Investigation of Liquid Liquid Phase Separation in Immunoglobulin G Solutions

Author: Lovisa Jansson

Supervisor: Foivos Perakis

Co-Supervisor: Anita Girelli

Department of Physics
Bachelor Degree project 15 credits
Bachelor programme in Physics 180 credits
Spring term 2023
Abstract

Liquid-liquid phase separation (LLPS) is an important phenomenon in soft condensed matter that explains many properties of membraneless organelles in living cells. The research on this topic is, therefore, a field with a wide range of applications such as biopharmacy and biomaterials. In this project, we investigate the LLPS of the antibody protein Immunoglobulin G (IgG) by analyzing the liquid dynamics of IgG solutions at a wide range of temperatures with dynamic light scattering (DLS). It was found that the slow component of the autocorrelation function increases with decreasing temperature below 0°C. This can be attributed to either the number of protein clusters increasing as the sample approaches phase separation or LLPS droplets forming in the solution. LLPS was detected through optical microscopy, visualising the droplet formation in the IgG solution. This work confirms that LLPS can be detected for bovine IgG solutions without the presence of cosolvents and without water freezing in the sample.
Acknowledgements

First of all, I sincerely thank my supervisors Foivos Perakis and Anita Girelli for their unwavering interest and dedication to my work and for always sharing their knowledge in every aspect of the project. I also thank Anita for the countless hours spent in the lab together.
Furthermore, I want to give a special thanks to Sharon Berkowicz for always coming to the rescue and assisting me with the DLS.
Finally, thanks to the entire SDAQS group for continuous support throughout the project, for creating such a supportive environment and for always lending me a helping hand with big and small problems.
1. Introduction

1.1 Liquid-Liquid Phase Separation 2
1.2 Immunoglobulin G 4

2. Materials and Methods 6

2.1 Ultraviolet-visible Spectroscopy 6
2.2 Dynamic Light Scattering 7
2.3 Microscopy 10
2.4 Sample Preparation 11

3. Results and Discussion 12

3.1 UV-VIS Results 12
3.2 Dynamic Light Scattering Results 14

3.2.1 Intensity 14
3.2.2 Autocorrelation Function 15
3.2.3 Diffusion 19

3.3 Imaging Liquid-Liquid Phase Separation 22

4. Conclusion and Outlook 25

References 26

APPENDIX A 29
APPENDIX B 30
APPENDIX C 31
APPENDIX D 33
APPENDIX E 34
1. Introduction

1.1 Liquid-Liquid Phase Separation

The research on liquid-liquid phase separation (LLPS) has been a field on the rise for the past 20 years. It was anticipated already in the late 1800s that the cytoplasm in our cells is an emulsion, in other words, a mixture of immiscible liquids [1]. In principle, LLPS means that a solution which under certain conditions is in one phase can transition into two or more coexisting phases as those conditions change. Today there is a wide range of biological processes that are known to be linked to condensates and therefore completely dependent on LLPS dynamics [2]. Living cells have a very high protein concentration, usually about 40 % of the volume, which means that both hydrodynamic interactions and direct interactions between proteins strongly affect the diffusion processes in the cell [3]. One particularly interesting application is the connection between LLPS in proteins and human immune response. A recent example of this is the intense research on pathogen infections following the global pandemic in 2019. SARS-CoV-2, a virus that causes COVID-19, consists of four different proteins. One of these proteins, nucleocapsid protein, can phase separate with the RNA in human cells and create condensates which in turn allow the virus to transcript. Simply put, the virus uses LLPS to attack our cells. This knowledge indicates that the answer to how we cure COVID-19 and other similar diseases could lie within the field of protein liquid dynamics [1].

From a physical perspective, the one-phase state would represent a high entropy state with molecules randomly distributed within the solution and the two-phase state would be an ordered state with lower entropy. According to the second law of thermodynamics, the entropy must either increase or remain constant throughout spontaneous processes [4]. This means that during the process of phase separation, there have to be some driving forces that allow spontaneous demixing. One explanation is the interaction energy between particles in the solution. If some interactions have lower energy than others, these will be more favourable [5]. Proteins are multivalent interactors, meaning there are several different interaction opportunities between the molecules. The protein chains fluctuate and fold and give rise both to inter and intra-chain interactions. Depending on the structure of the protein different interactions can generate phase separation. The true nature of phase separation in proteins is in many ways still enigmatic, but the occurrence of LLPS in protein solutions is today a well-known fact within biological research [6].
A phase diagram describes the phase behavior of a system by illustrating under which conditions a certain phase of a material is stable. It can be used for more visually obvious phase transitions like liquid water and steam, as well as for mixtures of different liquid phases. Figure 1.1 displays an example of a phase diagram for LLPS with concentration on the x-axis and conditions such as temperature or pH on the y-axis. The area under the graph in figure 1.1 a), the 2-phase regime, visualizes the conditions under which the system can phase separate. Below the binodal curve (figure 1.1 b)), also known as the coexistence curve, the two phases can coexist. Hence it is equally thermodynamically favorable for the system to be in either of the phases and separation may occur. The spinodal curve is a metastability curve and within this area, one will always find the system to be phase separated. Figure 1.1 b) shows three different cases of phase separation under different conditions. In the two different cases of nucleation, a dense phase can form as droplets within a dilute phase or vice versa. In the third case, spinodal decomposition arises when the system is out of equilibrium and the LLPS starts immediately when it reaches the spinodal region. The critical point, also referred to as the critical temperature $T_c$, is the point above which we can not see a phase separation under any condition, but the system can exhibit locally critical fluctuations in the proximity of the critical point [5].
1.2 Immunoglobulin G

