence of SAs. Furthermore, although a hydrogel forms at 65°C, the value of storage modulus at the respective time is way less than the value seen without stearic acid. Therefore, one can draw the conclusion that stearic acid would increase the thermal stability of serum albumin and may change the denaturation temperature of the protein.

ATR-FTIR Spectroscopy (PCA Analysis):

gBSA$_T$(65,7.0,360)

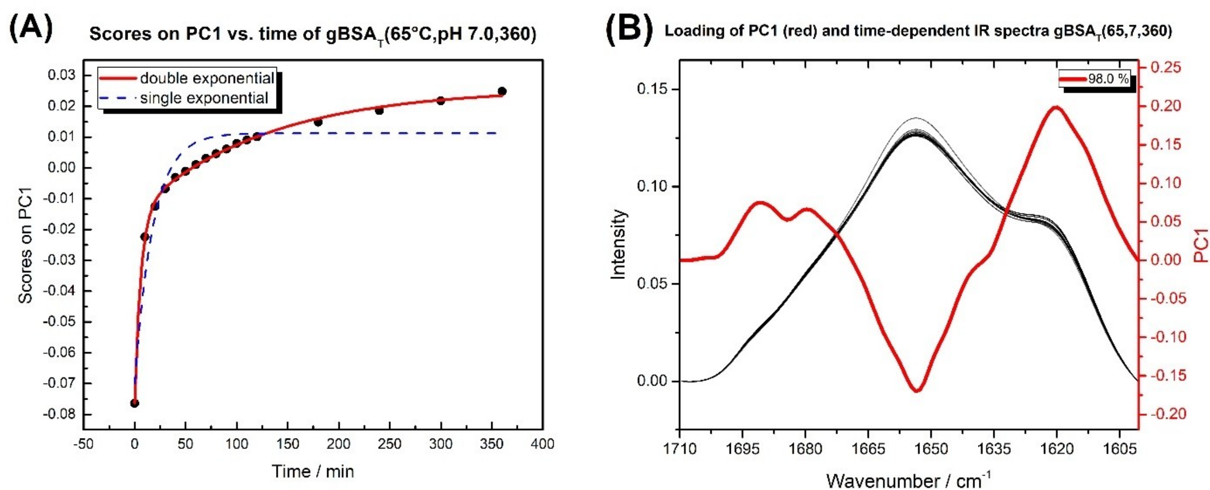


Figure S3. (A) Diagram of PC1 scores against time for *g*BSA$_T$(65,7,360). (B) the time-dependent IR spectra of *g*BSA$_T$(65,7,360) and the loading of PC1 in red.

At t = 0 the beta sheet content is already quite high. This is consistent with the rheological data as the hydrogel formation at 65°C starts immediately. The temperature of the precursor solution of the hydrogels is raised to 65°C from room temperature instantly, there is no preheat treatment.

***g*HSA$_T$(50,7.0,360)**

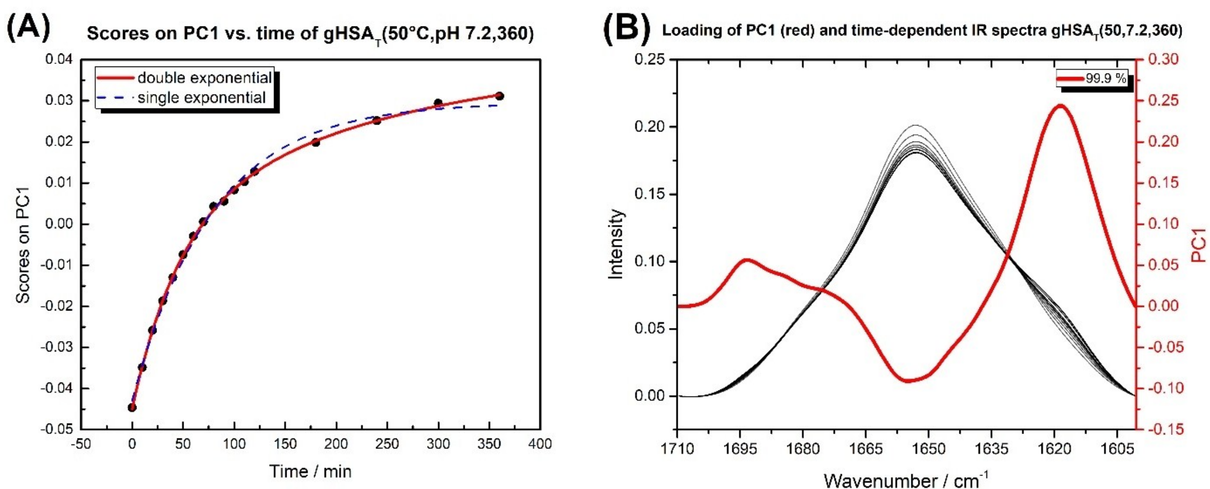


Figure S4. (A) Diagram of PC1 scores against time for *g*HSA$_T$(50,7,360). (B) the time-dependent IR spectra of *g*BSA$_T$(50,7,360) and the loading of PC1 in red.

