Research Article

Gelation of Gelatin: An Introduction to the Physics of Gels by Means of Rheological Investigations

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Introduction

Aim of the Experimental Course

Gels are common materials in our everyday life: in food products, in cosmetics and in health care. Among the most common and useful gels are gelatin based gels which are obtained through denaturation and hydrolysis of collagen, an important structural protein in animals [1]. Depending on the extraction in acidic or basic media one can distinguish between gelatin A and gelatin B respectively. Gelatin A has an alkaline isoelectric point and gelatin B has an isoelectric point close to 5. The isoelectric point of a protein corresponds to the pH value where the protein does not migrate in the presence of an electric field, meaning not the absence of charges but an equal amount of positive and negative charges.

There are unfortunately very few experimental courses aimed to introduce physical characterization methods of gels and in particular of the gelation kinetics, with some exceptions however [2].

The experimental course presented herein is typically designed for third year university students having already received an introductory course in polymer physics and chemistry and should last over two days. It is more suited for students in biochemistry/chemistry or students in biotechnology than for students in physics. Indeed, the physical part in the given explanations will be reduced to a minimum (essentially for reasons of time during the experimental course), but the macroscopic qualitative and the quantitative aspects of gelation kinetics will be emphasized. At the end of the course, the experimental findings have to be related to molecular aspects of the gelation process.

We will focus on gelatin based gels because they rely on biosourced product and because the gelation process is mainly controlled by temperature changes. The gelation process is nevertheless influenced by the solution pH and the ionic strength but in any case, when the temperature is higher than a critical level no gelation occurs [3]. In case of non-availability of gelatin or for some ethical or religious reasons, gelatin may be replaced by agar-agar, a polysaccharide extracted from red algae.

Theoretical Background

During the first half day of the course, the students are invited to read a fundamental reference about gels and gelation processes. Among others, I suggest ref. [4]. The most important point at this level is to clarify the notion of a gel: it is a liquid entrapped in a polymer mesh which is able to swell (volume increase) and to deswell (volume decrease) under specific external conditions. There are physical and chemical gels, the cohesion of the former being ensured by junctions due to weak intermolecular forces whereas the cohesion of the later is ensured by a variable amount of covalent bonds. The milieu undergoing gelation is called a "sol" a terminology which should be distinguished from "solution". Indeed, the material undergoing gelation, a protein in the case of gelatin, should be considered as a colloidal suspension in a solvent (water in the present case).

The main part of the investigation will rely on the use of a shear rheometer, whose principle can also be found in the literature (even on the web). But other methods could be used for example swelling experiments just requiring a precision balance.

In a rheometer the gel, or sol undergoing gelation, is sandwiched between two plates, the lowest one is related to a thermostated bath and is immobile, the higher plate is under periodic oscillation and is related to a dynamometer allowing to measure the applied force and to calculate a torque. If the applied torque is a sine wave of the time, the measured signal-the shear stress transmitted through the gel- will be a periodic wave with a contribution in phase with the applied signal and a part with a phase angle shift. The first contribution will allow to calculate the storage modulus (G') of the investigated material, the second contribution will allow to calculate the loss modulus (G") of the investigated material. G' is related to the materials elasticity whereas G" is related to the dynamic viscosity of the material (the mathematical relationships are not required at this level).

The instructor could give a little introductory lecture about those concepts during the first 15-30 min of the lecture. Otherwise, the students could be invited to read the specialized literature, indeed some time is available between the experimental runs all along the two days of the experimental course [4].

Experimental Part

To get the given data, I used bovine skin gelatin of type B from Sigma Aldrich (ref: C6650) without any modification or purification step. It has to be noted that the obtained quantitative results, in terms of gel elasticity (storage modulus) are extremely sensitive on the source of the used gelatin (major difference between fish and porcine or bovine gelatin for instance) [3]. The main parameter, in addition to temperature, will be the gelatin concentration which will be expressed in % w/v.