Immunoglobulin G (IgG) is an antibody protein that can be found in human blood cells and plays an important role in the adaptive immune system. In this work, the IgGs come from bovine blood. IgG is a Y-shaped molecule with a hydrodynamic radius around 5.5 nm [8, 9]. The molecule consists of two heavy and two light chains and has two antigen binding sites as part of the variable regions called Fab whose amino acid sequence strongly determines the proteins’ biological functions [10, 11].

In previous studies of LLPS in IgG solutions [10, 12], the temperature dependence of the phase separation for different concentrations has been investigated with and without the presence of polyethylene glycol (PEG). PEG is a substance that increases the temperature where LLPS is possible at a certain concentration by introducing depleting forces without changing the nature of the protein-protein interactions in the solution. Two different examples of this are shown in figure 1.3. These results indicate that the phase diagram for IgG has an upper critical solution temperature (UCST). Thus above the global maximum of the coexistence curve the IgG solution is completely miscible.
Besides deriving phase diagrams for IgG, Wang et al. [10] also visualized the LLPS of IgG with microscopy imaging, here included as figure 1.4. One can see the formation of the dense droplets within the dilute phase in figure 1.4 b).

In this project, the aim is to investigate the liquid dynamics of IgG in a single-component solution without the presence of co-solvents like PEG. The goal is to detect phase separation both through light scattering measurements and microscopy.
2. Materials and Methods

2.1 Ultraviolet-visible Spectroscopy

Ultraviolet-visible spectroscopy (UV-VIS) was performed in this project with a Cary 60 UV-Vis Spectrometer with serial number MY22119235 from Agilent. UV-VIS is a useful method to determine the concentration of protein solutions. A beam of monochromatic UV radiation is directed at the solution and the absorbance by the sample can be determined by comparing the initial and the detected intensities. Figure 2.1 shows a schematic picture of the UV-VIS setup [13].

![Figure 2.1: Schematic picture of UV-VIS setup [13].](image)

The Lamberts-Beers law [14] (equation 1) relates the absorbance (A) of the sample to its concentration (C):

\[
C = \frac{A}{\varepsilon}
\]

(1)

ε is the molar extinction coefficient, it is a measure of how strongly a substance absorbs light at a specific wavelength. For IgG ε is 1.4 mg\(^{-1}\)ml cm\(^{-1}\) [15]. This means that the absorbance of 1.4 at wavelength 280 nm represents 1 mg of IgG.
2.2 Dynamic Light Scattering

Dynamic light scattering (DLS) is a method used to determine the size distribution of particles in a colloidal solution to gain information about its dynamic behavior. The basic principle is that a laser beam is directed at a sample, usually using a beam splitter and lenses, that will be scattered by the particles in the solution sample. The intensity of this scattered light will then be measured by single photon counts at a detector, located at a specific scattering angle \( \theta \). In this project an LS spectrometer from LS instruments was used, a drawing of this setup can be seen in figure 2.2. The setup also includes a chiller with a temperature range from -11 to 25 °C.

\[
q = \frac{4\pi n}{\lambda} \sin \frac{\theta}{2}
\]  

(2)

Where \( \theta \) is the scattering angle, \( \lambda \) is the wavelength of the incoming light and \( n \) is the dimensionless refractive index of the solvent. In this work, the solvent is water which has a refractive index of 1.33 [17].
From the scattered intensity at a certain point in time at a certain angle \( I(q, t) \), the autocorrelation (also called \( g_2 \)-function) can be defined as [18]:

\[
g_2(q, \tau) = \frac{\langle I(q, t)I(q, t + \tau) \rangle}{\langle I(q, t) \rangle^2}
\]  

Here \( \tau \) represents the delay time between two measurements. The angular brackets denote the expectation value of the intensity, i.e. the average value over time. Two intensities measured only a short time apart will be unchanged since the particle that reflected the light could not have moved much during this time. However, at longer timescales, the correlation function decays exponentially. The way to interpret this is that we do not have any correlation between the different states/positions our particle was in at different times. This decay rate is what depends on the particle size and we can distinguish differently sized particles in a solution by analyzing the different scattering intensities [13]. For the larger particles we see that the decay is slower than for the smaller particles in figure 2.3 b).

