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# Programmed Ephemeral Gels: New Types of Biomaterials

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**Summary:** In various biological processes, a solid gel phase is produced which later dissolves. Similar ephemeral protein gels were obtained in vitro using two antagonistic enzymes, one generating and the other cleaving covalent bonds. Alternate sol/gel and gel/sol transitions should occur within such a system, generating transient gel phases. An experimental system consisting of gelatin, transglutaminase and thermolysin was first tested. The various gels obtained were programmed to dissolve after a determined time, without any change in temperature or medium composition, and constitute a completely new type of material which we term ''Enzgels''. Varying temperature, but also the protein nature and concentration, the protease specificity, the ratio of the two antagonistic enzymes and, the activity of each enzyme, we were able to generate a full range of ephemeral gels with controlled life times and mechanistic properties.

**Keywords:** dynamics; enzymes; gelatin gels

## Introduction

Many biopolymers display gelling properties in aqueous media. Gelation is a complex operation and theoretical models have been elaborated to describe the macroscopic sol/gel transition.[1] *In vivo*, protein biopolymers are dense networks which undergo enzyme-catalyzed changes in composition leading to important variations at the macroscopic scale, including gel transition.[2,3] Enzymes usually catalyze very specific reactions often implying one single chemical group.[4] However, in some cases, these subtle modifications at the molecular scale cause important variations at the supramolecular level. Among the 3500 characterized enzymes, a few are particularly efficient at catalyzing phase transitions. These include proteases which may dissolve insoluble protein phases.[5] The implication of proteases acting as destructive catalysts is of crucial importance in various physiological and pathological events[6–8] where the major rationale is that proteolysis would disorganize the biological tissue.

In contrast, proteases contribute to the generation of a new solid phase in wound healing or blood coagulation. Through zymogen activation, they generate a viscous solution or a soft gel phase which is later stabilized by transglutaminases. This second type of enzymes create covalent intermolecular protein cross-linking[9] giving rise to irreversible gel networks. Here, the two types of enzymatic reactions coexist and participate synergistically[10,11] to the formation of a solid phase from macromolecules in solution. However, in other biological events, these two enzymes can be considered as antagonistic ones as proteases cleave peptide bonds while transglutaminase generate isopeptide bonds. At a supramolecular level, as far as the physical state of a proteic gel is concerned, transglutaminase contributing to the cross-linking of the protein lattice may be considered as a reverse protease. In the extracellular matrix (ECM) remodeling,[3,12] these two types of

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enzymes act conversely. Oscillating concentrations for soluble proteins and insoluble lattices were calculated in a model considering proteases and transglutaminases as antagonistic catalysts of a futile cycle in which ECM proteins and their soluble proteolysis fragments are interconverted.[13]

*In vitro*, phase transitions are due to a change in the medium composition or in the physicochemical parameters, rather than to enzymes. We wanted to reproduce *in vitro* the same scheme as observed *in vivo* where the two types of reactions coexist and a solid phase is first obtained from macromolecules in solution and is later dissolved. We suggest the use of two antagonistic enzymes to catalyze sol/gel followed by gel/sol transitions in order to obtain the successive polymerization and solubilization of proteins without further addition of reactants and without any change in temperature or pressure. Here, the transglutaminase is considered as a sol-gelase enzyme while protease acts as a gel-solase. This presupposes the capacity to ''program'' the global behavior of a mixture containing at least one protein and two enzymes.

Among the proteins and polypeptides able to give rise to a gel, gelatin is an interesting model. It is not found in nature but derived from collagen by hydrolytic treatment and has the capability to form thermally reversible networks.[14] Below the sol-gel transition temperature, part of the gelatin coils gives rise to triple helices reminiscent of the native collagen[15] and solution turns into gel. Physical protein gels may be stabilized by the further addition of covalent bonds due to transglutaminase reaction. Physical, Chemical, Chemical – Physical and Physical – Chemical gels were obtained by cooling the gelatin solution and/or by transglutaminase reaction. It has been shown that the overall properties as well as the dynamics inside the gels are dependent upon the order of formation and on the relative amount of triple helices and covalent bonds.[16] Using gelatin, we first focused on the kinetics of antagonistic enzymes, elaborating a mathematical model based on a transglutaminase/protease cycle interconverting soluble proteins and an insoluble network. This model allows the prediction of protein bonding/dissociation and of the resulting phase transitions.[17]

Based on the model, we were able to obtain gelatin ephemeral gels experimentally, examples of which are described in the present paper. We thus used various proteases and obtained ephemeral gels with a large amount of properties.