gHSA$_{T}$(65,7.0,360)

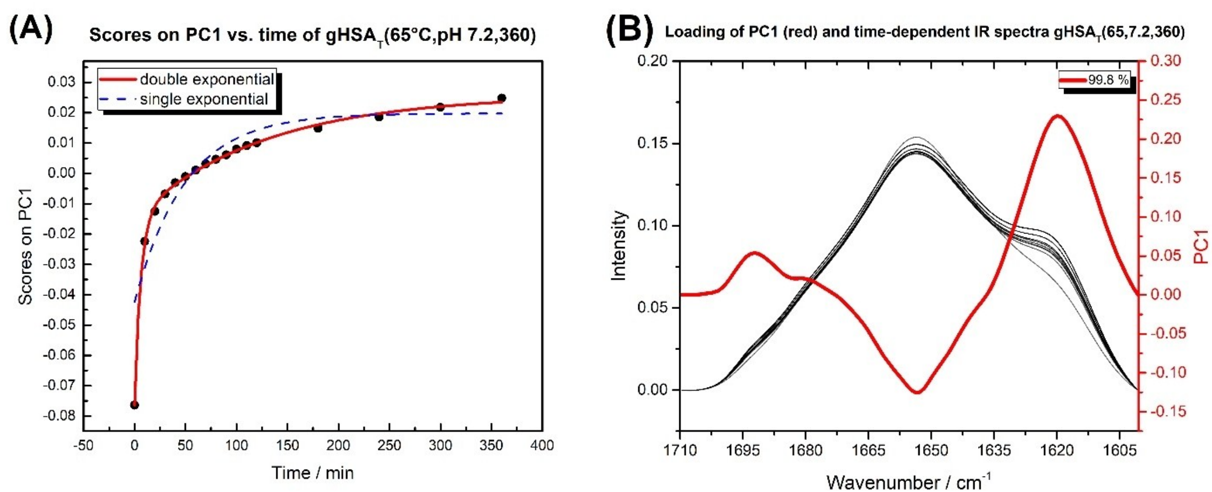


Figure S5. (A) Diagram of PC1 scores against time for gHSA$_{T}$(65,7,360). (B) the time-dependent IR spectra of gHSA$_{T}$(65,7,360) and the loading of PC1 in red.

gHSA$_{p}$(37,3.5,360)

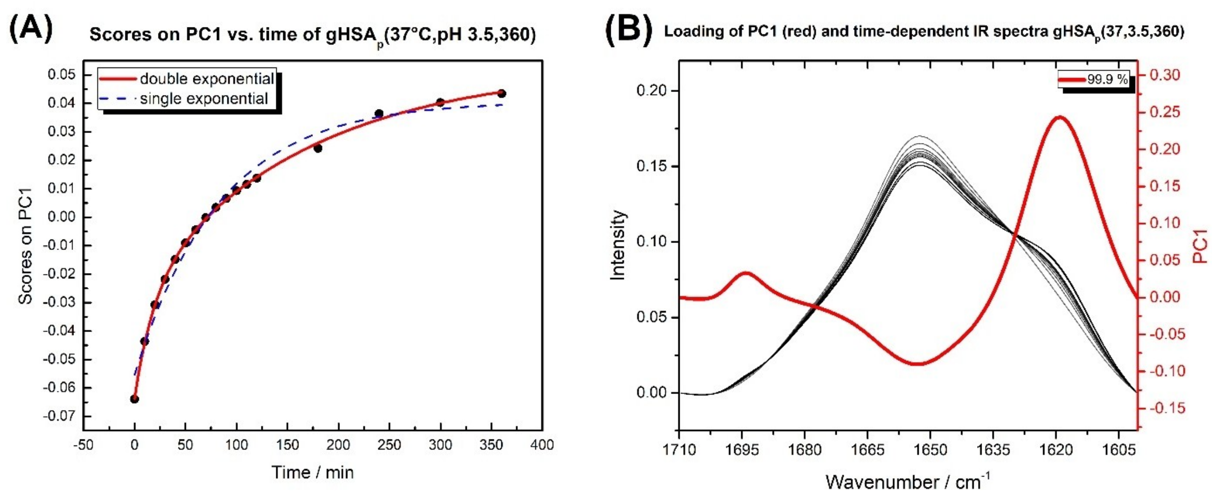


Figure S6. Diagram of PC1 scores against time for gHSA$_{p}$(37,3.5,360). (D) the time-dependent IR spectra of gHSA$_{p}$(37,3.5,360) and the loading of PC1 in red.

