CHAPTER 7

Diffusivity of Whey Protein and Gum Arabic in their Coacervates*

ABSTRACT

Structural properties of whey protein (WP) / gum arabic (GA) coacervates were investigated by measuring the diffusivity of WP and GA in their coacervate phase as a function of pH by means of three different complementary techniques. The combination of these measurements revealed new insights into the structure of coacervates. Nuclear magnetic resonance (NMR) measured the self-diffusion coefficient of the GA in the coacervate phase prepared at various pH values. Fluorescence recovery after photobleaching (FRAP) was measured using a confocal scanning laser microscope (CSLM). The WP and GA were covalently labeled with two different dyes. The time of fluorescence recovery, related to the inverse of the diffusion coefficient, was evaluated from the measurements and the diffusivity of the WP and GA on a long time scale could be individually estimated at each pH value. Diffusing wave spectroscopy (DWS) combined with transmission measurement was carried out in the coacervate phase and the diffusion coefficient corresponding to the averaged diffusion coefficient of all particles that scattered in the system was calculated as a function of pH. Independently of the technique used, the results showed that the diffusion of the WP and GA within the coacervate phase was reduced as compared to a diluted biopolymer mixture. NMR, DWS and FRAP measurements gave similar results, indicating that the biopolymers moved the slowest in the coacervate matrix at pH 4.0 - 4.2. It is assumed that the diffusion of the WP and GA is reduced because of a higher electrostatic interaction between the biopolymers. Furthermore, FRAP results showed that in the coacervate phase WP molecules diffused 10 times faster than GA molecules. This result is very relevant as it shows that WP and GA move independently in the liquid coacervate phase. Finally, DWS measurements revealed that the coacervate phase rearranged with time, as evidenced by a decrease of the diffusion coefficient and a loss of the turbidity of the sample. A more homogeneous transparent coacervate phase was obtained after few days / weeks. Faster rearrangement was obtained at pH 3.0 and 3.5 than at higher pH values.

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Considered for publication in Langmuir
INTRODUCTION

Coacervation is a term used to describe an associative liquid / liquid colloidal phase separation. The phase separation leads to the formation of a phase rich in the colloids, called coacervate, and a remaining phase which is poor in colloids [Bungenberg de Jong, 1949a, 1949b; Schmitt et al., 1998; Turgeon et al., 2003]. In the case of complex coacervation, the coacervate is obtained due to the ability of two attracting molecules to form a complex, e.g. two oppositely charged polymers. The complexes concentrate into coacervate droplets that will sediment and coalesce to form a separate phase. The coacervate phase exhibits specific properties that distinguish it from the original solution. It is usually more viscous and more concentrated than the initial polymer solution [Nairn, 1995]. Complex coacervation was first discovered by Tiebackx (1911). Later, Bungenberg de Jong (1949a, 1949b) collected a large amount of data on the complex coacervation of gelatin / gum arabic systems. Based on their experimental work, Overbeek and Voorn (1957) developed a first theoretical model for complex coacervation.

From a biological point of view, complex coacervation is a very interesting phenomenon. Oparin (1953) suggested that coacervates could play a role in the appearance of life on earth. The nature, formation and structure of coacervate systems can help to understand the mechanism of biological processes. Furthermore, due to their particular properties, coacervates are applied in foods, cosmetics, pharmaceutical and medicines [Schmitt et al., 1998]. The main application of complex coacervation, used in industry since the 1950’s, is microencapsulation. Insoluble materials (e.g. flavors, drugs) can be coated by a layer of coacervate [Burgess, 1994; Chilvers and Morris, 1987; Daniels and Mittermaier, 1995; Ijichi, 1997; Luzzi, 1970]. Coacervates are also used as fat replacers or meat analogues in food products, or biopackaging [Tolstoguzov, 1974; Soucie and Chen, 1986; Kester and Fennema, 1986; Shih, 1994]. From both a fundamental and an applied point of view, there is a need to a better understanding of coacervate systems. The formation of the electrostatic complexes between two polymers can be influenced by many physico-chemical parameters. Indeed, pH and ionic strength play a key role in the strength of the electrostatic interaction [Bungenberg de Jong, 1949b; Overbeek and Voorn, 1957]. Studies revealed that complexation appeared as a two-step process upon pH changes: first
intrapolymeric soluble complexes are formed and then the occurrence of interpolymeric complexes and insoluble complexes leads to macroscopic phase separation [Dubin et al., 1994; Kaibara et al., 2000; Mattison et al., 1995; Girard et al., 2002; Weinbreck et al., 2003a]. Recent work from Leisner and Imae (2003) showed that the poly(glutamic acid) / dendrimer coacervates are randomly branched gels with a sponge-like morphology and compact inhomogeneities larger than 20 nm. The structure of the â-lactoglobulin / gum arabic system was studied by confocal scanning laser microscopy (CSLM) and it appeared that the coacervate contained vacuoles of entrapped solvent [Schmitt et al., 2001a]. Sanchez et al. (2002a) explained that the presence of the solvent vacuoles might be due to the presence of residual uncharged gum arabic at the interface of the coacervate droplets, which facilitates the entrapment of solvent when the droplets coalesce into a coacervate phase. In a purely synthetic system of zwitterionic Gemini surfactants in water, Menger et al. (2000; 2002) reported the structure of coacervates as sponge-like vesicles. These recent studies already give new insights into the complexity of coacervate systems and more work is necessary to understand the structure of coacervates.

