Biopolymer folding driven nanoparticle reorganization in bio-nanocomposites†

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In this paper we report the influence of biopolymer folding on nanoparticle spatial distribution in two typical bio-nanocomposite hydrogels. These systems consist of negatively charged nanosized fillers (polyoxotungstate clusters and silica particles, 2.2 nm and 23.0 nm in diameter, respectively) dispersed at low volume fractions in a positively charged gelatin hydrogel. The filler state of dispersion is investigated during triple helix folding by combining small-angle neutron scattering (SANS) and polarimetry experiments. Neutron contrast matching/polarimetry correlations indicate that the nanoparticle spatial distribution is clearly modified during triple helix folding for the two systems. In the first case, polyoxotungstate clusters are initially arranged in small finite size aggregates that grow with increasing triple helix rate: \( \Delta R_G = +150\% \) and \( \Delta L(q \to 0) = +250\% \) for \( \Delta [\text{helix}] = +40\% \). In the second case, silica particles initially form a connected network that undergoes a significant densification through gelatin conformational transition. In the two cases, the kinetics of triple helix folding is only slightly affected by the presence of the nanoparticles and their state of dispersion. In our experimental conditions, these two processes are almost thermo-reversible following triple helix unfolding.

1 Introduction

Bio-nanocomposites result from the assembly between a biopolymer continuous phase and a dispersed phase, which has at least one dimension of the order of nanometres. These nanostructured materials are receiving increasing interest due to their low environmental cost and high versatility toward a broad range of applications1–3 such as for instance sensors, a optical transducers, k remote controlled drug delivery or actuators in microfluidics devices. n

An additional motivation arises from the outstanding ability of biopolymers to direct the assembly of biogenic nanoscale components via weak physical interactions into controlled and sophisticated structures, thanks to their self-organization properties and the synergy observed between the inorganic colloids and the organic components.4 Of particular value would be bio-inspired methods that could be applied to non-biogenic nanoscale components with interesting electronic, magnetic or optical properties since controlling the spatial distribution of the nanoparticle is one of the cornerstone challenges for optimizing the macroscopic properties of such nanostructured materials. This idea is not very new, but only recently has started to gain favour.5,6 Among the different processes that have been explored, a typical route to make bio-nanocomposites consists in dispersing charged nanosized nanoparticles in a semi-dilute solution of an oppositely charged bio-polyelectrolyte that can subsequently undergo a sol–gel transition induced by a physical/chemical stimulus (pH, multivalent ions, temperature). The as-obtained soft-bionanocomposite (i.e. gel or coacervate) can be subsequently dried in a controlled manner to obtain a monolithic “hard-bionanocomposite”. In contrast to chemical gels, most of these physical networks are reticulated by anisotropic network junctions, such as helix or “egg-box”, which can connect biopolymer segments on more than two different chains via weak interactions such as electrostatic or hydrogen bonding.7 At the meso-scale, this gelation by “microcrystallization” of chain segments should have profound effects on the nanoparticle network topology. Surprisingly, knowledge on the relative positions and motions of nanoparticles during such kinds of physical reticulation has not been available so far, even if indications of possible inhomogeneities were reported.8 Therefore,
a fundamental understanding and control of the nanoparticle state of dispersion as a function of the biopolymer conformational behavior seems to be of critical importance in developing new truly tailored bionanocomposite involving for instance gelatin, gellan, alginate, agarose, agar–agar, pectin or even DNA as organic matrices.

In this context, the initial questions that we have tried to address here are: (i) whether biopolymer folding transition can induce a detectable modification of the inorganic particle state of dispersion? and (ii) if this probable evolution can lead to the formation of aggregates? Considering this last scenario, it would be interesting to state on the reversible character of this process and to relate the nanoparticle aggregate size and eventual organization level to the extent of folding. To answer these questions, we have considered two systems composed of positively charged gelatin, which is considered as a model biopolymer for physical gelation studies11–13 in electrostatic interaction with anionic polyoxotungstate clusters or spherical metal oxide nanoparticles respectively. We have examined the structure of these mixtures by combining light scattering, small angle neutron scattering (SANS) and optical rotation measurements during gelatin triple helix folding and melting at fixed pH and ionic strength. A key point of this study was that, owing to neutron contrast matching,14 we were able to analyze the nanoparticle average state of dispersion on representative volumes (~cm³) at the pertinent length scales. Gelatin chains are soluble in water at temperatures above the temperature of gelification (Tgel = 27 °C for a mammalian gelatin) and form reversible physical gels below Tgel. Above Tgel peptide chains are random coils, though there may be a significant number of β-turn structures.15,16 On cooling, transparent gels containing extended physical cross-links are formed by partial reversion to ordered triple-helical segments. The cross-links are separated along the chain contour by peptide residues still in the random coil configuration.17 Unlike most biopolymers, the coil–helix transition is slow in gelatin18 due to the high activation energy (~72 kJ mol⁻¹) of cis to trans isomerization reactions at prolyl peptide bonds.19–21 This should allow the detailed study of the nanocomposite structure evolution during triple helix folding using SANS and polarimetry.

Polynucleotide clusters (POMs) are molecular metal–oxide clusters with size from about one to several nanometres. Oxide nanoparticles display diameters generally above 5 nm. These two kinds of nanoparticles can be used in the field of bio-nanocomposite materials.1,17,8,20–24 We have chosen one representative entity of each category to appreciate the general character of the process under our attention and to consider the case where the nanoparticle dimension is higher or lower than the biopolymer mesh size (ζ). Besides these aspects, we have taken into account the compatibility of the nanoparticle scattering length density with gelatin one in order to obtain enough neutronic contrast at relatively low nanoparticle concentration thus avoiding artifacts provided by potential dynamics of phase separation. For these reasons, we used crown-shaped polyoxotungstate clusters ([H₂P₂W₁₈O₆₄]¹⁺) with dimensions (Dext = 2.2 nm, Dax = 0.9 nm, width = 1.0 nm) close to gelatin persistence length Lp/Lp/Rext = 2). This POM is a well-known superlaccanian polyanion (Fig. S1 of the ESI†) highly stable in the 1–8 pH range,25 which is expected to have applications in numerous domains, especially for electro-catalysis.26 We also used spherical SiO₂ nanoparticles with a diameter (D = 23.0 nm) significantly higher than gelatin persistence length (Lp/R ≈ 0.2). It is interesting to note that the binding mode of gelatin chains on the inorganic entities will probably vary from “point contact” at Lp/R = 2 to wrapping at Lp/R = 0.2. It was anticipated that the sensitivity of the inorganic species toward biopolymer folding will vary between these two situations.

In this paper, we show that the spatial distribution of the two kinds of nanoparticles is clearly modified during helix folding. Indeed, the contrast matching method enables the study of the nanoparticle network independently from biopolymer contribution. As anticipated, when starting from polyoxotungstate clusters initially arranged in small finite size aggregates dispersed in the gelatin interpenetrated network, we point out a continuous growth process induced by the triple helices formation and a direct relationship between the average size of the aggregates and the triple helix rate. In the case of silica, when starting from a connected particle network, we evidenced a significant densification of the inorganic structure through gelatin conformational transition. The kinetics of triple helix folding is only slightly affected by the presence of the nanoparticles and their state of dispersion. Under our experimental conditions, the different nanoparticle network evolutions are almost thermo-reversible following triple helix unfolding. We believe that in the future this reorganization process should be taken into account in the analysis of bio-nanocomposite properties if anisotropic network junctions are involved in the organic matrix reticulation.

2 Materials and methods

2.1 Materials

Gelatin extracted from porcine skin (type A with a bloom of ~175 g and an isoelectric point, IEP, close to 8 according to the supplier), sodium chloride (NaCl, purity ≥ 99.5%), hydrochloric acid ([HCl] = 37%) and Ludox® AM-30 colloidal silica dispersion (30 wt% of SiO₂ in water) were purchased from Sigma-Aldrich and the same batch of each species has been used for all of our studies. Deuterium oxide (D₂O, purity ≥ 99.9%) was purchased from Euriso-top. All these chemicals were used without further purification. In all the experiments, we used water from a Millipore system (resistivity > 18 MΩ).