The gelatin is dissolved under magnetic stirring in a defined volume of distilled water which is heated between 50 and 60°C in a closed vessel to minimize the influence of evaporation. In addition, experiments could be performed replacing the distilled water by sodium chloride solutions of varying concentrations to investigate the influence of the ionic strength on the gelation kinetics.

The optimal rotation speed of the magnet should be adjusted to ensure homogeneous agitation without excessive foaming. Some foam will anyway be observed and the supervisor should ask to the students why such a phenomenon occurs (the denaturated gelatin has a tensio-active behavior in a similar manner than a surfactant). In the case of the experiments presented herein (see Figure 2) 10 mL of water was used and the mass of added gelatin was changed to get sols between 1 % and 20 % w/v. In this investigation, gelatin sols at 1, 3, 5, 8, 10, 12, 15 and 20 % were investigated. Too high concentrations of gelatin will require longer time to get an homogeneous gel. It has not be forgotten, anyway, that the solvent is the major species in a gel.

As soon as there are no observable gelatin particles is suspension, the gelation kinetics can be investigated. The students will be asked to note the time required to obtain a homogeneous sol. This is absolutely mandatory to get reproducible results.

In the next step, the students will investigate the gelation from a qualitative point of view: a vial partially filled with the gelatin sol, retired from the heating plate should be put at ambient temperature and observed by regular up-side down rotation of the vial. As soon as the sol does not flow down, gelation has occurred. By such experiments, done during the first morning (at the same time as used for reading from a book like ref [4]) of the experimental course, the students will define the threshold concentration for gelation. In the case of the experiments presented herein, the minimal gelation concentration lies around 5 % w/v. But for other gelatins, particularly those having a higher molecular mass, the minimal concentrations required to get gelation could be shifter to lower values. The occurrence of a gelation threshold is however a robust observation.

Having found this critical concentration, the students will use a rheometer to investigate the gelation kinetics in more detail and from a quantitative point of view. In the particular case of the data shown in this article the data were acquired with a Kinexus Ultra rheometer (Malvern, United Kingdom). Of course, all kinds of other commercially available devices will be perfectly suited. It is of major importance to use a cone plate geometry which ensures a homogeneous shear rate across the gel. The

supervisor of the experimental course has to discuss with the students about such experimental details. Herein the lower plate and the upper cone were made of stainless steel. The upper cone had a radius of 2.0 cm and an apex angle of 176 °. This particular rheometer is designed in a way such that the apex of the cone is 150 µm away from the lower plate (Figure 1). By this way the volume of investigated gelling solution was equal to 1.3 mL. Of course, cones of an other size can be used and the gap between the apex of the cone and the lower plate will also change.
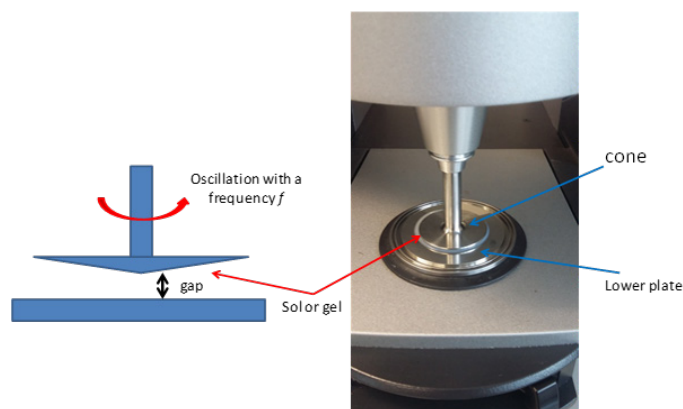
Figure 1: Illustration and picture of the experimental configuration in cone-plate rheological experiments. The cone and the lower plate are both made of stainless steel. On the picture, the gelling solution is slightly overflowing below the cone.

Before deposition of the hot gelatin sol on the lower plate, both plates should be carefully cleaned. Hot tap water (which allows to dissolve adhering gelatin gel residues), followed by distilled water and finally ethanol should be used. The plates should be allowed to dry before starting a new experiment.

