Gelatin-Polysaccharide Mixed Biogels: Enzyme-Catalyzed Dynamics and IPNs

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Summary: Various gelatin gels were studied by rheology in the presence of polysaccharides. Hyaluronic acid dissolved in the sol phase of gelatin gels modifies their viscosity but not the protein network. Using a hydrolase, the properties of the solution were modified in a controlled way. Similar experiments were realized with alginate and gave qualitatively identical results. Mixtures of alginate and gelatin were then studied as a function of different parameters, such as temperature and biopolymer concentrations. Under certain conditions, the formation of interpenetrating polymer networks of alginate and gelatin were obtained, the physical gelatin network being almost reversible inside the alginate one.

Keywords: biopolymers; gels; interpenetrating network; phase separation; rheology

Introduction

Biopolymer mixtures have been extensively studied for the last 20 years.[1–3] When two biopolymers (proteins and / or polysaccharides) are mixed together, three different behaviors can occur.[4–7] In some rare cases, the biopolymers are miscible and co-exist in a single, thermodynamically stable phase.[8] Most often, mixing two biopolymers results in phase separation, which can be associative (the first phase being enriched in both polymers, the second one in solvent) or segregative (each phase being enriched with one of the two biopolymers). The situation becomes more complex if one or two polymers undergo a sol-gel transition.

With more than one gelling agent, filled, complex or mixed gels can be obtained.[9] Critical parameters are the limit of co-solubility of the two biopolymers and their critical gelation concentration. Filled gels are constituted by a network filled with another non-gelling polymer. In complex gels, both polymers gel and interact with each other. Mixed gels consist in two relatively independent 3-dimensional networks and occur when the concentrations of each polymer are higher than the critical gelation concentration. Synergistic or antagonist effects can exist between the two biopolymers.

Gelatin is a protein obtained by the denaturation of the triple helix of collagen. In solution at moderate temperature, the gelatin chains are random coils, which associate into helices and gel when temperature decreases.[10,11] The behavior of mixtures of gelatin and various polysaccharides, such as maltodextrin,[1,2,12,13] pectin,[14–16] or alginate[9,14,17,18] has been studied.

In the present paper, we used rheology and polarimetry to characterize gelatin gels obtained in the presence of various polysaccharides. First, filled gels were realized in the presence of hyaluronic acid. Both the mechanical properties and the effect of polysaccharide on the gelation of gelatin were investigated. The implication of hyaluronic acid degradation by a hyaluronidase on the macroscopic properties of the gel was studied. Similar experiments were realized with alginate and alginate lyase. Then, mixed gels of gelatin and

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alginate were studied and the influence of experimental conditions, such as the temperature and the biopolymer concentrations, on the behavior of the mixture were investigated to highlight the contribution of both polymers during the gelation process. Finally, we studied the dynamics of an interpenetrating network formed by these two different biopolymers.

Influence of Hyaluronic Acid on Gelatin Gel Properties

Gelatin, type A1, Sigma G2500, extracted from pig skin by an acid process (p$I = 8.8$), bloom 300 is used at a final 7% (w/v) concentration. Hyaluronan (HA) from *Steptrococcus Equi species* (Fluka, 53747, M_w about 1 000 000 g mol^{-1}) was mixed with the 7% gelatin solution and the temperature was reduced from 40 to 27 °C (−0.5 °C min^{-1}), allowing the mixture to form a gel. Rheology measurements were performed using an apparatus with a cone/plate geometry (60 mm/2), with a strain of 0.05 and a frequency of 6.83 rad · s^{-1} (1 Hz). The viscoelastic behavior of the system at ω is characterized by the storage modulus, $G'(\omega)$, and the loss modulus, $G''(\omega)$, which respectively characterize the solid-like and fluid-like contributions to the measured stress response. Storage modulus G′ and loss modulus G″ were recorded as a function of time.

Figure 1 shows the temporal evolution of the rheological parameters, G′ and G″, for the mixtures containing 0.0%, 0.5% and 1% polysaccharide. Whatever the conditions, the system turns to a gel at 27 °C, since rheological moduli increase and G' becomes higher than G″. After 150 minutes, G′ and G″ reach values of 680 Pa and 135 Pa, respectively, in the presence of 1% hyaluronic acid as compared to the values of 225 Pa and 16 Pa, respectively, without the polysaccharide. The evolution of viscoelasticity parameters is dependent on hyaluronic acid concentration following exponential relations (data not shown). The influence of polysaccharide is higher on the loss modulus (G″, related to the liquid phase viscosity) than on the storage modulus (G', related to the elasticity of the solid network).