2.2 Sample preparation

Gelatin solutions. Gelatin solutions were prepared by swelling the gelatin granules in an aqueous solution for a minimum of 3 h at 5 °C. Gelatin was then dissolved at 50 °C using a magnetic stirrer for 30 min at 300 rpm. Except when noted (phase diagram experiments), the pH was adjusted to about 3.4 with an aqueous HCl solution (2 M) and the ionic strength was fixed at 0.1 M with NaCl. In this domain of pH and temperature, gelatin macromolecules are supposed to be in a random coil conformation with fully protonated amine (pK₁ NH₃⁺/NH₂ = 10.5) and carboxylic acid functions (pK₃ COOH/COO⁻ = 4) giving rise to a global positive charge (IEP ≈ 8). Considering that the positive charges are mainly given by arginine and lysine amino acids, one can estimate that 7.6 residues per 100 residues are positively charged according to the standard composition of
gelatin extracted from bones via an acid treatment.27 Thus, gelatin behaves like a weak polyelectrolyte that is not subjected to Manning counter-ion condensation.28 In all the experiments the final gelatin concentration is fixed at 10 wt% by taking into account the weight proportion of water (~10 wt%) in the commercial powder measured by drying at 150 °C. For SANS experiments, the samples were prepared following the same procedure, except that D2O was used instead of H2O. Taking into account the possibility of gelatin hydrolysis,29 we performed DLS measurements repeatedly over 24 h on a gelatin dilute sample ([gelatin] = 1 g L−1, pH 3.4) maintained at 50 °C and checked the presence of a single relaxation mechanism associated with an apparent hydrodynamic radius R_{H,app} = 14.8 nm during the whole period.

Silica dispersions. Silica dispersions of the desired concentration were obtained by dilution of the required quantity of commercial dispersion ([SiO2] = 30 wt%) in 0.1 M NaCl solution. The pH was then adjusted to 3.4 with an aqueous HCl solution (2 M). In Ludox AM, trivalent aluminium ions ([Al2O3] = 0.2 wt % in our dispersion according to the supplier) have been substituted for part of the tetravalent silicon ions on the surface of the particles. Therefore, these modified silica particles carry a more pronounced negative surface charge density over a wide pH range giving rise to broader stability against variation of pH.30

Polyoxotungstic cluster solutions (i.e. POM solutions). The salt K28Li5H7[P2W48O184]−92H2O has been prepared in three steps as previously described by Contant and Tézé25 and checked by FT-IR and 31P NMR in D2O/H2O mixture (a single line at −6.6 ppm). [H3P2W48O184]3− solutions of the desired concentration were obtained by dilution of the required quantity of powder in 0.1 M NaCl solution. The pH was then adjusted to 3.4 with an aqueous HCl solution (2 M).

Mixtures preparation. Two solutions, one of gelatin and one of clusters or nanoparticles, were first prepared (see above) separately at 50 °C and at twice the final desired concentrations. Two equal volumes of each solution were then quickly mixed and homogenized under vigorous stirring at 300 rpm for 20 min before measurements.

Sol–gel transition. For SANS and optical rotation measurements the same thermal protocol was applied (ESI†).

2.3 Turbidimetry

The stability of the different mixtures was evaluated by optical transmission measurements using a Turbiscan LabExpert setup (Formulaction, France). This device is mainly composed of a mobile head displaying a transmitting pulsed near-infrared light source (λ = 850 nm) and a detector analyzing the transmitted light. This reading head is situated in a thermo-regulated chamber and is moving up/down along a cylindrical cell, acquiring scattering data every 40 µm. We performed optical transmittance measurements at 50 °C or 10 °C in a range of sample height between 4 mm and 20 mm (from bottom to top) to avoid edge effects.

2.4 Static and dynamic light scattering

Static Light Scattering (SLS) and Dynamic Light Scattering (DLS) measurements were performed using a 3D DLS spectrometer (LS Instruments, Fribourg, Switzerland) equipped with a 25 mW HeNe laser operating at λ = 632.8 nm, a variable-angle detection system, and a temperature-controlled index matching vat. Solutions were directly filtered through a 0.2 µm cellulose filter into the cylindrical scattering cells and the temperature was fixed at 25.0 ± 0.1 °C or 50.0 ± 0.1 °C depending on the experiments.

In the SLS experiments, the excess of scattered intensity R(q) was measured with respect to the solvent, where the magnitude of the scattering wave vector q is given by

\[ q = (4\pi n l \sin(\theta/2)) \]  

where n is the refractive index of the solvent (1.34 for water at 25 °C), and θ is the scattering angle. In our experiments, θ was varied between 30° and 130°. The absolute scattering intensities I (in cm−1) were deduced by using a toluene sample reference.31–33

In the dynamic light scattering (DLS) experiments, the experimental signal is the normalized time autocorrelation function of the scattered intensity:34,35 g2(q,t).

The latter can be expressed in terms of autocorrelation of the concentration fluctuations, g1(q,t) through:

\[ g2(q,t) = 1 + \alpha + \beta g1(q,t) \]  

where α is the baseline (varying between 1 × 10−4 and 2 × 10−4 in our experiments) and β the coherence factor, which in our experiments is equal to 0.8–0.9.

For dilute solutions of polymer or nanoparticles, values of the diffusion constant D were obtained by the cumulant analysis. The latter is related to the average apparent hydrodynamic radius R_H of the diffusing species through the Stokes–Einstein relation

\[ D = \frac{kT}{6\pi\eta_{S}R_{H}} = \left( \frac{1}{q^2} \right) g_{1=0} \]  

where k is the Boltzmann constant, η_S the solvent viscosity, and T the absolute temperature.

2.5 Optical rotation

Gelatin is an optically active material in both the coil and helical states. Due to coherent chiral ordering helical domains rotate the plane of light polarization much more strongly than the individual chiral amino acids in the coil state. Thus, the coherent optical activity gives a direct indication of the fraction of the monomers in the helical states. Optical rotation was measured on a Perkin-Elmer 341 polarimeter. The glass cell has an optical path of 1 cm. The temperature is regulated by a programmable external circulating bath (Julabo F518). The measurements were performed with the same wavelength: λ = 436 nm after checking the validity of the Drude relation.34 The procedure of triple helix fraction determination has been previously described and detailed elsewhere.38,39 We recall that the helix amount (χ) is defined as the ratio between the number of residues in helical
conformation and the total number of residues. $\chi$ is derived from the following expression:

$$\chi = \frac{[\alpha]^{\text{exp}}_h - [\alpha]^{\text{coil}}_h}{[\alpha]^{\text{collagen}}_h - [\alpha]^{\text{coil}}_h}$$  \hspace{1cm} (4)$$

where $[\alpha]^{\text{exp}}_h = acl$ is the specific optical rotation of the gelatin in solution, $c$ is the concentration (in g cm$^{-3}$), $l$ is the optical path (dm), $\alpha$ is the optical rotation angle (degrees) measured experimentally, $[\alpha]^{\text{collagen}}_h$ is the specific optical rotation of native soluble collagen ($\chi = 1$), which contains only triple helices, and $[\alpha]^{\text{coil}}_h$ is the specific optical rotation of the coils ($\chi = 0$). An average value of 100 g mol$^{-1}$ per amino acid was considered. At high temperatures, it can be assumed that all the chains are in a random coil conformation. The specific optical rotations for the different gelatins in coil conformation were derived directly from the measurement in solutions at high temperatures. The helix rates plotted in Fig. 12 and 13 correspond to the averaged value on 1 h 30 min to account for SANS data acquisition.