After the deposition of the hot sol on the plate (thermostated at 25 ± 0.1 °C), the cone is lowered down to reach the final separation from the lower plate. Immediately after the gelation kinetics should be started by putting the cone in rotation at a given frequency and for a given rotation amplitude. The gelation kinetics presented herein have been acquired at a rotation frequency of 1 Hz and a shear strain of 1 %. The gelation kinetics have to be stopped after 1 h of data acquisition (herein: one data point every 15 s) not only to be able to perform at least 4 experiments in half a day of experimental work but also to minimize the influence of solvent evaporation which would induce a systematic bias in the obtained results. Indeed, when the solvent evaporates, the gelatin concentration increases as well as the measured storage modulus.

Possibility of Complementary Experiments

After completion of the gelation kinetics, some frequency/amplitude sweep or temperature ramp experiments can be performed. In a frequency sweep experiment, performed a constant shear strain, the storage and loss moduli are measured for different rotation frequencies of the cone, typically from 10 to 0.1 Hz. If a material or gel displays a constant value in the storage modulus G', this means that it behaves as an elastic material in this frequency range. It could happen that the G' and G'' versus frequency curves intersect in the low frequency domain. This

typical frequency is the reciprocal of the characteristic time of the junctions ensuring the stability of the physical gel. These considerations are however out of the scope of the present short time experimental course aimed mostly for students in biochemistry/chemistry.

In an amplitude sweep experiment, the frequency is maintained at a constant value and the amplitude of the imposed cone oscillations (shear strain) is changed. It might well be that the storage modulus changes upon a critical shear strain and it is also possible to observe a change in the loss modulus G" in the investigated frequency range. Typically, as G" is related to the dynamic viscosity of the liquid present in the gel, the frequency sweep experiment is a method to investigate if this fluid is a newtoniam (shear viscosity independent of the shear rate) or a non-Newtonian fluid. The supervisor of the experimental section should discuss with the students about such considerations after a frequency or an amplitude sweep has been performed.

Temperature Ramp Experiments: Characterization of the Gel-Sol Transition

Finally, after gelation completion (after 1h of a kinetic run) a temperature ramp experiment will be performed: in such an experiment the temperature is ramped (at scan rate of x °C/min) between the initial temperature at which the gelation kinetics has been followed to a final temperature above the gel-sol transition. Typically, the temperature sweep rate x can be changed between 0.1 °C.min^{-1} to 5 °C.min^{-1}. The lowest temperature scan rate means the longest experimental time. Such experiments could also be performed by decreasing the temperature, starting form the sol state (immediately after deposition of the sol on the plate maintained at high temperature), at a given rate to the initial temperature of 25 °C in order to investigate the reversibility of the sol-gel transition.

These additional experiments, either the temperature ramp or the frequency sweep or amplitude sweep experiments should be performed during the second day and should be adapted to the time remaining.

The last 4 hours of the day should be dedicated to the data interpretation, in particular to the plots of the data in an illustrative manner (see the results and discussion section).

Results and Discussion
Qualitative Observation of the Gelation Process
The vials containing the freshly prepared gelatin sols are removed from the heating plate (central picture in Figure 2) and regularly switched up-side down. In the case where the sol flows on the bottom, we are still in the presence of a sol state (left picture in Figure 1). However, when the material is able to withstand its own weight (right picture in Figure 2), the sol-gel transition has occurred.
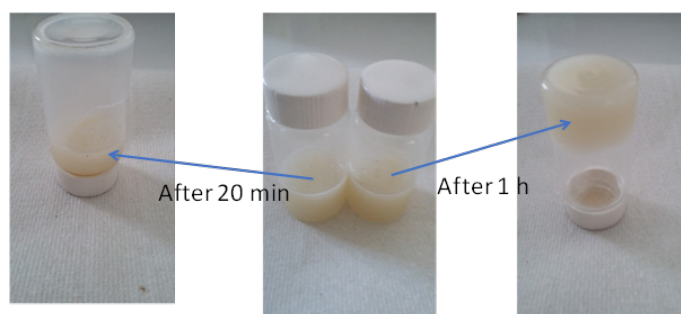
Figure 2: Central picture: gelatin sols (at 10 %w/v) immediately after homogenization at 70°C during 30 min and under agitation (magnetic stirrer at 400 rpm). Left picture: the tube is turn upside down after 20 min: the gel is not yet formed. Right picture: the tube is turned upside down after 1h: the gel is formed and is able to sustain its own weight.