## Various Gel Properties

Using rheology and polarimetry,[16] we first characterized the differences between physical gels and chemical gels. When a gelatin solution is cooled, protein coils locally assemble into triple helices, the emergence of such a physical network was followed through the viscoelastic properties of the gel (Figure 1 squares, Table 1). The triple helix amount increased to 15% in 150 minutes after the beginning of the cooling ramp (Table 1), a gel being obtained for 9% helices. Raising back the temperature leads to a gel-sol transition (Figure 1). For the chemical gel, the time dependence of the storage modulus (Figure 1, circles) indicates a fast sol-gel transition due to the rapid formation of intra- and intermolecular isopeptidic bonds catalyzed by transglutaminase. No helix was formed at 40 °C, then, the temperature was decreased to 27 °C and helix formation was observed in both physical and chemical gels (Table 1).

For the chemical gel, the presence of a covalent network before helix formation strongly limited, but did not totally prevent triple helix propagation. This indicates that macromolecular dynamics was allowed inside the covalent gel. The obtained gel is a Chemical-Physical gel.

To estimate gel time, $t_0$ was considered as the beginning of the cooling ramp. For the Chemical and Physical – chemical gels, $t_0$ corresponds to transglutaminase addition. Gel time was estimated when

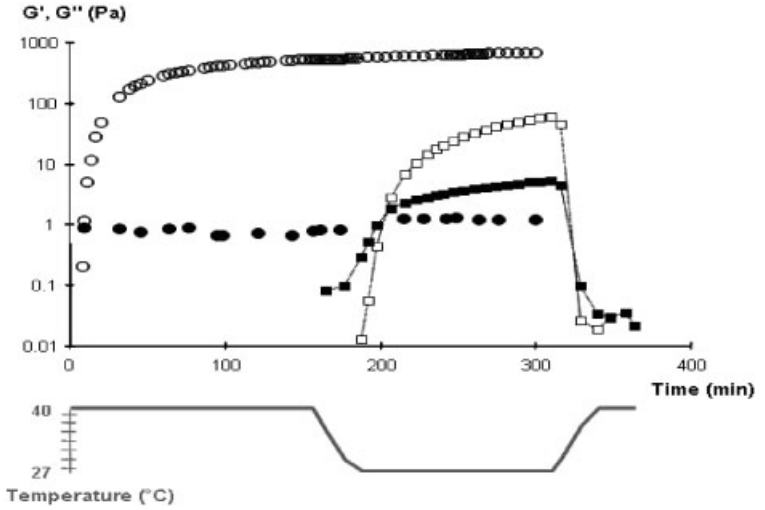



***Figure 1.***
Storage (open symbols) and loss modulus (filled symbols) measured at 40, 27 then 40 °C as a function of time for Physical gel (squares) and Chemical then Chemical - physical gel (circles). Gelatin concentration is 5% (W/V).

$G' = G''$. $G'$, $G''$ and helix % were measured at $t_{150\ min}$.

From these observations, a scheme may be proposed to describe the different supramolecular organizations of the various gels.

These differences in the gel networks have been correlated to the variations in hydrolysis rates by thermolysin.[16] It has been confirmed that the history of the gel is of crucial importance for its macroscopic properties. Moreover, a network with both helices and covalent bonds could be protected from the enzymatic hydrolysis which is of biological and industrial relevance.

Then, the transglutaminase concentration was varied.