2.6 Small angle neutron scattering (SANS) and small angle X-ray scattering (SAXS)

SANS experiments were carried out on the PACE spectrometer in Léon Brillouin Laboratory at Saclay (LLB, France). The chosen incident wavelength, $\lambda$, depends on the set of experiments, as follows. For a given wavelength, the range of the amplitude of the transfer wavevector $q$ was selected by changing the detector distance, $D$. Three sets of sample-to-detector distances and wavelengths were chosen: $D = 4.7$ m, $\lambda = (10 \pm 0.5) \text{ Å}; D = 2.5$ m, $\lambda = (10 \pm 0.5) \text{ Å}; D = 1.2$ m, $\lambda = (6 \pm 0.5) \text{ Å}$ so that the following $q$-ranges were respectively available: $4.3 \times 10^{-3} < q (\text{Å}^{-1}) < 4.52 \times 10^{-2}, 7.5 \times 10^{-3} < q (\text{Å}^{-1}) < 7.99 \times 10^{-2}, 2.62 \times 10^{-2} < q (\text{Å}^{-1}) < 2.72 \times 10^{-1}$. Measured intensities were calibrated to absolute values (cm$^{-1}$), using normalization by the attenuated direct beam classical method. Standard procedures to correct the data for the transmission, detector efficiency, and backgrounds (solvent, empty cell, electronic, and neutronic background) were carried out. To combine data from light and neutron scattering it is necessary to rescale the SLS data to give the overlap with the SANS data. The light scattering intensity at each $q$ value was adjusted by the ratio between the contrasts of the two techniques so that the SLS data overlap with the SANS data in the region of comparable $q$. Each sample was achieved in a 100% D$_2$O solvent or in a 72%/28% H$_2$O/D$_2$O solvent which matches with the gelatin contribution so that the signal is only arising from the inorganic nanoparticles. Table 1 summarizes the different component characteristics considered in this study.

Small-angle X-ray scattering (SAXS) experiments were performed on the Swing beamline at SOLEIL synchrotron, Saint-Aubin (France). The incident beam energy was 12.0 keV ($\lambda = 1.35$ Å), and the distance from the sample to the Aviex CCD detector was 1435.8 mm. The corresponding scattering vector $q$ varied from 0.007 to 0.7 Å$^{-1}$. Experiments were performed at 25 °C.

3 Results and discussion

3.1 Single solute solutions characterization

In this section, we present detailed characterizations performed on each individual component before mixing to ascertain their dimensions and initial dispersion state in the following conditions: [NaCl] = 0.1 M, pH = 3.4 and $T = 50$ °C where gelatin is in the sol state.

i. Gelatin solution. The structure of gelatin macromolecules alone or in various mixed systems has been described in both dilute/semi-dilute states at pHs close to IEP by means of SANS since the pioneering study of Pezron et al.\textsuperscript{32} However, to the best of our knowledge, few detailed studies were dedicated to the gelatin structure at pHs where only positive charges are present on the chains and a polyelectrolyte solution character is expected. DLS experiments were first carried out in dilute gelatin solutions ([gelatin] = 0.1 wt%). Fig. 1 represents a typical

![Fig. 1 Scattered electric field autocorrelation functions $g^{(2)}(t)$ obtained at $\theta = 90^\circ$, $T = 50$ °C, [NaCl] = 0.1 M and pH = 3.4 for aqueous dispersions of: SiO$_2$ nanoparticles at 0.20 wt% (open lozenges); [H$_2$P$_4$W$_{20}$O$_{80}$]$^{13-}$ clusters at 0.37 wt% (open triangles); dilute gelatin at 0.10 wt% (open circles) and semi-dilute gelatin at 0.10 wt% (open squares). Dashed lines correspond to the fit of the data using a monoeponential function (red) or a double exponential relaxation (blue).](image)

Table 1 Densities, neutron scattering length densities per unit volume and contrasts per unit volume of the different systems studied by SANS

<table>
<thead>
<tr>
<th></th>
<th>Gelatin</th>
<th>Silica particles</th>
<th>POMs</th>
<th>100% D$_2$O</th>
<th>72%/28% H$_2$O/D$_2$O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Density</td>
<td>1.44</td>
<td>2.30</td>
<td>3.85</td>
<td>1.11</td>
<td>1.03</td>
</tr>
<tr>
<td>$\rho/10^n$ g cm$^{-3}$</td>
<td>1.69</td>
<td>3.60</td>
<td>4.60</td>
<td>6.40</td>
<td>1.69</td>
</tr>
<tr>
<td>$(\Delta\rho)_{D_2O}/10^3$ g cm$^{-3}$</td>
<td>22.2</td>
<td>7.84</td>
<td>3.24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$(\Delta\rho)_{H_2O/D_2O}/10^3$ g cm$^{-3}$</td>
<td>0</td>
<td>3.65</td>
<td>8.47</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
correlation function \( g^{(1)}(t) \) obtained for a scattering angle \( \theta = 90^\circ \) at \( T = 50^\circ C \). The scattered electric field autocorrelation function is characterized by a single diffusive relaxation with a characteristic time inversely proportional to \( q^2 \). With relation (3), we obtain an apparent hydrodynamic radius \( R_{H,\text{app}} \) equal to 15 ± 2 nm for gelatin. SLS experiments were carried out in the same sample (Fig. S2a of the ESI†) to determine its molecular mass (\( M_w \)) and apparent radius of gyration (\( R_{G,\text{app}} \)). According to Guinier’s law, we have determined \( M_w = 20 000 \pm 1000 \) g mol\(^{-1} \) and \( R_{G,\text{app}} \approx 18 \pm 4 \) nm (Fig. S2b of the ESI†), so that \( R_{G,\text{app}}/R_{H,\text{app}} \approx 1.2 \). At a gelatin concentration of 10 wt%, that is, in the semi-dilute range where gelatin chains are interpenetrated, the autocorrelation function of the scattered electric field becomes bimodal as shown in Fig. 1. To analyze the scattering data within this context, \( g^{(1)}(t) \) was fitted by the sum of two exponentials:

\[
g^{(1)}(q, t) = A_f(q)e^{-t/\tau_f} + A_S(q)e^{-t/\tau_S} \quad (5)
\]

with \( A_f(q) + A_S(q) = 1 \). Both the fast and slow relaxation times, \( \tau_f \) and \( \tau_S \), vary as \( q^2 \), which is the signature of collective diffusion processes. The best fit of the data with eqn (5) was obtained for \( \tau_f = 0.05 \) ms and \( \tau_S = 5.00 \) ms (Fig. 1).

The two modes are respectively assigned to be the collective motion of the network meshes and the diffusion mode of gelatin clusters.\(^{13,14,38,39} \) Concerning the fast mode, according to de Gennes scaling theory for semi-dilute solutions in good solvents,\(^{40} \) the dynamical behavior of the solution can be described in terms of a single characteristic length, that is, the correlation length (\( \xi \)) or the blob size which is the mesh size of the network of interpenetrated chains. We have estimated this size to \( \xi \approx 40 \) A with eqn (3). This value agrees with measurements performed elsewhere with different types of gelatin in the same range of concentration.\(^{13,41–44} \) The slow mode has already been extensively studied by several authors using light scattering techniques.\(^{13,14,38,39} \) We briefly remind that the origin of the corresponding gelatin associations, also called inhomogeneities, is not yet fully understood even if the implication of hydrophobic interactions between apolar lateral groups seems probable.\(^{38} \) Indeed, the typical size of these structures (\( a \)) is independent of gelatin concentration, temperature increase, redilution, coacervation, and ionic strength but varies with the concentration of an added surfactant.\(^{38,43} \) Considering that inhomogeneities are embedded in the semi-dilute gelatin network of macroscopic viscosity \( \eta_{\text{gelatin}}^{10\text{wt}}% \approx 0.015 \text{ Pa s} \), we can estimate the apparent size, \( a \), of these associations using:

\[
a = \left( \frac{kT}{6\pi\eta_{\text{gelatin}}^{10\text{wt}}%} \right)^{\frac{1}{2}} q^2
\]

We found \( a \approx 276 \) A, a value in very good agreement with measurements performed elsewhere with different types of gelatin at pH generally close to IEP meaning that these associations are also resistant to pH variations. This result holds with the assumption that inhomogeneities could arise from hydrophobic interactions between apolar groups.

Fig. 2 displays the SANS scattering pattern (low-q SLS is also presented after rescaling to SANS at the ratio between the contrasts of the two techniques\(^{49} \)) for a 10 wt% gelatin semi-dilute solution at 50 °C.

