

## A rheological study to analyse the mixed gelation of a protein with a polysaccharide with the use of a cross-linker

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### ABSTRACT

We have studied the gel formation kinetics for mixed gels based on crosslinking human serum albumin (HSA) and high methoxyl pectin (HMP) with N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC), mixtures which have recently been patented for use as wound dressings<sup>1</sup>. Small deformation oscillatory rheology has been used to investigate the effect of pectin and EDC concentration on gelation and allowed us to provide a hypothesis for the gelation mechanism.

### INTRODUCTION

Wound dressings are a commonly used health care product which may serve one or more of the following functions: Protection of the wound from external influences, absorption of wound exudates, compression, and promotion of immobilisation as well as being a more acceptable sign of an injury than an open wound. For chronic wounds, the wound dressing also tends to serve as a delivery vehicle for wound healing drugs and typically gel-based dressings are used. Classical gel-based dressings are composed of a single or mixed polysaccharide and/or protein gel system based on, for example, starch, dextran, carboxymethylcellulose, pectin, heparin, collagen, chitosan, alginate, gelatine, and specific salts of alginic acid<sup>2,3</sup>.

More recently, dressing products for chronic wounds based on the protein human serum albumin (HSA) have been patented<sup>1,4</sup>. These novel dressings can be formed by chemically crosslinking HSA with the classical crosslinking agent N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC)<sup>5</sup>. Chemical crosslinking retains the native configuration of the protein and therefore its immunogenicity<sup>6</sup>. The material properties such as adhesiveness and brittleness of pure HSA gels, however, are unsuitable for its use as a wound dressing<sup>1</sup>. To improve these important characteristics for a wound healing dressing, one can introduce a second polymer such as the polysaccharide high methoxy pectin (HMP) and form a mixed gel system. HMP is preferred over other polysaccharides for several reasons such as water binding capacity and acceleration of wound healing, etc, as claimed in the this work underlying patent<sup>1</sup>. The presence of carboxylic acid groups is also crucial as explained below.

A HSA molecule consists of 585 amino acids of which 36 aspartic acids, 62 glutamic acids and 59 lysine are located at the surface. The joint total of 98 carboxylic acid groups of the former two and the amine group of each lysine participate in the polymerisation reaction with EDC leading to gel formation. The water-soluble crosslinker EDC was selected for its property to not introduce an atom into the

crosslinked molecules. It solely acts as an agent to enable covalent bonding between two molecules. Initially, it reacts with the carboxylic acid groups to create a highly reactive intermediate which then reacts with the amine groups (of another HSA molecule) to form covalent amide bonds accompanied by the release of the by-product isourea. One mole of EDC reacts with one mole of carboxylic acid group and then one mole of amine group. The optimal reaction medium for EDC has been described to be at a pH between 4.7 and 6<sup>5</sup>.

In the mixed HSA:HMP system investigated, EDC not only serves as a crosslinker between two HSA-based binding sites, it also potentially crosslinks HSA and HMP initiated by the presence of carboxylic acids in the molecular architecture of HMP with its  $\alpha$ -(1-4)-linked D-galacturonic acid backbone. In this work we test the hypothesis that a mixed gel network of HSA and HMP is formed based on the reaction scheme depicted in Fig. 1 by following the gelation kinetics rheologically using small deformation oscillatory studies. Our hypothesis of how gel formation proceeds for HSA its own, which we studied initially, and a mixed HSA:HMP is depicted schematically in Fig. 2.