![Figure 2.3](image)

*Figure 2.3: a) Intensity \( I(q,t) \) as a function of time (t) and its characteristic shape for large and small particles. b) \( g_2 \) function over \( \tau \) for the two different \( I(q,t) \). [19]*
In a solution, the particles undergo Brownian motion, which describes the thermal/random movement of the particles suspended in a liquid medium. If assumed that the particles do not interact with each other through collisions or other types of energy exchange, then the diffusion in the solution is described by the Stokes-Einstein equation:

\[ D = \frac{k_B T}{6\pi \eta R_H} \]  

(4)

Where \( D \) is the diffusion coefficient, \( k_B \) is the Boltzmann constant, \( T \) is the absolute temperature, \( \eta \) is the viscosity of the liquid and \( R_H \) is the hydrodynamic radius of the particles [18]. Even if IgG molecules, as mentioned in section 1.2, have a very distinct Y shape it is not uncommon when using a model like this to assume that the molecules have a spherical shape. In that case, the theoretical hydrodynamic radius of IgG is about 5.5 nm [8, 9]. The Stokes-Einstein equation indicates that larger particles have a slower diffusion than small particles in a solution with a specific viscosity.

The \( g_2 \)-function decays over time and needs to be fitted to extract the characteristic relaxation times. In this work, the fit will be to a double exponential where the second part is a stretched exponent, according to equation 5:

\[ g_2 = a_1 \exp(-2\Gamma_1 t) + a_2 \exp(-2\Gamma_2 t^{c_2}) \]  

(5)

Coefficients \( a_1 \) and \( a_2 \) are the amplitudes of the first and second decay respectively and \( \Gamma_1 \) and \( \Gamma_2 \) are the decay factors. For the stretched exponent \( c_2 \) is the Kohlrausch-Williams-Watts (KWW). More than one decay can be distinguished in the \( g_2 \)-function if there are large variations in the particle sizes in the sample. In this case, the first decay would be a fast decay representing smaller particles, and the slower stretched decay would indicate the presence of larger particles.

The diffusion coefficient \( D \) can also be derived in terms of the correlation function \( g_2 \) according to equation 6:

\[ \Gamma = \frac{1}{q^2} \rightarrow \Gamma = Dq^2 \]  

(6)

\( \Gamma \) is the decay factor of the \( g_2 \)-function, making it possible to calculate properties like \( D \) and \( \eta \) from DLS data.
In this experiment, IgG samples were measured at a variety of angles from 30°-150° and temperatures ranging from -11 °C to 20 °C. For full measurement script, see Appendix A.

2.3 Microscopy

To visually detect LLPS of IgG solutions, samples were examined and photographed with a UI industrial camera from iDS imaging and corresponding software UEye cockpit. The sample holder was mounted inside a cryostat model VPF-100 from JANIS research company, connected to a LakeShore 335 Temperature controller according to figure 2.4. An external light source was also added to ensure visibility.

To measure the microscopy images a grid with 600 μm circles was inserted in the sample holder and imaged in the same way as the protein sample. An image of the grid is included in Appendix E.

Figure 2.4 Microscope and cryostat setup.
2.4 Sample Preparation

The protein source is a powder with $\geq 99\% \gamma$-globulins from bovine blood containing 80\% IgG, 10\% IgM and <10\% IgA [20] produced by Sigma-Aldrich (G5009, batch number: SLCM8673). A 20 mM HEPES buffer solution with pH 7 was prepared with the use of NaOH. A stock solution was created by dissolving 1.5 g of IgG powder in 5 ml HEPES buffer. The concentration of this solution was determined by UV-VIS measurements with 3 different samples that were made according to table 2.1.

<table>
<thead>
<tr>
<th>Concentration (mg/ml)</th>
<th>Protein (mg)</th>
<th>Sodium Chloride, 500 mM (ml)</th>
<th>Water (ml)</th>
<th>Total Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.04</td>
<td>0.3</td>
<td>0.66</td>
<td>1</td>
</tr>
<tr>
<td>0.5</td>
<td>0.02</td>
<td>0.3</td>
<td>0.68</td>
<td>1</td>
</tr>
<tr>
<td>0.01</td>
<td>0.004</td>
<td>0.3</td>
<td>0.696</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 2.1: Sample preparation for UV-VIS.

For the DLS measurement, three different sample concentrations were prepared according to table 2.1. Approximately 0.5 ml of the solution was inserted into a capillary and set in the DLS sample holder.

<table>
<thead>
<tr>
<th>Sample Concentration (mg/ml)</th>
<th>Protein (ml)</th>
<th>Buffer (ml)</th>
<th>Total Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0.472</td>
<td>0.528</td>
<td>1</td>
</tr>
<tr>
<td>150</td>
<td>0.707</td>
<td>0.293</td>
<td>1</td>
</tr>
<tr>
<td>75</td>
<td>0.354</td>
<td>0.646</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 2.2: Sample preparation for DLS. Protein is 212 mg/ml IgG solution and Buffer is 20 mM HEPES.