Simultaneous PCA on all three different gelation mechanism for HSA:

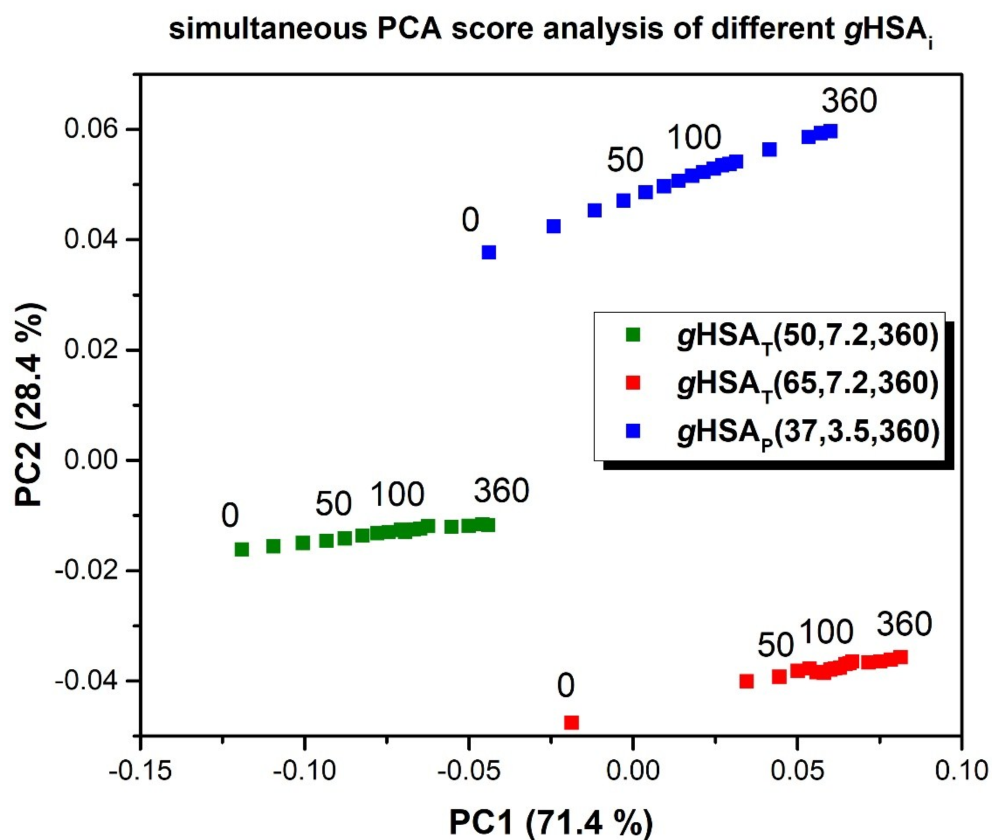


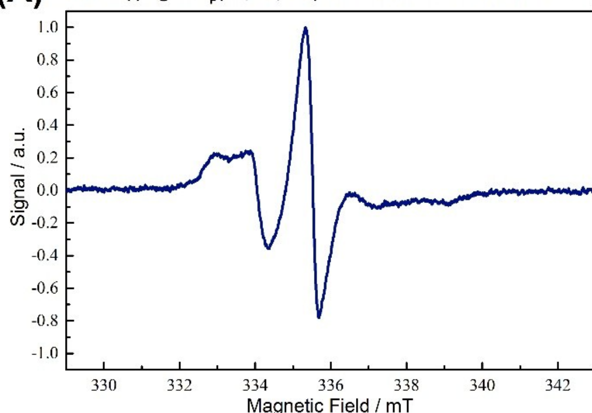
Figure S7. Diagram of PC1 against PC2 components during the gelation process of HSA. Blue squares depict the process of $g\text{HSA}_p$(37,3.5,360) preparation on the ATR-IR crystal. The green and red squares depict the $g\text{HSA}_T$(50,7.2,360) and $g\text{HSA}_T$(65,7.2,360) respectively. The PC2 component becomes relevant only for $g\text{HSA}_p$, where the pH is lowered to 3.5 by using 2 M HCl.