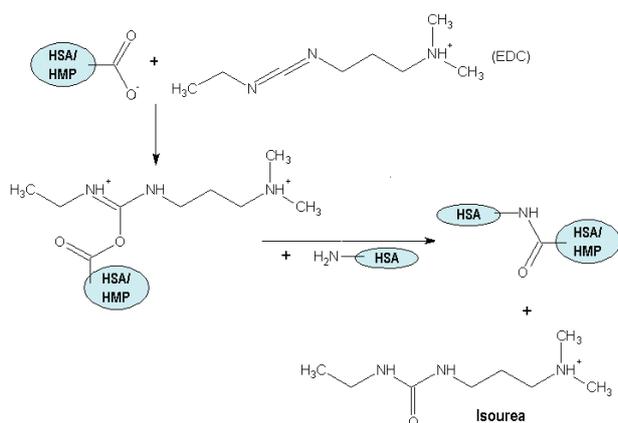


Figure 1: Crosslinking reaction scheme

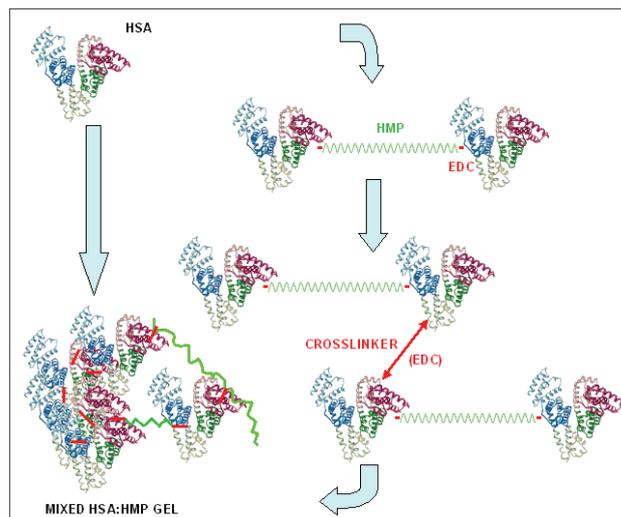


Figure 2: Schematic of hypothesised gel formation process. EDC: short straight line —; HMP: long zigzag line  $\wedge\wedge\wedge$  (The picture of HSA was obtained from Curry S. *et al*, 1998)<sup>7</sup>

## MATERIALS AND METHODS

Clinical grade HSA in 20% w/v aqueous solution in which the purity of dry HSA is greater than 95% was purchased from Grifols (Cambridge, UK). According to the supplier, the solution contains between 130-160mmol/l of sodium and not more than 2mmol/l of potassium. HMP in form of citrus pectin with an acclaimed DE>60% and no added sugar (classic CU 401 USP standard) was obtained from Herbstreith & Fox (Neuenbürg, Germany). EDC with  $\geq 99.0\%$  purity was purchased from Apollo Scientific (UK). Hydrochloric acid, sodium hydroxide, and low viscosity mineral oil were of reagent grade and obtained from Sigma-Aldrich (UK). All materials were used as obtained.

All samples were prepared at room temperature (25°C). Mixtures of HSA and HMP were prepared by dissolving an appropriate amount of HMP powder into 20% w/v HSA solution on a magnetic stirrer to obtain final levels of HMP in the mixed system of 0.8% w/v or 1.6% w/v. Subsequently, the pH of the mixture was adjusted using 1M HCl and 1M NaOH to

pH6.9 which corresponds to the natural pH of the pure HSA system investigated. This pH is not within the optimum pH range for EDC, and future studies will include evaluation of the effect of pH on the gelation kinetics. To induce gelation, an appropriate volume of aqueous solution of EDC at 6.9% w/v was added to result in a specific molar proportion of EDC:HSA in the final sample which was 30 moles of EDC to 1 mole of HSA for most of our work. This corresponds to a theoretical total of half of all possible crosslinking sites being involved in the polymerisation reaction and it results in a free standing gel. Typically, 250 $\mu$ l of EDC solution were added to 1ml of the biopolymer mixture using an Eppendorf pipette followed by rapid mixing on a vortex agitator. The sample was then loaded onto the rheometer.

Small deformation rheological experiments were carried out using a stress controlled dynamic shear rheometer (MCR301 ex Anton Paar, Austria) fitted with a 50mm smooth parallel plate geometry. The gap height was 900 $\mu$ m and the rim of the sample was covered with low viscosity mineral oil in order to prevent solvent loss during the measurements. Dynamic oscillatory shear tests at a single frequency of 1Hz and a constant strain amplitude of 1% were conducted isothermally at 25 $^{\circ}$ C to follow the gelation of the HMP:HSA mixture as induced by EDC. The strain amplitude was carefully controlled (using the direct strain oscillation control at the MCR301) and we confirmed that 1% strain corresponds to working in the linear viscoelastic domain as soon as a value for the storage modulus was measurable.