For the microscopy new samples were prepared from the same stock solution with the same protein/buffer ratio as in table 2.2, but with a total volume of 0.5 ml. 15 $\mu$l of the protein solution was put between two calcium fluoride windows with a 0.01 mm plastic foil spacer.
3. Results and Discussion

3.1 UV-VIS Results

Three different solutions were analyzed with UV-VIS and prepared according to table 2.1. Figure 3.1 displays the detected absorbance over the wavelength for each of the concentrations. There is a clear decrease in absorbance with decreasing concentration in the peak at 279 nm, marked with a black dashed line in the figure. This is in agreement with the linear relation between absorbance and concentration in Lamberts Beers law (equation 1).

![UV-VIS](image)

*Figure 3.1: Absorbance over wavelength in nm from UV-VIS measurement of three different IgG solutions with concentrations 0.01 mg/ml (red), 0.5 mg/ml (green) and 1 mg/ml (blue). The analyzed absorbance peak is at 279 nm, marked with a black dashed line.*

The absorbance value was extracted from the peak at 279 nm and the concentration was calculated using equation 1. Table 3.1 shows the expected and calculated concentrations for each of the samples.
Table 3.1: Expected concentration of sample from the preparation recipe in table 2.1, the detected absorbance at 279 nm and the calculated concentration using Lambert Beers law with extinction coefficient $\varepsilon = 1.4 \text{ mg}^{-1}\text{ ml cm}^{-1}$.

<table>
<thead>
<tr>
<th>Expected concentration (mg/ml)</th>
<th>$\lambda$ (nm)</th>
<th>Absorption</th>
<th>Calculated concentration (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>279</td>
<td>1.203</td>
<td>0.85</td>
</tr>
<tr>
<td>0.5</td>
<td>279</td>
<td>0.591</td>
<td>0.42</td>
</tr>
<tr>
<td>0.01</td>
<td>279</td>
<td>0.108</td>
<td>0.0077</td>
</tr>
</tbody>
</table>

The lower limit for the UV-VIS absorption sensitivity is 0.3, hence all values below this value are assumed to be inaccurate. In this case, that means the value of 0.01 has to be excluded due to low absorption. From a dilution factor, corresponding to how many times each sample was diluted compared to the original solution, the concentration of the stock solution is calculated to be:

Calculated concentration $\cdot$ dilution factor = Sample concentration

$0.85 \cdot 250 = 214 \text{ mg/ml}$

$0.42 \cdot 500 = 211 \text{ mg/ml}$

$0.077 \cdot 2500 = 192 \text{ mg/ml}$ (excluded)

Stock solution concentration:

\[
\frac{211 + 214}{2} \approx 212 \text{ mg/ml}
\]
3.2 Dynamic Light Scattering Results

3.2.1 Intensity

All data from the DLS measurements were analyzed with Python. Every measurement was performed according to the respective measurement script in Appendix A.

At room temperature, the IgG solution is a clear liquid for all the analyzed concentrations. As temperature decreases during the DLS measurement, the turbidity of the sample increases drastically, as shown in figure 3.2.

![Figure 3.2: a) IgG 75 mg/ml in DLS capillary at room temperature. b) IgG 75 mg/ml in DLS capillary at approximately -11 °C (262 K).](image)

For decreasing temperatures an increase in the detected intensity $I(q)$ was observed for 75 mg/ml. The $I(q)$ values are all normalized with the laser intensity for each measurement, 0.05 for 293-273 K and 0.001 for 273-262 K (see measurement script in Appendix A). Each data point is also divided by the $I(q)$ value for the highest $q$-value $q_0 = 24 \ \mu$m$^{-1}$. In figure 3.3 we see a continuous increase of the $I(q)$ as the sample cools down and at the lowest temperature, 262 K, a distinct change appears in the shape of the curve. This can indicate that we have larger particles forming in the solution as it approaches the phase separation. It is also typical that the concentration fluctuations arising with the phase separation cause visual turbidity and increased scattering. Here an important point is that from image 3.2 b) we see that there is no freezing in the sample at -11 °C, and the sudden change in $I(q)$ then arises from other changes within the liquid.
3.2.2 Autocorrelation Function

From the $I(q,t)$ data the autocorrelation function ($g_2$-function) was calculated for each temperature and $q$-value according to equation 3 and then fitted with a double exponential as shown in equation 5. Figure 3.4 illustrates the correlation functions for all $q$-values and their respective exponential fit at six different temperatures. At 293 and 283 K, the decay seems to be dominated by the fast component and only the lowest $q$-value has a distinctly visible slow decay. At 273 K the decays adjacent to the lowest $q$-value start increasing in amplitude and at 268 K the rise is even more pronounced. The amplitude of the slow decay reaches the highest value at 263 K and there is a distinct difference in the shape of the $g_2$ -functions with the highest and lowest $q$-values. The overall trend is that the second component of the decay increases with decreasing temperature except for the lowest temperature in this measurement, 262 K. Here the amplitude of the slow decay drops drastically compared to 263 K. Looking carefully at the intermediate $q$-values (green/ yellow) it appears that the shape of these also changed suddenly at 262 K.
Figure 3.4: Correlation function as a function of time (t) calculated from DLS measurement of 75 mg/ml IgG solution at six different temperatures. The Red dashed line is a double exponential fit of the decay.