These experiments should be performed for sols of different concentration in gelatin…several runs can of course be performed in parallel. The student team (typically three students) should work in a collaborative manner to organize the weighing of gelatin, pipetting and heating of water, preparation of the sol, taking some pictures to justify the formation of gels (as in Figure 2). As an advice, the students should start with the highest concentration for which the gelation is the fastest. As soon as gelation is observed, a rheological characterization should be undertaken for the same gelatin concentration. The lower the gelatin concentration is the longer the gelation time is …this gelation time will be more quantitatively defined from the rheological experiments. Anyway, below a threshold concentration no gelation will occur even at the end of the day-provided the solvent does not evaporate.

The instructor should then ask the students why the gelation is so strongly concentration dependent. The answer to this question requires some knowledge about the conformation of a coiled polymer chain and about the influence of the increase in polymer concentration: the polymer coils will start to interact (which kinds of interactions?) and constitute species of larger size. We will come back to this point later on.

The monitoring of the gelation kinetics is extremely instructive (Figure 3). For the gelatin, used in this investigation, the initial loss modulus G" (related to the dynamic viscosity) is always higher than the initial storage modulus G' related to the gel's elasticity. This means that the freshly prepared and hot sols behave mostly like liquids. But after a given time in contact with the lower plate maintained at constant temperature (herein 25.0 ± 0.1 °C), a crossover between the two curves occurs where G'=G". This corresponds to the onset of gelation, and the corresponding duration will be called the gelation time τ. It has to be noted that there is some uncertainty in the determination of τ, the higher so the shorter τ is. Indeed, there is a time lag between the removal of the hot sol (70°C) from the hot plate, its deposition on the lower plate and the beginning of the data acquisition (it is estimated to 60s).

For longer durations G' remains always larger than G". As explained in the experimental section, the experiments should be stopped after 1 h of measurement. The students should observe

the slope of the gelation kinetics at the end of the data recording. It is indeed not equal to zero even for the highest investigated solution (15 % w/v in the data shown in Figure 3) and remains larger for the smallest used concentrations. This means that the gelation process is not yet achieved after this time. Even if the experiments would be performed for longer durations, but minimizing the water evaporation with the help of a solvent trap one would still find the same trend: even if the gelation process slows down, it never stops totally. This highly suggests that the formation of the gel is not an equilibrium process. Some reading in specialized textbooks could be welcome at this level [4].

It is also apparent from data as those shown in Figure 3:
i. that the gelation time decreases when the gelatin concentration in the initial sol is increased (black arrow in Figure 3).
ii. That the final G' and G'' values increase with the initial gelatin concentration.
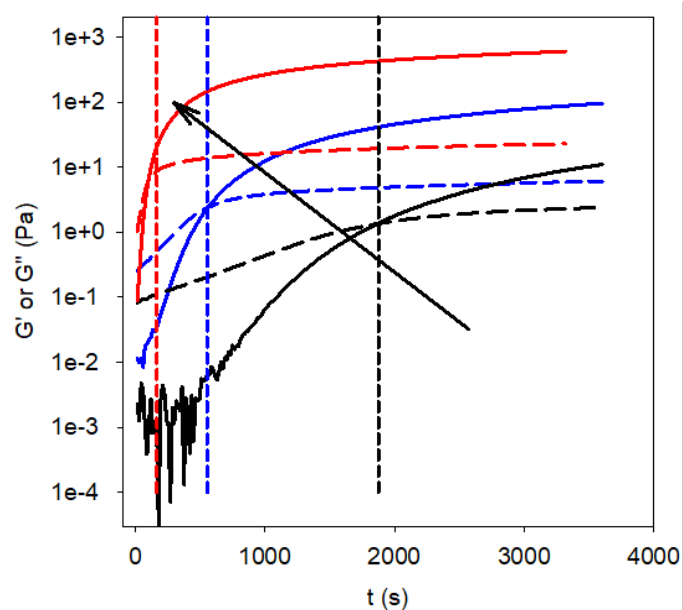