## RESULTS AND DISCUSSION

At the pH of the mixture, pH 6.9, the HSA molecules show little conformational change<sup>8,9</sup>. HSA and HMP are negatively charged since their isoelectric point (IEP) and pKa value is 4.8<sup>8</sup> and 3.55<sup>10</sup> respectively. For BSA:pectin mixtures

formation of single phase mixtures at pH values above the BSA IEP has been reported<sup>11</sup>. Based on centrifugation for 90 min at 2600g, we also found no evidence of phase separation in the liquid HSA:HMP mixtures. This of course, does not imply that the system remains single phase during gelation.

In the following we first show results for the EDC induced chemical gelation of HSA on its own before discussing the behaviour of HSA:HMP mixtures.

### Gelation kinetics for HSA as induced by addition of EDC

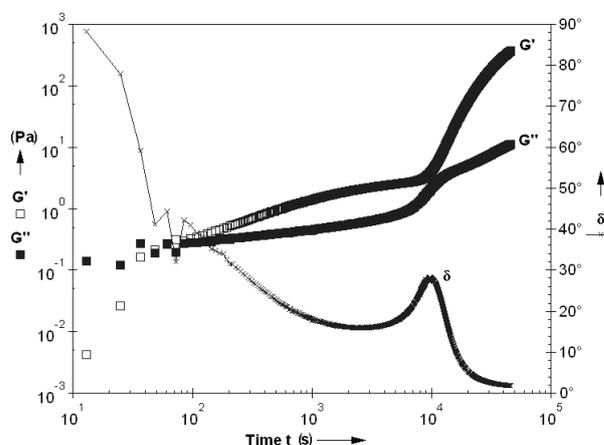


Figure 3:  $G'$ ,  $G''$  and phase angle recorded during gelation of 16% w/v HSA in presence 30:1 moles of EDC:HSA and at pH6.9 ( $f = 1\text{Hz}$ ,  $\gamma = 1\%$ ,  $T = 25^{\circ}\text{C}$ )

Fig. 3 shows the evolution of the dynamic moduli  $G'$  and  $G''$  during gelation of a 16% w/v HSA solution induced by EDC. The molar concentration of EDC is 30 fold the molar concentration of HSA and the pH of the mixture is 6.9. To facilitate the discussion of the results, the phase angle has been plotted on the right-hand ordinate.

First, we can note that the data for the phase angle are initially not smooth and data for  $G'$  are extremely low. This indicates that the system is dominated by liquid behaviour, prior to the gel point.

About 100s into the measurement, the phase angle assumes values below  $45^\circ$  followed by a continued decrease before showing a maximum at about  $10^4$ s. This maximum is due to an apparent increase of the loss modulus relative to the storage modulus. Following the maximum in  $\delta$ , the observed increase in  $G'$  is steeper than the increase in  $G''$ . Both continue to increase for the remaining time of the experiment and therefore  $\delta$  continues to decrease. It asymptotically reaches a phase angle close to zero which indicates that the gel network is highly elastic with little viscous components.

The data suggest a two-step gelation behaviour with a first and a second gelation event at  $\sim 100$ s and  $10^4$ s respectively. Based on the reaction scheme presented in Fig. 1, one could argue that observation of a two-step gelation process is not surprising. However, the time-lag between the 2 gelation events makes this unlikely which leads us to hypothesise that the gelation event at 100s is a consequence of the full two-step chemical gelation process. Individual protein aggregates start to form and they start dominating the rheological response. These primary particles grow in volume as time proceeds and they will reach a critical volume so they start interacting with each other and coherent gel network formation is initiated. This is related to the second gelation event at about  $10^4$ s. Aggregate interaction has significantly proceeded from the starting nuclei and prior to a coherent network being formed a slight network rearrangement takes place, at the time  $\delta$  reaches a maximum, followed by a coherent gel network development, when the storage modulus of the system increases at a faster rate than the loss modulus. We hypothesise that this second gelation event is akin to physical gelation in a sense that the closest distance between primary particles has decreased such that covalent cross-linkage between is enabled.