The figure shows that hyaluronic acid accelerates the sol/gel transition of gelatin. This can be attributed to an excluded volume effect. This hypothesis is reinforced by the reduction of the water availability for gelatin due to the water shell necessary for the polysaccharide solvation.

The kinetics of triple helix formation was followed by polarimetry (Figure 2) on

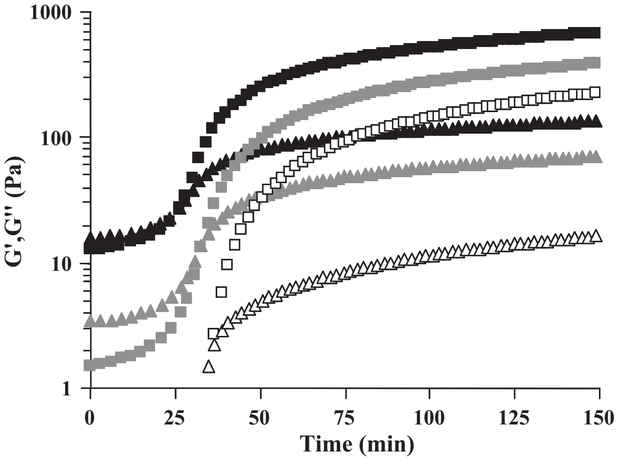

Figure 1.
Evolution of viscoelasticity parameters (G′: square, G′′: triangle) of 7% gelatin gels containing 0.0% (white symbols), 0.5% (grey symbols) or 1% hyaluronic acid (black symbols).

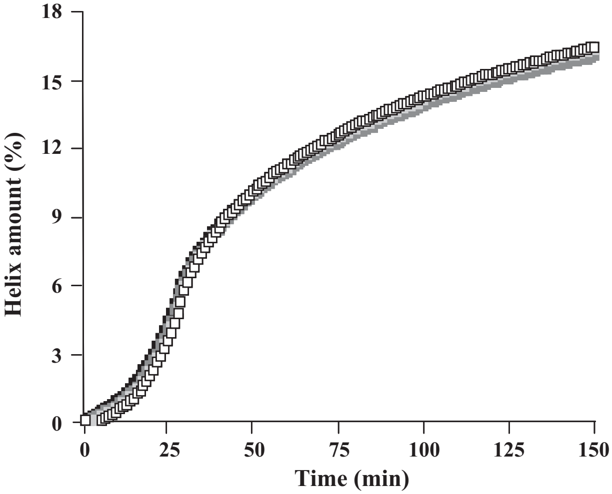

Figure 2.
Influence of hyaluronic acid on gelatin triple helix formation. Gelatin alone (white square) or with 0.3% hyaluronic acid (light grey square), 0.7% hyaluronic acid (dark grey square) and 1% hyaluronic acid (black squares).

mixtures containing 7% gelatin and various concentrations of hyaluronic acid. The percentage of helices is derived from the specific optical rotation. The helix fraction χ is derived from:

$$\chi = \frac{[\alpha]_\lambda{}^{\text{helix}} - [\alpha]_\lambda{}^{\text{coil}}}{[\alpha]_\lambda{}^{\text{collagen}} - [\alpha]_\lambda{}^{\text{coil}}}$$

The helix percentage is equal to 100 χ and expressed in %. λ is the wavelength ($\lambda = 435$ nm), $[\alpha]_\lambda = \alpha/lc$ is the specific optical rotation of the protein in solution, c is the concentration (g cm^{-3}), l is the optical path (0.1 dm), α is the optical rotation angle (degrees) measured experimentally, $[\alpha]_\lambda{}^{collagen}$ is the specific optical rotation of native soluble collagen ($\chi = 1$), which contains only triple helices, and $[\alpha]_\lambda{}^{coil}$ is the specific optical rotation of the coils ($\chi = 0$). $[\alpha]_{435nm}^{collagen} = -800$ deg cm^3 g^{-1} dm^{-1} at 27 °C, $[\alpha]_{435nm}^{coil} = -274$ deg cm^3 g^{-1} dm^{-1} at pH 7.4 and 40 °C.

