Segregative phase separation in aqueous mixtures of polydisperse biopolymers

Segregatieve fasescheiding in mengsels van polydisperse biopolymeren in water

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Abstract

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Keywords: biopolymer, gelatine, dextran, PEO, phase separation, polydispersity, molar mass distribution, SEC-MALLS, CSLM.

The temperature-composition phase diagram of aqueous solutions of gelatine and dextran, which show liquid/liquid phase segregation, were explored at temperatures above the gelation temperature of gelatine. It turned out that the compositions of coexisting phases show practically no dependence on temperature between 40°C and 80°C. Also, the total polymer concentration at which phase separation occurred was found to be nearly independent on temperature. These observations suggest an entropy driven phase separation.

Phase separation is found to be accompanied by strong fractionation of the molar mass distribution in the two phases. Molar mass distributions in coexisting phases were investigated using Size Exclusion Chromatography with MultiAngle Laser Light Scattering. The molar mass of the native material and concentration appeared to be the only factors that affected the final molar mass distributions, temperature having no effect. The results show that in the molar mass range where fractionation is the strongest, i.e. roughly below the maximum in the distribution, fractionation is governed by a Boltzmann factor $\exp(^{-\Delta G}/_{kT})$, where ΔG denotes the free energy involved in transferring a polymer with a certain length from the enriched to the depleted phase, and in this case turns out to be proportional to the molar mass.

The phase behaviour of aqueous mixtures of poly(ethylene oxide) (PEO) and dextran is also studied. From the temperature dependence we conclude that the phase separation between PEO and dextran is partly caused by sterical interactions. From the equilibrium phase volumes of the phase separated mixture and the shape of the temperature–composition phase diagram of PEO and dextran we conclude that also the decrease of solvent quality of water for PEO at increasing temperatures is involved. It is suggested that the characteristics of the PEO-water interaction can affect the degree of fractionation. This suggestion is based on the observation that the degree of fractionation is not a simple exponential function of the molar mass.

Adsorption of high molar mass dextran at the interface between gelatine and dextran during the phase separation of aqueous mixtures of these two polymers is also studied. This adsorption is observed after macroscopic phase separation as well as during the phase separation. In the last case, the system is studied with Confocal Scanning Light Microscopy. For this purpose, the dextran is covalently labelled with fluorescein 5-isothiocyanate (FITC). It turns out that the adsorption of high molar mass dextran is highly affected by the labelling. The adsorption of labelled dextran leads to a stable film between the two coexisting phases, thus preventing dextran droplets to coalesce. If the degree of labelling increases above a certain level, the labelled dextran does not lead to a stable film, but is preferentially present in the gelatine-rich phase.

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Chapter 1

Introduction

1.1 General introduction

Mixing of polymer solutions may result in phase instabilities. These phase instabilities may have an attractive or an repulsive origin. An example of the former case is that polymers with an opposite charge stick together and form insoluble complexes. An example of the latter case is when a mixture of neutral or equally charged polymers segregates into separate phases. The focus of this thesis is on the latter form of phase instability: segregative phase separation.

Apart from a fundamental interest in this type of phase separation, it is also relevant to applications in e.g. foods and pharmaceutics. Food applications follow from the fact that many food systems contain biopolymers. Apart from their nutritional value, these polymers are used as thickening or stabilising agents and to give products their desired textural properties. Often one uses mixtures of polymers to widen the sensory window of products, but this also causes undesired phase instabilities under processing/storaging conditions. Thus, understanding of segregative phase separation aids in controlling undesired phase instabilities. In contrast to avoiding instabilities, phase instabilities have also been purposely utilised to create anisotropic structures.

The phenomenon of thermodynamic incompatibility of biopolymers is already known since 1896, when Beijerinck discovered the separation of gelatine and soluble starch [1]. During the past century, the incompatibility of biopolymers has been the subject of many studies which resulted in a large quantity of experimental data [2,3]. These data mainly give a qualitative description of the phase separating conditions. It is shown that phase separation of biopolymer mixtures often is induced by electrostatic interactions between polymers with a similar sign of charge. This implies that the chemical structure of the polymers, the pH and the ionic strength are important parameters for the phase separation. It is also shown that aggregation of one of the polymers may induce phase separation. In these cases, the phase separation is often due to the fact that the solvent quality for one of the polymers changes. In contrast to the number of qualitative descriptions, quantitative descriptions on phase separation are relatively scarce. This is due to the fact that most biopolymers are very polydisperse in their molar mass and often have the tendency to form a gel already at a low concentration or at moderate temperatures [4-9]. The formation of a gel arrests the phase separation. Gelation also increases the apparent molar mass of the gelling polymer, which in turn speeds up the phase separation [10]. Apart from the experimental data of the phase behaviour, theoretical models describing the phase behaviour of polymers have been developed [11-16]. However, it is not clear to what extent these theories are valid for the phase separation of mixtures of biopolymer solutions.

The aim of this thesis is to obtain more quantitative insight in the segregative phase separation of biopolymer mixtures by experimentally studying the effects of temperature,

molar mass, polydispersity and concentration on the occurrence of phase separation and on the molar mass distribution of the constituents in the separating phases. Gelation of the systems was avoided. The experimental results are put in the context of already available theories.

1.2 Phase separation

When a solution of polymer *A* is added to a solution of polymer *B*, two things might happen; the two solutions will mix or the solutions will not mix and the system separates into two phase. Which of these two processes will happen depends on the sign of the change of the Gibbs free energy of mixing, ΔG_{mix}, which is given by

$$\Delta G_{mix} = \Delta H_{mix} - T\Delta S_{mix} \tag{1.1}$$

in which ΔH_{mix} and ΔS_{mix} denote the enthalpy of mixing and entropy of mixing respectively. T denotes the absolute temperature. If $\Delta G_{mix} \leq 0$, the system will mix, whereas in the case of $\Delta G_{mix} > 0$, the system will separate into separate phases.

1.2.1 Flory-Huggins

The Flory-Huggins theory describes a mean field lattice model in which the distribution of molecules on a lattice is calculated. The first version of the model describes the distribution of a single polymer species in a solution [11-14]. However, this model is extended to describe the distribution of more than one polymer species in a solution [17]. The assumptions that are made in this model are i) each lattice site is occupied by either a polymer segment or a solvent molecule, ii) the polymers are flexible and iii) the interactions are restricted to nearest-neighbour pair interactions in the lattice. This results in the following general expression for the Gibbs free energy of mixing per unit volume:

$$\frac{\Delta G_{mix}}{RT} = \sum_i \frac{\phi_i}{V_i} \ln \phi_i + \left(\frac{1}{2}\right) \sum_i \sum_j \chi_{ij} \phi_i \phi_j \tag{1.2}$$

where R is the gas constant, T is the absolute temperature, ϕ_i denotes the volume fraction and V_i the volume of component i, and χ_{ij} denotes the Flory-Huggins pair interaction parameter. This pair interaction parameter is a function of the energies of the interaction between segments of component i and j when these occupy neighbouring positions in the lattice. If fully entropic, this interaction parameter is inversely dependent on the temperature. The first term in the expression for $\Delta G_{mix}/RT$ is the combinatorial entropy part of the expression whereas the second term is the interaction part.

1.2.2 Depletion

The depletion model gives the thermodynamic explanation for the phase separation of colloids and non-adsorbing polymers in a solution on the basis of excluded volume [15,16]. The system can be pictured as in figure 1.1. Around the colloids, a depletion zone is shown. This is a zone from which the mass centres of the polymers are expelled. This expulsion is caused by the loss of conformational entropy of the polymer chains in the presence of a wall. When two colloids approach each other sufficiently close, the depletion zones will overlap. The polymers in the system are expelled from this overlap volume, $V_{overlap}$. This results in an osmotic pressure difference, $\Delta\Pi$, between the overlap volume and the bulk phase. This osmotic pressure results in an effective attraction of the colloids. Due to this attraction of the colloids, the volume which is available for the polymers increases with $V_{overlap}$. This increase of the volume by $V_{overlap}$ results in a lowering of the free energy of the system, ΔG, by $-\Pi V_{overlap}$.

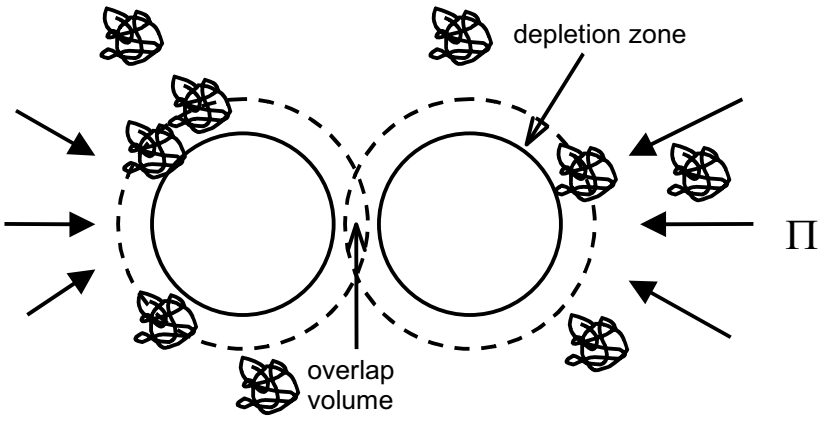

Figure 1.1: Mechanism of the depletion interaction.

The situation as depicted in figure 1.1 shows colloidal particles which are larger than the polymer chains. However, in the literature this model is also used for other systems such as the description of the phase separation in which the polymers are larger than the colloidal particles [18,19].

1.3 Materials

For the study on the segregative phase separation of aqueous (bio)polymers, which is described in this thesis, a model system is chosen which consists of an aqueous solvent and two dissimilar (bio)polymers. In this section, a more detailed description is given of the polymers used for this study.

Most of the work presented in this thesis is performed on aqueous mixtures of gelatine and dextran. This system is chosen mainly because of its good experimental accessibility. In order to obtain a more general view on the phase separation of aqueous mixtures of two (bio)polymers, the phase separation of a second model system is studied. This model system consists of a mixture of poly(ethylene oxide) (PEO) and dextran in water.

1.3.1 Gelatine

Gelatine is the denatured form of collagen. Collagen comprises 30% of the total protein of mammals. It can be found, for instance, in skin, bone and muscle [20], where it is organised in mechanical strong fibrils, specified to the function of the tissue [21]. To convert collagen into gelatine, the collagen is heated in the presence of water under acid or alkaline conditions. During this process, non-covalent as well as covalent inter- and intramolecular bonds are broken and the structured collagen is transformed in a more amorphous form: gelatine. The conditions of the denaturation process are determinative for the final properties of the gelatine such as molar mass and iso-electric point.

The gelatine molecule is a linear chain of amino acids and has, depending on the pH of the solution, a negative (pH > pI) or a positive (pH < pI) net charge. If gelatine is dissolved in water, it forms a random coil. However, if the temperature of the solution is dropped below the gelation temperature, the chains will form helices. For the formation of one helix, three molecules are required. If the concentration of helices is above a critical one, a network is formed. If the temperature is increased again, the helices are broken and the chains will form a random coil formation again. However, if the temperature becomes too high, the gelatine will decompose. The peptide bonds between the amino acids are hydrolysed and the molar mass of the gelatine decreases. This hampers network formation [22].

1.3.2 Dextran

Dextran is a polysaccharide which is found in slimy layers around bacteria of the species *Leuconostoc mesenteroides*. In this layer, the dextran is involved in the protection of the cell and the adhesion of the cell to surfaces [23]. The synthesis of dextran is catalysed by the extracellular enzyme dextransucrase. This enzyme hydrolyses sucrose into glucose and fructose and binds the glucose monomers to a glucan. The chain which then arises is mainly linear and the glucose monomers are bounded by α-1,6-linkages, whereas branches start with α-1,3-linkage. The degree of branching of the chains is dependent on the origin (i.e. strain of the bacteria) of the dextransucrase [23]. From the dextran that is used in this study, it is known from literature that approximately 95% of the linkages in the chain are of the α-1,6-type and 5% are of the α-1,3-type [23-25]. The length of the branches varies up to 50-100 monomers [25].

In an aqueous solution, dextran is a random coil. Dextran is not able to form a gel, it only increases the viscosity of a solution. The viscosifying effect depends on the concentration and molar mass of the dextran and the degree of branching of the dextran chains [24,25].

1.3.3 Poly(ethylene oxide)

Poly(ethylene oxide) (PEO) is a synthetic polymer. The net formula of the polymer is $(CH_2\text{-}CH_2\text{-}O)_n$. PEO is used as one of the key components in aqueous two-phase systems for the separation and purification of biological materials such as proteins [26-28]. High molar mass PEO (>5 MDa) is also used as a flocculant in applications such as papermaking [26].

The poly(ethylene oxide) chain has no charge and as it is hydrophilic, it dissolves readily in water, at least at moderate temperatures [26].

1.4 Outline of the thesis

In this thesis, an experimental study to the phase separation of aqueous (bio)polymer mixtures is described. Three chapters in this thesis deal with mixtures of gelatine and dextran and one with the mixture of PEO and dextran. The work presented in chapter 2 to 4 describes the equilibrium state of the phase separation whereas the results presented in chapter 5 mainly describe non-equilibrium conditions.

In chapter 2 the phase separation of gelatine and dextran is studied. In this chapter, the phase behaviour is mainly explored as function of temperature and molar mass. From the results of the experiments, conclusions are drawn on the mechanism of the phase separation of biopolymer mixtures.