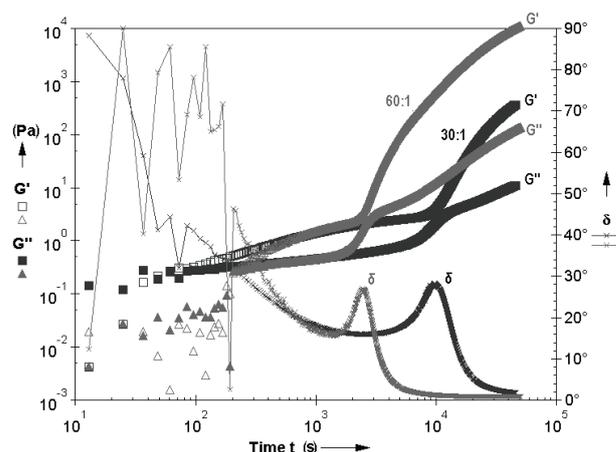


Figure 4:  $G'$ ,  $G''$  and phase angle recorded during gelation of 16% w/v HSA in presence of variable molar ratio of EDC:HSA (30:1, 60:1) and at pH6.9 ( $f = 1$ Hz,  $\gamma = 1\%$ ,  $T = 25^\circ\text{C}$ ).

Further insight can be obtained from changing the levels of EDC in the system. Fig. 4 for example shows the gelation kinetics at twice the molar ratio of EDC. The first gelation event is delayed whereas the second gelation event is observed earlier. The gel moduli at the final stages of the experiment are roughly one order of magnitude higher. This concentration dependence on EDC points towards the formation of smaller protein aggregates at the first gelation stage which can be compared to the formation of a larger number of nucleation sites. The protein aggregates will also be denser since the rate of the chemical reaction is faster leading to a smaller overall volume fraction of particles at equivalent times. We hypothesise that a minimum volume fraction of aggregates is required before we obtain a measurable value for  $G'$  in the oscillatory test, hence, the later observation of the first gelation event. The nuclei continue to grow in volume and the comparatively larger number of them enables the formation of a coherent gel network at an overall smaller aggregate size. Therefore, the second gelation event is observed earlier. Also, the smaller size and

larger number goes along with a larger interaction potential due to the increased surface area resulting in a finer network structure detected as higher gel moduli at comparable later times during the experiment. In support of this statement the increase in cooperativity of interaction can be noted from a narrowing of the peak width around the phase angle maximum.

### HSA:HMP mixtures

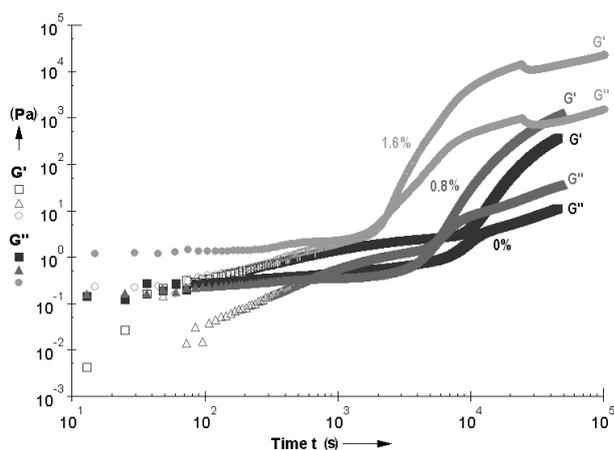


Figure 5:  $G'$ ,  $G''$  recorded during gelation of 16% w/v HSA in presence of variable concentration of HMP (0% w/v, 0.8% w/v, 1.6% w/v), 30:1 moles of EDC:HSA and at pH6.9 ( $f = 1\text{Hz}$ ,  $\gamma = 1\%$ ,  $T = 25^\circ\text{C}$ )