In this work, complex coacervation of whey protein / gum arabic (WP/GA) was investigated. Previous results indicated under which conditions coacervates are obtained, i.e. pH, ionic strength, protein to polysaccharide (Pr:Ps) ratio [Weinbreck et al., 2003a; Schmitt et al., 1999]. The content in WP, GA, and water of the coacervate phase was found to be very much dependent on these physico-chemical parameters; the stronger the electrostatic interaction, the more concentrated the coacervate phase in biopolymers was [Weinbreck et al., 2004a]. The strength of the electrostatic interactions was estimated from the absolute product of the measured zeta potential of the WP and the GA, and it appeared to be maximum at pH 4.0. Investigations of the structural properties of the coacervate phase were carried out by small angle X-ray scattering (SAXS) and it appeared that, for a Pr:Ps = 2:1, the coacervate phase was the most structured at pH 4.0, the pH at which the strength of the electrostatic interaction and the concentration of the polymer in the coacervate phase were maximal [Weinbreck et al., 2004a]. Furthermore, rheological measurements on the WP/GA coacervate phase indicated that the highest viscosity was obtained at pH 4.0 [Weinbreck et al., 2004b]. The viscosity of the coacervate was 45 times higher than the viscosity of a blank prepared at the same biopolymer concentration (Cp), but at a pH
where no electrostatic interaction took place (pH 7). On the other hand, for other pH values, on either side of pH 4.0, the viscosity of the coacervate phase was between 1 and 15 times higher than their respective blanks (same Cp, pH 7). The high viscosity was just not only due to a higher Cp in the coacervate phase, but it was mainly due to a stronger attractive interaction between WP and GA. Taken together, the results show that at the pH of optimum coacervation (pH 4.0 for Pr:Ps = 2:1), the strength of electrostatic interaction is the strongest, giving rise to a very dense concentrated and viscous coacervate phase. It is thus expected that the mobility and the diffusion of WP and GA would be limited under those conditions. The focus of this study was on the diffusivity of WP and GA within their coacervate phase prepared at various pH values. The diffusion of WP and GA was monitored individually in order to investigate whether the WP and GA diffused together or individually in the coacervate phase. Finally, the long-time behavior of the coacervate phase was investigated by following the evolution of the diffusion coefficient during several days. In order to fulfill these goals, three different techniques were used. Nuclear magnetic resonance (NMR) was used to measure the diffusion coefficient of GA within the coacervate phase. Fluorescence recovery after photobleaching (FRAP) measurements were carried out with a CSLM, and the diffusion of the labeled WP and labeled GA could be individually estimated. Finally, diffusing wave spectroscopy (DWS), combined with transmission measurements, was used to obtain quantitative values of the diffusion coefficient corresponding to the averaged diffusion of all particles that scattered in the system. The three techniques measure translational diffusion coefficients. NMR and FRAP measurements monitor long-time diffusion, whereas DWS monitors short-time diffusion. NMR, FRAP and DWS measurements were found to be very suitable methods for the investigation of highly concentrated systems. Each technique used here had its advantages and limitations. Thus, the use of three independent techniques would give complementary results on the structure of the WP/GA coacervates as a function of pH.
EXPERIMENTAL SECTION

Materials

Bipro is a whey protein isolate (WP) consisting mainly of â-lactoglobulin (â-lg), and á-lactalbumin (á-la) - from Davisco Foods International (Le Sueur, USA). Residual whey protein aggregates were removed by acidification (at pH 4.75) and centrifugation (1 h at 33000 rpm with a Beckman L8-70M ultracentrifuge, Beckman instruments, The Netherlands). The supernatant was then freeze-dried (in a Modulo 4K freeze-dryer from Edwards High Vacuum International, UK). Finally, the resulting powder was stored at 5°C. The final powder contained (w/w) 88.1% protein (N x 6.38), 9.89% moisture, 0.3% fat and 1.84% ash (0.66% Na+, 0.075% K+, 0.0086% Mg2+, and 0.094% Ca2+). The protein content of the treated Bipro is: 14.9% â-la, 1.5% BSA, 74.9% ß-lg, and 3.2% immunoglobulin (IMG).