In chapter 3 the effect of the phase separation on the molar mass distribution of gelatine and dextran is studied. The fractionation of the molar mass as result of the phase separation is studied by determining the molar mass distributions of gelatine and dextran in the coexisting phases. Also consequences of the fractionation in molar mass are discussed.

Chapter 4 provides a more general view on the mechanism of the phase separation of (bio)polymer mixtures. In this chapter the phase separation of PEO and dextran is studied and compared to the results of the mixture of gelatine and dextran as they are presented in chapter 2 and 3.

Chapter 5 deals with effects of the polydispersity of the polymers during the phase separation. The observations are mainly made by using Confocal Scanning Light Microscopy (CSLM).

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Chapter 2

Compatibility of Gelatine and Dextran in Aqueous Solution*

ABSTRACT

The temperature-composition phase diagram of aqueous solutions of gelatine and dextran, which show liquid/liquid phase segregation, were explored at temperatures above the gelation temperature of gelatine. It turned out that the compositions of coexisting phases show practically no dependence on temperature between 40°C and 80°C. Also, the total polymer concentration at which phase separation occurred was found to be nearly independent on temperature. These observations suggest an entropy driven phase separation. An explanation in terms of depletion, reversible clustering, and subsequent transient network formation of gelatine at temperatures well above the temperature of gelation is suggested. Phase separation is found to be accompanied by strong fractionation of the molar mass distribution in the two phases.

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2.1 Introduction

As is commonly the case for mixtures of biopolymers, gelatine and dextran are incompatible in aqueous solution. At sufficient concentration (typically above 3% (w/w) of both components) segregative phase separation takes place, resulting in gelatine-rich and dextran-rich domains. Such phase separation processes are often the basis for structuring processed food. In the literature a large quantity of experimental data is available on the compatibility of food biopolymers [1,2]. Because of the polydispersity and the tendency to form gels above a certain concentration and below a certain temperature, quantitative descriptions of biopolymer phase separation are relatively rare [3-8]. Gelation, if it occurs, prevents the phase separation from proceeding beyond a certain point. At this point, the system usually consists of microscopic phase regions, that do not necessarily have the composition characteristic of thermodynamic equilibrium.

The work presented here is part of an effort to provide a detailed description of the equilibrium phase diagram of mixing of an aqueous mixture of two biopolymers above the gelation temperature of both components.

The gelatine/dextran system was chosen because it is experimentally accessible and it is representative for gelling and phase separating mixtures of biopolymers. Moreover, it enables one to study the kinetics of phase separation in the absence and in the presence of gelation [9]. As a consequence, the complicated interplay between phase separation and gelation can, in principle, be unravelled. Above the gelation temperature (about 30°C) of gelatine, the segregation appears to proceed like 'normal' liquid – liquid phase separation in contrast with the case where that gelation of gelatine takes place (below 30^{o}C). In the latter case, the thermodynamic driving force for segregation becomes stronger, at least partly because the molecular weight of gelatine effectively increases due through aggregation. At the same time, a viscosity difference between the gelling gelatine domains and the non-gelling dextran domains is established. This viscosity difference probably has a profound influence on the segregation kinetics [10,11]. Eventually, gelation will halt the process of segregation leaving the system in a kinetically frozen, metastable state. Recently, the crucial role of the presence of an disorder – order transition of one the polymers in driving phase separation was demonstrated [12,13] to be not unique for gelatine containing biopolymer mixtures.

Here, we are principally interested in answering the questions of why gelatine and dextran are incompatible above the gelation temperature of gelatine and to what extent the phase diagram of mixing can be understood in terms of a disorder – order transition, as is the case below the gelation temperature of gelatine. Commonly, a difference in solvent quality is assumed to play an important role in biopolymer compatibility [1,2]. However, almost 30 years ago, it was suggested that self-aggregation of gelatine was a driving force

for segregation from other polymers, especially near the iso-electric point (IEP) of gelatine [14]. This would imply a deviation from the predictions of mean field theory, as such predictions do not take into account concentration fluctuations. However, self-aggregation of sodium caseinates as determining factor for phase separation in mixtures with polysaccharides could be explained qualitatively [15] by the classical condition for phase instability

$$\frac{\partial^2 G_{mix}}{\partial c_2^2}\frac{\partial^2 G_{mix}}{\partial c_1^2} - \left(\frac{\partial^2 G_{mix}}{\partial c_1 \partial c_2}\right)^2 < 0 \qquad (2.1)$$

in which $G_{mix}(c_1,c_2)$ is the Gibbs free energy of mixing solutions of polymers 1 and 2 at concentrations c_1 and c_2, respectively.

Recently, self-aggregation or clustering of gelatine quenched in a clustered state was shown to affect its compatibility with locust bean gum in the way expected on the basis of its larger molecular mass [16,17]. It was also suggested that steric interactions between gelatine clusters and locust bean gum might be the dominant reason for phase instability at high ionic strength. In that work, gelatine aggregates were prepared by cooling a dilute gelatine solution below the gelling temperature. Anticipating the results of the present work, gelatine aggregates, reversibly forming at temperatures well above the gelation temperature of gelatine, might interact sterically with dextran, causing incompatibility.

Our data suggests the applicability of a depletion model, recently introduced by Odijk [18,19], based on an observation by de Gennes [20]. The compositions of coexisting gelatine-rich and dextran-rich phases appear to be in line with the predictions of this model. The Flory-Huggins appoach is deemed less suitable here because of two reasons. First, a mean field approximation might not be valid in a semi-dilute mixture of two polymer solutions, and second, the approach does not allow an interpretation in terms of molecular detail, in contrast with the depletion model of Odijk.

Experiments exploring the compatibility of gelatine and dextran have been carried out. All experiments were performed at temperatures above the gelation temperature of gelatine, where one can obtain full (equilibrium) phase separation. Fully phase-separated mixtures were investigated with respect to the volumes and compositions of coexisting phases. In addition, the phase separation temperature as a function of composition was determined.

2.2 Experimental

2.2.1 Materials

Porcine skin gelatine (IEP ± 8.7, bloom 305 g, $M_w \approx 170$ kDa) was kindly provided by Degussa Biosystems (Centre de Recherches 50500 Baupté, France). Dextran (148, 282, 484 and 2000 kDa, with these values verified by SEC-MALLS) was purchased from Sigma Chemicals. The ingredients were used without further purification. Solutions for the determination of phase diagrams were prepared by gravimetrically adding solvent (0.1 M NaCl and 0.02% sodium azide) to the proper amount of material. Dextran dissolves readily at room temperature. Gelatine was dissolved by stirring the mixture over night with a magnetic stirrer at ± 50°C.

2.2.2 Salt and pH dependence

The compatibility of 5% (w/w) gelatine/5% (w/w) dextran was tested as a function of NaCl concentration at 20°C and 60°C. It was found that salt favours phase separation. Above 0.01 M NaCl, phase separation takes place at both temperatures, and below this salt concentration, the solution stays transparent at both temperatures. The pH was about 6. Considering the IEP of gelatine is 8.7, gelatine will be positively charged, and salt will screen repulsive interactions between gelatine chains. To study the effect of gelatine self-aggregation on compatibility, it was considered to be useful to minimise the effect of long-range electrostatic interactions. Therefore, 0.1 M NaCl was chosen as the solvent.

2.2.3 Determination of the temperature–composition phase diagram

To determine the temperature–composition phase diagram, mixtures of 5% (w/w) gelatine and 5% (w/w) dextran in 0.1 M NaCl were made by adding equal volumes of 10% solutions. Approximately 10 gram of the mixture was transferred to a plastic tube. Paraffin oil was put on top of the meniscus to prevent evaporation of water. The tubes were held in a water bath for ± 20 hours to reach equilibrium phase separation. Equilibrium was assumed to be reached when a sharp meniscus between the transparent gelatine-rich and dextran-rich fluid layers was observed. Hereafter, the heights of the upper and lower phase were measured, to calculate the volume of the phases. Samples of these phases were taken with a hypodermic syringe.

To determine the concentration of the polymers in the upper and lower phases, the samples taken from the upper and lower phases were diluted 40 times. Optical rotation was measured at two different wavelengths (365 and 578 nm) [21]. With the use of the optical rotation per unit concentration $\alpha_{sp,g}$ and $\alpha_{sp,d}$, determined from calibration lines, the concentrations in the diluted phases were calculated by solving the system

$$\begin{aligned}\alpha_{meas}(c_g, c_d, \lambda = 365nm) &= \alpha_{sp,g}(\lambda = 365nm)c_g + \alpha_{sp,d}(\lambda = 365nm)c_d \\ \alpha_{meas}(c_g, c_d, \lambda = 578nm) &= \alpha_{sp,g}(\lambda = 578nm)c_g + \alpha_{sp,d}(\lambda = 578nm)c_d\end{aligned} \quad (2.2)$$

in which the subscripts *meas*, *sp*, *g* and *d* mean measured, specific (per % (w/w)), gelatine and dextran, respectively. Calibration lines to obtain $\alpha_{sp,g}$ and $\alpha_{sp,d}$ were measured in the range from 0 to 1% (w/w) at 80°C and at wave lengths of 365 nm and 578 nm. This method is valid only if gelatine and dextran contribute to the total optical rotation in proportion to their concentration. To probe this simple additivity of optical rotation contributions, for each wavelength the optical rotation of five symmetrical (50%/50%) mixtures of gelatine and dextran were measured (total polymer content = 0, 0.5, 1.0, 1.5 and 2.0% (w/w)) and compared with those of solutions of only gelatine and only dextran. The results were in excellent agreement with the assumption of additivity, as long as the temperature was higher than 50°C. All determinations of concentration were therefore carried out at 80°C. The weights of the phases were derived from the heights of the phases by assuming that both phases had the same density, equal to 1 g ml^{-1}.

2.2.4 Determination of the molar mass distribution of gelatine and dextran

Size exclusion chromatography (SEC) equipped with a multiangle laser light scattering (MALLS) detector, a refractive index (RI) detector and an optical rotation detector was used to determine the molar mass distribution of gelatine and dextran. For mixtures of gelatine and dextran, equation (2.2) was used to calculate the gelatine and dextran concentrations in the eluent. Instead of values of the optical rotation at two wavelengths, the optical rotation at one wavelength and the refractive index were used.

For gelatine a $LiNO_3/KH_2PO_4/K_2HPO_4$ (pH 6.68) buffer was used as eluent, for dextran 0.1 M $NaNO_3$. The flow rate was 1 ml min^{-1}. Typically, 4 mg of dry material in 200 µl was injected, resulting in a concentration of 0.2% (w/w) in the detector cells.

2.2.5 Determination of the phase separation temperatures

A mixture containing 7% (w/w) gelatine and 7% (w/w) dextran was heated to 70°C in a water bath. Such a solution is phase separated. Then, solvent (0.1 M NaCl) was added drop wise with stirring. This addition was continued until interruption of stirring was not followed by the appearance of a haze in the solution within ca. 5 minutes. No appearance of a haze was interpreted as compatibility. Lowering the temperature again caused a haze to appear, and the procedure was repeated. After every addition of solvent, the container plus solution were weighed.

2.3 Results

Figure 2.1 presents the temperature–composition phase diagram of mixtures of 5% (w/w) gelatine and 5% (w/w) dextran for molar masses of dextran of 282 and 148 kDa. No significant effect of the temperature and an insignificant effect of dextran molar mass on the phase compositions were found in the temperature region studied. It is expected that lowering the total polymer concentration (i.e. adding solvent) will bring the system closer to the miscible state. Therefore, upon approach to this state, the phase compositions should become more similar. Such is observed in figure 2.2 which is the 3D analogue of figure 2.1 for different total concentrations of solute. Only at 3.5%/3.5% (w/w) is a temperature found (about 60°C) above which no phase separation takes place. At lower concentrations, phase separation occurs near or below the gelation temperature of gelatine, and no full segregation can be achieved.

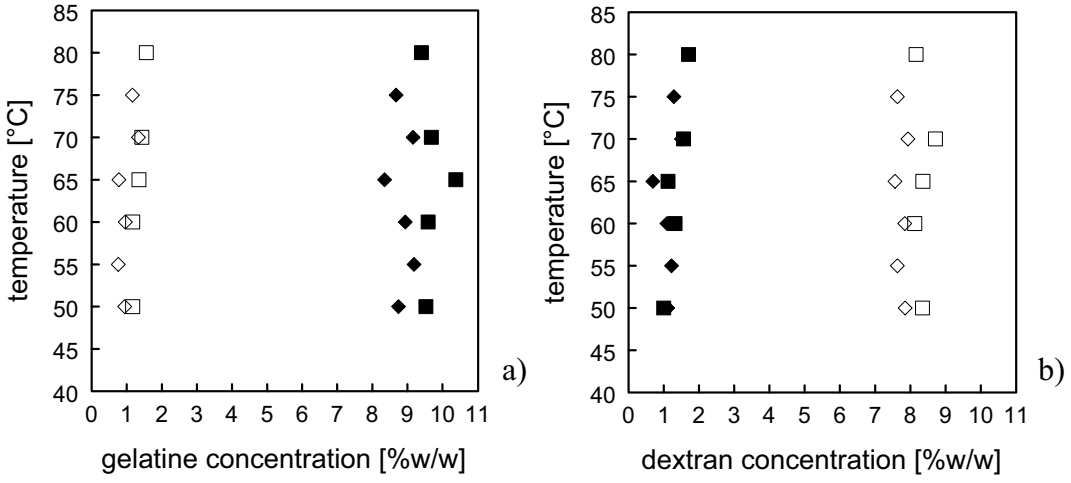

Figure 2.1: Coexisting phase compositions after full phase separation in 5% (w/w) gelatine/5% (w/w) dextran/0.1 M NaCl for dextran of molar mass 282kDa (◆) and 148kDa (■). Open symbols: dextran-rich phase, closed symbols: gelatine-rich phase. a) gelatine concentrations and b) dextran concentrations.