In any case, the triple helix rate reaches a value of 16.5% after 150 min. Moreover, the kinetics is nearly superimposable, indicating that hyaluronic acid does not influence the association of random coil gelatin chains into triple helices.

These results show that the mixture of gelatin and hyaluronic acid leads to a filled gel: the gelatin network is formed independently of the polysaccharide which is simply in solution in the sol phase of the protein gel. The presence of the large polysaccharide molecules (average Mw = 10^6 Da) mainly modifies the properties of the sol phase of the gel.

To determine the influence of hyaluronic acid degradation on the characteristics of a filled gelatin gel, a solution of hyaluronidase (bovine testicular, Sigma H3506, 451 U mg^{-1}) at various concentrations (1, 5 and 10 U mL^{-1}) was added to a solution containing 7% gelatin and 1% hyaluronic acid. Gelation of this mixture was induced by a temperature decrease from 40 to 27 °C and the evolution of the rheological parameters were followed by rheology over 900 min (Figure 3).

Hyaluronidase induces a variation of viscoelasticity parameters before gelation as shown by a decrease of the G′ and G″ values compared to those observed without the hydrolase in Figure 1. However, after gelation, there is no difference between the evolution of the various samples and after 900 min, whatever the quantity of hyaluronidase used, G’ values tend to a common

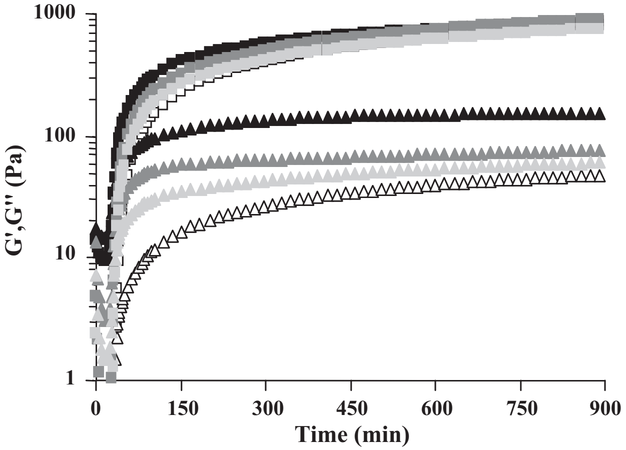

Figure 3.
Influence of hyaluronic acid degradation on viscoelasticity (G′: squares, G″: triangles) evolution of a 7% gelatin gel containing 1% hyaluronic acid. 1 U mL^{-1} of hyaluronidase (black symbols), 5 U mL^{-1} of hyaluronidase (dark grey symbols), 10 U mL^{-1} of hyaluronidase (light grey symbols). Gelatin alone (white symbols).

value of 840 Pa. The loss modulus is also depending on the quantity of enzyme used: the higher the hyaluronidase concentration, the lower the G″ value. However, in each case, after the enzyme has reacted, the loss modulus value is close to that of the simple gelatin gel. The enzyme is thus able to influence both the elasticity of the system and its viscosity.

The hyaluronic acid remodelling by hyaluronidase leads to a protein-oligosaccharide system whose rheological characteristics are equivalent to the simple gelatin gel without polysaccharide. Using hyaluronic acid and hyaluronidase, it was thus possible to modulate the mechanical properties of a gelatin gel with time and in a controlled way.

Influence of Alginate on Gelatin Gel Properties

First, a similar experiment was realized with alginate from *Macrocystis pyrifera* (Sigma A7253) and alginate lyase from *Flavobacterium sp.* (Sigma A1603, 38730 U g^{-1}) instead of hyaluronic acid and hyaluronidase. The interest of alginate resides in its capability to give rise to a gel phase in the presence of Ca^{2+}. Gels were realized at 27 °C with 5% gelatin containing 0% or 1% alginate under conditions such as alginate does not undergo sol/gel transition. A gelatin gel containing alginate molecules in solution is obtained which may be considered as a filled gel. Whatever the alginate concentration tested, the gel point occurs for the same helix content of 9% indicating that alginate is not part of the network. Then various mixtures were compared through their gel time, G′ and G″ values after 4 hours at 27 °C (Table 1).