The higher the transglutaminase concentration, the faster the sol/gel transition and the higher the elasticity of the gel. From a meticulous analysis of the curve, it was shown that despite the viscosity increase in the solution, the transglutaminase reaction is not diffusion-controlled before the sol/gel transition while the evolution of macroscopic properties is strongly reduced after the gel point. A non linear relation was obtained in the gel phase between the enzymatic reaction and enzyme concentration which is characteristic of diffusional constraints.[17] We consider here that as the covalent bonding increases inside the gel, the diffusion of the enzyme inside the protein network becomes increasingly limited. This diffusion-reaction mechanism was taken into account for further calculations and experiments.

## Ephemeral Gels

Having previously observed the hydrolysis of the gels with thermolysin,[16] we tried to add the protease inside the gelatin solution

***Table 1.***
Viscoelastic properties and helix % in different types of gel.

| Gel type | Tgase, Temperature | G′ (Pa) at 150nm | Bonds |
|---|---|---|---|
| Physical | −, 27 °C | 60 | Triple helix (15% after 150 min) |
| Chemical | +, 40 °C | 527 | Covalent bonds |
| Chemical-Physical | +, 40 → 27 °C | 722 | Covalent bonds + Triple helix (3%) |
| Physical-Chemical | +, 27 °C | 907 | Covalent bonds + Triple helix (9%) |

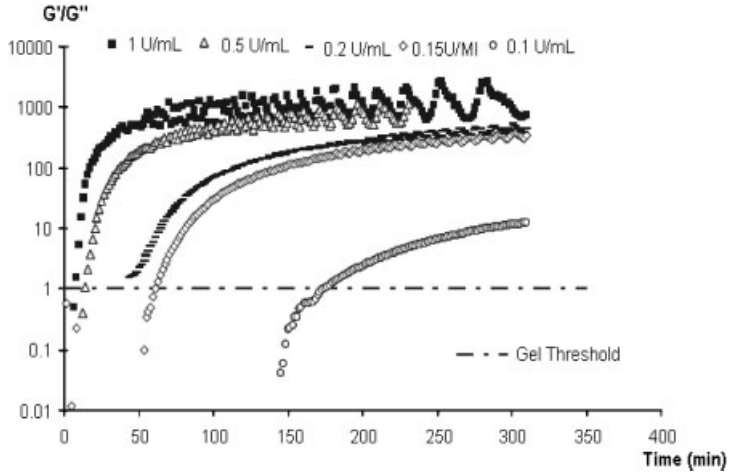



***Figure 2.***
$G'/G''$ as a function of time for different transglutaminase concentrations. (■: 1 $U.mL^{-1}$, △: 0.5 $U.mL^{-1}$, ■ : 0.2 $U.mL^{-1}$, ◇: 0.15 $U.mL^{-1}$, ○ : 0.1 $U.mL^{-1}$). A gel is defined here when $G'/G'' > 1$. Gelatin concentration is 5% (W/V).

before the formation of a physical gel (Figure 3).

A gel is rapidly obtained after the gelatin solution is cooled, then under the protease action the gel is solubilized. Polarimetry measurements show that helices are not destroyed by thermolysin reaction. A second cooling of the hydrolyzed solution at 20 °C allows the gel to form again, this gel/sol transition is concomitant with the appearance of new helices. This experiment shows that experimentally a gel can undergo alternate gelation transitions when modifying the physicochemical conditions and adding a protease.

Gels were then realized in the presence of the two antagonistic enzymes, i.e. transglutaminase and thermolysin. In the given example (Figure 4), the two enzyme reactions were carried out at 40 °C, a temperature where no physical gelatin gel can be obtained, so that only transglutami-