From the above discussion of characteristic times, it seems appropriate to treat the gelatin scattered intensity with an usual approach regarding at \( q < 0.025 \) Å\(^{-1} \) \( \xi^{-1} \) (according to DLS), as the sum of two separate contributions arising respectively from the thermal concentration fluctuations and from the spatial inhomogeneities. This choice of decomposition is motivated by considering that the spatial scale of index fluctuations, due to inhomogeneities, is large compared to the one of concentration fluctuations in solution or gel. Indeed, in our case, the characteristic relaxation times (\( \tau_{\text{slow}} \approx 5.0 \) ms and \( \tau_{\text{fast}} \approx 0.05 \) ms) estimated from DLS measurements support this assumption. Thus, the total scattered intensity can be decomposed in two additive contributions as follows:

\[
I(q) = I_{O-Z}(q) + I_{D-B}(q) = \frac{I_{O-Z}(0)}{1 + q^2 \xi^2} + \frac{I_{D-B}(0)}{(1 + q^2 a^2)^2} \quad (6)
\]

where \( I(q) \) is the total scattered intensity, \( I_{S}(q) \) is the intensity scattered by the ‘homogeneous’ entangled network and \( I_{D-B}(q) \) is the intensity scattered by ‘inhomogeneities’. At an intermediate \( q \) range (i.e. \( q \approx \xi^{-1} \)) such semi-dilute solution can be fully described by a single parameter \( \xi \) and the pair-correlation function should follow an Ornstein–Zernike (O–Z) form.\(^{40,46} \) After Fourier transform, this assumption leads to the following Lorentzian form of the scattered intensity:

\[
I_{O-Z} = \frac{I_{O-Z}(0)}{1 + q^2 \xi^2}
\]

At a very low \( q \) range (i.e. \( q \ll \xi^{-1} \)), the contribution of the inhomogeneities is supposed to be greatly dominant and the data are regarded assuming a random distribution of the inhomogeneities, as the scattering from a two-phase random medium described by the Debye–Bueche (D–B) form. In this frame, the inhomogeneities spatial correlation, of average length \( a \), is
damped according to the following exponential correlation function \(\gamma(r) = \exp(-r \lambda_0)\).

In reciprocal space, the related expression of the scattered intensity is:

\[
I_{D-B} = \frac{I_{D-B}(0)}{(1 + q^2a^2)^\frac{3}{2}}
\]

In Fig. 2, the red continuous line represents the best fit of the data with eqn (6) where \(\xi\), \(a\) and \(I_{O-Z}(0)\) behave as free parameters, while the green and blue dashed lines represent the corresponding contributions of the O–Z and D–B functions respectively. One can observe the fair agreement achieved between experimental data and eqn (6) for \(\xi = 34\ \text{Å};\ a = 256\ \text{Å}\) and \(I_{O-Z}(0) = 1.15\ \text{cm}^{-1}\). At first glance, these values are compatible with those previously determined by DLS and those already reported in the literature\(^{47,48}\) even if previous studies were mainly performed at pHS close to IEP and concentrations below or equal to 5 wt%.

To ascertain this statement and facilitate the scaling analysis we have plotted in the log–log scale (Fig. 3a) the concentration dependence of the correlation length by combining the present result and previous ones obtained in the sol state (\(T > 40^\circ\text{C}\)). The data suggest an exponential close to \(-0.50\) with magnitude somewhat less than that predicted \((-0.77\) for such semi-dilute solution in good solvent.\(^{49}\) This value is consistent with the weakly fluctuating semi-dilute regime dominated by two-point collisions or in other words “marginal solvent”\(^{47}\) that was already reported for gelatin solutions by Pezron \textit{et al.}\(^{32}\)

ii. Silica dispersion. The silica nanoparticles were characterized by DLS, SANS and SAXS under the same experimental conditions for [SiO\(_2\)] = 0.2 wt%. The correlation function \(g^{11}(r)\) depicts a monoexponential cooperative relaxation mode (Fig. 1) written as:

\[
48\quad I(q) = (\Delta \rho)^2 V_{SiO_2} \phi_{SiO_2} P_{SiO_2}(q) S_{SiO_2}(q)
\]

The polydispersity in size is described by a log-normal distribution \(l(r,R_0,\sigma)\) where \(r\) is the radius, \(R_0\) the mean radius and \(\sigma\) the variance:

\[
l(r, R_0, \sigma) = \frac{1}{\sqrt{2\pi\sigma r}} \exp\left(-\frac{1}{2\sigma^2}\ln^2\left(\frac{r}{R_0}\right)\right)
\]

Finally, the global scattering intensity was satisfactorily fitted by the following relation:

\[
I(q) = (\Delta \rho)^2 V\phi \int_0^\infty P_{SiO_2}(q, r) l(r, R_0, \sigma)dr
\]

It is seen that the best agreement between experimental and calculated data is fair for \(R = 8.9\ \text{nm}\) and \(\sigma = 0.12\). In summary,
SiNPs are almost monodisperse and well dispersed in the absence of gelatin.

iii. POM solutions. POMs were characterized following the same procedure with $[H_3P_2W_{28}O_{81}]^{13-} = 0.37$ wt%. As shown in Fig. 1, the correlation function $g^{(1)}(r)$ depicts a very short relaxation consistent with a dilute solution of very low sized clusters. According to the cumulant analysis the short relaxation corresponds to $R_{H,app} = 1.2$ nm in rough agreement with the expected size for individual objects (see Fig. S1 of the ESI†). We present in Fig. 4b the results of a SAXS experiment performed in a similar dilute solution. As above, neglecting cluster interactions, we have plotted on the same graph the best fit of the data with eqn (7) and by considering the form factor of a monodisperse hollow cylinder (eqn (11):

$$P(q) = V_s(\Delta \rho)^2 \int_0^1 \Psi^2[qS(1-x^2)^{1/2},R_c(1-x^2)^{1/2}]dx$$

(11)

where $\Psi(q,y,z) = (1/(1-\chi^2))A(qy) - \gamma A(qz)$, $A(w) = 2J_1(w)/w$, $\gamma = R_C/R_S$, $V_S = \pi(R_S^3 - R_C^3)L$, $K = (\sin(qHS)qHx^2)$, $R_c$ is the core radius, $R_S$ is the shell radius, $L = 2f$ is the cylinder length and $J_1$ is the first-order Bessel function. The integral over $x$ is the orientational average and the returned form factor is scaled to units of cm$^{-1}$. The agreement between experimental and calculated data is quite fair for $R_C = 0.45$ nm, $R_S = 1.10$ nm and $L = 1.00$ nm. One can note that the form factor of hollow sphere is also in good agreement with this scattering profile probably due to the small cylinder length. These dimensions suggest that the POMs are well dispersed without any aggregation effect.

3.2 Macroscopic phase behavior

In this section, most of the observations are done at high temperatures, namely 50 °C, where we expect no triple helix formation. We have investigated the stability of gelatin/SiO$_2$ mixtures at fixed gelatin concentration ([gelatin] = 10 wt%) to define the optimal conditions for our scattering study. In the first set of experiments, we have varied the silica nanoparticle concentration between 0.01 wt% and 18 wt%, keeping all others parameters constant ([gelatin] = 10 wt%, [NaCl] = 0.1 M, pH = 3.5, and $T = 50 \, ^\circ C$).

After 20 minutes of mixing, three different liquid states can be distinguished (Fig. 5): (i) region (1) at low concentrations: a slightly white transparent solution; (ii) region (2) in the intermediate concentration range: turbid solutions with a pale white color; (iii) and finally at high concentration region (3): macroscopically biphasic samples composed of a dilute solution of both silica nanoparticles and gelatin floating above a denser white viscous phase. This lower phase exhibits a viscous liquid character at 50 °C according to a qualitative flow test. Considering the turbid nature of the samples in region (2), we assume the transition between regions (1) and (2) as due to a mesoscopic phase separation leading to the formation of colloidal structures that strongly scatter light. Meanwhile, the second transition between regions (2) and (3) will be considered as a macroscopic phase transition. To ascertain quantitatively the limits between regions (1) and (2), we have performed optical transmission ($\delta$) measurements. As shown in Fig. 6a, the different samples are homogeneous since $\delta$ is constant all along the sample height for each mixture. We have then plotted in Fig. 7a the evolution of the average $\delta$ over sample height as a function of the nanoparticle concentration in order to appreciate the shape of the transition between regions (1) and (2). Considering this plot, one can observe that $\delta$ decreases progressively as the nanoparticle concentration is increased until $\delta = 0.01\% \pm 5\%$ for [SiO$_2$] $\geq 0.4\%$. We have defined the boundary between regions (1) and (2) at the lower nanoparticle concentration for which $\delta = 0.01\%$. For regions (1) and (2), we have performed optical transmission.
versus $I$ evolution of the average optical transmission that could balance the loss of conformational entropy of the biopolymer. To further investigate the mechanisms that drive the condensation process from low nanoparticle concentration (region (1) and possibly the beginning of region (2)) the system evolution is mainly enthalpically driven with the formation of polyelectrolyte/colloids complexes. Thus, these complexes interact via long-range electrostatic repulsions which are strongly modulated by the screening of all the ions present in solution.