Gum arabic (GA; IRX 40693) was a gift from Colloides Naturels International (Rouen, France). The powder contained (w/w) 90.17% dry solid, 3.44% moisture, 0.338% nitrogen, 0.044% Na+, 0.76% K+, 0.20% Mg2+ and 0.666% Ca2+. Its weight average molar mass (M_w = 520 000 g/mol) and its average radius of gyration (R_g = 24.4 nm) were determined by size exclusion chromatography followed by multiangle laser light scattering (SEC MALLS). SEC MALLS was carried out using a TSK-Gel 6000 PW + 5000 PW column (Tosoh Corporation, Tokyo, Japan) in combination with a precolumn Guard PW 11. The separation was carried out at 30°C with 0.1 M NaNO_3 as eluent at a flow rate of 1.0 mL min⁻¹.

Stock solutions of 3% (w/w) were prepared by dissolving the powder in deionized water.

Fluorescent labels, namely fluorescein-5-isothiocyanate (FITC) and 5- (and 6-) carboxyfluorescein succinimidyl ester (FAM-SE) were purchased from Molecular Probes (Leiden, The Netherlands) and deuterium oxide (D₂O) from Aldrich Chemical Company Inc. (Milwaukee, USA).

Preparation of the coacervates

The stock solutions of 3% (w/w) WP and GA were mixed at a protein to polysaccharide (Pr:Ps) ratio of 2:1 (w/w). Sodium azide (0.02% w/w) was added to prevent bacterial growth. The pH of the mixtures was set (± 0.05 pH-unit) at the desired value (pH range between 3.0 and 4.5) using 0.1 M and 1 M HCl. After acidification, the samples were
placed in a separatory funnel at 25°C. All the experiments were carried out on the coacervate (lower) phase that was collected after 24h of phase separation.

**Nuclear magnetic resonance (NMR)**

1D-NMR and DOSY (DiffusionOrdered SpectroscopY) spectra were taken on a Bruker DRX500 spectrometer operating at 500.13 MHz for the DOSY experiments. A pulse sequence, using stimulated echo, longitudinal eddy current delay and bipolar gradient pulses, was applied. All experiments were performed at 22°C. Typical values for the parameters were: 8 s for the relaxation delay, 400 ms for diffusion time (Δ) and 3 ms for the gradient pulse length (δ). The gradient strength was varied linearly from 10 to 500 mT/m. Gradient calibration was based on a solution of SDS, glucose and ATP in D2O [Morris and Johnson, 1993]. Processing of the data was carried out using the DOSY module of the XWinNMR software.

The coacervate samples were prepared as described above, except that D2O was used as a solvent instead of deionized water. The pH was adjusted with DCl and NaOD. The pH values were taken from the reading of the pH meter and were not corrected for the influence of the D2O. NMR measurements were carried out on the WP/GA coacervate phase at pH 4.5, 4.2, 4.0, and 3.8 in order to determine the diffusion coefficient of the GA within the coacervate phase. It was not possible to determine the diffusion coefficient of the WP because of severe signal broadening.

**Fluorescence recovery after photobleaching (FRAP)**

**Labeling procedure**

WP (5% w/w at pH 7.0) was covalently labeled with FITC (1 ìmol / g WP) and GA (5% w/w at pH 8.0) with FAM-SE (4 ìmol / g GA). Both mixtures were left overnight under stirring in the dark. The mixtures were then freeze dried after removal of the free label by exhaustive dialysis against deionized water. Fluorescein was the fluorophore of choice because it readily photolyzes under relatively low illumination intensities [Gribbon and Hardingham, 1998; Chen et al., 1995]. Turbidity experiments as described in a previous paper were carried out with the labeled polymers and it was found that the pH at which complex coacervation occurred was not affected by the labeling procedure [Weinbreck et al., 2003a]. Coacervates with labeled-WP/GA and WP/labeled-GA were prepared as described above. FRAP measurements were carried
out on the labeled-WP/GA coacervates at pH 4.3, 4.2, 4.0, 3.8, 3.6, 3.5, and on the WP/labeled-GA coacervates at pH 4.0, 3.8, 3.6, 3.5.