In figure 2.3, examples are shown of the dependence of the phase transition temperature on the total polymer concentration of mixtures with a constant mass ratio of gelatine and dextran (1:1). A very sharp dependence of the transition temperature on the overall polymer concentration is found at temperatures well above the gelation temperature of gelatine. In contrast, in the lower temperature regime, in which gelatine gels, the transition temperature is hardly affected by the total polymer concentration. The inflexion point is found at higher total concentration for lower molar masses of dextran. However, the temperature of the inflexion point is always 32°C. In other words, below 32°C , phase transitions take place in a broad range of concentrations, whereas at higher temperatures,

the phase separation temperature is very hard to determine accurately, because of its extreme sensitivity to the overall concentration.

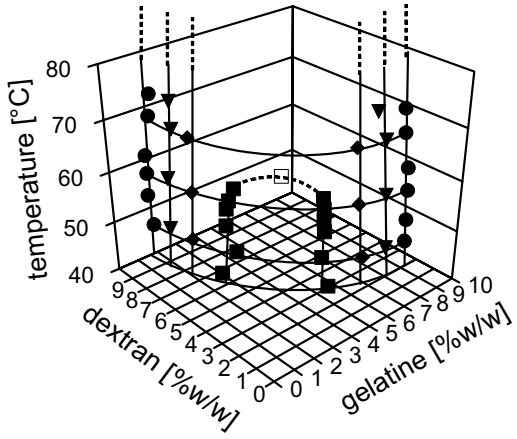

Figure 2.2: Coexisting phase compositions in gelatine/dextran/0.1 M NaCl for a 282-kDa dextran and a 170-kDa gelatine. Overall compositions gelatine/dextran: 3.5%/3.5% (■), 4%/4% (◆), 4.5%/4.5% (▼) and 5%/5% (●) (all (w/w) percentages).

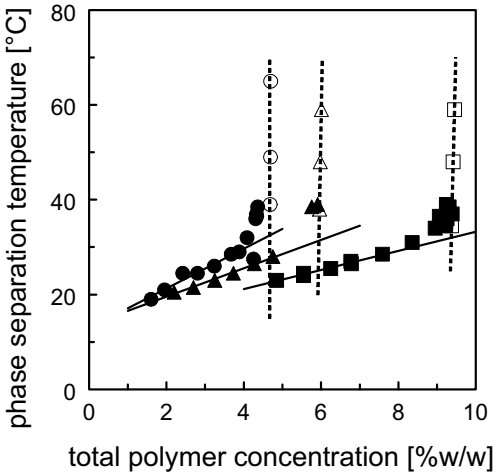

Figure 2.3: Phase separation temperature vs. total polymer concentration. The polymer component is 50% gelatine/50% dextran (w/w). M_w of dextran is 2000 kDa (■), 282 kDa (▲) and 37 kDa (●), M_w of gelatine is 170 kDa. Lines are drawn as an aid to the eye.

In figure 2.4, the volume fraction filled by gelatine-rich phase is plotted against the mass fraction of gelatine in the total mass of dissolved polymer. This was done for fully phase separated gelatine/dextran mixtures at 60°C. Because the volume is largely made up of solvent, this method of presenting the data shows the distribution of solvent over the two phases. There are data points for different values of the molar mass of dextran, total concentration, and pH. The molar mass, total concentration and pH were found to have no

effect on the distribution of solvent over the phase volumes. Temperatures of 70°C and 50°C (data not shown) give very similar results. The general conclusion from figure 2.4 is that, whatever the molar mass, temperature, pH, total concentration, the same phase volume ratio is established, as determined by the dry gelatine/dextran weight ratio.

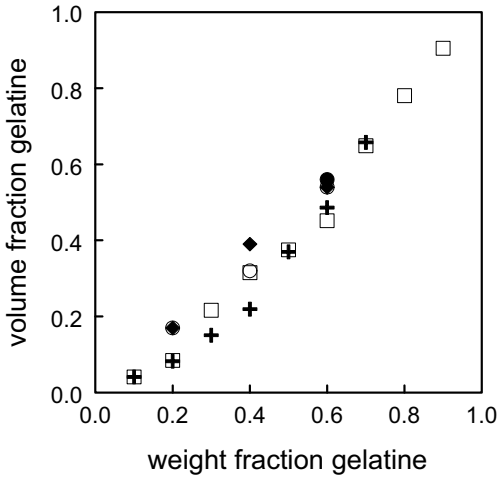

Figure 2.4: Volume fraction filled by the gelatine-rich phase versus weight fraction of gelatine in the dry solute (gelatine + dextran). Total concentration of polymer equals 20% (+, □, ○, ◆), $M_{w,dextran}$ is 148 kDa (+), $M_{w,dextran}$ is 2000 kDa (□, ○, ◆, ●), pH 6 (+, □, ●), pH 5.6 (◆) and pH 6.5 (○).

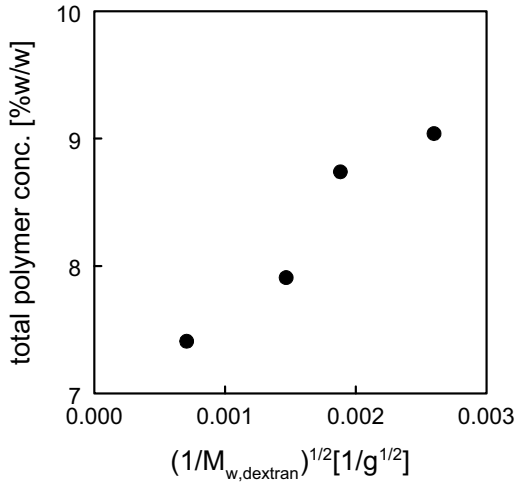

Figure 2.5: Total polymer concentration above which phase separation takes place vs. $M_{w,dextran}^{-1/2}$. Temperature = 70°C. Gelatine/dextran dry mass ratio 50%/50%, M_w gelatine = 170 kDa.

The effect of dextran molar mass is demonstrated in figure 2.5, in which at a fixed temperature, fixed 50/50 gelatine/dextran dry mass ratio, and fixed gelatine molar mass the total phase transition concentrations are plotted against $M_w^{-½}$ of dextran. A linear

dependence is found, which is in line the mean field prediction [20]. The number of data points and the polydispersity do not allow for a discrimination of between the mean field exponent of $^1/_2$ and the strong segregation prediction of $^4/_5$. It is, however, clear that concentration induced phase separation shows no obvious anomalous behaviour as a function of dextran molar mass.

The size exclusion chromatography multiangle laser light scattering (SEC-MALLS) results for the molar mass distribution of the gelatine used here are depicted in figure 2.6. These results were obtained under non-denaturing conditions (0.1 M $LiNO_3$, pH 6.7), similar to those in the phase separating systems studied in this work (0.1 M NaCl). The molar mass distribution shows two peaks, which become more equal in height as the temperature is decreased from 80°C to 40°C. This bimodal distribution has maxima at values of the molar mass that differ by a factor of 2. Considering the existence of an isobestic point, dimerization was found to occur to some extent. Special care was taken to ensure that all material injected was recovered at the end of the column. Doubling of the molar mass of gelatine on cooling above the gelation temperature was observed before [22] by static light scattering and low-shear viscometry. The increase in molar mass was shown to be reversible: the details of the reversibility will be the subject of a future publication.

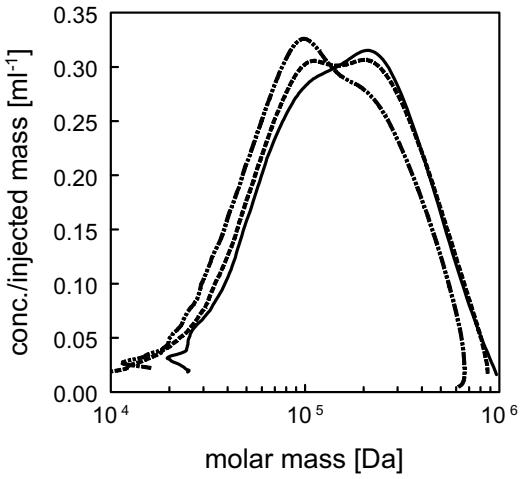

Figure 2.6: SEC-MALLS molar mass determination of gelatine at various temperatures. At 40°C (—), 60°C (- -) and 80°C(— ··). Detection by refractive index.

In table 2.1, the values of M_w, the mass average molar masses, are listed as a function of temperature. Included in table 2.1 are the values of α in the relation

$$R_g = b\left(\frac{M_w}{M_{st}}\right)^{\alpha} \tag{2.3}$$

where R_g is the radius of gyration, b the statistical length, and M_{st} the mass of this length. R_g could only be reliably determined above M=200 kDa. Below this value R_g was smaller than 10 nm.

Table 2.1: Molar mass, the parameter α of equation (2.3) of gelatine and the SEC performance at various temperatures. The statistical error in M_w is estimated at 5%, in α at 10%.

T [°C]	M_w [kDa]	α	recovery [%]
40	177	0.36	97
50	184	0.37	99
60	175	0.43	99
70	167	0.44	100
80	147	0.47	100

An example of the fractionation caused by phase separation is shown in figure 2.7. Here, the molar mass distributionsof gelatine and dextran in both coexisting phases are shown. Strong fractionation occurs, especially for dextran. The molar mass of dextran in the dextran-rich and dextran-poor phases differs by a factor of roughly 6. For gelatine, this difference is a factor of about 3. Both components have about the same average mass in their poor phases.

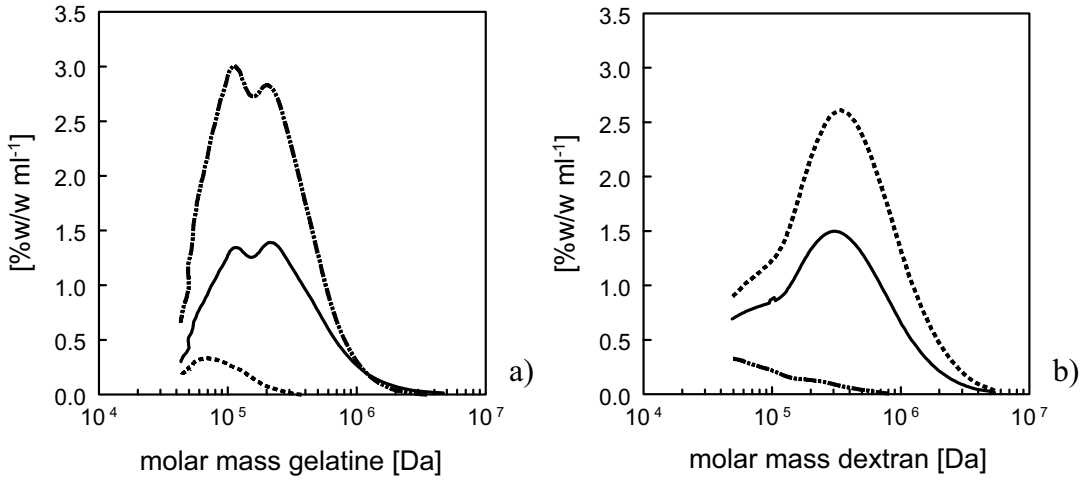

Figure 2.7: Molar mass distribution of a) gelatine and b) dextran in a fully phase separated 5%/5% (w/w) gelatine/dextran mixture at 50°C. Native material prior to phase separation (—), dextran-rich phase (- -) and gelatine- rich phase (— ··).

2.4 Discussion

The present work is an exploration of the incompatibility of gelatine and dextran at temperatures above the gelation temperature of gelatine. Below this temperature, phase separation is driven by gelatine ordering and found to be correlated to the extent of helicity of gelatine [12]. Gelation of gelatine acting as a strong promoter of phase separation is confirmed by figure 2.3: at phase transition temperatures below the gelation temperature of gelatine (25-30^{o}C), the total concentration for which phase separation is found might be as low as 1%, whereas above the gelation temperature of gelatine, phase separation occurs only above about 4%.

The effect of polydispersity is significant, as is seen in figure 2.7. Especially for dextran, material in its poor phase has little in common with that in its rich phase. The width of the coexistence regions in figures 2.1 and 2.2 at fixed temperature is dominated by polydisperisity, because material in its poor phase originates from low molar mass wing of the parent distribution. This wing contains molecules that are hardly involved in the phase separation but are still detected in the determination of the composition. The material in its rich phase is nearly the same as the starting material. This should be an important consideration in a quantitative analysis of the effect of polydispersity on phase composition. Such an analysis is outside the scope of the present work. It is the subject of a considerable amount of literature [23-29].