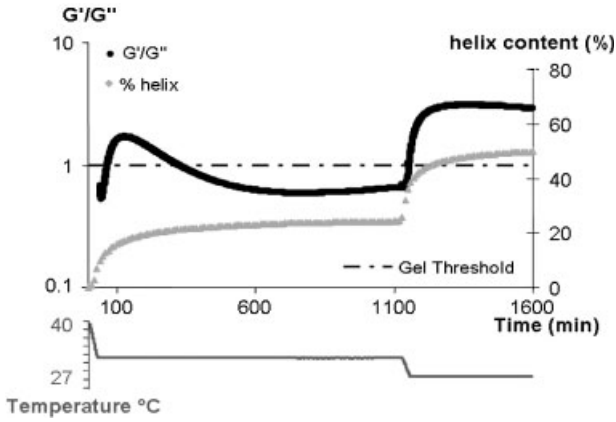



***Figure 3.***
Viscoelastic behaviour and helix content of a physical gel containing a protease as a function of time. The temperature protocol (—) is as follows : 40 °C → 27 °C → 20 °C. A gel is defined here when $G'/G'' > 1$. Gelatin concentration is 5% (W/V).

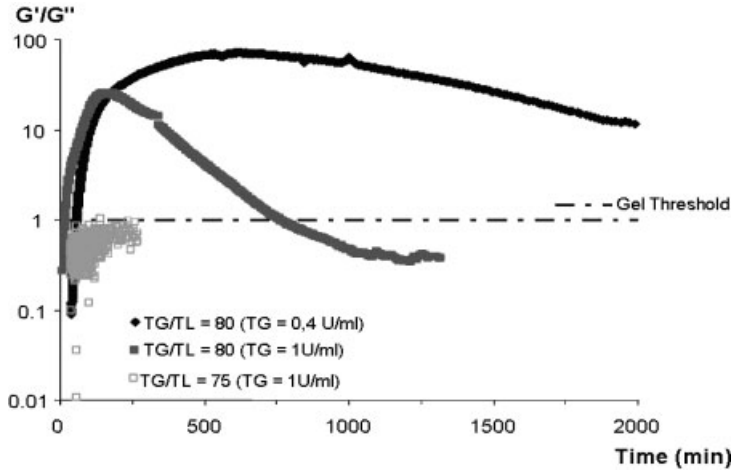



***Figure 4.***
Evolution of the viscoelastic properties of ephemeral gels as a function of time (min) at 40 °C for T/P = 80 with T = 0.4 U.mL$^{-1}$ (◆) or T = 1 U.mL$^{-1}$ (■) and for T/P = 75 with T = 1 U.mL$^{-1}$ (○). A gel is defined here when G′/G″ >1. Gelatin concentration is 5% (W/V).

nase-catalyzed covalent bonds are responsible for the gelation process. The properties of the gel were followed by rheology, as previously described.[16] Under these conditions, as a functional reference, a gel is considered when the storage modulus G′ becomes higher than the loss modulus G″, that is to say when $G'/G'' > 1$. At a constant gelatin concentration (50 g. L$^{-1}$), various transglutaminase to protease ratios (T/P) were tested and also the enzyme activity was varied for a constant T/P ratio. It is important to mention that during the whole experiment, the temperature is kept constant.

The analysis of the results on Figure 4 indicates that the gelation process is an accurate phenomenon under these conditions. With a T/P ratio of 75 no gel was obtained (the experiment was carried out over 80 hours), while increasing this value to 80 allowed gelling to proceed. This first phase transition occurs in 19 minutes, which is slower than for a gel without protease (8.5 minutes). As predicted by a mathematical model,[18] the gel is evolving: its viscoelasticity increases to reach a maximal value ($G'/G'' = 26$) in 165 minutes, then dissolution begins, being achieved in 760 min. The process is irreversible, no spontaneous gelling was observed beyond this point. This result is consistent with the hypothesis of small fragments development due to the protease reaction on soluble gelatin chains.

The overall behavior of the preparation is also strictly dependent on the enzyme activity. A slight decrease in the enzyme concentrations (0.4 unit instead of 1 unit transglutaminase, T/P ratio constant) results in an increase in the gelling time (56 min instead of 19 min), higher visco-elastic properties ($G'/G'' = 71$ after 10 hours), and a longer gel phase (solubilization needs 80 hours).

A physical-chemical ephemeral gel vas realized at 27 °C. At this temperature, the enzymatic reaction is concomitant with the triple helix formation. The obtained gel is due to both weak interactions and covalent bonding. Varying temperature, completely different properties were observed (Figure 5).