In our case, when the NaCl concentration is increased from 0.1 M to 1 M, the Debye screening length ($\lambda_D$) decreases from $\sim$1 nm to $\sim$0.3 nm and as a consequence the kinetics of primary complex growth via a multiple collision process should be favored. In contrast, increasing the pH in situations (B) and (C) shifts the onset of phase separation toward a higher nanoparticle concentration. It seems reasonable to attribute this effect to the deprotonation of some carboxylic acids ($pK_a$ COOH/COO$^-$ = 4) giving rise to negative charges on the gelatin chain thus lowering the ‘electrostatic affinity’ between gelatin and silica. This statement is ascertained by the disappearance of the phase separation process when the pH is increased toward the gelatin isoelectric point (IEP = 8 corresponding to situation (C)). We recall that the silica nanoparticles considered in this study carry a pronounced negative surface charge density in the whole studied pH range. To clarify the influence of the electrostatic interactions, we have estimated the charge ratio ([+][−])$^{38}$ corresponding to the onset of mesoscopic phase separation at ([+][−])$^{38}$ ≈ 7.5 (Fig. 7a). In this estimation, we assume on the basis of ref. 30 that, at pH = 3, 2/3 of the negative charge concentration is ensured by the aluminium atoms ([Al] = 0.2 wt% in the mother nanoparticle solution) while the silicium is responsible for 1/3 and that all the positive charges arise from arginine and lysine amino acids which represent 7.6 residues per 100 residues according to ref. 27 and 29. To determine whether this threshold depends on other parameters, we have then analyzed the effect of time and temperature on samples prepared at [gelatin] = 10 wt%, [NaCl] = 0.1 M, pH = 3.4, $T = 50$ °C and various nanoparticle concentrations.

As shown in Fig. 6e, the samples of region (1) keep their homogeneous character for at least three days and the onset of mesoscopic phase separation is not modified during this period. However, this region is metastable because some sedimentation has been evidenced in region (1) after two months. Considering the time scale of our experiments (two days), we will consider the samples of this region as kinetically stable. Finally, we have considered the influence of temperature on the system stability by cooling the samples from 50 °C to 5 °C. This thermal treatment induces a sol/gel transition in all the samples except for the supernatant solution encountered in region (3), which is experimental procedure as above ($T = 50$ °C) and similar [gelatin]/[SiO$_2$] ratio, we have successively considered three other situations: (A) [NaCl] = 1 M and pH = 3.4; (B) [NaCl] = 0.1 M and pH = 5.5; (C) [NaCl] = 0.1 M and pH = 8.0. As shown in Fig. 6b–d and 7b, when either the pH or the ionic strength is increased the onset of mesoscopic phase separation is significantly shifted toward higher or lower nanoparticle concentration respectively thus revealing the key role of electrostatic interactions. The driving interaction should involve the fully protonated amines mainly given by arginine and lysine amino acids present on the gelatin chains and the negative charges mainly ensured by the aluminates functions at such low pH on the nanoparticle surface. The lowering of the transition boundary observed in situation (A) can be understood by considering that the primary complexes behave like charged colloids due to partial ion-pairing between gelatin and silica at the surface of the primary complexes. Thus, these complexes interact via long-range electrostatic repulsions which are strongly modulated by the screening of all the ions present in solution.

We just remind that counter-ions seem to play a key role during this last stage since their complete release from the core of the globules may induce an increase of translational entropy that could balance the loss of conformational entropy of the biopolymer. To further investigate the mechanisms that drive the evolution of our system, we have analyzed the effect of pH and ionic strength in a second set of experiments. Following the same measurement. Similar liquid–liquid phase separations following a two-step process have often been observed in mixed systems involving polyelectrolytes and oppositely charged colloids and were generally discussed in the frame of a complex coacervation process, or associative phase separation. Several theoretical descriptions and numerical simulations exist, but up to now, there was no complete theory for such system phase diagrams. Nevertheless, it seems clear that at low nanoparticle concentration (region (1) and possibly the beginning of region (2)) the system evolution is mainly enthalpically driven with the formation of polyelectrolyte/colloids complex through weak non-specific attractive interactions. At higher colloid concentrations the condensation process from almost neutral nanometric complexes toward micrometric liquid drops is still a matter of debate.

Fig. 7 (a) Evolution of the average optical transmission and of the charge ratio ([−]/[+] versus SiO$_2$ nanoparticles concentration at pH 3.5, $I = 0.1$ M and [gelatin] = 10 wt%, after: 20 min at 50 °C (full squares), 15 h at 50 °C (open circles) and 24 h at 5 °C (open triangles). (b) Evolution of the average optical transmission versus SiO$_2$ nanoparticles concentration at 50 °C and [gelatin] = 10 wt% for: $I = 0.1$ M, pH = 3.5 (full squares); $I = 1$ M, pH = 3.5 (open circles); $I = 0.1$ M, pH = 5.5 (open triangles) and $I = 0.1$ M, pH = 8.0 (open lozenges). The dashed lines correspond to simple exponential or polynomial fits and should be used as a guide for the eyes.

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probably too dilute.\(^5\) Moreover, as shown in Fig. 6f and 7a, the onset of phase separation is not sensitive to rapid temperature sweep, at least between 50 °C and 5 °C.

In summary, we have shown that the gelatin/SiO\(_2\) mixtures are subject to a two-step associative phase separation process. The onset of mesoscopic phase separation should be driven by electrostatic interactions between the anionic nanoparticles and the positively charged gelatin chains. This phase transition boundary is independent of time for at least two weeks and is insensitive to the concentration of the positively charged gelatin chains. This phase transition boundary is subject to a two-step associative phase separation process. The gelatin/SiO\(_2\) phase transition boundary is independent of time for at least two weeks and is insensitive to the concentration of the positively charged gelatin chains. This phase transition boundary is subject to a two-step associative phase separation process.

In the following, we will limit our investigations to samples of region (1) prepared in the following conditions where the system is stable in the time scale of our experiments: [SiO\(_2\)] = 0.20 wt%, [gelatin] = 10 wt%, [NaCl] = 0.1 M, pH = 3.4, T = 50 °C. The nanoparticle concentration has been chosen in order to maximize the neutron scattering intensity. Following similar experimental strategy, we have observed the same behavior for the gelatin/POMs clusters and we have chosen to perform our measurements with \([\text{H}_7\text{P}_8\text{W}_{48}\text{O}_{184}]^{33-}\) = 0.37 wt%, [gelatin] = 10 wt%, [NaCl] = 0.1 M, pH = 3.4 and T = 50 °C.

### 3.3 Structure of the nanocomposite solutions at T = 50 °C

Here also we work at a high temperature (T = 50 °C), i.e. above the melting point of all triple helices.

Fig. 8a displays the scattering patterns in pure D\(_2\)O solvent for two mixtures ([H\(_7\)P\(_8\)W\(_{48}\)O\(_{184}\)]\(^{33-}\) = 0.37 wt%/gelatin) = 10 wt% and [SiO\(_2\)] = 0.20 wt%/gelatin) = 10 wt%). For gelatin, the low-q SLS data are also represented after rescaling to SANS by the contrast ratio of the two techniques. At large q (q > 0.06 Å\(^{-1}\)) all the curves overlap with the reference gelatin data. This result can be understood by considering the negligible character of the nanoparticle scattering in this domain (Fig. S3 of the ESI\(^\dagger\)) and the high intensity level of the incoherent background (I = 0.1 cm\(^{-1}\)) of gelatin at 10 wt%. In the intermediate q-range the scattering from both nanocomposites is larger than that of gelatin suggesting that a nanoparticle complexation is occurring without modifying the gelatin chain section. At low q (q < 0.009 Å\(^{-1}\)), two different situations are observed depending on the nanoparticle:

- in the presence of \([\text{H}_7\text{P}_8\text{W}_{48}\text{O}_{184}]^{33-}\) the scattered intensity converges toward the pure gelatin one when q decreases, suggesting the absence of large aggregates involving clusters and that the gelatin inhomogeneities are not disturbed by the clusters.