A surprising observation in figures 2.1 and 2.2 is the total absence of temperature dependence in the phase compositions in the range 40° to 80^{o}C. Even at 3.5%/3.5%, which is the only composition for which phase separation occurs on cooling rather than on concentrating the system (within the temperature window between gelation of gelatine and breakdown of gelatine), no temperature dependent width of the coexistence region was found. From the homogeneous state at 62^{o}C, the 3.5%/3.5% system splits into two phases at 60^{o}C which stay the same on cooling to 40^{o}C. Phase compositions of 4%/4%, 4.5%/4.5% and 5%/5% are unaffected by temperature between 40^{o}C and 80^{o}C. This insensitivity to temperature of the phase compositions is in accordance with the absence of a temperature dependence in the phase volumes. It is tempting to relate this temperature independence of phase properties to the extremely steep dependence of the phase separation temperature on the total polymer concentration. This steep dependence is directly seen in figure 2.3 and more indirectly in figure 2.2, where closing of the miscibility gap at 62^{o}C is observed for the 3.5%/3.5% composition, but no closing of the gap is seen at all below 75^{o}C for the slightly higher concentration of 4%/4%. Therefore, the phase transition appears to be determined by the concentration and hardly at all by the temperature. This observation suggests an entropic driving force for phase separation. The transition observed here is reminiscent of the order-disorder transition in lyotropic liquid

crystals and crystallization in hard sphere systems, which are driven by concentration changes.

Recently, a model for entropically driven phase separation has been proposed by Odijk [18,19]. It is based on the assumption that depletion interactions dominate phase stability, and it applies to mixtures of small protein particles in a semidilute polysaccharide solution. A large size difference between gelatine and dextran chains exists only for a part of the molar mass distributions of both components. However, as will be shown, this fact does not appear to affect the agreement between the model prediction and the data. Odijk's theory was found to be successful in the cases of β-lactoglobuline/pullulan, α-lactoalbumine/pullulan and several other comparable systems [19]. Starting from the observation of de Gennes [30] that the depletion of polymer segments around a small hard sphere in a semidilute polymer solution has a length scale of the order of the radius of the sphere, Odijk [18,19] introduced the following expression for the Helmholtz free energy of mixing F

$$\frac{F}{k_B TV} = \rho \ln \rho - \rho + K\rho\varphi + c_1 \xi_1^{-3} \varphi^{\alpha} \tag{2.4}$$

where k_B denotes Boltzmann's constant, T is the temperature, V is the volume, ρ is the concentration of protein particles, φ is the volume fraction of polysaccharide, and c_1 is a numerical constant. Here, use has been made of the correlation length given by $\xi = \xi_1 \phi^{-\alpha/3}$, where ξ_1 is a constant, $\alpha = {}^9/_4$ for a good solvent and

$$K = \frac{8\ pa}{3d} \tag{2.5}$$

which is not dependent on protein or polysaccharide concentration and which is a measure of the degree of incompatibility of the small protein particles and the semidilute polysaccharide solution. p denotes the persistence of the polysaccharide chain ($p = A_K / d$, A_K is Kuhn's length), d is the chain thickness and a is the radius of the protein particle.

It can then be derived from the equality of the chemical potentials of protein in the coexisting phases that

$$\ln\left(\frac{\rho_1}{\rho_2}\right) = d_m \frac{v_{mol} K}{M_{mol}} (\varphi_{m,2} - \varphi_{m,1}) \tag{2.6}$$

where subscripts 1 and 2 refer to coexisting phases 1 and 2 respectively, ρ_i is the volume fraction of gelatine in phase i, d is the mass density, M_{mol} is the molar mass of dextran, v_{mol} is the molar volume of dextran, and $\varphi_{m,i}$ is the mass fraction of dextran in phase i. From replotting figure 2.2 (figure 2.8) according to equation (2.6) it can be seen that our data are consistent with the above equation [30], i.e. the interaction constant K is independent of concentration.

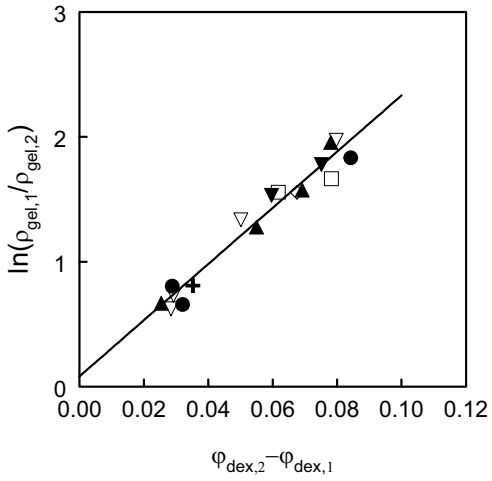

Figure 2.8: Logarithm of the ratio of gelatine mass fractions vs. the difference in dextran mass fractions in coexisting phases of phase separated aqueous mixtures of gelatine and dextran. For 45°C (+), 50°C (▲), 55°C (●), 60°C (∇), 62°C (◇), 70°C (□) and 75°C (▼). The slope of the best linear fit (line) is 22.4 (After Odijk [18,19])

The reason why temperature has so little influence, both on phase compositions and on the total phase separation concentration, remains to be explained. Despite the fact that depletion appears to be important, temperature is expected to play a role in the phase composition and the total concentration at which phase separation occurs. In other words, concentration-induced phase separation close to the phase boundary should be counteracted by increase of temperature. This is manifestly not the case, as shown in figure 2.3. Presently, the explanation of the absence of temperature dependence is unclear. We propose that it may be related to co-operative clustering of gelatine molecules increasing the value of K in equation (2.4). Clustering is tentatively concluded from the SEC-MALLS results in figure 2.6, which show that, under non-denaturing circumstances (i.e. no additives such as SDS, that disrupt physical bonds) significant clustering takes place at temperatures well above the melting temperature of gelled gelatine. The observation of clustering is consistent with the low values of α in equation (2.3) (See table 2.1, measured at 0.2% (w/w)). Values below 0.5 indicate chains more compact than random walks, unstable in the dissolved state and, therefore, with a tendency to cluster. This clustering is

suggested as an explanation for the absence of temperature dependence when this clustering proceeds towards large - possibly percolating - transient structures, the size of which would strongly enhance phase separation from dextran. The formation of these structures would then have to be co-operative in order not to be destroyed by a temperature increase.

The dependence of the phase transition temperature on the total concentration in mixtures of maltodextrin and gelatine and maltodextrin and iota-carrageenan was recently reported [13,31]. Qualitatively, the same picture as in figure 2.3 was obtained: a weak dependence on concentration below the ordering transition of either gelatine or carrageenan, with a transition to a steeper dependence above this transition. In these cases, however, the dependence in the disordered regime is much less extreme than in the case of gelatine/dextran. The most important difference between maltodextrin and dextran with respect to phase separation is expected to be the molar mass. M_n of maltodextrin was 9 kDa, whereas the data in figure 2.3 were obtained for dextrans of much larger molar mass. The weaker concentration dependence of the phase separation temperature in the case of maltodextrin is not unexpected if phase separation is strongly influenced by the exclusion of foreign polymer by self-associating gelatine chains. The lower the molar mass of the foreign polymer (maltodextrin, dextran) the more soluble it will be in an environment of clustering gelatine.

2.5 Conclusions

The temperature-composition phase diagrams of aqueous mixtures of gelatine and dextran have been explored. Experimental results indicate that polydispersity leads to significant fractionation, more so for dextran than for gelatine. The data on overall phase composition suggest consistency with predictions based on a recently developed depletion model by Odijk [18,19]. We have also observed clustering of gelatine to occur well above its gelation temperature. In view of the depletion model, this clustering is thought to play a role in the phase stability as a function of concentration. The clustering may eventually lead to - possibly percolating - transient clusters of gelatine, enhancing phase separation. The phase diagrams, together with the consistency with the depletion model, suggest a temperature independent steric incompatibility of dextran with transient, thermally reversible, gelatine clusters.

2.6 Acknowledgement

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Chapter 3

Phase separation induced fractionation in molar mass in aqueous mixtures of gelatine and dextran*

ABSTRACT

An overview of the effects of phase separation of aqueous mixtures of gelatine and dextran on the fractionation in molar mass of these two components is given. Molar mass distributions in coexisting phases were investigated using Size Exclusion Chromatography with MultiAngle Laser Light Scattering. The initial molar mass of the native material, concentration and temperature were varied. The results show a strong fractionation in molar mass for both components. The molar mass of the native material and concentration appeared to be the only factors that affected the final molar mass distributions, temperature having no effect. The results show that in the molar mass range where fractionation is the strongest, i.e. roughly below the maximum in the distribution, fractionation is governed by a Boltzmann factor $e^{-\Delta G/kT}$*, where* ΔG *denotes the free energy involved in transferring a polymer with a certain length from the enriched to the depleted phase, and in this case turns out to be proportional to the molar mass. Comparison of the results of phase separation with results on dialysis shows that water affinity is not the driving force for the phase separation of gelatine and dextran in aqueous solution. The gelation properties of gelatine in both phases were also determined. The gelation properties of the gelatine in the coexisting phases differ from those of native gelatine. In particular, the gelatine in the gelatine-poor phase shows strong differences compared to the native material.*

* *Physical Review E*, **67**(2) 2003

3.1 Introduction

At sufficient concentration (above 3% (w/w) of each component), segregative phase separation takes place in aqueous mixtures of gelatine and dextran. This phase separation results in a gelatine-rich and a dextran-rich phase. Such phase separation processes are often the basis for structuring processed foods. In the literature, a large quantity of experimental data is available on the compatibility of food biopolymers [1]. Often biopolymers, certainly extracellular bacterial and cell-wall biopolymers, used in food products are polydisperse in their molar mass. Due to this polydispersity, fractionation in molar mass takes place during phase separation. This results in different molar mass distributions in the coexisting phases relative to the original mixture. The denser phase favours larger particles [2]. Fractionation was studied in the past, e.g. in emulsions [3] and in mixtures of β-lactoglobulin aggregates and κ-carrageenan [4]. However, quantitative theories only exist for a narrow molar mass distribution [5].

In spite of the presence of fractionation, in several studies the mechanical properties of the mixed gels are compared with [6-9] or even calculated with [10] the properties of the native material. We show that the functional properties, such as gel strength and specific viscosity, of the material in both phases differ from each other and from the native material.

The work presented here gives an insight into the fractionation of the molar mass due to phase separation of aqueous mixtures of gelatine and dextran in different conditions. The initial molar mass distributions, concentration and equilibrium temperature were varied. The system gelatine/dextran was chosen because of its good experimental accessibility. We note that in this paper phase separation always took place at temperatures above the gelation temperature of gelatine (≈ 30-35°C), in order to enable full equilibrium phase separation. So in this paper we do not consider cases of phase separation in conjunction with gelation.

The role of the low molecular mass part of the distribution in the establishment of an osmotic pressure equilibrium was studied using dialysis experiments, in which the phase separation was imposed by a semi-permeable membrane.

3.2 Experimental

3.2.1 Materials

Porcine skin gelatine (IEP ± 8.7, bloom 305 g, $M_w \approx 170$ kDa) was kindly provided by Degussa Biosystems (Centre de Recherches 50500 Baupte, France). Gelatine with M_w of 43 kDa (bloom 93 g) and 74 kDa (bloom 281 g) were kindly provided by DGF Stoess, Germany. Dextran with M_w of 148 kDa and 282 kDa was purchased from Sigma

Chemicals. The ingredients were used without further purification. Solutions were prepared by gravimetrically adding solvent (0.1 M NaCl and 0.02% NaN_3 to prevent bacterial growth) to the proper amount of material. Dextran dissolves readily at room temperature. Gelatine was dissolved by stirring the mixture over night on a magnetic stirrer at approximately 50°C.

3.2.2 Determination of the temperature–composition phase diagram

To determine the temperature–composition phase diagram, mixtures of equal weight concentration of gelatine and dextran in 0.1 M NaCl were made. For this purpose two equal weights of a dextran solution and a gelatine solution with the same weight percentage polymer were mixed. Approximately 10 gram of the mixtures was put into a plastic tube. To prevent evaporation of the solvent, paraffin oil was added on the top of the meniscus. The tubes were held in a water bath at a temperature above the gel temperature of gelatine for approximately 20 hours to reach equilibrium of the phase separation. Equilibrium was assumed to be reached when a sharp meniscus between transparent gelatine-rich and dextran-rich fluid layers was observed. Hereafter, the heights of the gelatine-rich and dextran-rich phase were measured to calculate the volume of the phases. Samples of these phases were taken with a syringe with hypodermic needle.

To determine the concentration of gelatine and dextran in the coexisting phases, samples taken from the gelatine-rich and dextran-rich phases were diluted 40 times in 0.1 M NaCl. Optical rotation was measured at two different wavelengths (365 and 578 nm) at 80°C. With the use of calibration lines, the concentrations in the diluted phases were calculated by solving the system:

$$\begin{aligned} \alpha_{meas}\left(c_g, c_d, \lambda = 365nm\right) &= [\alpha]_{g,\lambda=365\,nm} c_g + [\alpha]_{d,\lambda=365\,nm} c_d \\ \alpha_{meas}\left(c_g, c_d, \lambda = 578nm\right) &= [\alpha]_{g,\lambda=578\,nm} c_g + [\alpha]_{d,\lambda=578\,nm} c_d \end{aligned} \tag{3.1}$$

where subscripts *meas*, *g* and *d* mean measured, gelatine and dextran. $[\alpha]$ is the specific rotation (per % (w/w)) at a definite wavelength. Calibration lines to obtain $[\alpha]_g$ and $[\alpha]_d$ were obtained in the range from 0 to 1% (w/w) at 80°C and at the two different wavelengths. This method is valid only if gelatine and dextran contribute to the optical rotation proportionally to their concentration. To probe this simple additivity of the contributions to the optical rotation in a mixture, for each wavelength the optical rotation of five symmetrical mixtures (i.e. solute consisting of 50% gelatine and 50% dextran) of gelatine and dextran was measured and compared with that of the sum of the pure components. The results were in satisfactory agreement with the assumption of additivity.