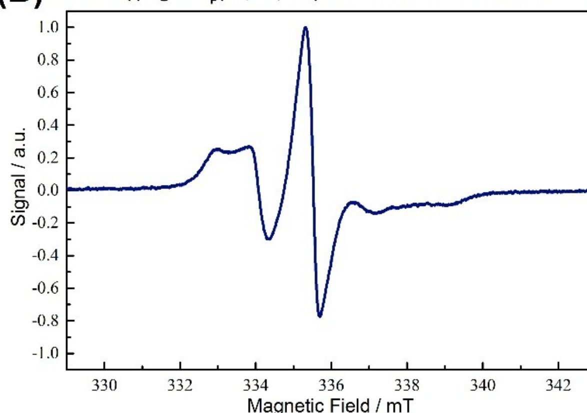
CW EPR Spectroscopy *g*BSA_p:

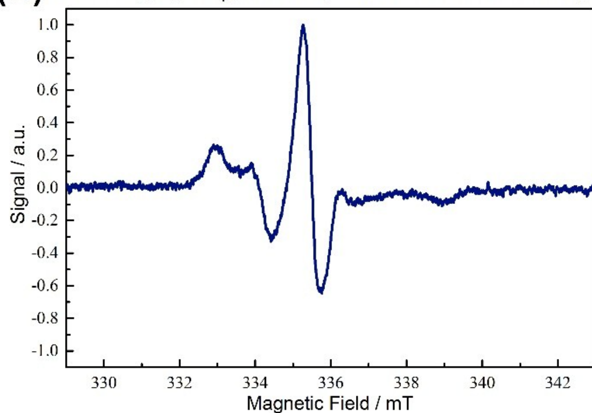
The pH induced albumin hydrogels would dissolve in water after formation. In order to make these hydrogels stable, there is another stage need to be done called acid leaching. The purpose is to neutralize the hydrogels by using a solution that extracts the acid while the remainder stays intact. It also increases the stability of the hydrogel in various other chemical environments. It is worth mentioning that this is the case when 2 M HCl is used for synthesizing the gels. If one uses another acid e.g. H2SO4 the hydrogels may be stable in water even without the leaching.

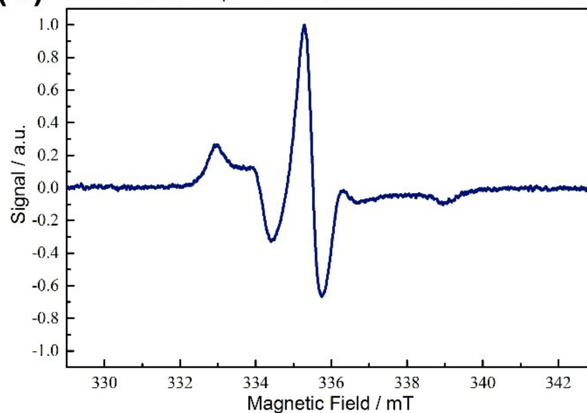
The hydrogels are prepared, acid leaching is completed and then FAs are introduced (method 3). (E) and (F) hydrogels at 1:1 and 2:1 16-DSA:BSA molar ratio. The hydrogels are prepared, and then FAs are introduced (method 3) and afterward acid leaching is done.

PBS buffer solution (pH 7.4) was used for acid leaching. The stop point was when the pH of the buffer solution in contact with the hydrogels remained at 7.4. The exact needed time for the completion of the acid leaching stage depended on the volume ratio of the buffer solution and the hydrogel and the interfacial area. It is also a function of the number of times the buffer solution in contact with the hydrogel is replaced with fresh PBS buffer solution.