Figure 3: Representative gelation kinetics followed at 25°C at a constant frequency of 1Hz and a shear strain of 1% for gelatin at 8 % w/v (black lines), gelatin at 10 % w/v (blue lines) and gelatin at 15 % w/v (red lines). In all cases, the full lines correspond to the storage modulus and the dashed lines to the loss modulus. The vertical dotted lines correspond to the gelation time for each composition and the black arrow is aimed to represent the evolution of the curves when the gelatin concentration increases.

As a remark, the gelation times determined by rheometry are markedly lower than the qualitative times after which the sol could be switched up-side down without flow. The qualitative experiments also give a gelation time, not only with less precision than by rheometry but also in different conditions. The volume of the gels used in the qualitative experiments displayed in Figure 2 correspond to 10 mL, whereas the rheology data displayed in Figure 3 correspond to 1.3 mL of gel in contact with a much larger surface area at a well- controlled temperature. The gelation corresponding to the appearance of a solid like behavior is related to the formation of at least one continuous mechanical pathway between the bottom plate and the upper plate, allowing to transmit the mechanical stress between the two plates (Scheme 1). In physics, it is said that a percolation threshold is

reached at this level of the gelation process.

When all the data are plotted as a function of the gelatin concentration in a usual linear representation, Figure 4A, the data just reflect the marked decrease in the gelation time and the increase in G' (after 1h of gelation) with the gelatin concentration (Figure 4A, right hand axis). A much more informative picture of the gelation process could be acquired by plotting the same data in a double logarithmic representation (Figures 4 B and C): some straight lines are obtained.

This means that τ scales as $C^{-\alpha}$ (with α =3.60 in this case) (Figure 4B) that G' scales as C^{β} (with β = 4.6 in this case) and that G'' scales as C^{γ} (with γ=3.2 in this case) (Figure 4C). These power laws with exponents much larger than 1 (corresponding to a linear dependence) are common place in the literature. The power law for the G' versus gelatin concentration and for the G'' versus gelatin concentration are however not the same (Figure 4C).
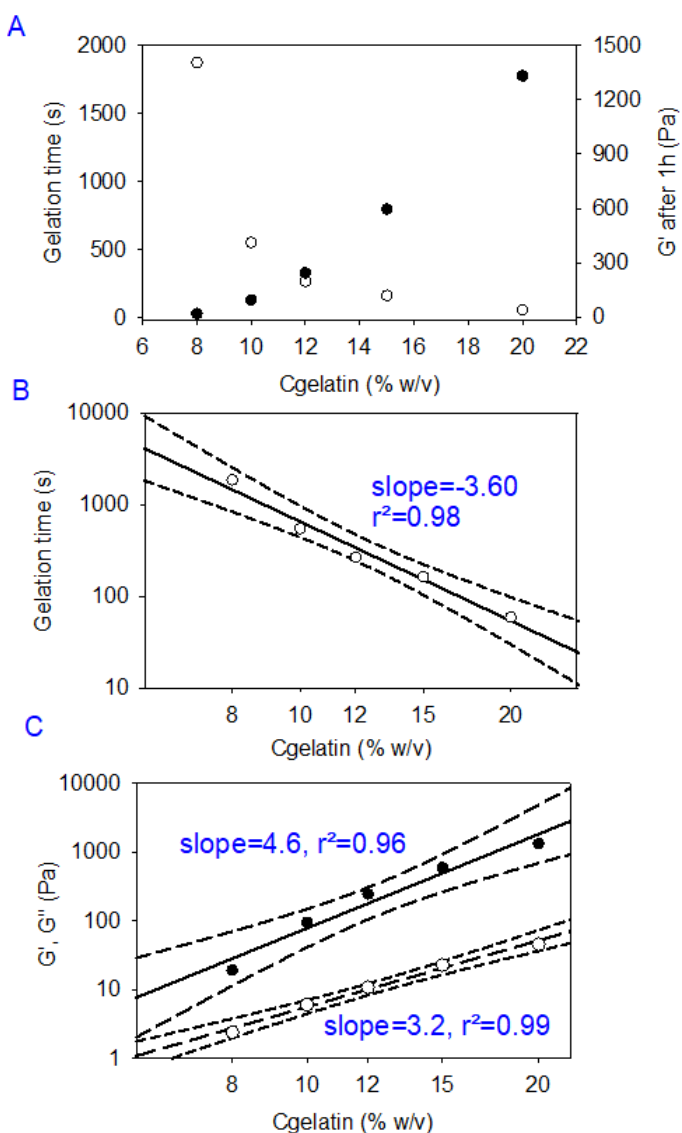