The weight of the phases was derived from the height of the phases. To calculate the weight fraction of a phase, the approximation was made that both phases had the same density.

3.2.3 Dialysis of gelatine solution against dextran solution

Dialysis tubing with a pore size of 12-14 kDa was first boiled in water. Two solutions were made, one of 8.0% (w/w) gelatine in 0.1 M NaCl and one of 8.0% (w/w) dextran (282 kDa) in 0.1 M NaCl. 10 gram of the gelatine solution was put in the dialysis tubing. This tubing was immersed in 500 gram of the dextran solution for 20 hours. The gelatine as well as the dextran concentrations were measured.

3.2.4 Determination of the molar mass distribution of gelatine and dextran

Size exclusion chromatography (SEC) equipped with a multiangle laser light scattering (MALLS) detector and a refractive index (RI) detector was used to determine the molar mass distribution of gelatine and dextran. For mixtures of gelatine and dextran, an additional detector monitoring optical rotation (OR) was applied at 365 nm. By combining the signals from the RI detector and the OR detector, the contribution from dextran and gelatine to the two signals can be unravelled using the set of equations in equation (3.1). The only difference is that $[\alpha]_g$ and $[\alpha]_d$ at λ=578nm were replaced by the refractive index increments of, respectively, gelatine and dextran. Thus:

$$\begin{aligned} \alpha_{meas}\left(c_g, c_d, \lambda = 365\ nm\right) &= [\alpha]_{g,\lambda=365\ nm}\, c_g + [\alpha]_{d,\lambda=365\ nm}\, c_d \\ \Delta n_{meas}\left(c_g, c_d\right) &= \left(\frac{dn}{dc_g}\right) c_g + \left(\frac{dn}{dc_d}\right) c_d \end{aligned} \tag{3.2}$$

with Δn the difference relative to the buffer solution and (dn/dc_i) the refractive index increment due to concentration c_i of species i. The values of (dn/dc_i) in the buffer used were 0.159 for gelatine and 0.130 for dextran.

A $LiNO_3/KH_2PO_4/K_2HPO_4$ (pH 6.7) buffer was used as eluent. The flow rate was 1 ml min^{-1}. The columns that were used were a combination of TSK guard + TSK G5000PW + TSK G3000PW. Typically 4 mg of dry material in 200 µl was injected, resulting in a concentration of 0.2% (w/w) in the detector cells.

For the determination of the molar mass distribution of gelatine and dextran, samples were taken from the gelatine-rich and dextran-rich phases after equilibration of ca. 20 hours (see table 3.1). In addition to these mixtures, also the pure components were determined. All samples from the coexisting phases were diluted 40 times in the eluent and put in vials. Before detection, the vials were stored for 30 minutes at 80°C. The

temperature of the SEC column and the MALLS detector was 50°C. The OR detector cell had a temperature of 40°C and the RI detector cell was not temperature controlled.

Table 3.1:Overview of the samples studied. (concentrations in [% (w/w)], temperature in [°C]).

sample code	conc. gelatine 170 kDa	conc. gelatine 43 kDa	conc. gelatine 74 kDa	conc. dextran 148 kDa	conc. dextran 282 kDa	T_{ps} [a]
gel 170	5.0					-
mix gel		2.5	2.5			-
dex 148				5.0		-
dex 282					5.0	-
mix dex				2.5	2.5	-
A1	5.0				5.0	50.0
A2	5.0				5.0	60.0
A3	5.0				5.0	70.0
B	4.5				4.5	60.0
C	4.0				4.0	60.0
D	5.0			5.0		60.0
E	5.0			2.5	2.5	60.0
F		2.5	2.5		5.0	60.0

[a] T_{ps} = temperature at which phase separation was made to take place.

The expressions used to calculate the molar mass averages are the following:

$$M_n = \frac{\sum_i c_i}{\sum_i \frac{c_i}{m_i}} \tag{3.3}$$

$$M_w = \frac{\sum_i c_i m_i}{\sum_i c_i} \tag{3.4}$$

$$M_z = \frac{\sum_i c_i m_i^2}{\sum_i c_i m_i} \tag{3.5}$$

with c_i in g ml^{-1}, the concentration of molecules with molar mass m_i. The polydispersity is defined as (M_w/M_n).

3.2.5 Determination of the mechanical properties of gelatine

In order to measure the mechanical properties of gelatine in the two phases, gelatine has to be extracted from these phases. For this purpose a mixture of 5.0% (w/w) gelatine (M_w 170 kDa) and 5.0% (w/w) dextran (M_w 282 kDa) in 0.1 M NaCl was left to phase separate for 20 hours at 60°C. Samples of the upper and lower phases were taken. The sample of the dextran-rich phase was cooled down so that phase separation continued and gelled droplets of gelatine were formed. This phase was centrifuged (60 min, 12,000 g) until the gelled gelatine droplets had formed a precipitate. The concentrations of gelatine and dextran in this precipitate and in the upper phase were measured using polarimetry. Both contained less than 1% (w/w) dextran. The upper phase as well as the precipitate were diluted with 0.1 M NaCl to a gelatine concentration of 2% (w/w). A solution of 10% (w/w) native gelatine in 0.1 M NaCl was made and stored for 20 hours in a water bath at 60°C. This solution was also diluted to a concentration of 2% (w/w) gelatine. Small-deformation oscillatory measurements were performed on a strain controlled rheometer (Rheometrics, Fluids Spectrometer RFS II) using a cone and bob system at 1% strain and 1 Hz. First, the gelatine solution was cooled from 50 to 15°C. After that, the temperature was held at 15°C for 1 hour and the ageing of the gel was followed. Finally a strain sweep was performed between 1 and 100% strain.

3.3 Results

3.3.1 Phase diagram

Figure 3.1 is the temperature–composition phase diagram of mixtures containing 5.0% (w/w) gelatine and 5.0% (w/w) dextran, for dextran molar masses of 148 and 282 kDa. There turns out to be no significant effect of either the temperature or the dextran molar mass on the phase composition in the temperature region that was studied. It is expected that lowering the initial total polymer concentration will bring the system closer to the miscible state. In other words, by diluting the system, the phase compositions should become more similar. This is indeed observed in figure 3.2. In this figure is also indicated the only composition (3.5% (w/w)/3.5% (w/w)) for which a temperature induced phase transition could be observed in the temperature range between the gelation of gelatine (ca. 30°C) and 80°C, above which gelatine decomposes.

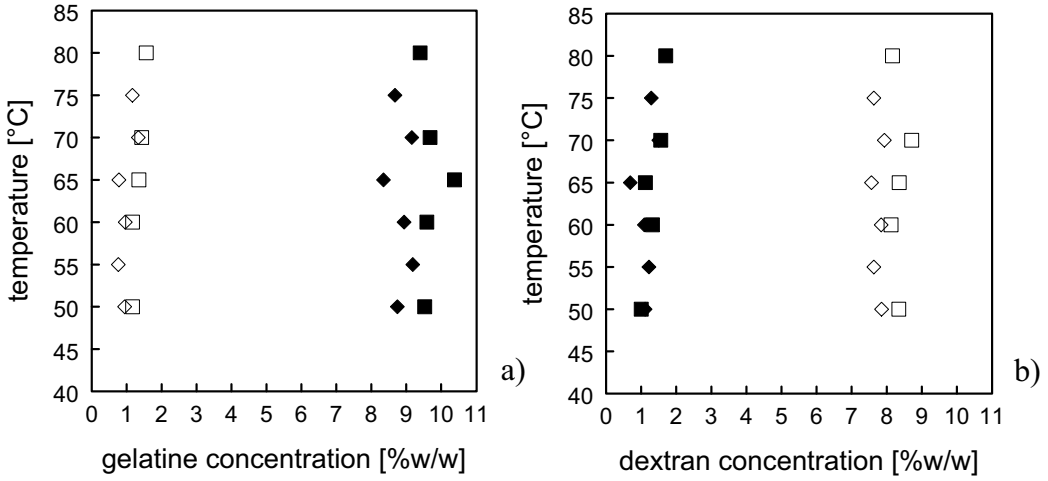

Figure 3.1: Coexisting phase compositions in gelatin/dextran/0.1 M NaCl for M_w of dextran 148 kDa (■) and 282 kDa (◆). a) gelatine concentrations and b) dextran concentrations. Initial compositions gelatine/dextran: 5% (w/w)/5% (w/w). Open symbols represent dextran-rich phase, closed symbols represent gelatine-rich phase.

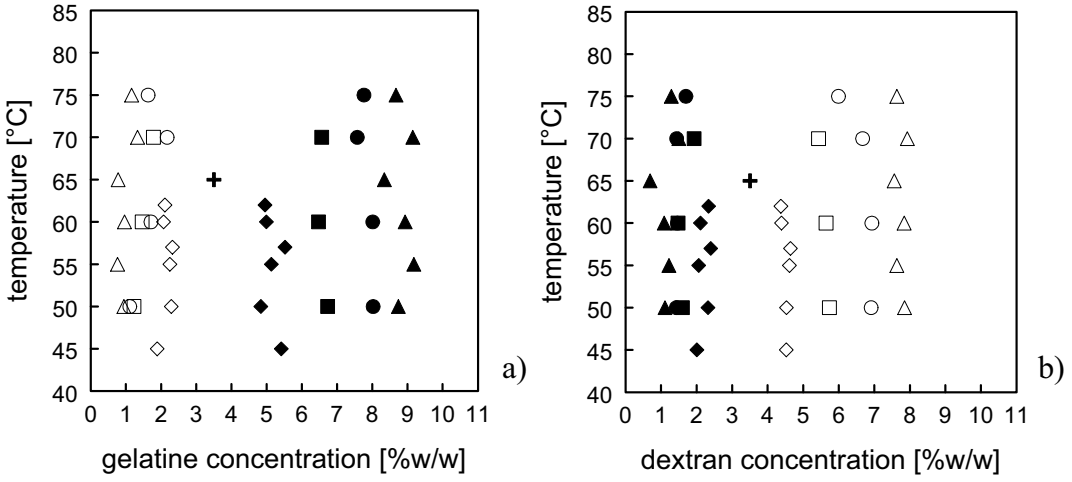

Figure 3.2: Coexisting phase compositions in gelatin/dextran/0.1 M NaCl for M_w of dextran 282 kDa. a) gelatine concentrations, b) dextran concentrations. Initial compositions gelatine/dextran: 3.5%/3.5% (◆), 4%/4% (■), 4.5%/4.5% (●) and 5%/5% (▲) (all percentages (w/w)). Open symbols represent dextran-rich phase, closed symbols represent gelatine-rich phase. + temperature where the mixture 3.5% gelatine and 3.5% dextran did not show phase separation anymore.

Figure 3.3 shows the composition–composition phase diagram for a range of temperatures above the gelation temperature of gelatine. This figure also shows the temperature independence of the phase diagram.

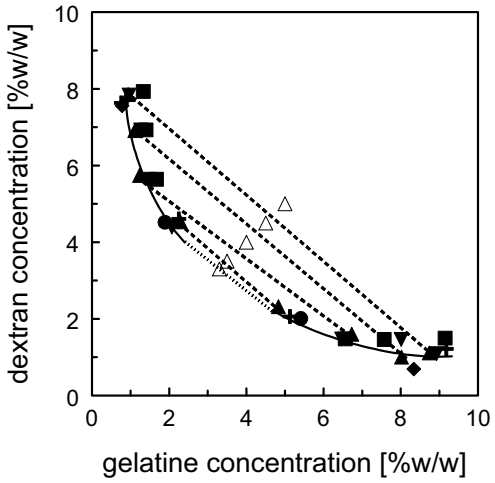

Figure 3.3: Composition – composition phase diagram of gelatine and dextran 282 kDa in 0.1 M NaCl. The closed symbols represent the coexisting phases at 45°C (●), 50°C (▲), 55°C (+), 60°C (▼), 65°C (◆) and 70°C (■). The open triangles represent the initial mixtures at 50°C.

3.3.2 Molar mass distributions

An overview of the molar masses of the pure components is given in table 3.2. Figure 3.4 to 3.7 show the molar mass distributions of gelatine and dextran in coexisting phases, as well as the distributions prior to phase separation. The curves are normalised to the concentration of the original sample (using the elution volume as the quantity on the horizontal axis) i.e. the areas under the curves are equal to the concentrations in the coexisting phases. The area under the curves prior to phase separation of gelatine and dextran is 5.0% (w/w), i.e. the overall concentration in the phase separating mixture. From the SEC-MALLS data of the pure components, the elution volume was converted to the molar mass of each component. The lowest molar mass which could be reliably detected was 40 kDa.

Molar mass effects

Comparing the phase diagrams in which the molar mass of dextran is varied (figure 3.1), it can be concluded that the molar mass of dextran hardly influences the composition of the phases. Figure 3.4 shows the influence of the molar mass of dextran on the molar mass distribution of the two components in the two phases.