At a temperature where both triple helices and covalent bonds contribute to the network, the gel resistance is increased. These results are consistent with the ones previously observed when adding protease outside the gel.[16] One may remark that

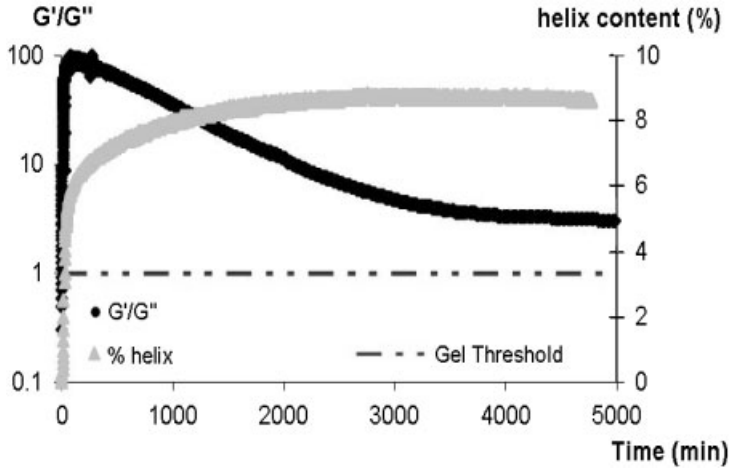



***Figure 5.***
Evolution as a function of time of the viscoelastic properties and triple helix content of a physical-chemical ephemeral gel at 27 °C for T/P = 80. Gelatin concentration is 5% (W/V).

helices are not degraded during the gel/sol transition. This is due to the particular choice of the protease, thermolysin which preferentially recognizes hydrophobic residues with large lateral chains,[18] as they are rare in helices, thermolysin should preferentially act on the random coil part of the gel chains.

Next, an ephemeral gel was realized at 40 ° C based on a chemical gel, and then, after the gel/sol transition the obtained solution was cooled to 27 °C to induce the formation of triple helices (Figure 6).

We can observe that, in this case, despite the occurring of an amount of triple helices sufficient to induce a sol/gel transition (9%), no physical gel was obtained after the dissolution of the chemical gel. This particular effect is due to the high rate of hydrolysis realized by thermolysin. The

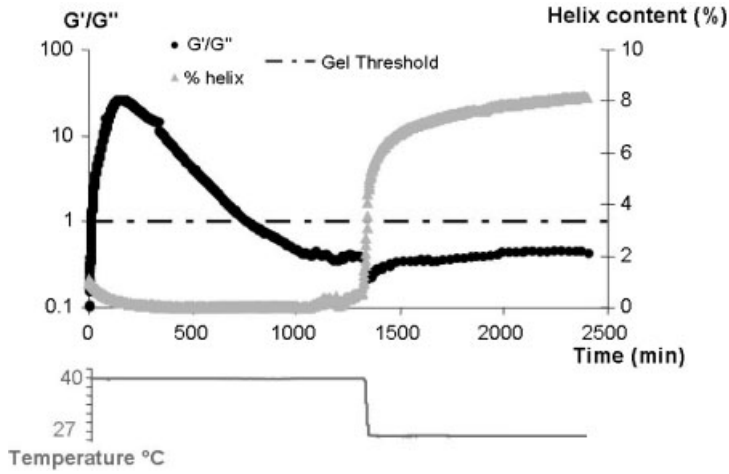



***Figure 6.***
Evolution as a function of time of the viscoelastic properties and triple helix content of a chemical ephemeral gel thus cooled at 27 °C. T/P = 80. The thermal protocol is 40 °C → 27 °C.