- in the presence of SiO\(_2\) nanoparticles, the scattered intensity shows much higher magnitude than that of the gelatin and with steep variation described by a power law with an exponent close to -2.6, suggesting the formation of large aggregates. The exponent value may reflect the fractal structure of these aggregates. In this view, aggregates are not compact but present a somehow open structure, though the exponent is much higher, meaning more compaction, than the 1.8 values for Diffusion Limited Aggregation, and closer to the value (2.2) found for Reaction Limited Aggregation, observed in the presence of electrostatic attraction.\(^5\)

To complete and clarify the picture of the nanoparticle distribution we present in Fig. 8b scattering experiments performed in a 28% D\(_2\)O/72% H\(_2\)O mixture where gelatin scattering is matched (see ESI\(^\dagger\)). In the high q-range, the poor counting statistics combine with the incoherent background perturbation in preventing any detailed data analysis. Therefore, we will restrict our discussion to the low/intermediate q domain. In the case of POMs clusters, the scattering pattern is close to the one of individual clusters at medium and large q, but clearly shows a decrease at low q. This is however a smooth variation, analogous to a Guinier regime, suggesting a slight aggregation of the nanoparticle into a small finite size structure. This feature is often encountered when charged nanoparticles are dispersed in an oppositely charged semi-dilute network even at low volume fractions. \(I(q)\) can be fitted by the sum of the two Guinier fits:

\[
I(q) = I_1(q \to 0)e^{(qR_{1,G})^2/3} + I_2(q \to 0)e^{(qR_{2,G})^2/3}
\]

(12)

At low q, the first Guinier fit characterizes aggregates of POMs with \(R_{1,G} \approx 7.3\) nm and \(I_1(q \to 0) \approx 0.09\) cm\(^{-1}\). At higher q, the best adjustment is obtained for \(R_{2,G} \approx 1.5\) nm and \(I_2(q \to 0) \approx 0.07\) cm\(^{-1}\) which is in fair agreement with the characteristics of individual POMs (Fig. 1 and 3). This last result suggests that individual POMs may coexist with aggregates and/or that POMs keep their individual character inside the aggregates. Moreover, it is interesting to note that the very small size of these aggregates is compatible with the homogeneous character of the system from the turbidimetric point of view.

In the case of SiO\(_2\), the scattered intensity at low q depicts a similar behavior to that observed in pure D\(_2\)O with the presence of power-law decay; the apparent exponent, however, is slightly

---

**Fig. 8** (a) Variation of the scattered intensity, \(I\), with q in pure D\(_2\)O solvent at T = 50 °C for the reference gelatin sample ([gelatin] = 10 wt%, full squares) and for the nanocomposite samples: \([\text{H}_7\text{P}_8\text{W}_{48}\text{O}_{184}]^{33-}/\text{gelatin}\) (circles) and [SiO\(_2\)]/gelatin) (triangles). (b) Scattering in mixed H\(_2\)O/D\(_2\)O solvent at T = 50 °C for the nanocomposite samples: \([\text{H}_7\text{P}_8\text{W}_{48}\text{O}_{184}]^{33-}/\text{gelatin}\) (green circles) and [SiO\(_2\)]/gelatin) (blue triangles).
smaller, 2.3 instead of 2.6. This result ascertains our belief that SiO₂ nanoparticles form almost branched and open structures at 50 °C in the presence of gelatin in coil conformation.

In summary, using contrast matching is indeed useful; it allows us to remove the low q contribution of the gelatin inhomogeneities and access the nanoparticle contribution. We then see that after mixing at 50 °C the two kinds of nanoparticles are interacting with the oppositely charged gelatin chains. Depending upon the kind of inorganic entity, two different situations occur. The [H₇P₈W₄₈O₁₈₄]³³⁺ clusters are arranged in small finite sized aggregates which possibly coexist with individual POMs while the silica nanoparticles form connected networks at large length scale. In the latter case, the apparent fractal dimension of the flock suggests a branched character compatible with a flocculation process mediated by the gelatin chain interface.⁹⁹

3.4 Kinetics of gelatin renaturation at T < 50 °C

We have complemented the SANS investigations by performing optical rotation measurements following the same thermal procedure to establish the dependence of the helical content with time and temperature. The measurements were mainly performed in 28% D₂O/72% H₂O since the solvent composition was found to slightly influence the renaturation kinetics that is based on hydrogen bonding. Fig. 9 represents the helix amount, χ (i.e. the percentage of residues in helical conformation, see ESI†), vs. time during our thermal protocol for the different samples. It appears that the presence of nanoparticles has a weak influence on triple helix renaturation in the first part of the thermal protocol. This feature suggests that the renaturation process may first involve gelatin residues that do not interact with nanoparticles. In the second part, when the samples are cooled from 27 °C to 11 °C, the presence of fillers tends to significantly hinder the helix formation. This effect is more important for silica than for POMs. Since silica particles are larger, one possible origin is steric hindrance. A second origin could be gelatin wrapping occurring around silica nanoparticles (since Rₕ silica/Lₚ = 5) thus decreasing the concentration of ‘free’ gelatin available for triple helix renaturation.

These results are consistent with previous investigation performed on chemically cross-linked gelatin⁹⁹ but in partial contradiction with a recent study focusing on physical cross-links.⁹⁰

In this study we have shown that the physical cross-linker (vanadeate oxo clusters 1 nm in diameter) favors triple helix nucleation in a first stage before limiting the triple helix network extension above a certain gelatin concentration. At this point, it is hard to explain this discrepancy but the larger size of the present nanoparticles, their aggregation behavior, and the higher cooling rate used here⁹⁸ may play key roles in the gelatin–nano-object interface. Indeed, one can imagine that very small particles act like punctual and labile cross-links which favor the gelatin chain local alignment and close contact thus smoothing the way toward triple helix formation while bigger and aggregated particles may rather act as more spacey spacers that hinder chain/chains association. It is also important to notice that the process is fully reversible, as checked via a final heating ramp until 50 °C.

We have summarized in Table 2 the averaged helix amounts at different times corresponding to the SANS measurements.

3.5 Structure of the nanocomposite hydrogels: T < 50 °C

In this section we investigate the influence of gelatin renaturation on the two nanoparticles dispersions, during cooling from 50 °C to 11 °C. Let us first observe the scattering in pure D₂O, and start with pure gelatin. Fig. 10a shows that the overall shape of the scattered intensity is not deeply affected by the helix transition; neither are the characteristic sizes (ξ, a) given by a fit to eqn (6). However, except at very low q, some changes are clear: at 27 °C the intensity increases in two q ranges, one at low q and another in the high q. A further increase at lower temperature (11 °C) makes the intensity appear as shifted by an almost constant factor (in the log–log plot) except at very low q. This is compatible with the formation of gelatin triple helices, id est of more compact parts of chains which scatters more at large q and increases the linear density of chains influencing the lower q range.

Still in D₂O, in the presence of nanoparticle (Fig. 10b and c), the temperature effect is very similar on the shape and the enhancement of the intensity, so that it looks dominated by the triple helix formation in gelatin. However, at lower q, the intensity increases more with POMs than for pure gelatin. This is less obvious for silica, but can be seen by comparing the signals of pure and filled gelatin at all stages of cooling as in Fig. S3 of

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Triple helix amount (χ) of the studied systems at different times corresponding to the beginning of SANS measurements. These values were averaged on 5400 s after the beginning of the SANS measurement to take into account the delay of data acquisition. The values of tᵣ and the corresponding temperatures are given in the Materials and methods section</th>
</tr>
</thead>
<tbody>
<tr>
<td>t₀</td>
<td>t₁</td>
</tr>
<tr>
<td>XGelatin(%)</td>
<td>0.0</td>
</tr>
<tr>
<td>XPOM-Gelatin(%)</td>
<td>0.0</td>
</tr>
<tr>
<td>XSilica-Gelatin(%)</td>
<td>0.0</td>
</tr>
</tbody>
</table>
and should be used as guide for the eyes.

for (a) the reference gelatin sample ([gelatin] = 10 wt%) and for the nanocomposite samples: (b) [H₇PO₄W₄O₁₈₄]³³⁻/[gelatin]; (c) [SiO₂]/[gelatin]. In (a) the inset represents the mesh, determined from eqn (6), as a function of helix amount. The continuous lines correspond to linear fit and should be used as guide for the eyes.