Table 3.2: M_n, M_w and M_z and the polydispersity for (a) gelatine and (b) dextran for the native material and the material in the different phases for the different mixtures (concentrations in [% (w/w)], molar mass averages in [kDa]).

(a) gelatine

	GELATINE IN GELATINE-RICH PHASE					GELATINE IN DEXTRAN-RICH PHASE				
	conc. gelatine	M_n	M_w	M_z	$\frac{M_w}{M_n}$	conc. gelatine	M_n	M_w	M_z	$\frac{M_w}{M_n}$
gel 170		97	184	422	1.9		97	184	422	1.9
mix gel		69	147	379	2.1		69	147	379	2.1
A1	10.2	105	201	534	1.9	1.0	62	72	86	1.2
A2	10.2	97	175	410	1.8	1.2	62	74	92	1.2
A3	9.7	87	150	359	1.7	1.4	59	67	79	1.1
B	8.2	98	183	472	1.9	1.4	68	86	119	1.3
C	6.7	102	200	549	2.0	1.8	75	102	154	1.4
D	10.4	96	169	353	1.8	1.3	62	73	94	1.2
E	10.2	97	180	265	1.9	1.2	61	71	86	1.2
F	10.8	76	145	304	1.9	1.8	46	57	76	1.2

(b) dextran

	DEXTRAN IN GELATINE-RICH PHASE					DEXTRAN IN DEXTRAN-RICH PHASE				
	conc. dextran	M_n	M_w	M_z	$\frac{M_w}{M_n}$	conc. dextran	M_n	M_w	M_z	$\frac{M_w}{M_n}$
dex 148		60	146	391	2.4		60	146	391	2.4
dex 282		64	299	993	4.7		64	299	993	4.7
mix dex		63	261	915	4.1		63	261	915	4.1
A1	1.2	25	49	109	1.9	7.9	73	332	1020	4.5
A2	1.2	28	58	135	2.1	7.8	73	348	1050	4.8
A3	1.3	26	56	150	2.1	7.9	73	328	1000	4.5
B	1.4	29	80	231	2.8	8.0	75	382	1120	5.1
C	1.6	33	107	312	3.3	7.9	76	400	1160	5.3
D	1.4	52	130	416	2.5	7.5	62	73	94	1.2
E	1.4	29	65	157	2.3	7.7	96	250	821	3.6
F	2.2	31	128	542	4.1	6.5	65	330	1050	5.1

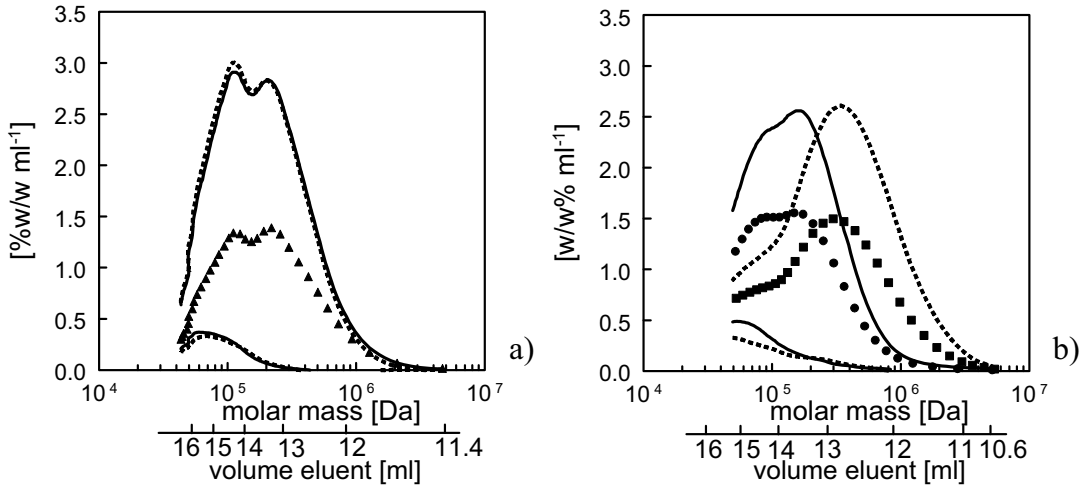

Figure 3.4: The effect of dextran molar mass on the molar mass distributions in coexisting phases at 60°C. a) gelatine and b) dextran. Lines upper set: rich phase, lines lower set: poor phase. Sample A2 (- -) and sample D (—). Symbols: native material. Gelatine 170 kDa (▲), dextran 148 kDa (●) and dextran 282 kDa (■). Overall concentration of both gelatine and dextran is 5% (w/w).

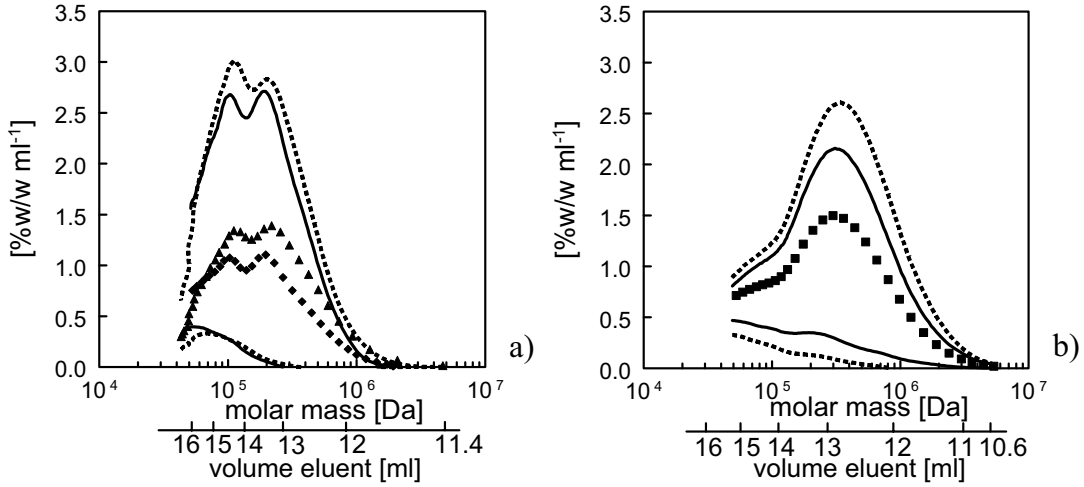

Figure 3.5: The effect of gelatine molar mass on the molar mass distributions in coexisting phases at 60°C. a) gelatine and b) dextran. Lines upper set: rich phase, lines lower set: poor phase. Sample A2 (- -) and sample F (—). Symbols: native material. Gelatine 170 kDa (▲), mixture gelatine 43 kDa and gelatine 74 kDa (◆) and dextran 282 kDa (■).

Let us first look at the results for gelatine in both phases (figure 3.4a, table 3.2a). Figure 3.4a shows that the molar mass of dextran has no influence on the distribution of gelatine in both phases. From the data for the gelatine-rich phase in table 3.2a the same conclusion can be drawn.

The results for dextran in the dextran-rich phase show a downward shift of the molar mass distribution and the average molar mass values with decreasing initial molar mass of dextran. The opposite happens in the dextran-poor phase: all values of dextran increase with decreasing initial dextran molar mass. In table 3.2b it can be seen that the average

molar mass values of the mixture of dextran in sample *E* lie between the values of samples *A2* (dextran 282 kDa) and *D* (dextran 148 kDa).

Table 3.2 also gives the results for mixture *F*. From table 3.2 it is clear that the mixture of two gelatines that is used in this sample has lower molar mass values than gelatine 170 kDa, which is used in all other samples. Figure 3.5 shows the molar mass distributions of samples *F* (mixture of gelatine) and *A2* (gelatine 170 kDa) before and after phase separation. For gelatine, it turns out that decreasing the molar mass of the native material results in a decrease of the average molar mass in both coexisting phases. Together with this decrease in average molar mass values, the peaks of the distributions in both phases shift to lower molar mass values.

Comparing the molar mass distribution of dextran from sample *F* with that of sample *A2* shows that the distribution in the dextran-rich phase is not affected by the molar mass change of gelatine. The distribution in the dextran-poor phase however, is wider. The same conclusions can be drawn from the data in table 3.2b. In the dextran-rich phase, hardly any changes occur to the dextran molar masses, while in the dextran-poor phase the molar mass of dextran increases with decreasing gelatine molar mass. It is remarkable that the final concentration of dextran in its rich phase decreases and in its poor phase increases compared to that in sample *A2* in which only dextran 170 kDa is used.

Summarising the effects of the molar mass of the two components, it appears that in coexisting phases, gelatine as well as dextran does not affect the molar mass distribution of the other component, nor its average values of the molar mass in the enriched phase of this other component. On the other hand, the molar mass of the opposite component does affect the molar mass in the depleted phase. By increasing the molar mass of component A, the molar mass of component B decreases in its depleted phase. The influence of varying the molar mass of a component on its own distribution is the strongest in its own enriched phase.

Concentration effects

The phase diagram in figure 3.2 shows the effect of diluting the system on the phase composition. It shows that if the total initial polymer concentration decreases, the composition of the phases become more similar. This is reflected in figure 3.6. The area under the curves of gelatine and dextran in their rich phase decreases when the initial concentration polymer decreases. On the other hand, the area under the curves of gelatine and dextran in their poor phase increases on decreasing the initial concentration.

The molar mass distributions of gelatine as well as dextran in their enriched phases hardly change as function of the concentration (see figure 3.6). In contrast, the peak values of the polymers in their depleted phases increase on decreasing the initial concentration.

This shift in peak values results in an increase of the values for M_n, M_w and M_z of all components in all phases. The small increase of the molar mass values of the components in their rich phases is also due to the shift in peak value in the poor phases: the more material with a low molar mass moves to the poor phase, the higher the average molar mass in the rich phase will be.

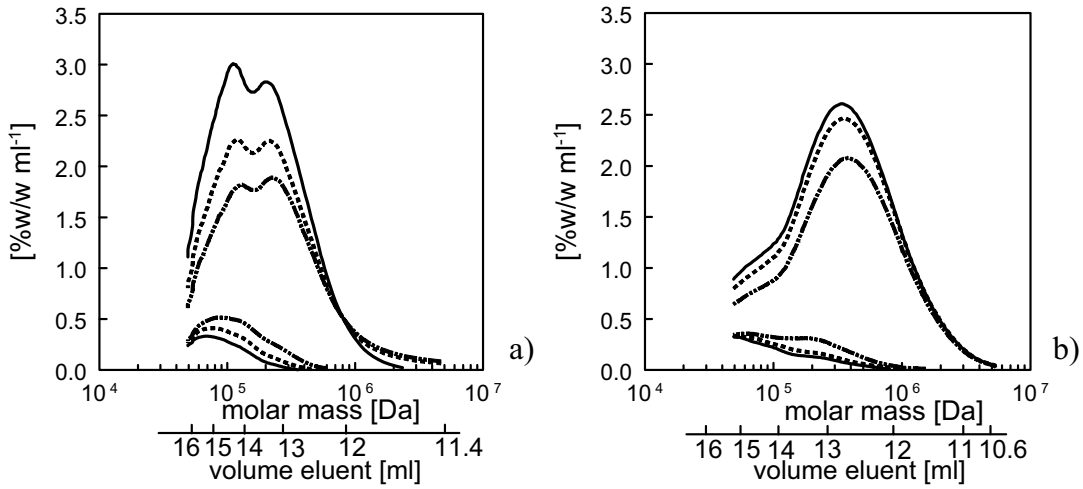

Figure 3.6: Molar mass distributions in coexisting phases at 60°C, for various total polymer concentrations. a) gelatine and b) dextran. Upper set of lines: rich phase, lower set of lines: poor phase. Sample A2 (—), sample B (- -) and sample C (— · ·).

Temperature effects

The results of the fractionation on the molar mass distribution as function of the temperature is given in figure 3.7. For gelatine (figure 3.7a) in the rich as well as in the poor phase a decrease of the molar mass with increasing temperature is observed. This effect is the strongest for the sample at 70°C. This decrease in molar mass is probably due to temperature induced degradation of the gelatine which is a result of the method used. From the literature [11] is known that gelatine shows this degradation and before the samples were taken, the tubes were in a water bath for 20 hours.

For dextran in its rich phase the temperature has no influence on the molar mass distribution and subsequently on the values of the molar mass. In the gelatine-rich phase the molar mass distribution of dextran becomes broader with increasing temperature. The molar mass values also increase slightly with increasing temperature.

Gelatine peak shape

All figures of the molar mass distribution of gelatine show a bimodal distribution of the gelatine in its rich phase, probably corresponding to a monomer – dimer equilibrium [12,13]. Apart from an overall downwards shift, which is probably due to some thermal degradation, the peak also changes with respect to its shape as a function of temperature

and concentration (see figure 3.6a and 3.7a). Decreasing the concentration results in a shift of the peaks to a slightly higher molar mass. It also influences the ratio of the heights of the two peaks. It appears that the lower the concentration, the higher the dimer peak compared to the monomer peak. On the other hand, the molar mass of the gelatine in its depleted phase shifts to the molar mass of the monomer peak. Apparently, the gelatine from the monomer peak in its rich phase shifts to its depleted phase with decreasing starting concentration. This results in a smaller monomer peak and a relatively higher dimer peak in the gelatine-rich phase. Increasing the temperature also increases the amount of material with a lower molar mass in the gelatine-rich phase.