Figure 4: Analysis of the results displayed in Figure 3.
Panel A: variation of the gelation time (O, left hand axis) and storage modulus after 1h of gelation (●, right hand axis) as a function of the gelatin concentration.
Panel B: representation of the variation of the gelation time as a function of the gelatin concentration on a double logarithmic

scale. The full line and dotted lines correspond to the linear regression and the limits of the 95 % confidence interval.

Panel C: representation of the storage (●) and loss (O) moduli as a function of the gelatin concentration after 1h of gelation on a double logarithmic scale. The linear regression lines and the limits of the 95 % confidence intervals are also represented.

Finally, as complementary informations, when temperature scans are performed at a temperature scan rate of x °C.min⁻¹, it appears that both G' and G" decrease as the temperature increases but in a different manner. For a given temperature the G' and G" values again become equal and above this gel-sol transition temperature, the material behaves again as a sol with G">G'. Figure 5 displays a temperature scan performed on a 12 % w/v gelatin gel at a scan rate of 0.5 °C.min⁻¹. If this experiment would have been performed at a different scan rate or on a different gel prepared from a different gelatin concentration, the transition temperature would have been different. This is an illustration of the non equilibrium state of the investigated gels.
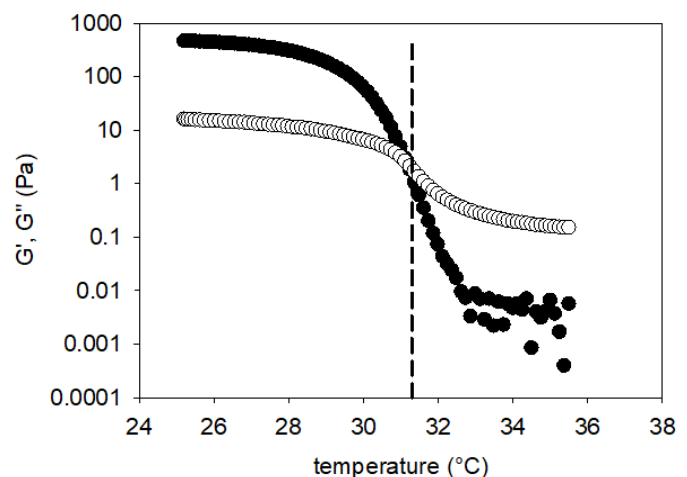
Figure 5: Temperature sweep experiment for a 12 % w/ v gelatin gel after 2 h of gelation at a temperature increase rate of 0.5 °C.min⁻¹. The dashed vertical line corresponds to the gel-sol transition at 31.3 °C in these experimental conditions.