Alginate accelerates the sol/gel transition of gelatin, i.e. its association into triple helices. The variations here observed cannot be attributed to a simple mass change. When a 6% gelatin gel is compared to a 5% gelatin gel containing 1% alginate in the liquid phase, the gel time at 27 °C and the G′ value at 240 minutes are similar (8.5 vs. 11 minutes and 252 vs. 256 Pa), but the G″ value increases from 17 to 44 Pa indicating that the sol phases are different. When 1% oligoalginates (previously obtained from alginates by alginate lyase catalyzed hydrolysis) are included into a 5% gelatin gel, the gel time drops to 6.5 min and the G′ value is the same as for a gel containing 1% alginate. As oligoalginates are shorter polysaccharidic chains, the viscosity of the solution estimated through G″ values is lower than in the presence of the alginate.

Table 1.
Properties of gelatin gels formed in the presence of alginate or oligoalginates.

	Gel Time (min)	G′ value$^{240\,\mathrm{min}}$ (Pa)	G″ value$^{240\,\mathrm{min}}$ (Pa)
Gelatin 5%	22	102 ± 6	9 ± 0.6
Gelatin 6%	8.5	252 ± 14	17 ± 1
Gelatin 5% + Alginate 1%	11	256 ± 15	44 ± 3
Gelatin 5% + Oligoalginate 1%	6.5	264 ± 16	21 ± 2

Then, the gelation of gelatin was followed simultaneously with alginate hydrolysis. A 5% gelatin solution containing 1% alginate and 0.2 U mL^{-1} of alginate lyase was used. In these experiments, temperature was maintained at 27 °C for 4 hours to allow gelatin gel formation, then raised to 40 °C to melt the gel and the whole process was repeated three times with the same sample (Figure 4).

A new gel is formed each time the temperature is decreased to 27 °C; however, the gel time decreases from 9–10 minutes to 7 and to 5.5 minutes. As previously shown, reducing the alginate chain length reduces the gel time. These results confirm that alginate and oligoalginates favor the gelatin network formation. The viscoelastic properties of the gel are identical in the 3 successive runs; the process for the protein gelation is perfectly reversible.[18] The gel described here can be considered as a semi-interpenetrating network where the network is a physical gel due to hydrogen bonds.

Gelation Behavior of Alginate-gelatin Mixtures

The next step was to obtain an interpenetrating network where both gelatin and alginate were allowed to form their own independent network. Several combinations were assayed. It was impossible to generate an alginate gel inside the previously formed gelatin gel. Finally, the formation of a protein network inside a calcium-alginate gel was tested. However, the formation of the alginate/gelatin co-gel is very sensitive to experimental conditions which can modify the behavior of both polymers.[19]

In the presence of gelatin, the formation of alginate gel is dependent on temperature, as described in Figure 5. Addition of D-Glucono-δ-lactone (GDL, 180 mM) to a solution containing alginate, gelatin and calcium ions chelated by EDTA (CaEDTA, 180 mM) leads to a slow release of Ca^{2+} ions, which allows the formation of a homogeneous alginate gel. At 20 and 27 °C, the alginate gel is normally formed

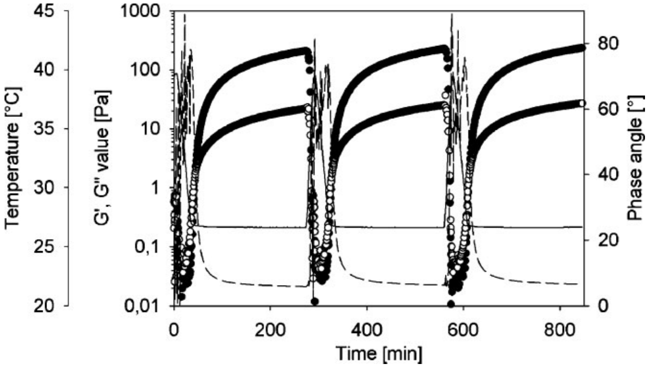

Figure 4.
Rheological profiles of 5% gelatin gels supplemented with 1% alginate and 0.2 U mL^{-1} alginate lyase. G′ value (closed circle), G″ value (open circle), phase angle (dashed line), temperature (line).