**Equipment**

Photobleaching experiments were carried out on a LEICA TCS SP confocal scanning laser microscope (CSLM), equipped with an inverted microscope (model Leica DM IRBE), used in the single photon mode with an Ar/Kr visible light laser. The following Leica objective was used: 63x/UV/1.25NA/water immersion/PL APO (63w). The laser emitted at 488 nm, within the excitation spectrum of FITC and FAM-SE. 1 mL of sample was poured into a spherical cavity microscope slide covered by Parafilm and a coverslip to avoid drying. Digital images files were acquired in 1024 x 1024 pixels resolution. A prebleached image of dimension 80 x 80 μm was taken with a reduced laser intensity (about 40% of the maximum laser power). Then a block of dimensions 20 x 20 μm with a height of approximately 1 μm, centered on the observed region and selected by the zoom control of the instrument, was bleached at maximum laser intensity for 30 s (laser power 9 mW). For fluorescence recovery, time series were collected in 2 dimensions (1 image per minute) at the reduced laser intensity that was used for the prebleached image.

**Data analysis**

The intensity of the fluorescence of an area was represented by the average value of the pixels in this area. Fluorescent recovery curves were obtained by plotting the intensity of the bleached area divided by the intensity of the background as a function of time. Normalization on the background intensity was necessary because of fluctuations in the laser power. The recovery curves have an exponential shape according to the following equation:

\[
\frac{I_{\text{norm}}(\infty) - I_{\text{norm}}(t)}{I_{\text{norm}}(\infty) - I_{\text{norm}}(t_0)} = e^{-\frac{t}{\tau}}
\]

Eq.(1)

in which \( I_{\text{norm}}(t) = \frac{I_{\text{bleached area}}(t)}{I_{\text{background area}}(t)} \) and \( \tau \) is the time constant of the system and is inversely proportional to the diffusion coefficient (D). The values of \( \tau \) were calculated at various pH values for the labeled-WP and the labeled-GA. All measurements were repeated at least 4 times. The values reported in this study are the averaged values and their respective standard deviation. The averaged \( \tau \) values at various pH values
were compared by doing a Scheffe test with significant marked differences at p < 0.05 (Statsoft, Inc. (2001). STATISTICA, version 6).

The diffusion coefficient of the molecule (D) in one direction is related to the $\hat{\phi}$ value and can be expressed as:

$$ D = \frac{a^2}{2\tau} \quad \text{Eq.(2)} $$

where $a^2$ corresponds to the mean squared displacement in one direction (which depends on the size of the bleached area).

**Diffusing wave spectroscopy (DWS)**

DWS measurements were carried out on an experimental setup. The expanded laser source was an NEC Corp. 50 mW He/Ne laser emitting at wavelength $\lambda = 633$ nm through an optical fiber. Coacervate samples were prepared as described above at various pH values and were poured into a 0.5 cm optical path length cuvette and covered by Parafilm to avoid drying of the samples. The samples were stored at room temperature and were measured every day for 22 days. The temperature was controlled at 25°C. The measurements were carried out in the transmission mode. The scattered light intensity was processed with 50R Flex Instruments software, and the intensity autocorrelation function $g_2(t)$ of each sample was collected. The field autocorrelation function $g_1(t)$ is defined as [Weitz and Pine, 1993]:

$$ g_2(t) \equiv |g_1(t)|^2 \quad \text{Eq.(3)} $$

The field autocorrelation function $g_1(t)$ can also be defined as [Weitz and Pine, 1993]:

$$ g_1(t) = \frac{\left( \frac{L}{l^*} + \frac{4}{3} \right) \sqrt{\frac{6t}{\tau_0}}} {\left( 1 + \frac{8t}{3\tau_0} \right) \sinh \left( \frac{L}{l^*} \sqrt{\frac{6t}{\tau_0}} \right) + \left( 4 \sqrt{\frac{6t}{\tau_0}} \cosh \left( \frac{L}{l^*} \sqrt{\frac{6t}{\tau_0}} \right) \right)} \quad \text{Eq.(4)} $$

where L is the sample width (L = 0.5 cm), $l^*$ is the transport mean free path of a photon, $\hat{\phi}_0$ is defined as:

$$ \tau_0 = (D \times k_0^2)^{-1} \quad \text{Eq.(5)} $$

where D is the diffusion coefficient and $k_0$ is defined as:

$$ k_0 = \frac{2\pi}{\lambda} \quad \text{Eq.(6)} $$

and $\lambda$ is defined as:
\[ \lambda = \frac{\lambda_{\text{laser}}}{n} \quad \text{Eq. (7)} \]

where \( \lambda_{\text{laser}} \) is the wavelength of the laser light in vacuum, \( n \) is the refractive index of the medium and for water \( n = 1.33 \).

**Transmission measurements**

Transmission measurements were carried out on each coacervate sample with a CARY 4000 spectrophotometer (Varian, The Netherlands) in order to obtain \( I^* \). The same cuvettes as for DWS measurements were used. \( I^* \) was calculated from transmission \( (T) \) measurements according to [Kaplan et al., 1993]:

\[ T = \frac{5 \, I^*}{3 \, L} - \frac{4 \, I^*}{3 \, L} \quad \text{Eq. (8)} \]

Thus, from the fit of the data of the correlation function \( (g_1(t)) \) given in Eq. (4) using the transmission data, a value of the diffusion coefficient \( D \) could be calculated.