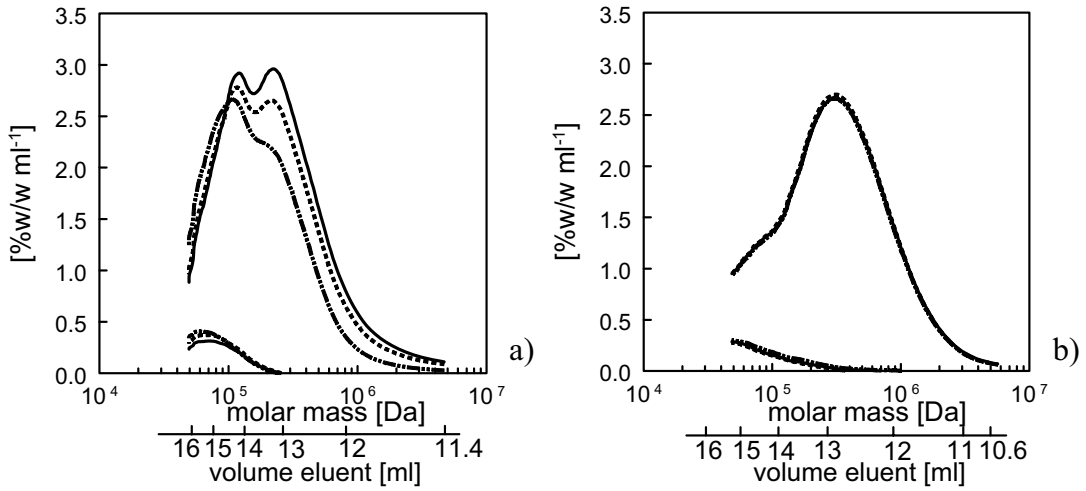

Figure 3.7: The effect of temperature on the molar mass distributions in coexisting phases. a) gelatine and b) dextran. Upper set of lines: rich phase, lower set of lines: poor phase. Sample A1 (—), sample A2 (- -) and sample A3 (— · ·).

3.3.3 Phase separation compared with dialysis

As a result of the phase separation, molar mass distributions become fractionated between the coexisting phases in equilibrium. To investigate the influence of the fractionation on this equilibrium, dialysis experiments were carried out. With use of a dialysis membrane, the water in the system, and not the polymers, was forced to establish the equilibrium. This approach bears on the assumption that the interface between coexisting phases formed by phase separation, can be considered as a semipermeable 'liquid' membrane, permeable to water and small polymers. For the dialysis experiments as well as the phase separation experiments, the water concentrations in both 'phases' were determined. Figure 3.8 shows the results. The initial concentrations were chosen such that the concentrations in the coexisting phases were nearly the same as the concentrations inside and outside the dialysis tube. This figure shows, considering the experimental error of 10% in the determination of the concentration gelatine and dextran, that the suppression

of fractionation does not significantly affect the distribution of water over the two phases, neither does temperature.

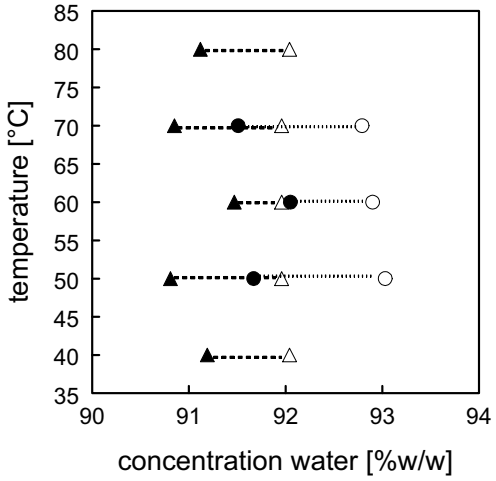

Figure 3.8: Concentration of water after dialysis and phase separation in gelatine-rich (closed symbols) and dextran-rich (open symbols) phases. Dialysis performed with 8 w/w% gelatine against 8 w/w% dextran (▲). Phase separation performed with 4 w/w% gelatine and 4 w/w% dextran (for final polymer concentrations, see Fig. 2) (●). Dotted lines connect final concentrations in coexisting phases.

3.3.4 Mechanical properties of gelatine after phase separation

Figure 3.9 shows the results of the rheological measurements with the native gelatine and the gelatine of both phases.

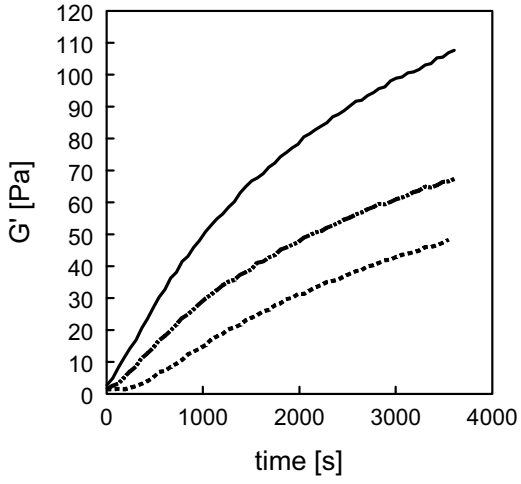

Figure 3.9: G' of the gels made from gelatine after 'quench' to 15°C. Native gelatine (——), gelatine from the enriched phase (— ‥) and gelatine from the depleted phase (- -).

These results show that the gelatine from the enriched phase is able to form a stronger gel than the gelatine from the depleted phase. The reason that the native gelatine forms a

stronger gel than the gelatine from both phases is probably due to the fractionation. This fractionation can be both the fractionation in molar mass as well as a chemical fractionation.

3.4 Discussion

3.4.1 General

The study presented in this paper focuses on the effect on molar mass distribution as a result of phase separation of gelatine and dextran in aqueous solution. The general picture that emerges is that as a result of the phase separation both components of the biopolymer mixture get fractionated with respect to their molar mass. The enriched phases of both components contain preferentially the particles from the high molecular mass part of the distribution, while the depleted phases mainly contain the small particles from the distribution. It appears that the fractionation is the strongest for dextran. As a result of this fractionation it is expected that the (functional) properties of both polymers in both phases change, relative to their native material. As an example, the gel properties of gelatine taken from each of the phases were compared. It turns out that the gelatine fractions have significantly different gel strengths. This result of the fractionation should be taken into account in understanding the gel strength of gelled biopolymer based water in water emulsions [10,14].

The temperature has no influence on the composition of the two coexisting phases. This suggests that the phase separation is induced by excluded volume effects, as discussed in a previous paper [12]. A model for the entropically driven phase separation of small protein particles in a semidilute polysaccharide solution is given by Wang *et al*. [15]. This model could be successfully applied to the system gelatine/dextran [16]. Although one condition for the applicability of the model only exists for a small part of the distribution, i.e. a large size difference between gelatine and dextran chains, the experiments seem to be in good agreement with this model [12].

It appears that the molar mass does not affect the concentrations of gelatine and dextran in the coexisting phases. Forciniti *et al.* observed for the system poly(ethylene glycol)/dextran that with increasing molar mass, the effect of increasing the molar mass on the concentrations in the coexisting phases disappeared [17]. Apparently, the molar masses we used in this study were sufficiently large that we did not find an effect on the concentrations in the coexisting phases. However, the overall polymer concentration does affect the molar mass in the coexisting phases. Decreasing the concentration results in an increase of molar mass of gelatine as well as dextran in both phases. This is probably due to the fact that a decrease of concentration results in a decrease of excluded volume, which enables larger molecules to be present in the depleted phase. This concentration dependent

molar mass distribution is also determined by Croguennoc *et al.* [4] for the system κ-carrageenan/β-lactoglobulin/water. They observe that with increasing κ-carrageenan concentration in the system, there is a decrease in the smallest size of the β-lactoglobulin aggregates that phase separates.

Comparing the results of the dialysis experiments and the phase separation experiments, the following conclusion can be drawn. Figure 3.8 shows that, whether the coexisting phases are pure solutions of gelatine and dextran separated by a dialysis membrane, or the coexisting phases are the result of phase separation without a dialysis membrane, the water concentration in the coexisting phases is the same for both cases. The 'contamination' of the two phases with the opposite component (as is the case for phase separation without a membrane) turns out not to affect the water distribution. If there would be a strong difference in water affinity between the two polymers, one would expect a difference in water distribution between the membrane and non-membrane cases. Such a difference is not found. Therefore we conclude that differences in water affinity are not the main driving force for the phase separation.

3.4.2 Quantitative interpretation

In order to quantify the degree of fractionation, we introduce the quantity $(c_{x,poor,m}/c_{x,rich,m})$, in which $c_{x,poor,m}$ and $c_{x,rich,m}$ are the concentrations of component x (gelatine or dextran) with a degree of polymerisation m in the depleted ('poor') and the enriched ('rich') phase respectively (see figure 3.10 for an example). The value of m=1 corresponds to a monomer. For gelatine, the monomer mass is taken to be 90 Da (the average mass of an amino acid in the gelatine used) and for dextran 162 Da (the mass of a glucose repeating unit). Figure 3.10 was derived by dividing, for each molar mass of the distribution, the polymer concentration in the depleted phase by the concentration of polymer in the enriched phase and plotting this value against the corresponding mass of the polymer in number of monomers.

We find that fractionation of gelatine and dextran takes place for all molar masses of the distribution. For molar masses up to approximately 1000 monomers, the fractionation is found to depend exponentially on the degree of polymerisation, m, for each species x with polymerisation m according to:

$$\frac{c_{x,\,poor,m}}{c_{x,rich,m}} = \frac{V_{x,rich} n_{x,poor,m}}{V_{x,poor} n_{x,rich,m}} \approx C_x e^{-Am} \tag{3.6}$$

where C_x is a constant, $V_{x,rich}$ and $V_{x,poor}$ are the volumes of the two coexisting phases, $n_{x,rich,m}$ and $n_{x,poor,m}$ are the number of particles of species x with m monomers in the rich

and depleted phases respectively and A is a constant that depends only on the initial polymer concentration. Above 1000 monomers per chain, no reliable determinations were possible. Figure 3.10a shows that the slope of the dextran curve is steeper than the slope of the curve of gelatine. This implies a stronger fractionation for dextran than for gelatine. This is in good agreement with figure 3.4 to 3.7 and table 3.2a and 3.3b. These figures and tables also suggest that the fractionation is stronger for dextran than for gelatine: the peaks of the distributions and the average molar mass values of dextran in the two phases differ more than those of gelatine. With respect to the effect of concentration, figure 3.10b shows (for dextran) that the lower the initial concentration, the less steep is the slope of the curve. The same can be seen for gelatine (figure not shown). This implies that the fractionation is less strong if the concentration decreases. We also observed that the degree of fractionation shows no temperature dependence (not shown).

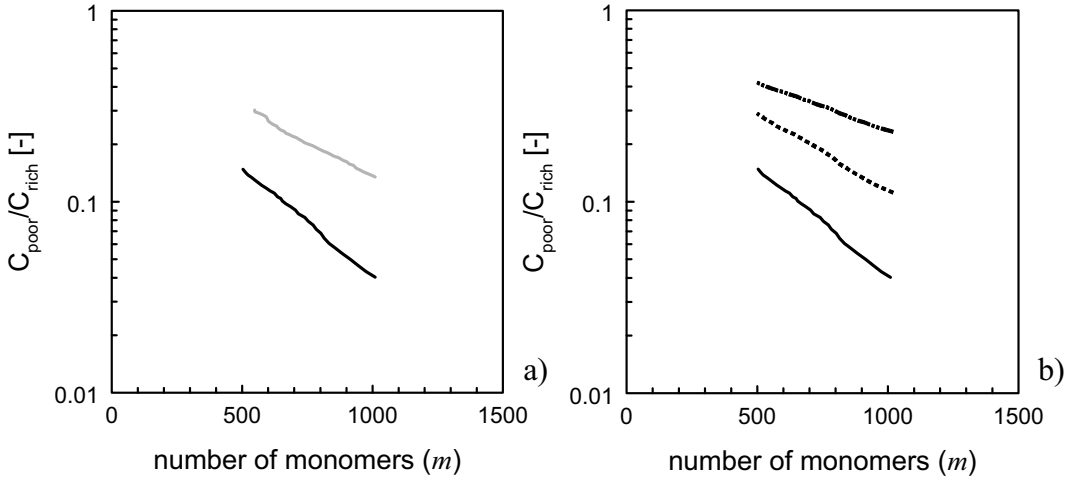

Figure 3.10: ($c_{x,poor,m}/c_{x,rich,m}$) as function of the number of monomers in a polymer. a) for sample A2 (5 w/w% gelatine 170 kDa + 5 w/w% dextran 282 kDa, T_{ps} = 60°C). Grey line: gelatine, black line: dextran. b) effect of concentration for dextran at T_{ps} = 60°C. sample A2 (5%/5%) (──), sample B (4.5%/4.5%) (- -) and sample C (4%/4%) (─ ‧ ‧) (all percentages (w/w)).

The exponential dependence of the fractionation on molar mass, according to equation (3.6), was put forward in the literature decades ago [18-21]. In general the fractionation can be described by a Boltzmann factor:

$$\frac{c_{x,poor,m}}{c_{x,rich,m}} = e^{-\frac{\Delta G}{RT}} \tag{3.7}$$

where ΔG denotes the free energy increase involved in transferring one mole of polymers (with m monomers) from the enriched to the depleted phase. Bawn [19] correctly derived equation (3.7) on the basis of a free energy containing a heat term and an entropy of

mixing term, while Brönsted erroneously ignored the latter, though arriving at the same result [20,21].