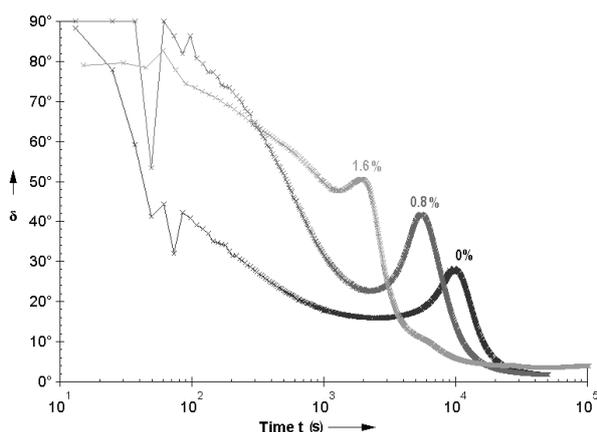


Figure 6: Phase angle data for moduli presented in Fig. 5

Fig. 5 and Fig. 6 shows the dynamic moduli and the phase angle respectively for the gelation of HSA:HMP mixtures. The concentration of HSA and the pH in the mixtures are equivalent to the pure HSA gels discussed above, 16% v/w and pH6.9 respectively. The molar concentration of EDC:HSA was also 30 and the two concentrations of HMP in the mixed systems were 0.8% w/v and 1.6% w/v. The results for zero addition of HMP are included in the graphs to facilitate the discussion.

The results clearly indicate that presence of HMP in the system affects both, the initial gelation kinetics as well as the final gel moduli. The differences in gelation kinetics are most evident from the evolution of the phase angle depicted in Fig. 6. The time elapsed when the phase angle first assumes a value below  $45^\circ$  increases with increasing concentration of HMP. In fact, in presence of 1.6% w/v HMP the phase angle decreases to a value just above  $45^\circ$  and values below  $45^\circ$  are actually not assumed as a consequence of what we previously termed the first gelation event. For this system, 1.6% w/v HMP, the first gelation event corresponds to the minimum of the phase angle before it goes through a maximum. We observed this maximum behaviour for the pure HSA gel, and also see it for the mixture containing 0.8% w/v HMP, and we argued that the maximum in  $\delta$  at this later stage of the experiment indicates a second gelation event. Its dependence on HMP concentration, with otherwise constant conditions (pH, molar ratio of EDC:HSA), is such that it occurs earlier with increasing HMP concentration. At the same time the value of the maximum phase angle is higher, and above  $45^\circ$  for the mixture containing 1.6% w/v HMP. Following the maximum in  $\delta$  it asymptotically reaches a value of zero. The final phase angles recorded for HSA and the mixed system containing 0.8% w/v HMP are similar (just below  $2^\circ$ ). The mixed

system based on 1.6% w/v HMP shows a higher late stage phase angle ( $4^\circ$ ).

The dynamic moduli for the mixtures (see Fig. 5) show the same overall evolution with time as the pure HSA system. However, the data for the mixed system containing 1.6% w/v HMP show a discontinuity some time after the 2<sup>nd</sup> gelation event. Based on the current evidence we can only speculate that this is in an indication of a longer time rearrangement of the gel network.

The delay in the first gelation event with increasing HMP concentration and the fact that the rheological response of the system with the higher of two investigated HMP concentrations is dominated by the viscous signal leads us to the hypothesis that the presence of HMP slows down the initial chemical reaction leading to protein aggregates. This could simply be a viscosity effect impacting on diffusion rates, as an ungelled pectin solution, depending on concentration, would show a phase angle greater than  $45^\circ$ . Whether HMP molecules are part of the initial gelation reaction is not possible to deduce from these data. The formation of the coherent gel network associated with the second gelation event is accelerated in presence of HMP which may indicate participation of HMP in the formation of the global network. We hypothesise that HMP plays a major role in 'connecting' (bridging or crosslinking) the primary particles which is supported by the fact that the relative magnitude of the viscous component,  $G''$ , is higher compared to the relative magnitude of the elastic component,  $G'$ . Also, an increased HMP concentration imparts a small but measurable viscous component to the final gel detected as a small but finite phase angle. However, an increase in HMP concentration does not have the same effect on the cooperativity of the 2<sup>nd</sup> gelation step, as evidenced by an increase in the EDC concentration.

## CONCLUSIONS AND FUTURE WORK

The results on the gel formation of HSA:HMP as chemically induced by the presence of EDC indicate that controlling the reaction rate by the level of the EDC crosslinker and the concentration of the interacting polysaccharide HMP affects the gel(ation) properties and therefore it can be expected that these parameters impact on the performance of the system as wound healing dressing. This work is ongoing with the current being on investigating the parameters affecting the reactivity of EDC which are the relative molar concentration of EDC and the pH of the system. The gels will also be visualised using microscopy techniques to see if the variable parameters will impact on the fine structure of the gels produced.

## ACKNOWLEDGMENTS

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