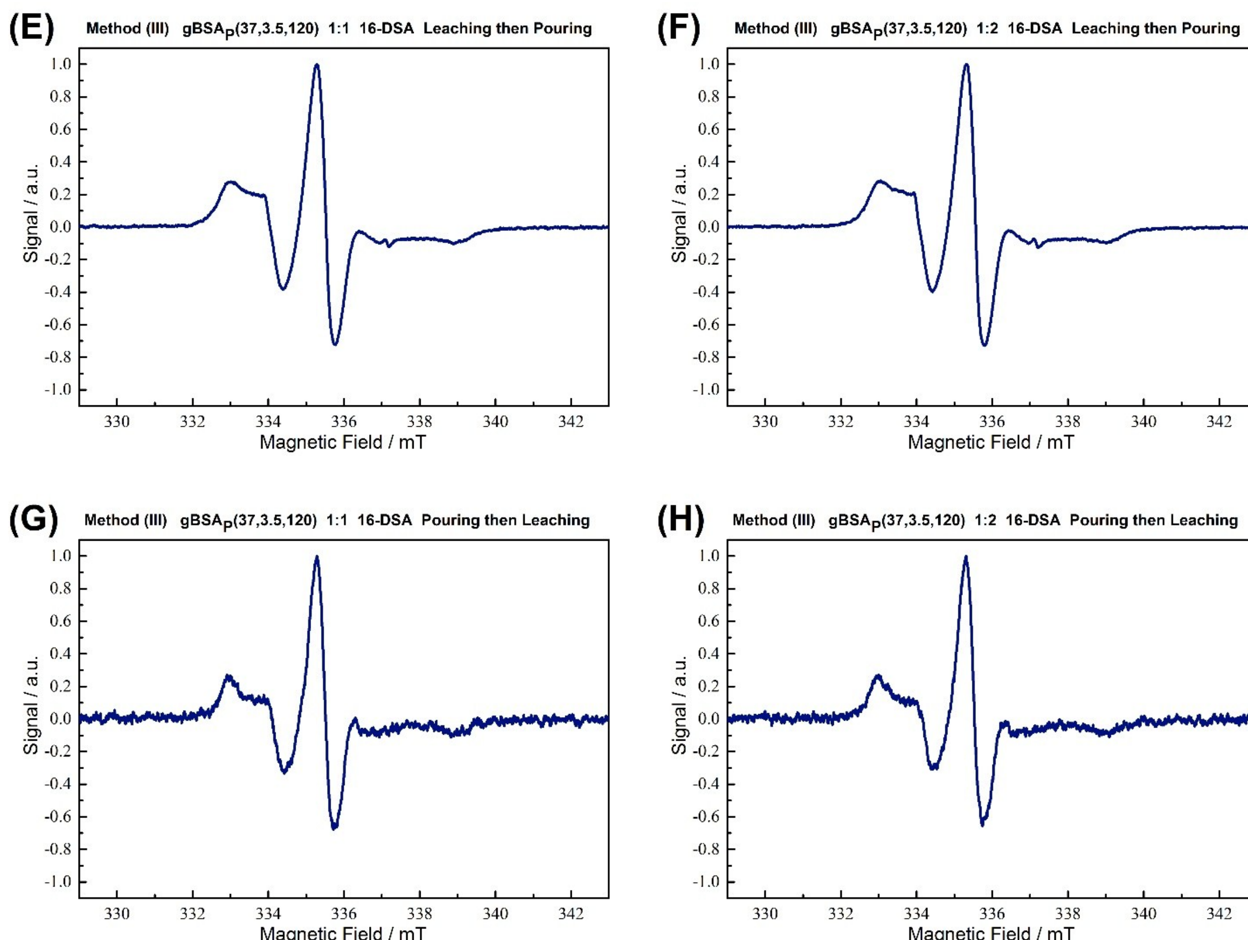


Figure S8. gBSAp(37,3.5,t) spectra (A) at 1:1 (a) and 2:1 (b) 16-DSA:BSA molar ratio without leaching (method 1). (C) and (D) hydrogels at 1:1 and 2:1 16-DSA:BSA molar ratio respectively, the spectra are obtained after leaching is completed (method 1).

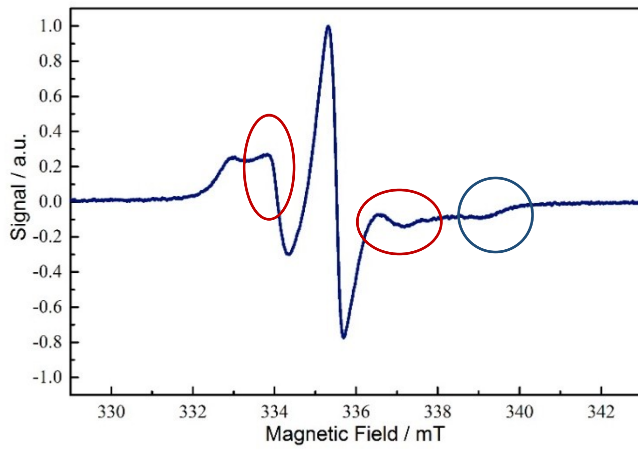
After the leaching has been completed the hydrogel would be stable in water (pH 7.3), PBS (pH 7.4), NaOH-H2O (pH 10.3) and HCl-H2O (pH 4.0) for more than three months; contrarily, the gBSAp hydrogel without the leaching dissolves in water very fast. It has been reported that gBSAp would completely degrade in 17 hours in 8 M urea or 10% SDS (Sodium Dodecyl Sulfate) even after leaching.