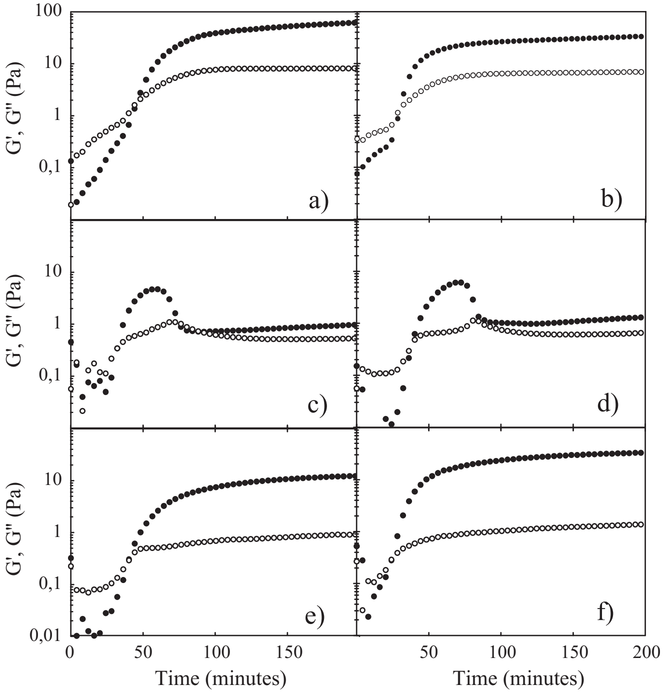

Figure 5.
Effect of temperature on the gelation of alginate, for a mixture with alginate 1%, gelatin 1.5%, CaEDTA 180 mM, GDL 180 mM. (a) 20°C; (b) 27 °C; (c) 40 °C; (d) 45 °C; (e) 50 °C; (f) 60 °C. G′ (closed circles), G″ (open circles).

(Figure 5 a and b). At 40 °C and 45 °C (Figure 5 c and 5 d), G′ and G″ begin to increase up to respectively 5 and 1 Pa (40 °C) or 6 and 1 Pa (45 °C), but then G′ decreases sharply to values close to 1 Pa. The elastic modulus G′ remains higher than the viscous modulus G″, suggesting that the mixture is in a weak gel state. Visually, the mixtures are viscous, heterogeneous and sticky solutions. At 50 and 60 °C (Fig 5 e and 5 f) the alginate gel formation becomes possible again. The value of G′ at 50 °C is lower than those at 27 °C and 60 °C, suggesting that 50 °C constitutes an intermediate situation between the collapse of alginate gel around 40 °C and the optimal gel formation. The values of G″ above 50 °C are smaller than that at 27 °C, perhaps because of a smaller viscosity of gelatin at these high temperatures.

For alginate (1%) and gelatin (1.5%) mixtures, at 4 °C or 20 °C, a gel is formed after respectively 30 minutes and 3 hours, with or without GDL. This suggests that this gel is a result of the formation of a gelatin network. On the other hand, when the mixture is thereafter placed at 40 °C, a gel state is still observed, despite the melting of the gelatin gel, and is probably constituted by an alginate network. It can, therefore, be assumed that at 4 °C or 20 °C, both alginate and gelatin networks are formed at the same time.

Both alginate and gelatin concentrations were thus modified to investigate the effect of polymer concentrations on alginate gelation at 40 °C. First, the alginate concentration was kept constant (1%) and the gelatin concentration varied from 0 to 2%. Then, the same experiment was done with a constant gelatin concentration (1%) and increasing concentrations of alginate (from 1% to 1.5%). By comparing the obtained results, it appears that the total biopolymer concentration is the key parameter in the alginate gel break. If the total concentration is lower than 2%, the alginate solution can gel, but above 2.25% a further evolution of the gel into a viscous heterogeneous solution is observed.

Interpenetrating Alginate-Gelatin Networks

Using the conditions determined above and elsewhere [19], a co-gel of alginate and gelatin was formed at 27 °C. It can be considered as an interpenetrating polymer network constituted of two different gels. The alginate gel was first obtained by complexation of alginate with calcium ions, and then the gelatin gel was formed by cooling the mixture (Figure 6). Further temperature cycles from 27 to 10 °C induce successive melting and gelation of gelatin. Each time the gelatin is melted at 27 °C, the gel recovers the G′ and G″ values of the calcium-alginate gel (~300 and ~24 Pa, respectively). In contrast, the G′ value after 480 min at 10 °C decreases (from 787 to 579 Pa after two runs) each time a new gelatin network is formed. In consequence, the gelatin physical network may be formed into the alginate gel, but it is more difficult to form after melting phases.