To further investigate the changes in the correlation functions, the parameters of the exponential functions were analyzed in more detail. Figures 3.5 and 3.6 a) show the same $g_2$-functions as in figure 3.4 at 293 and 263 K respectively, with the second stretched decay added as a dotted line for each $q$-value. As expected from figure 3.4 the amplitude of the slow decay ($a_2$) is close to zero for all except the last two $q$-values in figure 3.5 a). In figure 3.6 a) $a_2$ is increased for all the $q$-values, but the increase is most pronounced for the lowest $q$-values.

Figures 3.5 and 3.6 b) - d) include the different parameters of the exponential fit to the decay. Because the full double exponential fit is performed for every $q$-value some restrictions have been set on the parameters in the following plots to avoid presenting irrelevant data. The limit is to not include data from the slow decay if $a_2$ is smaller than 0.2. If $a_2$ is above 0.8 however, the data from the fast decay is excluded. This assumes that the fast decay dominates when the amplitude of the slow decay is small and vice versa if the amplitude of the slow decay is large.

In figure 3.5 b) the $\Gamma$ factor is plotted as a function of $q^2$ and fitted with a linear function. According to equation 6, the slope represents the diffusion coefficient $D$. Here only two
decays satisfy the applied conditions for the slow decay. Figure 3.5 c) shows the amplitude of the slow decay normalized by the total amplitude \((a_1 + a_2)\). This graph clearly illustrates the decrease in \(a_2\) for increasing \(q\) accordant with figure 3.5 a). The two data points that fulfill the conditions for the slow decay are shown again in figure 3.5 d), here with the KWW over \(q\)-values. Both values are within the error bars of the other and close to 0.5.

From figure 3.6 b) more of the datasets collected exhibit a slow decay with amplitude over 0.2 and can therefore be included in the fit. Here a linear fit is performed with reference to equation 6. In this case, the fast decay deviates from this fit for high \(q\)-values meanwhile it is within the error bars for lower \(q\). Similar to 293 K, we see \(a_2\) increasing with decreasing temperature in figure 3.6 c). However, the maximum amplitude is almost doubled here compared to at 293 K. The KWW in figure 3.6 d) also appear to have larger values at 263 K and decreases with rising \(q\)-values.

![Figure 3.5: Data from DLS measurement of 75 mg/ml IgG solution at 293K. a) \(g_2\)-function over time (t). The dashed line shows a double exponential fit and the dotted line visualizes only the second part of the decay. b) decay factor \(\Gamma\) over \(q^2\) for the fast decay (red) and slow decay (blue). The black line is a linear fit to the points. c) Amplitude of the slow decay over \(q\)-value. d) KWW of the slow decay over \(q\)-value.](image)

\[ T = 293.0 \text{ K} \]
The parameter analysis presented in this work qualitatively shows that the slow decay is more pronounced at lower temperatures, confirming that this component increases while approaching the phase separation. With regard to figure 2.3, we attribute the fast component of the exponential fit to small particles expected to be single IgG molecules in the solution. With the same reference, the slow decay is due to larger protein clusters forming in the sample. The second component is to some extent present at all measured temperatures, indicating that protein clusters are always present in the solution due to protein interactions, even without phase separation [20]. The increase of the slow decay can therefore be attributed to aggregates forming as the sample approaches phase separation or to the phase separation itself. At 293 K the solution appears to consist mainly of small particles in Brownian motion whilst in the interpretation that the second decay is attributed to clusters at 263 K it seems that the number of clusters increases, as indicated by the increase in amplitude. The KWW around 0.5 suggests that these clusters are polydisperse in size.
If the sample has undergone LLPS and formed two new phases it is not possible from this data to determine the new protein concentrations for each of them. Since the amount of IgG molecules in the solution remain constant throughout the measurement, the phase separation will not only change the slow decay by introducing dense clusters, it will also affect the fast decay as the characteristics of the dilute phase are established. One possible explanation for the sudden change in the slow decay at 262 K is that the laser beam (100 μm diameter) at this time hit a part of the sample that mostly consists of the dilute phase with smaller particles. That could be possible both if the sample exhibits nucleation or spinodal decomposition.

Plots for all the temperatures measured with DLS but not included in this section are shown in Appendix C.