**RESULTS AND DISCUSSION**

The main purpose of this work was to get more insights into the structure of the whey protein (WP) / gum arabic (GA) coacervate phase by monitoring the diffusion of the WP and the GA within the coacervate itself. As previous results showed, the coacervate phase is very viscous and very concentrated in polymer (up to 20 – 30% w/w) [Weinbreck et al., 2004a, 2004b]. Various techniques were used and results were compared.

**Nuclear Magnetic Resonance (NMR)**

Translational diffusion of molecules was studied by means of pulsed field gradient NMR techniques. A magnetic field gradient was applied in combination with a spin-echo pulse sequence. If diffusion occurs, the NMR signal is reduced depending on the value of the diffusion coefficient and the strength of the field gradient. With diffusion-ordered 2D NMR (DOSY) experiments, usually the strength of the magnetic field gradient is varied. A 2D spectrum was obtained with the chemical shift on the abscissa and the self-diffusion coefficient on the ordinate [Morris and Johnson, 1993].
From an analysis of 1D-NMR spectra of a WP sample, a GA sample and a WP/GA coacervate sample, the signals characteristic for WP and GA could be assigned. GA showed a sharp peak at 1.27 ppm and a characteristic envelope around 3.9 ppm (Figure 7.1). WP in the absence of GA showed characteristic signals at 0.9 ppm (-CH$_3$ groups) and in the aromatic region from 6 to 11 ppm. In the coacervate phase the signals of WP were strongly broadened and could not be monitored in the DOSY experiment. The signals of GA were broadened too but were still clearly detectable in the DOSY spectra. Consequently, for WP/GA coacervates prepared at pH 4.5, 4.2, 4.0, and 3.8 only the self-diffusion of the GA could be determined.

The results of the diffusion coefficients of GA as a function of pH are presented in Figure 7.2. The diffusion coefficient of the GA in a dilute system at pH 7 ($D_{pH7} = 10.8 \times 10^{-12}$ m$^2$s$^{-1}$) was higher than the diffusion coefficient of GA in the coacervate, since GA molecules were electrostatically bound to WP in a very concentrated biopolymer phase. The diffusion coefficient of GA in the coacervate was pH-dependent and showed a minimum close to the pH range 4.0 – 4.2. The use of D$_2$O as a solvent might induce some changes in the behavior of the coacervate phase as compared to a coacervate prepared in H$_2$O. Hydrophobic interactions are enhanced in D$_2$O, but the dielectric constant remains the same. However, the pK$_a$ values of glutamine residues shifts +0.1 unit in D$_2$O and since pD = pH (meter reading) + 0.4 - pK$_a$, the dissociation would shift as well [Bundi and Wüthrich, 1979]. Therefore, the exact position of the minimum in the

![Figure 7.1: $^1$H-NMR spectra of GA sample, WP sample, and WP/GA coacervate sample at pH 4.0.](image-url)
The diffusion coefficient of GA within the coacervate phase may not exactly coincide with that in H$_2$O. It might shift the maximum 0.2 – 0.3 units towards more acidic pH values if H$_2$O is used as a solvent. From previous measurements, it was found that pH 4.0 corresponded to the pH at which the strength of interaction, the viscosity, and the concentration in polymer were all at maximum [Weinbreck et al., 2004a, 2004b]. Thus, it is expected that the diffusion of polymers is slowest around this pH value. The diffusion of WP could unfortunately not be determined from NMR measurements. Another method, fluorescence recovery after photo bleaching (FRAP), was therefore used to measure independently the diffusion of WP and GA.

**Fluorescence recovery after photo bleaching (FRAP)**

FRAP is a technique used to investigate molecular mobility within cells and membranes systems in situ. In this study, a confocal scanning laser microscope (CSLM) was used to study diffusion of labeled-WP and labeled-GA in the coacervate phase.
**Diffusion of labeled-GA**

GA molecules were labeled with FAM-SE on the proteic part present in the GA molecules. Because the amount of protein in the GA sample is low (2%), a stronger fluorescein label was used (FAM-SE) in a higher concentration than that used for the labeling of WP with FITC. Coacervates containing WP (non labeled) and labeled-GA were prepared at pH 4.0. Pictures of the sample were taken at 1 min intervals for at least 1.5 h. A characteristic FRAP time series is presented in Figure 7.3. Even before bleaching, the coacervate phase appeared heterogeneous in the partition of GA. Indeed, some bright yellow spots indicated that large aggregates of GA were present in the sample. As reported by Sanchez *et al.* (2002a), these aggregates are known to be always present in GA samples. Some round black spots were also visible on the pictures, probably inclusion of solvent droplets entrapped in the coacervate. After photo bleaching, a black square appeared on the picture fading away with time. The intensity profile of the background area (\(I_{\text{background}}\)) and the bleached area (\(I_{\text{bleached area}}\)) was monitored with time. The intensity of the bleached area was initially low and then increased to reach the same intensity as the background because of the diffusion of GA. Overall variations in intensity were due to fluctuations in the laser power.