If the same system is studied by polarimetry at 435 nm, a large optical rotation shift follows each of the three temperature decreases from 27 to 10 °C due to helix formation. Helix concentrations reach successively 40.5% (6 mg mL^{-1}), 37% (5.5 mg mL^{-1}) and 33% (4.9 mg mL^{-1}). Less and less helices are formed but they are theoretically sufficient to generate an infinite network (> 4.5 mg mL^{-1}).

Adding an alginate lyase to this system led to an increase in gel time, a sharp decrease of G′ and G″ (9.6 vs. 309 Pa and 3.4 vs. 25 Pa, respectively). Applying temperature cycles to this mixture resulted in various gel properties. In this system, gelatin gel was fully reversible as successive metling-gelling cycles gave the same G′ and G″ values at 27 °C.[18]

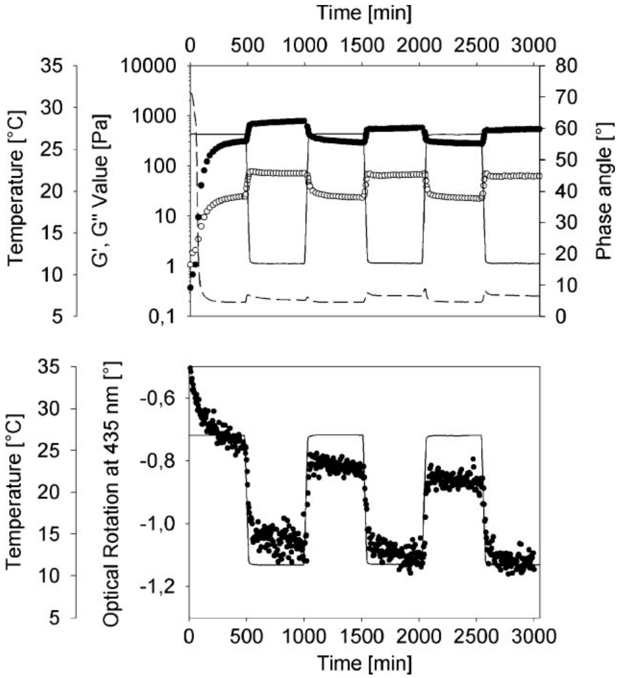

Figure 6.

Reversible gelatin (1.5%) physical network formation inside a calcium-alginate gel (1%) followed by rheology (up: G′ value (closed circle), G″ value (open circle), phase angle (dashed line), temperature (line)) and polarimetry at 435 nm (down: Optical rotation (closed circle), temperature (line)).

Conclusion

This study provides evidence for the complex behavior of polysaccharide and gelatin mixtures, and highlights conditions when either one or the other polymer gives a gel or when the formation of a mixed gel is possible. The addition of either hyaluronic acid or alginate to the liquid phase of the gelatin gel led to qualitatively similar results. For both filled gels, the presence of a polysacharide in the liquid phase does not affect the formation of the gelatin triple helix and does not cause an increase of the elasticity of the gel. Polysaccharides do not interfere with the protein network except that they accelerate gel formation. Moreover, they increase the viscosity of the liquid phase of the gel. Those filled gels behave as semi-interpenetrating networks. In both cases, it was possible to hydrolyze specifically the polysaccharide without affecting the protein network; this result is very promising for drug delivery as the time after which the viscosity decreases and diffusion increases is perfectly under control. When both gels can form (high polymer concentrations and a temperature ranging from 35 to 45 °C), in some cases the alginate gel collapses. This collapse could be caused by phase separation in the mixture or by an over-association of alginate favored by a high calcium and total biopolymer concentration. In other particular conditions, a mixed gel of gelatin and alginate is formed, the alginate and gelatin gels being respectively irreversible and reversible upon temperature cycles. The additional use of enzymes able to specifically degrade one of the two networks enlarges the dynamic properties of interpenetrated networks based on two biopolymers of different chemical nature.

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