3.2.3 Diffusion

The Stokes-Einstein equation (equation 4) states the diffusion of a particle in Brownian motion and the viscosity (\(\eta\)) in this equation has a temperature dependence [21]. To determine a theoretical value for the diffusion in an IgG solution a model for the temperature dependence of water viscosity was implemented. (The results can be found in Appendix B.) The viscosity of the IgG 75 mg/ml solution was then estimated to be \(4.5 \times \eta_{\text{water}}\) at each temperature.

Figure 3.7 a) Illustrate the diffusion coefficient \(D\) for the slow and fast decay respectively in 75 mg/ml. The dots are values determined from the DLS data using equation 5 and the solid lines are theoretical temperature dependence of the diffusion according to Stokes Einstein. For the red line, the hydrodynamic radius \(R_h\) is set as 5.5 nm to represent a single IgG molecule. The fast decay fits well to this model for high temperatures but the deviation grows larger below 273 K. The blue line represents the theoretic diffusion for larger particles with \(R_h = 360\) nm in Brownian motion. This is similar to the behavior of the particles causing the slow decay at high and intermediate temperatures. From 269 K it strongly diverges from the theoretical values by first decreasing and then increasing again from 265 K.

The temperature dependence was also investigated on the parameters from the exponential fit of the \(g_2\)-functions. Figure 3.7 b) displays both the amplitude and KWW of the slow decay for every measured temperature. The data points are the mean of the parameter value
at every temperature. In agreement with the data already presented in figure 3.5 and 3.6, the
values of these parameters increase with decreasing temperature. One possibility is that the
sample starts to phase separate as the diffusion starts to deviate from the theoretical values,
which is at the same temperature as the increase in the slow decay arises. Another
interpretation is that the phase separation occurs at the lowest temperature when we see a
fast increase in the diffusion coefficient, which could be a detection of a new diluted phase.
The drastic changes with the solution at 262 K is consistent with both the temperature
dependence of the $I(q)$ and $g_2$-functions presented in section 3.2.1 and 3.2.2, providing
strong indications of phase separation in the sample.

Figure 3.7: IgG solution 75 mg/ml a) Theoretical (line) and Experimental (dots) values of the diffusion
coefficient $D$ over temperature $T$. Theoretical values are estimated from the Stokes-Einstein equation with
hydrodynamic radius set to 5.5 nm for the fast decay (red line) and 360 nm for the slow decay (blue). b)
Amplitude (triangles) and KWW (squares) parameters from the exponential fit of the slow decay in $g_2$
-function over temperature ($T$).

According to Stokes-Einstein, larger particles have lower values for $D$. Physically this
means that protein clusters with large hydrodynamic radii are less able to move within the
liquid they are suspended in and therefore have a lower diffusion coefficient than single
molecules. As mentioned in section 3.2.2 some clusters are always present in the solution, this is also indicated in figure 3.7 a) by the diffusion coefficients corresponding to $R_h = 360$ nm. The diffusion behavior we see in figure 3.7 a) can be explained by the growth of these protein clusters. Another interpretation could be that if LLPS is starting in the sample as the decrease in $D$ is observed, it could correspond to droplets. It is not expected for these droplets to follow Brownian motion but as an estimate, $R_h$ of a droplet or cluster, causing the slow decay at 264 K is approximately 3 $\mu$m when calculated with the Stokes-Einstein equation. However, as the sample approaches the phase separation, intermolecular interactions in the sample can no longer be neglected and the nature of the diffusive behavior becomes more intricate, causing alternations in $D$.

Four weeks after the 75 mg/ml sample was prepared, that same sample was analyzed again. During these weeks the solution stayed inside the capillary sealed with a lid and parafilm and kept in a fridge at 5°C. A new DLS measurement was performed on the sample at this time with the same measurement script as the first measurement but with a higher laser intensity (see Appendix A). The goal was to check the reproducibility of previous results and a recreation of figure 3.7 with the data from the second DLS measurement of the 75 mg/ml sample is attached in Appendix D. In this measurement, the diffusion follows the theoretical value well for the fast decay down to 267 K where it starts to deviate. This is consistent with previous data except that the deviation from the theoretical value appears at lower temperatures. The data from the slow decay is also similar to the results presented in figure 3.7 a). It follows the theoretical values for high temperatures and equally to the slow decay the deviation from this estimate appears at lower temperatures for the second measurement. The value at 283 K stands out with very low diffusion meanwhile the other values are within a reasonable range of the theoretical model down until 265 K where we start to see a small increase in the diffusion coefficient that decreases again from 263 K.

The temperature dependence of the amplitude and KWW of the slow decay is in agreement with data already presented in figure 3.7 b) where both parameters increase with falling temperature. The exception is the maximum values of the amplitude and KWW that are far lower after four weeks than by the time of the sample preparation.

The many similarities confirm that there is reproducibility in the overall trend within the one-phase region. The indications for LLPS are also reoccurring in the second measurement, but only at a lower temperature, which possibly is due to the phase separations’ stochastic nature. At low temperatures, we also see a difference in the behavior for the slow decay. One explanation as to why these differences are observed could be the
aging of the sample. During the four weeks, protein interactions can change within the solution and give rise to different conditions than right after the sample preparation.