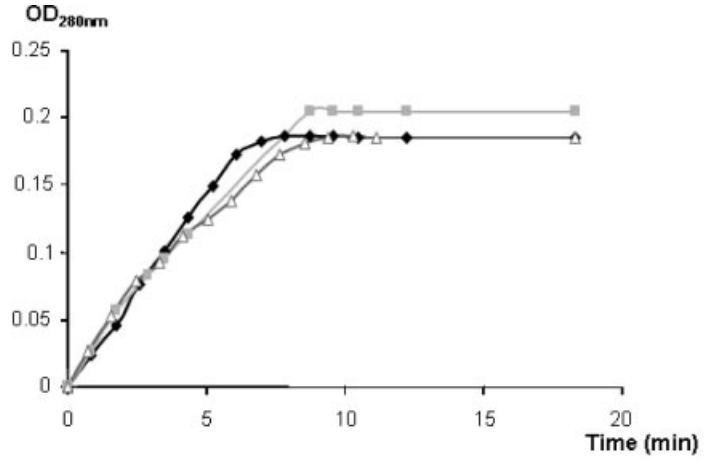



***Figure 7.***
Hydrolysis by collagenase of a physical gel (◆), a chemical gel (△) or a physical-chemical gel (■) as a function of time. Gelatin concentration is 7% (W/V).

medium contains too many small protein chains and peptides to allow the obtaining of a correct network although the molecular mobility of the protein chains allows the triple helix association. This result brings new insights on the relation between triple helices and gelation. Contrary to what was observed with native gelatin,[19–20] there is no direct relation in the present case between triple helix content and G′.

These results illustrate that varying the temperature, we were able to generate ephemeral gels with various life times and mechanistic properties. A full range of controlled properties were obtained by modifying the protein nature and concentration. We thus tested the role of protease specificity.

The second protease used was a collagenase which is specific of the Gly-X-Y sequence responsible for the triple helix formation (Figure 7).

When different gels were hydrolyzed with collagenase, the same hydrolysis rate

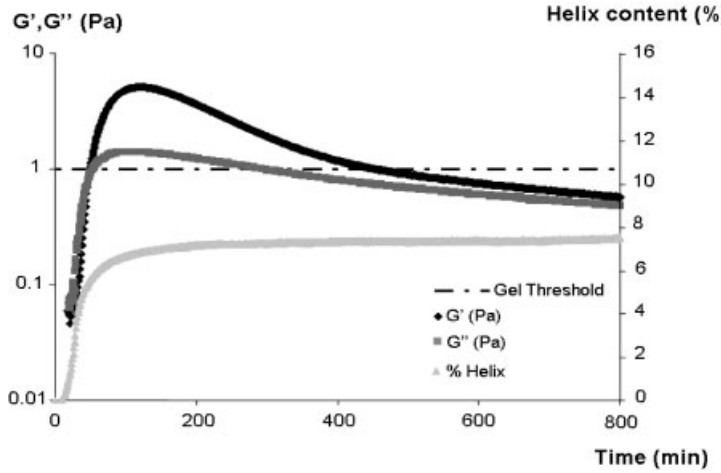



***Figure 8.***
Ephemeral physical –chemical gel behaviour with thermolysin as a function of time. G′ (◆), G″ (■) and helix % (▲) were followed. The reaction is carried out at 27 °C. Gelatin concentration is 7% (W/V).

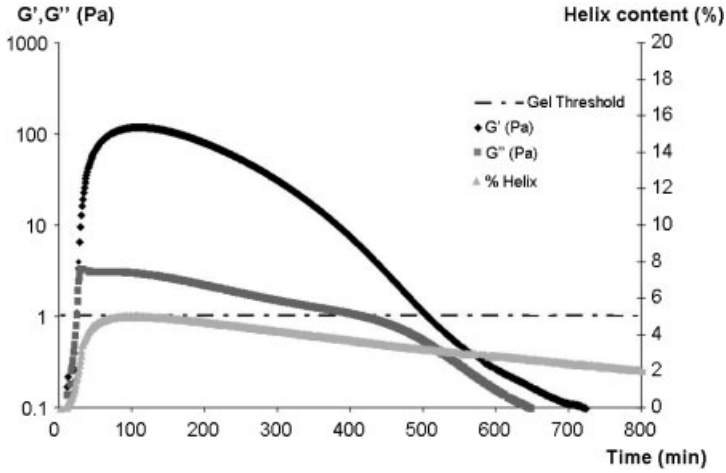
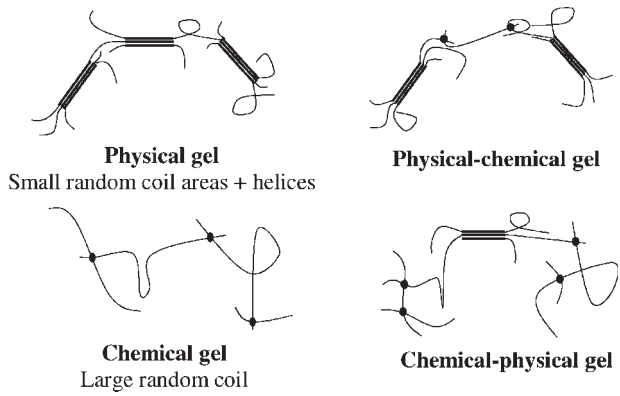