The significant enhancements of these characteristics upon cooling prove that a growing process occurs. The intermediate-q variations follow the same trend reaching at 11 °C, while at 50 °C the scattering intensity comes close to its initial level. This reversibility is confirmed by the curve fitting: \( R_{G1} \) is back to ~10.7 nm and \( I(\theta = 0°) \) is back to ~0.17 cm⁻¹. These results suggest that upon cooling, on average, individual clusters have disappeared to rearrange into very small aggregates of finite size and/or to join the larger aggregates. It is important to note the absence of any correlation peak. This is the case with small and polydisperse aggregates where the coordination number of the particles is low, as commonly observed for branched aggregates.⁵⁸

- for the silica nanocomposite (Fig. 11b), contrast matching shows the power-law behavior at low and intermediate \( q \) already observed at 50 °C. Upon cooling the power-law exponent as well decay toward a lower \( q \) range is observed when the temperature is decreased. This evolution has been quantified by fitting with the sum of two Guinier laws (eqn 12), as done above at 50 °C. At 11 °C, the low-q variation corresponding to POM aggregates is fairly well-fitted with \( R_{G1} = 18.4 \) nm and \( I(\theta = 0°) = 0.49 \) cm⁻¹.

Fig. 10 Variation of the scattered intensity, \( I(q) \), in pure D₂O with \( q \) at: \( T = 50 \) °C (black squares); \( T = 27 \) °C (red disks) and \( T = 11 \) °C (blue triangles) for (a) the reference gelatin sample ([gelatin] = 10 wt%) and for the nanocomposite samples: (b) [H₇PO₄W₄O₁₈₄]³³⁻/[gelatin]; (c) [SiO₂]/[gelatin]. In (a) the inset represents the mesh, red circles and the size inhomogeneities (a, black full squares), determined from eqn (6), as a function of helix amount. The continuous lines correspond to linear fit and should be used as guide for the eyes.

Fig. 11 Scattering in mixed H₂O/D₂O solvent at \( T = 50 \) °C (\( t_0 \)), \( T = 11 \) °C (\( t_1 \)) and \( T = 50 \) °C after one renaturation/melting cycle (\( t_5 \), for the nanocomposite samples: (a) [H₇PO₄W₄O₁₈₄]³³⁻/[gelatin] and (b) [SiO₂]/[gelatin]. The lines plotted in (a) correspond to eqn (16) using: \( I_{q=0.1} = 0.09 \) cm⁻¹, \( R_{G,1} = 7.3 \) nm, \( I_{q=0.2} = 0.07 \) cm⁻¹, \( R_{G,2} = 1.5 \) nm for \( t_0 \) (\( T = 50 \) °C); \( I_{q=0.1} = 0.41 \) cm⁻¹, \( R_{G,1} = 18.4 \) nm, \( I_{q=0.2} = 0.12 \) cm⁻¹, \( R_{G,2} = 3.8 \) nm for \( t_1 \) (\( T = 11 \) °C) and \( I_{q=0.1} = 0.17 \) cm⁻¹, \( R_{G,1} = 10.7 \) nm, \( I_{q=0.2} = 0.14 \) cm⁻¹, \( R_{G,2} = 2.6 \) nm for \( t_5 \) (\( T = 50 \) °C).

the ESI. Indeed, the nanocomposite scattering patterns always overlap the one for gelatin for \( q \) higher than 0.04 Å⁻¹, while at low \( q \) it increases more than for the biopolymer alone. This suggests the presence of a nanoparticle aggregation process since triple helix content at a given time is slightly lower to the case of pure gelatin as shown previously with polarimetry.

To observe more directly the filler evolution, we now use contrast matching of gelatin:

- for the [H₇PO₄W₄O₁₈₄]³³⁻ nanocomposite (Fig. 11a), a significant enhancement of the scattered intensity with a shift of the
the aggregate size and/or concentration. For \( q > 0.04 \text{Å}^{-1} \), we observe a strong decrease of \( I(q) \) at 11 °C, which can be explained by a lower interfacial area for more compact or larger aggregates.

To analyze these results with consistency, the question then arises of the dependence of the nanoparticle network characteristics with triple helix concentration for these two systems.

### 3.6 Correlation between the inorganic network evolution and the triple helix rate

In the following we check the correlation between SANS and optical rotation measurements. Fig. 12 displays the evolution of \( R_G \) and \( R(q \to 0) \) determined for [H\(_3\)P\(_2\)W\(_8\)O\(_{42}\)]\(^{10-}\) nanocomposites in matched solvent \textit{versus} the triple helix amount. At first glance, the trend of the data evolution shows that a reversible aggregation process is promoted by the triple helices formation. Remembering that we have a bimodal distribution of POM aggregates size (scattering fitted by the sum of two Guinier fits), we observe that the variation of the primary aggregates (Fig. 12a) is quite continuous and of significant amplitude with \( R_{1,G,\chi=40\%} \approx 2.5 \times R_{1,G,\chi=0\%} \) and \( I_{1,\chi=40\%}(q \to 0) - I_{2,\chi=40\%}(q \to 0) \approx 3.6 \times (I_{1,\chi=0\%}(q \to 0) - I_{2,\chi=0\%}(q \to 0)) \). For this population the process is thermo-reversible to a large extent.

In contrast, the size evolution of initially individual POMs (Fig. 12b) undergoes a smoother erratic evolution with \( R_{2,G,\chi=40\%} \approx 2.5 \times R_{2,G,\chi=0\%} \) and \( I_{2,\chi=40\%}(q \to 0) \approx 2 \times I_{2,\chi=0\%}(q \to 0) \) that is almost irreversible.

At this stage, it is interesting to calculate the mean number of aggregation in the primary aggregates (\( N_{1,\chi=0\%\text{-agg}} \)). In this issue, we first calculate the isolated POMs concentration at \( t_0(c_{2,\chi=0\%}) \) using:

\[
c_{2,\chi=0\%} = \frac{I_{2,\chi=0\%}(q \to 0)\rho^2N_A}{M_w(\Delta \rho)^2} = 0.25 \text{ g cm}^{-3}
\]

where \( \rho = 3.87 \text{ g cm}^{-3} \), the calculated volumic mass (g cm\(^{-3}\)), and \( M_w = 12021.4 \text{ g mol}^{-1} \), the calculated molecular mass of the clusters.

Then one can deduce the primary aggregates concentration \( (c_{1,\chi=0\%} = 0.37 - c_{2,\chi=0\%}) \) and calculate the molecular mass of the primary aggregates: \( M_{w,1,\chi=0\%} = 20436 \text{ g mol}^{-1} \) according to the following expression:

\[
M_{w,1,\chi=0\%} = \frac{(I_{1,\chi=0\%}(q \to 0) - I_{2,\chi=0\%}(q \to 0))\rho^2N_A}{c_{1,\chi=0\%}(\Delta \rho)^2}
\]

The ratio between the molecular mass of the primary aggregates \( (M_{w,1,\chi=0\%}) \) and of individual clusters \( M_w \) gives \( N_{1,\chi=0\%\text{-agg}} \approx 1.7 \). Unfortunately, one cannot determine the evolution of \( N_{1,\chi=0\%\text{-agg}} \) \textit{versus} the triple helix rate since primary aggregates and initially isolated clusters are each aggregated since the very first stage of the renaturation process \( (t_1) \) and should exchange building blocks in a unpredictable manner.