**Diffusion of labeled-WP**

WP molecules were labeled with FITC and a coacervate at pH 4.0 was prepared using labeled WP and (non labeled) GA. The same bleaching procedure was used as for the above sample. Figure 7.4 illustrates the time series pictures taken by CSLM. Before bleaching, the WP looked homogeneously distributed in the coacervate phase. Some dark spots corresponding to entrapped solvent were also visible. After 20 min, the bleached square had disappeared. The recovery of the bleached area seemed to be much faster than when GA was labeled, showing qualitatively that the diffusion of WP was faster than the diffusion of GA.

**Quantification**

The intensities of the bleached area and the background were monitored as a function of time. The recovery curve could be obtained by plotting \(\ln \frac{I_{\text{norm}}(\infty) - I_{\text{norm}}(t)}{I_{\text{norm}}(\infty) - I_{\text{norm}}(t_0)} \equiv \ln X\) as a function of time, and it is expected to be a straight line which slope = \(-1/\delta\). The recovery curves of the samples presented in Figures 7.3 and 7.4 are shown in Figure
Figure 7.3: CSLM micrographs of a WP/labeled-GA coacervate prepared at pH 4.0 at different times. The black rectangle corresponds to the bleached area. GA is labeled with FAM-SE.

Figure 7.4: CSLM micrographs of a labeled-WP/GA coacervate prepared at pH 4.0 at different times. The black rectangle corresponds to the bleached area. WP is labeled with FITC.
7.5. The recovery curve of the WP appeared to be a straight line which gave a $\delta = 9 \text{ min}^{-1}$. The slope corresponding to the recovery of GA was less steep, and $\delta = 53 \text{ min}^{-1}$. However, it should be noted that in some cases the recovery curves of the GA deviated slightly from the straight line, probably because of the heterogeneities in the GA mixtures, but the $\delta$ values of GA were always much larger than the WP values.

**Figure 7.5:** Recovery curves of the fluorescence with time from Figures 7.3 and 7.4, based on Eq. (1). Samples correspond to a coacervate of ( ): labeled-WP/GA and ( ): WP/labeled-GA.

The values of $\delta$ were calculated for a range of pH values for coacervates with labeled-WP and labeled-GA. In Figure 7.6a, the values of $\delta$ corresponding to the diffusion of WP are plotted as a function of pH. Considering statistical analysis, it seemed that diffusivity was lowest at around pH 4.0 and 4.2 and faster on either side of these values. Indeed, at pH 4.0 the viscosity and the strength of the electrostatic interactions were at maximum, paralleled by a slower diffusion of the molecules. For pH > 4.4, the coacervate phase did not appear as a homogeneous phase but some large coacervate droplets were still present, showing that phase separation was incomplete. FRAP experiments could thus not be carried out at pH > 4.4. On the other hand, for pH values below 3.5, the diffusion was so fast that most of the recovery of the fluorescence occurred during the bleaching step; quantitative analysis could thus not be performed.
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at pH < 3.5. The values of $\delta$ for GA were compared to the values of $\delta$ for WP in Figure 7.6b. The values of $\delta$ for GA were more difficult to calculate because of the heterogeneities in the sample, and reproducibility of the results was limited. However, even if the standard deviations were rather large, the values of $\delta$ of GA are significantly larger than the values of $\delta$ for WP, illustrating quantitatively that the diffusion of WP in the coacervate was faster than the diffusion of GA. Diffusion of the GA was also too fast at pH < 3.5 and could not be quantified by FRAP. Furthermore, at pH > 4.1, the samples containing labeled-GA showed a poor phase separation and coacervate droplets did not coalesce into a homogeneous phase. This limiting pH value was lower than the limiting value corresponding to samples prepared with labeled-WP. Although it was checked that the labeling did not influence the pH of complex formation, it remains possible that the fluorescent markers changed the interfacial properties of the coacervate droplets and thus their coalescence. Indeed, fluoresceins (FITC and FAM-SE) are hydrophobic molecules that are positively charged at pH below 6.4. It was already reported that FITC molecules were present at the gelatin / dextran interface [Edelman, 2003]. In the case of the coacervates prepared with labeled-GA, the concentration of FAM-SE label was higher than the concentration of FITC for labeled-WP, which would then enhance this interfacial phenomenon. Furthermore, it was also reported by Sanchez et al. (2002a) that the interface of the coacervate droplets was mainly made of GA molecules. In that case, the FAM-SE marker would also be located at the interface, preventing coalescence of the coacervate droplets.