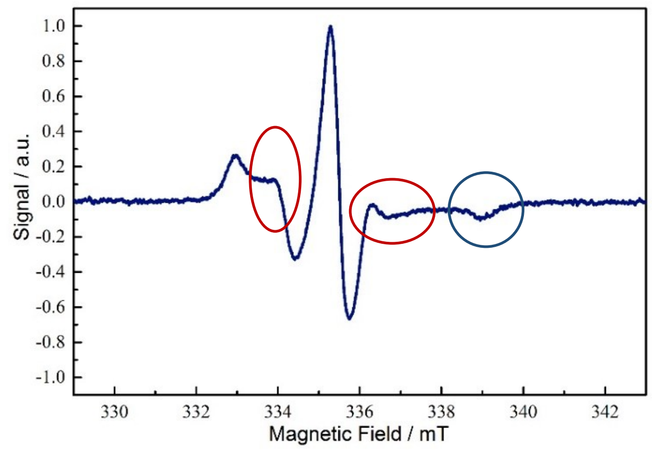
By comparing the spectra of (A) and (B) with (C) and (D) at 1:1 and 2:1 FA:BSA ratio respectively, one can understand that without leaching there are some FAs that tumble rather fast, a sign that they are not strongly bound to the protein, it is also obvious that some of the FAs, which were weakly bound to the protein are taken up during the leaching step and the ones that remained in the hydrogel are tightly bound to the protein. This is a robust proof that there are strong fatty acid binding sites inside the hydrogels formed with this mechanism.

In (E) and (F), we see that fatty acids diffuse to the hydrogel solution and bind themselves to the protein network. Only a very small amount of the 16-DSAs can tumble in fast regime. This shows that there are sites in the protein network which fatty acids would prefer to be there instead of aggregating or staying in solution. In (H) and (I),

remarkably, the spectra show that there are some FAs remains inside the hydrogels, a sign that there are very strong binding sites inside the gels.

Leaching take up weakly bounded FAs





CW EPR Spectroscopy using Tempo and 4-Hydroxy-TEMPO benzoate:

TEMPO and 4-Hydroxy TEMPO benzoate are smaller spin-probes compared to 16-DSA. To analyse the capacity of the hydrogels as drug delivery systems, we carried out EPR experiments using these two probes. For TEMPO, results are shown in Fig. S9, the TEMPO is water soluble and the powder of TEMPO was directly added to the precursor solution of the hydrogels. The results show that spin-probes are in fast tumbling region with the $\tau_c \cong 1.76\ ns$ and the Hyperfine splitting constant of 46.10 MHz**.** This was the case for all different gel preparation methods which can be regarded as characterization of how the trapped water in the hydrogels are immobilized.

For 4-Hydroxy TEMPO benzoate case, the results are significantly noteworthy. First of all, for sample preparation we dissolved the 4-Hydroxy TEMPO benzoate into Ethanol and made a 12 mM stock solution. Then according to the required molar ratio (BSA: 4-Hydroxy TEMPO benzoate) the 4-Hydroxy TEMPO benzoate solution was added to the precursor solution of the hydrogels (Method I). The results show that the spin-probes are in the fast or intermediate (see Table S1) region, However, we noticed that addition of Ethanol to the precursor solution change the mechanical properties of the hydrogels in remarkable way (see Fig. S11 and compare the results with Fig. 2). Not only do the ethanol decrease the concentration threshold of protein concentration for gel preparation but it also gives the possibility to make hydrogels at neutral pH (7.4) and 37°C. Besides, in contrast to 16-DSA, the 4-Hydroxy TEMPO benzoate increase the storage modulus value (and the difference between G´and G”) at relative time scales (see Fig. S11 (A) and (B)). These results should be seen as preliminary results and further investigation is required.