Similar data are available in the literature [5, 6]. If some time is remaining after such temperature sweep experiments the students could use the formed gel to perform a temperature sweep in the reverse direction for instance from 38 to 25 °C; without separating the cone from the lower plate. They would observe again a sol-gel transition but the critical temperature at which the G" and G' curves would cross will be different from the corresponding temperature measured in the previous (temperature increase) scan. The difference between both curves will markedly increase for higher temperature scan rates. This is a particular example of a *hysteresis*, characteristic of a non-equilibrium process. Djabourov et al. [5] showed an other property of gelatin sols which displays a similar hysteresis: the optical rotation. This reference should be available in the experimental room or downloaded during the experimental course
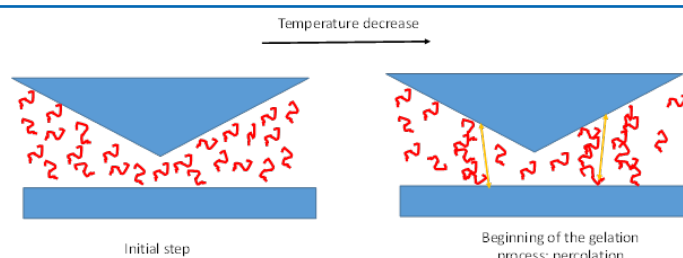
Scheme 1: Schematic representation of the gelation process starting from coiled gelatin molecules with the formation of intermolecular helical contacts upon cooling.

This will force the students to finally think about the molecular events underlying the macroscopic observations they made up to know. They could have great benefit to read the introduction of [6]: gelatins are a denatured form of collagen which is a structural protein present in the skin, bone, tendons, etc. In its native form it forms a triple helix from three left handed polypeptide chains. The presence of such ordered secondary (and tertiary) structures in a protein confers some optical rotation (in a more complicated way than the rotation of the polarization plane of linearly polarized light when it passes through a solution of a pure enantiomer of a chiral molecule). At high temperature, gelatin is coiled having lost most of the helical structure initially present in collagen. But upon cooling, some local helixes refold. If the gelatin concentration is too low, typically below the critical concentration required to form a gel, the helix formation is intramolecular. But above the critical gelation concentration, for which some continuous pathways appear between the lower plate and the cone in the configuration of the rheometer (right part of scheme 1), or across the whole volume of the gel, the helixes from preferentially between contacting molecules…the helix formation becomes an intermolecular process.

The students should then write a short report describing their experiments and should also answer how to increase the elasticity of the gelatin gels at a constant gelatin concentration (answers can be found in biochemistry textbooks considering the possible chemistry allowed by the side chains of the amino acids present in gelatin).

Conclusions
This short experimental course lasting over two days is designed for a group of three students (in biochemistry/chemistry) under the supervision of a teacher which can simultaneously supervise one or two other experimental courses in the teaching lab. It is aimed to get a first approach on the gelation process of a physical, thermoreversible gel like gelatin. The experiments start first with the phenomenological observation of the gelation process as a function of the gelatin concentration (Figure 2). These experiments will last other the first day and will be complemented with the recording of gelation kinetics recorded by a shear rheometer. This will be the opportunity for the students to understand the basic working principle of such an apparatus and to simultaneously apprehend the notion of gelation time, storage and loss moduli. The obtained data, which are clearly not linearly dependent on the gelatin concentration, Figure 4A, have to be plotted on a proper manner, namely on a double logarithmic representation as in Figure 4D and Figure 4C.

The second day of the experimental course should indeed be devoted to such a proper interpretation of the data and to performing additional frequency sweep, amplitude sweep and particularly temperature sweep experiments (at different gelatin concentrations and at different temperature scan rates) to better apprehend the thermal and non-equilibrium nature of the gel-sol or sol-gel transitions. The second day should be achieved by the writing of a short report and by a team discussion with the supervisor to try to relate the macroscopic observations to molecular events using knowledge from biochemistry lectures.

This course could be associated with biochemical experiments (degradation of the gels by enzymes) and could be extended to the design of chemical gels starting from "physical" gelatin based gels.

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