FRAP measurements give information on the diffusion of the molecules on a long time scale. Under these conditions, the diffusion of both WP and GA could be measured, and the main conclusion was that WP and GA move independently in the coacervate phase. From Eq. (2), the diffusion coefficients of WP and GA could be calculated. However, since diffusion occurs in three dimensions, the molecules would be expected to diffuse on a horizontal plane (the plane of the bleached square, 20 x 20 µm) but also - and mostly - in the vertical direction. Since the height of the bleached block is approximately 1 µm, this value was taken for $a$ and $D = \frac{a^2}{2\tau}$. Under those conditions the diffusion coefficients were comprised between $1.1 \times 10^{-11}$ m$^2$s$^{-1}$ and $3.4 \times 10^{-12}$ m$^2$s$^{-1}$ for WP and between $7.8 \times 10^{-12}$ m$^2$s$^{-1}$ and $2.6 \times 10^{-12}$ m$^2$s$^{-1}$ for GA. Considering that the diffusion coefficients measured by FRAP were an approximation (because of the uncertainty of $a$), the values were in good agreement with the NMR results. Finally, a
Figure 7.6a: Averaged $\dot{\phi}$ of WP as a function of pH, as calculated from the recovery curves for a labeled-WP/GA coacervate. Error bars represent standard deviations.

Figure 7.6b: Averaged $\dot{\phi}$ of WP and GA as a function of pH, as calculated from the recovery curves for a WP/labeled-GA coacervate. ( ): $\dot{\phi}_{WP}$; ( ): $\dot{\phi}_{GA}$. Error bars represent standard deviations. (A): CSLM micrograph of the WP/GA coacervate phase with GA labeled with FAM-SE at pH = 4.1; (B): CSLM micrograph of the WP/GA coacervate phase with WP labeled with FITC at pH = 4.4.
third method was used for monitoring mobility in order to determine the diffusion of all scattering molecular species: diffusing wave spectroscopy (DWS).

**Diffusing wave spectroscopy (DWS)**

*Quantification of the diffusion coefficient*

The passage of multiply scattered photons in a turbid medium can be treated as a (diffusive) random walk. The multiple scattering process is modulated by the diffusive motion of the colloidal particles. As a result, intensity fluctuations in the scattered light reflect the diffusivity of the colloids as in traditional dynamic light scattering [Weitz and Pine, 1993]. DWS measurements were carried out on the coacervate samples obtained after 24h of phase separation (i.e. at the same conditions as for FRAP and NMR measurements described above). For each sample a correlation function was monitored, as depicted in Figure 7.7. By increasing the pH from 3.0 to 4.5, the decay of the correlation function was shifted to shorter times. For each sample, a curve fitting allowed determination of the values of the parameters of the correlation function described in Eq. (4). However, the mobility of the particles in the coacervate phase could not be directly compared for the different pH values because the turbidity of the coacervate was different for each sample. Thus, transmission measurements were carried out with a spectrophotometer; the results are reported in Table 1. For pH values 3.0 and 3.5, the transmission of the coacervates was higher, showing that the samples were more transparent and that the scattering intensities would be different from the other pH values. The calculation of $l^*/L$ from Eq. (8) reveals that $l^*/L$ was always smaller than 0.1 (multiple scattering mode) for all samples, which is the main requirement for the use of DWS. From the values of $l^*/L$ and the curve fitting, the diffusion coefficient could be calculated for all the coacervate samples. The results are given in Figure 7.8 and are compared quantitatively to the previous diffusion measurements obtained by NMR and FRAP. The diffusion coefficients obtained by DWS measurements corresponded to the averaged diffusion of all particles that scattered in the coacervate, *i.e.* WP and GA. DWS preferentially measured the particles that scattered more and thus the largest particles. The values obtained by DWS are in very good agreement with previous NMR and FRAP results. A minimum in the diffusion coefficient was found around pH 4.0 and 4.2. On either side of this pH range, the molecules diffused faster.
Table 1

<table>
<thead>
<tr>
<th>pH</th>
<th>T (%)</th>
<th>I'/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.5</td>
<td>0.23</td>
<td>0.001404</td>
</tr>
<tr>
<td>4.2</td>
<td>0.22</td>
<td>0.001286</td>
</tr>
<tr>
<td>4.0</td>
<td>0.27</td>
<td>0.001632</td>
</tr>
<tr>
<td>3.5</td>
<td>0.55</td>
<td>0.003328</td>
</tr>
<tr>
<td>3.0</td>
<td>1.69</td>
<td>0.010231</td>
</tr>
</tbody>
</table>

![Figure 7.7](image)

**Figure 7.7:** Field autocorrelation function $g_1(t)$ of WP/GA coacervates prepared at various pH values: ( ): pH 4.5; ( ): pH 4.2; ( ): pH 4.0; ( ): pH 3.5; (+): pH 3.0. The lines correspond to curve fitting using Eq. (4).