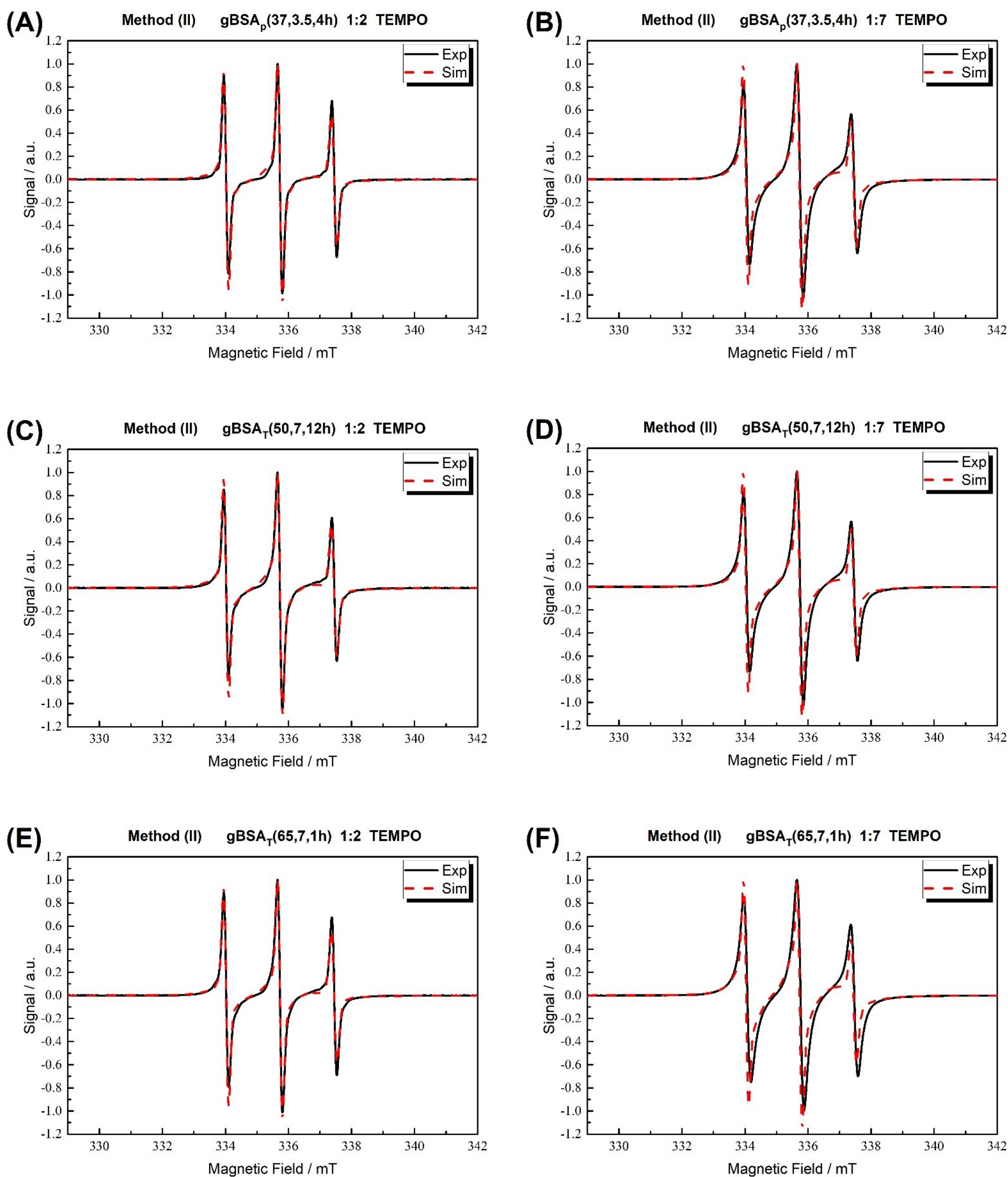


Figure S9. (A) $gBSA_p$(37,3.5,t) prepared after addition of TEMPO powder into the 20 wt. % precursor solution of BSA due to method(2), TEMPO:Albumin molar ratios of 1:2 (B)) $gBSA_p$(37,3.5,t) prepared after addition of TEMPO powder into the 20 wt. % precursor solution of BSA due to method(2), TEMPO:Albumin molar ratios of 1:7 (C) $gBSA_T$(50,7,t) prepared after addition of TEMPO powder into the 20 wt. % precursor solution of BSA due to method(2), TEMPO:Albumin molar ratios of 1:2 (D)) $gBSA_T$(50,7,t) prepared after addition of TEMPO powder into the 20 wt. % precursor solution of BSA due to method(2), TEMPO:Albumin molar ratios of 1:7 (E) $gBSA_T$(65,7,t) prepared after addition of TEMPO powder into the 20 wt. % precursor solution of BSA