Concerning SiO\(_2\) nanocomposites, we have plotted in Fig. 13 the evolution of the scattered intensity at \( q = 0.0075 \text{ Å}^{-1} \) \textit{versus} the triple helix amount (\( \chi_{\text{helix}} \)) as an indicator of the particles state of dispersion in the absence of well-defined Guinier variations. This plot reveals the same trend of the reversible compaction process as observed previously for POM nanocomposites.
3.7 Discussion and tentative local mechanism

These investigations evidence the effects of gelatin folding on the dispersion state of two kinds of inorganic entities. At first, let us consider the case of [H₃P₃W₁₈O₆₄]³⁻ clusters. From a structural point of view, the following observations have been done:

(a) In the absence of gelatin, POMs are well dispersed at 50 °C (and 11 °C) and pH 3.4.

(b) At t₀ (T = 50 °C), in the presence of gelatin, POMs are arranged in small finite sized aggregates (R₁,G ≈ 7.3 nm) that possibly coexist with individual POMs (R₂,G ≈ 1.5 nm).

(c) Upon cooling (T ≤ 27 °C), each of these two populations becomes significantly larger, in mass and size, suggesting that an aggregation process occurs. This evolution is continuous with the triple helix rate for the initially individual POMs and relatively discontinuous for the initially aggregated POMs.

(d) This process is almost thermo-reversible for the two populations.

(e) The system is below the phase separation threshold during the whole thermal process. Thus, macroscopically, we deal with liquid solution at T = 50 °C and hydrogels at T ≤ 27 °C.

The situation at the local scale for the subsequent stages can be described as follows. At t₀, the presence of nanoparticle aggregates is not very surprising close to the phase separation threshold. For instance, considering globular protein/poly saccharide complexation, Morfin et al. have recently evidenced the presence of lysozyme/hyaluronic acid (HA) single complexes dispersed in the semi-dilute HA network in the vicinity of the phase separation threshold.61,62 In contrast with this study here described aggregates do not display a rod-like behavior even if such an anisotropic shape could be expected. Indeed, gelatin wrapping around individual clusters seems hardly realistic because the radius of individual POMs is well below the gelatin persistence length: R_POM/L_p ≈ 0.5. This discrepancy may arise both from the low charge density and the flexible character of gelatin (1 charge per 8 residues, L_p ≈ 2 nm) compared to HA (1 charge per monomer, L_p ≈ 10 nm) that possibly hinders a large-scale cooperative straightening. Upon cooling, the triple helix renaturation occurs. At first glance, the formation of anisotropic junctions that most probably involve several chains (up to 3), due to the low molecular mass of used gelatin, may induce a contraction of the biopolymer network. As a consequence the initial aggregates of clusters and possibly the individual clusters should come locally closer on average if we assume that they are all in interaction with the whole biopolymer framework. This assumption seems reasonable since the gelatin chains are in large excess and the positive charge concentration is almost constant during the renaturation process. According to this scenario the aggregation process is a natural consequence of this contraction. The discussion of the more or less continuous variation of the initial aggregates size with triple helix amount is very difficult at this point. Very qualitatively, one can argue that the average number of gelatin chains connected to the initial aggregates should be higher than for initial individual POMs due to their size, making them more responsive to a modification of the biopolymer network. This argument may explain why the aggregate size evolution versus triple helix rate depicts a steep increase from the low triple helix concentration in contrast to individual clusters. In parallel, the reversible character of the association of initially aggregated POMs suggests that these objects are never in direct contact and/or do not significantly interact with one another maybe due to the presence of gelatin. At this point, we have no clear cut answer for these questions.

Turning our attention to silica nanoparticles, the following structural observations have been done:

(a) In the absence of gelatin, SiO₂ colloids are well dispersed at 50 °C (and 11 °C) and pH 3.4.

(b) At t₀ (T = 50 °C), in the presence of gelatin, the nanoparticles are aggregated and form almost elongated and open structures.

(c) Upon triple helix formation (T ≤ 27 °C), a densification of the fractal structure and/or an enhancement of the aggregate concentration are observed.

(d) The initial SiO₂ aggregates are recovered after triple helix melting.

(e) The system is below the phase separation threshold during the whole thermal process.

At t₀, the presence of nanoparticle aggregates can be analyzed in the same manner as for POMs by considering the proximity of the phase separation threshold. As mentioned in (c), the somehow open structure of these fractal aggregates (d_e ≈ 2.2) seems consistent with a RLA process between objects of similar charge.58 At this point it is not possible to distinguish if this aggregation results from ‘naked’ nanoparticles (silica–silica contact) or from nanoparticles coated with gelatin (gelatin–gelatin contact) since gelatin wrapping around nanoparticles seems plausible: R_Silica/L_p ≈ 5. However, it is clear that a cooperative effect exists between the nanoparticles and the gelatin since silica nanoparticles are well-dispersed at the same concentration in the absence of gelatin. Upon cooling, the contraction of the biopolymer network resulting from gelatin renaturation should bring the silica fractal aggregate locally closer on average and/or induce internal contraction of the aggregates themselves if gelatin is involved in their structure. Once again the reversibility of the process indicates that such a local densification of silica colloids does not involve strong inter-particular bridging, as a result of gelatin surface coating.

So that finally, when comparing POM and silica, we could crudely conclude that when the initial aggregation is weak, like with POM, the supplementary aggregation effect is stronger than when initial aggregation is already more important. But this should be thoroughly investigated using other situations and other inorganic species displaying for instance various shapes (i.e. discs, or rods). It is interesting to note that during gelatin folding the concentration of positive charges is constant but the charge distribution is different along the triple helix and at the end of the triple helix. In the first case, the linear charge density arising from the 3 gelatin strands is much higher than in the coil region and this feature should have an effect on the particle distribution. However, yet we have no clear cut understanding on the respective role played by the charge density along triple helices and gelatin single strand.

To summarize the different results and to clarify the global picture that emerges from this discussion, we have represented the tentative mechanisms in Scheme 1.
Scheme 1 Tentative sketches of the individual components (a) and of
the structure relative to gelatin semi-dilute solutions (T = 50 °C) and gels
(T ≤ 27 °C) filled with (b) [H₃P₆W₁₈O₆₄]⁺⁻ clusters and (c) SiO₂
nanoparticles.

4 Conclusions

We have shown that gelatin folding induces a reversible struc-
tural reorganization of nanometre-size particles initially
dispersed in gelatin semi-dilute solution.

Starting from polyoxotungstate clusters initially arranged in
small finite size aggregates, we point out a continuous growth
process induced by the triple helices formation and a direct
relationship between the aggregates average size/mass and the
triple helix amount. Starting from silica nanoparticles initially
connected in a somehow opened fractal network, we evidenced
a significant densification of the inorganic structure through
the gelatin conformational transition. The kinetics of triple helix
folding is not modified by the presence of inorganic entities for
triple helix rate χ ≈ 14.5% and slightly lowered down for χ ≥ 38%.
Under our experimental conditions, the observed reorga-
nizations are thermo-reversible following triple helix unfolding
suggesting that gelatin coating prevents strong local interactions
between the particles. We believe that in the future these obser-
vations could be extended to a wide range of nanocomposite
hydrogels where the organic matrix is reticulated by anisotropic
network junctions, such as helix or “egg-box”, as for instance:
gellan, alginate, agarose, agar-agar, pectin or even DNA.
Moreover, this reorganization process would be, at least, taken
into account in the analysis of such nanocomposite properties
and should be, at best, anticipated to design thermo-sensitive
nanocomposites based on the reversible aggregation of functional
coilooidal fillers.63

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40 We have estimated $D$ according to the following relation: $D = \left( \frac{1000N_A e^2}{\varepsilon_\text{vac} \varepsilon_\text{sol} k_B T} \sum_{i=1}^{n} C_i Z_i^2 \right)^{-1/2}$ where $\varepsilon_\text{vac}$ and $\varepsilon_\text{sol}$ are the vacuum and water permittivity (~78.5) respectively, $T$ is the temperature, $e$ is the elementary charge of an electron, $k_B$ is the Boltzmann constant, $N_A$ is the Avogadro’s number and $n$ is the number of charged solutes with a valence $Z_i$ and a concentration $C_i$. In this $D$ estimation, for simplicity, we only consider the salt contribution since the concentration of others, co-ions and counter-ions, is constant.