**Rearrangement with time**

Transmission and DWS measurements are non-invasive techniques which were used to follow samples for 22 days. Transmission of the coacervate at various pH values is plotted as a function of time in Figure 7.9. The samples at pH 3.0 and 3.5 turned from opaque to almost transparent in 2 to 5 days. In those conditions, DWS could not be carried out, since multiple scattering no longer occurred. At the other pH values, the
transmission increased slightly but remained at values below 0.4%, which was still in the multiple scattering mode. This change in turbidity as a function of time meant that rearrangement occurred in the samples. The coacervates became transparent, and thus more homogeneous, since the initial turbidity of dispersions was due to the presence of entities in the range of micrometer size. One could hypothesize that the rearrangement was due to the expulsion / redistribution of entrapped solvent. Because of the high viscosity of the mixtures, the rearrangement of the coacervate phase is a slow process. And since the viscosity was lower and the diffusion coefficient was larger at pH 3.0 and 3.5, the rearrangement occurred faster than at pH 4.0 and 4.2. For pH 4.5, it is possible that some residual unbalanced charges at the surface of the droplets prevented the coalescence, as depicted by CSLM in Figure 7.5b. Thus, rearrangement was slower. The rearrangement of the molecules within the coacervate phase had an impact on the diffusion coefficient. Indeed, as depicted in Figure 7.10, the diffusion of the molecules was slower after one week for all the pH values that could be measured. This result was another sign of the rearrangement of the coacervates with time. These results proved that the phase equilibrium of the coacervates was achieved very slowly. This factor needs to be taken into account in further studies on complex coacervation.

**Figure 7.8:** Diffusion coefficients in WP/GA coacervate after 24h of phase separation, using: ( ): NMR (diffusion of GA); ( ): DWS (diffusion of WP+GA); (+) FRAP (diffusion of WP); ( ): FRAP (diffusion of GA). Error bars correspond to standard deviations.
Figure 7.9: Transmission measurements of the WP/GA coacervate phases with time. ( ): pH 3.0; ( ): pH 3.5; ( ): pH 4.0; ( ): pH 4.2; (+): pH 4.5.

Figure 7.10: Diffusion coefficients of WP/GA coacervate using DWS: ( ): after 24h of phase separation ( ): after 8 days of storing at room temperature. Error bars correspond to standard deviations.
CONCLUSIONS

The complementary results for diffusivity of the biopolymers in a coacervate phase were obtained from three different techniques (i.e. NMR, FRAP, and DWS). All results were in good agreement with each other. The diffusion of WP and GA in the coacervate phase was much slower than in a dilute system. Because of the high concentration of polymers in the coacervate phase (20 – 30 % w/w) and because of the presence of electrostatic interactions at acidic pH, the diffusion of the biopolymers in the coacervate phase would be reduced. All the results indicated that the diffusion of the WP and GA was at minimum around pH 4.0 and 4.2, since it corresponds to the pH at which both the strength of the electrostatic interaction and the viscosity were maximum [Weinbreck et al., 2004b]. Furthermore, FRAP data showed that the diffusion of the WP in the coacervate was 10 times faster than the diffusion of the GA. It was concluded that WP and GA moved independently in the coacervate phase. This result is very relevant for the understanding of the structure of the coacervate, meaning that electrostatic bonds between WP and GA continually break and re-form, in a reversible way, and that WP can move along the GA chain. Finally, using DWS measurements, the behavior of the coacervate could be followed for several days and it was found that the coacervate phase slowly rearranged with time, as evidenced by the loss of turbidity of the samples and by the lower diffusion coefficient. Rearrangement occurred faster at pH 3.0 and 3.5; the samples looked transparent after a few days, preventing the use of DWS for further quantification. Thus, rearrangement of the coacervate phase occurs in order to form a more homogeneous transparent phase as a result of the diffusion of the polymers within the coacervate. This result opens new insights into the relatively unexplored field of coacervate structure.

ACKNOWLEDGMENTS

Friesland Coberco Dairy Foods (FCDF) is acknowledged for their financial support. The authors would like to thank Vincent Gervaise and Frédérique Sanzey for their contribution to the experimental work.