3.3 Imaging Liquid-Liquid Phase Separation

As seen in figure 3.2, the IgG 75 mg/ml solution is completely clear at room temperature. In accordance, the microscopy image is uniform at the same temperature, this is included in Appendix E for reference. From this state, the temperature controller was set to 259 K. The temperature controller had a ramp rate of 30 K per minute and the heater range was set to the maximum output of the controller. The first image in figure 3.8 was taken the moment the sensor reaches 259 K, this time is defined as $t=0$ minutes. In image 3.8 a) it is already possible to see some structures forming even though very undefined. After 5 minutes in which the temperature was kept at $259 \pm 0.5$ K image 3.8 b) was captured. In this picture, the structures formation is much more pronounced with bright and dark spots. After 15 minutes well-defined droplets have formed in the solution. This is a direct observation of the LLPS and how it develops over time at a fixed temperature below $T_c$. From this image, it is impossible to determine the concentrations of the different phases with certainty and decide if the droplets are dilute or dense. But if compared with the phase diagrams from previous studies (figure 1.3) 75 mg/ml appears to be close to or slightly to the left of the critical concentration and $T=259$ K is expected to be well below $T_c$ [10,12]. With this as a reference,
the hypothesis is that the sample is in a state with dense droplets within a dilute phase, but their specific concentrations remain unknown.

A previous study of phase separation in colloidal solutions by Jamie et al. presents images of the phase transition over time from the one-phase state into spinodal decomposition [22]. According to their data, the phase separation is still evolving 80 minutes after the droplets start to form within the solution. In a nucleation state, the LLPS would appear as different-sized droplets within the solution. Taking these results into consideration, it is possible that the domains of the different phases in the IgG sample could grow larger over time if the current conditions are within the spinodal region.

To determine the size of the droplets the microscopy images were analyzed in ImageJ, a Java image processing program [23]. By plotting the brightness across the diameter of the grid it shows how many pixels the radius represents in a 32-bit image. From the known grid radius, 1 pixel was determined to be approximately 1 μm. The grid images and analysis is attached in Appendix E.

Figure 3.9: Image of a droplet from 75 mg/ml IgG solution at 259K and the brightness along a line across the droplet over the pixel distance. The darkest pixel along the line is set to brightness= 0. Converting to true length scale, 1 pixel ≈1 μm.

Figure 3.9 shows a randomly selected droplet from the image in figure 3.8 c) and the brightness of each pixel over the pixel coordinates along the yellow line marked in the
If we define the diameter of the droplet as the distance between the two darkest spots along the line, this droplet has a diameter of 10 pixels. According to the reference grid, this represents around 10 μm. In the sample the droplet size is distributed around this value, see pixel scale in figure 3.8 c) for reference. From the same figure it is clear that the size of the droplets varies throughout the sample and this measure serves only as an estimate for the order of magnitude of the droplet size. This droplet diameter is consistent with the estimations from section 3.2.3 which said that the diffusion data indicated that we could have particles with hydrodynamic radius in the order of μm.

The sample thickness here is 10 μm because of the spacer and the droplets can be assumed to occupy all the available depth. This confinement might be limiting the spinodal decomposition [24]. Restricting the physical volume where the process takes place can simulate in vitro conditions and confinement appears to be essential to LLPS in living cells [25, 26]. In this work, the sample volume can therefore be a contributing factor to the appearance of LLPS both in microscopy and DLS investigations.

From 259 K the set point is changed to 290 K, executed with the same ramp rate and heater output as previously. The image in figure 3.10 b) is captured just as the temperature reading on the sensor reaches 279 K, meaning that the accuracy of the temperature is not very high. However, the image visualizes how the droplets dissolve as temperature increases. At 290 K the sample returns to its initial state before cooling (figure 3.10 c). This demonstrates that macroscopically the LLPS is reversible.

![Figure 3.10](image)

**Figure 3.10**: Remixing after phase separation of 75mg/ml IgG solution. From T=259 K the set point in the temperature controller was changed to 290 K, with a ramp rate of 30 K/min. a) Before heating b) At 279 K. c) At 290 K.
4. Conclusion and Outlook

The goal of this project was to investigate the liquid dynamics and phase separation of IgG protein solutions. This was done by different experimental methods; ultraviolet-visible spectroscopy, dynamic light scattering and optical microscopy. The protein solutions were analyzed at different concentrations and temperatures to detect LLPS and locate its positions in a phase diagram.

Combining results from the microscopy and DLS, we can conclude that LLPS was detected in the sample on multiple occasions. An increase in the turbidity of the sample and a simultaneous rise of a slow decay in the autocorrelation function strongly indicate that larger protein clusters form in the solution as temperature decreases. This is confirmed by the microscopy images visualizing the droplet formation over time and how the process is reversed with increased temperature. Phase separation was detected at several temperatures during different parts of the investigation, indicating that we do have located several points within the two-phase region of the phase diagram for IgG.