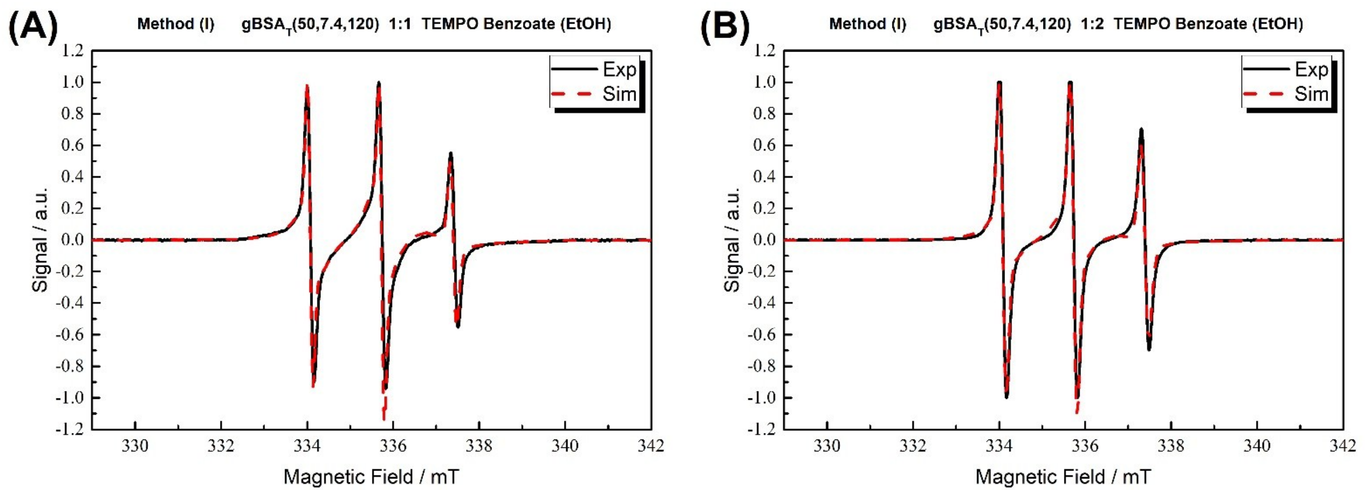


Figure S10. (A) gBSAT(50,7.4,t) prepared after addition of 4-Hydroxy TEMPO benzoate (12 mM solution dissolved in EtOH) due to method(1), TB:Albumin molar ratios of 1:1 (B) gBSAT(50,7.4,t) prepared once after addition of 4-Hydroxy TEMPO benzoate (12 mM solution dissolved in EtOH) due to method(1), TB:Albumin molar ratios of 2:1.

Table S1: Parameters of EPR Spectra of Figure S10.

Figure S10	Fraction	Correlation time τ_c (ns)	Hyperfine splitting constant a (MHz)
A			
	17%	0.17	46.78
	83%	2.6	44.1
B			
	31%	0.17	46.44
	69%	2.6	44.4

The influence of Ethanol and 4-Hydroxy-TEMPO benzoate on mechanical properties of the hydrogels:

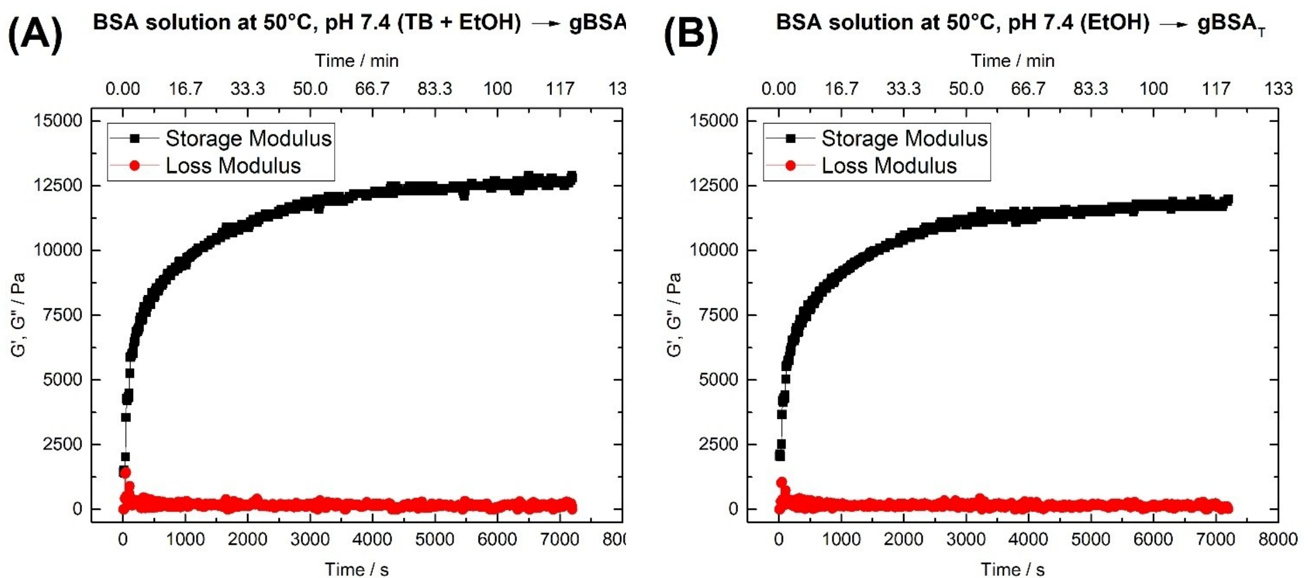


Figure S11. The influence of Ethanol and 4-Hydroxy TEMPO benzoate on the hydrogel formation. Time-dependent storage (G') and loss (G'') moduli of 20 wt. % (A) BSA precursor solution at 50°C, pH 7.4 without 4-Hydroxy TEMPO benzoate (B) BSA precursor solution at 50°C, pH 7.4 with 4-Hydroxy TEMPO benzoate (BSA 1:2 4-Hydroxy TEMPO benzoate molar ratio).