***Figure 9.***
Ephemeral physical –chemical gel behaviour with collagenase as a function of time. G′ (◆), G″ (■) and helix % (▲) were followed. The reaction is carried out at 27 °C. Gelatin concentration is 7% (W/V).

was observed whatever the type of bonds inside the gel. This shows that for this enzyme, contrary to what was observed with thermolysin, covalent bonds do not protect the network against the protease. Moreover, the formation of triple helices is not important for collagenase as the reaction rate is identical whether helices are formed (physical gel) or not (chemical gel). The main difference between the two enzymes is that collagenase mainly reacts inside the helix sequence while thermolysin acts outside it, where the covalent bonds are localized. So, protease specificity is of crucial importance for the hydrolysis of a protein gel network.

Then, this influence of protease specificity was studied on ephemeral gels (Figure 8 and 9).

Different properties were obtained with the two proteases. In the presence of collagenase, less triple helices are formed which are hydrolyzed by the protease. The gel obtained with collagenase is much more elastic than the one obtained with thermolysin and its profile is close to that of a chemical one. In this last case, collagenase cleaves helices, but the hydrolyzed chains




***Scheme 1.***
Proposed organization of the various gels.

are still locally associated and bound by covalent bonds network (see Scheme 1), a further hydrolysis is thus needed to solubilize the gel. With thermolysin, the hydrolysis does not affect the helix structures, but mainly the random chains bound by covalent bonds, so that one single cleavage may strongly destabilize the network. The differences in molecular recognition of the protein by the enzymes are of fundamental importance for the macroscopic properties of the gel and for its global behavior.

## Conclusion

A large amount of work has been dedicated in the past years to the catabolic action of proteases. Our main objective consisted in the concomitant introduction of transglutaminases: as these wide-spread enzymes can turn soluble proteins into insoluble lattices, their influence on the mechanical properties of biological gels appears to be the reverse of the proteases' action. We thus treated proteases and transglutaminases as reverse catalysts.

We have shown that two antagonistic enzyme activities, inside a mixed network, may generate a dynamic modification of protein network which induces a sol/gel transition followed by a gel/sol transition. Using both proteases and transglutaminases with a gelatin solution, we obtained a completely new type of material which we term ''Enzgels''. This is the first report in protein polymer literature where no modification in temperature or medium composition is required to dissolve a gel *in vitro*. The ''Enzgels'' are dynamic protein solutions able to spontaneously cycle from a sol/gel to a gel/sol transition. The interest of this type of material resides in their ability to be completely pre-programmed. The transition is depending on the conformation of proteins constituting the network, but the type of bonds forming the network itself is even more important.

By creating ''Enzgels'' we claim a major advance in biomaterial field. The ability to control the kinetics of both polymerization and solubilization reactions suggests a wide range of uses. ''Enzgels'' can provide a new proteic vehicle to deliver drug with fine time control. The fine control of both gel formation and cleavage reactions should lead to new applications in pharmaceutical industries, in dermal or surgery wound care market.

Moreover, these studies contribute to the knowledge of enzyme behavior in non conventional media. Finally, this work may play a part to the understanding of complex biological processes such as the matrix remodeling involved in fibrosis or cancer metastasis.

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