This work confirms the occurrence of LLPS within IgG solutions in different confinements without any co-solvents and shows that it is possible to reach low temperatures within the two-phase region without detecting freezing in the sample. The diffusion coefficient deviates from Stokes-Einstein estimations at low temperatures by decreasing faster than the theoretical curve, indicating larger protein clusters or droplets forming in the solution.

From the microscopy images, LLPS was observed as droplets that were measured to have a radius of 10 μm. In the interpretation that the increase of the slow component in the autocorrelation function corresponds to droplets within the solution, data from the DLS measurement indicates that these droplets have a hydrodynamic radius of around 3 μm. An interesting hypothesis for future investigations within this work is that a longer equilibration time could allow the sample to reach spinodal decomposition if such is thermodynamically allowed under current conditions.

The outlook for this project is to provide initial data for continuous work on LLPS in IgG solutions in the research group for Structural dynamics of aqueous solutions at Fysikum Stockholm University. The results of this thesis are to serve as a pilot project for future proposals for follow-up investigations at X-ray synchrotrons and X-ray Free Electron Lasers (XFELs).
References


APPENDIX A

Measurement script DLS

Measurement script DLS. At every temperature, each angle was measured 3 times for 6 seconds per measurement. The script was performed in 2 parts, first 20 to 0 °C with the laser intensity at a fixed value, and then 0 to -11°C with higher intensity. The entire script was repeated for both DLS measurements except for the different intensities according to columns 5 and 6.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Angles (degrees)</th>
<th>Duration time (s)</th>
<th>Number of measurements</th>
<th>Laser intensity first measurement (mW)</th>
<th>Laser intensity second measurement (mW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>30-150</td>
<td>60</td>
<td>3</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>15</td>
<td>30-150</td>
<td>60</td>
<td>3</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>10</td>
<td>30-150</td>
<td>60</td>
<td>3</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>30-150</td>
<td>60</td>
<td>3</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>0</td>
<td>30-150</td>
<td>60</td>
<td>3</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>0</td>
<td>30-150</td>
<td>60</td>
<td>3</td>
<td>0.01</td>
<td>0.2</td>
</tr>
<tr>
<td>−2</td>
<td>30-150</td>
<td>60</td>
<td>3</td>
<td>0.01</td>
<td>0.2</td>
</tr>
<tr>
<td>−4</td>
<td>30-150</td>
<td>60</td>
<td>3</td>
<td>0.01</td>
<td>0.2</td>
</tr>
<tr>
<td>−5</td>
<td>30-150</td>
<td>60</td>
<td>3</td>
<td>0.01</td>
<td>0.2</td>
</tr>
<tr>
<td>−6</td>
<td>30-150</td>
<td>60</td>
<td>3</td>
<td>0.01</td>
<td>0.2</td>
</tr>
<tr>
<td>−7</td>
<td>30-150</td>
<td>60</td>
<td>3</td>
<td>0.01</td>
<td>0.2</td>
</tr>
<tr>
<td>−8</td>
<td>30-150</td>
<td>60</td>
<td>3</td>
<td>0.01</td>
<td>0.2</td>
</tr>
<tr>
<td>−9</td>
<td>30-150</td>
<td>60</td>
<td>3</td>
<td>0.01</td>
<td>0.2</td>
</tr>
<tr>
<td>−10</td>
<td>30-150</td>
<td>60</td>
<td>3</td>
<td>0.01</td>
<td>0.2</td>
</tr>
<tr>
<td>−11</td>
<td>30-150</td>
<td>60</td>
<td>3</td>
<td>0.01</td>
<td>0.2</td>
</tr>
</tbody>
</table>
Water viscosity over temperature was implemented to estimate the viscosity of the IgG solution.
Recreation figure 3.7 with IgG solution 75 mg/ml 4 weeks after preparation.

a) Theoretical (line) and Experimental (dots) values of the diffusion coefficient $D$ over temperature ($T$). b) Amplitude (triangles) and KWW (squares) parameters from an exponential fit of the slow decay in $g_2$-function over temperature ($T$).
APPENDIX E

75 mg/ml at room temperature reference image:

Grid of circles with 600 μm diameter:
Grid Analysis:

The brightness of each pixel over the pixel coordinates along the line (magnified with a red dashed line) across a 32-bit image of the 600 µm grid.

In pixels, the distance between the darkest spots (minima on the graph) is: $704 - 60 = 644$ pixels. And the distance between the brightest spots (maximum on the graph) is: $673 - 102 = 571$ pixels. The diameter of the circle is estimated to be the average of these two distances; 

$$\frac{644 + 571}{2} = 607.5 \text{ pixels.}$$

Converting this to µm gives: 

$$\frac{607.5}{600} \text{ pixels/µm} = 1.0125 \text{ pixels/µm} \approx 1 \text{ pixel/µm}$$

→ 1 pixel = 1 µm.