Brönsted pointed out that for large, spherical molecules, *m* should be replaced by the surface area of the molecule [20]. Indeed, Albertsson derived an expression for the partitioning as a function of the particle surface area and the surface tensions, i.e. between the particle and the two separated phases [18]. Assuming Antonoff's rule [22], one may write the result of Albertsson as:

$$\frac{c_{x,poor,m}}{c_{x,rich,m}} = e^{-\frac{\gamma A_{surface}}{RT}} \tag{3.8}$$

where γ denotes the interfacial tension between the two separated phases and $A_{surface}$ the surface area of one mole of molecules with *m* monomers.

Table 3.3 shows the values of ΔG for the transfer of one mole of polymer consisting of 1000 monomers, from the enriched to the depleted phase at various temperatures and initial polymer concentrations. The value of 1000 monomers is chosen as a typical length. Table 3.3 shows that with decreasing concentration, the value of ΔG decreases. Assuming only entropy contributions, this would imply less difference in entropy for a polymer upon its transfer from the enriched to the depleted phase. This is certainly in line with the expectation that upon decrease of initial concentration each individual polymer will indeed have access to more free volume in both phases and therefore will experience a smaller difference in entropy upon transfer from the one phase to the other.

One may equate $\gamma A_{surface}$ to $\gamma m A_{monomer}$, where $A_{monomer}$ denotes the surface area of one mole of monomers. Using a typical value of $2 \cdot 10^{-18}$ $m^2 \cdot N_A$ for $A_{monomer}$ [23], with N_A Avogadro's number, and m=1000, and using the order of magnitude of ΔG in Table 3.3, one obtains a value for γ of 4 μN m^{-1}, which is an eminently reasonable value, considering the experimental values obtained in the range of 1 to 10 μN m^{-1} for similar systems [24].

Recently, the exponential behaviour of the fractionation was also observed in computer simulations [25]. The results of these simulations and the comparison with the experimental data will be the subject of a following paper.

With use of the values for A, C_x and ρ_x (with $\rho_x = V_{x,rich}/V_{x,poor}$), we can derive an expression for the number average molar mass of each polymer in each phase separately. Combining the following equation:

$$n_{x,m} = n_{x,rich,m} + n_{x,poor,m} \tag{3.9}$$

Table 3.3: Overview of the values for A, log(C/ρ) (with $\rho = V_{x,rich}/V_{x,poor}$) and ΔG for gelatine and dextran as function of the initial concentration and the temperature. The value of ΔG is per mole of polymers with a length of 1000 monomers.

		GELATINE			DEXTRAN		
T_{ps}[a] [°C]	initial concentration (sample) [% w/w]	A [-]	$\log\frac{C}{\rho}$ [-]	ΔG [J mol^{-1}]	A [-]	$\log\frac{C}{\rho}$ [-]	ΔG [J mol^{-1}]
50	5.0 + 5.0 (*A1*)	0.0018	-0.57	$6.36\cdot10^3$	0.0031	-0.53	$9.75\cdot10^3$
60	5.0 + 5.0 (*A2*)	0.0018	-0.57	$6.56\cdot10^3$	0.0031	-0.53	$10.05\cdot10^3$
60	4.5 + 4.5 (*B*)	0.0010	-0.32	$3.65\cdot10^3$	0.0019	-0.53	$6.73\cdot10^3$
60	4.0 + 4.0 (*C*)	0.0007	-0.12	$2.27\cdot10^3$	0.0013	-0.52	$4.85\cdot10^3$

[a] T_{ps} = temperature at which phase separation was made to take place.

in which $n_{x,m}$ is the total number of particles of species x with m monomers in the native material, with equation (3.6), we get:

$$n_{x,rich,m} = \frac{\rho_x \, n_{x,m,}}{\rho_x + C_x e^{-Am}}$$
$$n_{x,poor,m} = \frac{n_{x,m} C_x e^{-Am}}{\rho_x + C_x e^{-Am}} \tag{3.10}$$

Assuming that equation (3.6) is valid for all molar masses present in the system, we get from equation (3.10):

$$\frac{M_{x,n,rich}}{M_{mono,x}} = \frac{\sum_m n_{x,rich,m} m}{\sum_m n_{x,rich,m}} = \frac{\sum_m \frac{n_{x,m,} m}{\rho_x + C_x e^{-Am}}}{\sum_m \frac{n_{x,m,}}{\rho_x + C_x e^{-Am}}}$$
$$\frac{M_{x,n,poor}}{M_{mono,x}} = \frac{\sum_m n_{x,poor,m} m}{\sum_m n_{x,poor,m}} = \frac{\sum_m \frac{n_{x,m} m \, C_x e^{-Am}}{\rho_x + C_x e^{-Am}}}{\sum_m \frac{n_{x,m} C_x e^{-Am}}{\rho_x + C_x e^{-Am}}} \tag{3.11}$$

with $M_{mono,x}$ the monomer mass of polymer x. If the function of the molar mass distribution of the native material ($P(m)$) is known, equation (3.11) can than be written as:

$$\frac{M_{x,n,rich}}{M_{mono,x}} = \frac{\int_0^\infty \frac{P(m)m}{\rho_x + C_x e^{-Am}} dm}{\int_0^\infty \frac{P(m)}{\rho_x + C_x e^{-Am}} dm}$$
$$\frac{M_{x,n,poor}}{M_{mono,x}} = \frac{\int_0^\infty \frac{P(m)m\, C_x e^{-Am}}{\rho_x + C_x e^{-Am}} dm}{\int_0^\infty \frac{P(m)\, C_x e^{-Am}}{\rho_x + C_x e^{-Am}} dm} \qquad (3.12)$$

3.4.3 Practical consequences

The fractionation in molar mass also affects the mechanical properties of the gel that can be made from the gelatine from both phases. From the measurements of the storage modulus it turns out that the gelation properties of the gelatine are affected by the fractionation. As we expected, the gelatine from the poor phase is not able to form a gel that is as firm as the gel that can be made from the gelatine from the rich phase. Ferry and Eldridge already showed that the higher the molar mass of the gelatine, the higher the gel strength of the gel it forms [11,26]. The differences in the gel forming properties may be caused by either the amount of helices that can be formed or the length of the junction zones that are formed. Apart from physical fractionation (fractionation in molar mass), chemical fractionation can take place during phase separation. Due to this chemical fractionation, the gelatine in the two coexisting phases has a different chemical composition and, consequently, different gel forming properties. Surprisingly, the gel of the native gelatine forms a firmer gel than the gelatine in the two coexisting phases. This may be caused by chemical fractionation.

The gelatine graphs in figure 3.4 to 3.7 show a double peak for the gelatine in the gelatine-rich phase. From other studies [13] it is known that gelatine has a temperature dependent molar mass distribution even above the gelation temperature of gelatine (~30°C). Figure 3.4 shows that the shape of this bimodal distribution is affected by the fractionation. The fractionation apparently affects the monomer–dimer equilibrium of the gelatine. This change in the distribution of the gelatine-rich phase compared to the distribution of the pure gelatine may be related to the fact that the pure gelatine is able to form a firmer gel than the gelatine from the gelatine-rich phase.

The differences in gelation properties between the gelatine in the poor- and rich phase were not taken into account in earlier work [6-10]. However, the present work shows that

ignoring differences in the gelling properties of material in coexisting phases might lead to erroneous results for the calculation of the strength of mixed (phase separated) gels.

3.5 Conclusions

The phase separation of aqueous mixtures of gelatine and dextran results in a strong fractionation in molar mass of the two polymers. It appears that the overall concentration is the only factor that influences the molar mass distribution of the polymers in each phase. The temperature does not affect the distribution. For the lower molar mass part of the distribution (up to 1000 monomers), the fractionation is found to be exponential in the molar mass of the polymer. Interpreting this exponential ratio as a Boltzmann factor, the free energy involved in transferring a polymer with a certain length from the enriched to the depleted phase can be calculated. Using reasonable values for molecular dimensions we arrive at a reasonable value for the interfacial tension between the two phases.

It appears that the low molar mass part of the distribution has the same influence on the osmotic pressure whether it is in the depleted phase of a system in which species can move freely, or in the enriched phase of a forcibly separated system (using a dialysis membrane). This implies that water affinity is no driving force for the phase separation. In addition it is concluded that fractionation does not influence the total polymer concentration in the two coexisting phases. As a result of the fractionation, the gelling properties of the gelatine in coexisting phases change as compared to the properties of the native gelatine. This implies that the fractionation has to be taken into account when calculating the mechanical properties of a mixed phase separated gel containing gelatine.

3.6 References

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Chapter 4

Phase separation of aqueous mixtures of poly(ethylene oxide) and dextran*

ABSTRACT

Phase behaviour of aqueous mixtures of poly(ethylene oxide) (PEO) and dextran is studied as function of the polymer concentration, the PEO molar mass and temperature. The molar mass distributions of the two polymers in the coexisting phases are measured. From the temperature dependence we conclude that the phase separation between PEO and dextran is partly caused by sterical interactions. From the equilibrium phase volumes of the phase separated mixture and the shape of the temperature–composition phase diagram of PEO and dextran we conclude that also the decrease of solvent quality of water for PEO at increasing temperatures is involved. It is suggested that the characteristics of the PEO-water interaction can affect the degree of fractionation. This suggestion is based on the observation that the degree of fractionation is not a simple exponential function of the molar mass. The phase behaviour of the mixture PEO/dextran is compared to the previously studied phase behaviour of the aqueous mixture of gelatine and dextran.

* *Macromolecules, in press*

4.1 Introduction

Most foods contain mixtures of biopolymers. If the concentration of these polymers is sufficiently high, segregative phase separation might take place. This phase separation is often the basis for structuring foods. In literature a large quantity of experimental data is available on the segregative phase separation of biopolymers [1]. However, these data are only qualitative and give no information about the effect of the temperature on the phase separation. Therefore, it is not possible to obtain any information from these data about the mechanism of the phase separation.

In a previous paper [2], we chose the system of aqueous solutions of gelatine and dextran as a model system for food biopolymers to study the phase separation. We found that the concentration in the coexisting phases does not depend on temperature and that phase separation of this system can only be realised by increasing the total polymer concentration. This temperature independence of the phase separation suggests that phase separation between gelatine and dextran is driven by entropic interactions. Indeed, some features could be described using depletion theory [2-4]. Besides the phase diagram, also the molar mass distributions of the polymers after phase separation were measured [5]. It turned out that the phase separation results in a fractionation in molar mass and that the degree of fractionation depends exponentially on the molar mass.

To find out whether the mechanism of phase separation which we found for the system gelatine/dextran is common for mixtures of polymers or only specific for the system gelatine/dextran, we chose to study the phase behaviour of the system poly(ethylene oxide) (PEO)/dextran and compare this behaviour with that of the mixture gelatine/dextran. We chose PEO as replacement for gelatine because of its experimental advantages. Apart from this, it is just like most biopolymers a crude material in the meaning that it is polydisperse in its molar mass.

The main reason mentioned in literature for studying the system PEO/dextran is that it can be used for the partitioning of small biomolecules e.g. proteins. A lot of experimental data and theoretical descriptions of the effects of several parameters (e.g. polymer concentration and molar mass) on the partitioning of biomaterials are available [6-12]. However there are relatively few studies done on the influence of temperature and molar mass on the phase diagram. Forciniti *et al.* for example, performed a study on the effect of temperature and molar mass on the phase behaviour of the system PEO/dextran [13]. They found that with increasing molar mass of the polymers, the influence of temperature decreased. However, most of the PEO used in these studies had a molar mass smaller than 20 kDa.

For the study described in this paper we are interested in the phase behaviour of aqueous mixtures of PEO ($M_w \geq 100$ kDa) with dextran ($M_w = 282$ kDa). The molar mass

of PEO, the concentration of polymer and the temperature at which phase separation was established, were varied. Finally, the phase behaviour of the system PEO/dextran was compared to the phase behaviour of the aqueous system gelatine/dextran in order to obtain a more general view on the phase behaviour of aqueous (bio)polymer mixtures.

4.2 Experimental

4.2.1 Materials

Dextran with a M_w of 282 kDa was purchased from Sigma Chemicals. PEO with a M_w of 100 and 200 kDa were purchased from Fischer Scientific. The dextran was used without further purification. PEO was purified before use. This was done by dissolving the powder in Reversed Osmosis (RO) water by stirring on a magnetic stirrer at room temperature. After 24 hours, the solution was centrifuged (60 minutes, 11,000 g) and the supernatant was freeze dried. Before using this material, the molar mass distribution was measured with Size Exclusion Chromatography equipped with a Multiangle Laser Light Scattering detector (SEC-MALLS) (see table 4.1). Clear solutions were prepared by gravimetrically adding solvent (RO water with 0.02% sodium azide to prevent bacterial growth) to the appropriate amount of material. Dextran dissolves readily at room temperature. PEO was dissolved by stirring overnight.

Table 4.1. Overview of M_n, M_w, M_z and polydispersity (M_w/M_n) for PEO and dextran before phase separation (molar mass averages in [kDa]).

sample	M_n	M_w	M_z	$\frac{M_w}{M_n}$
PEO-100	39	104	253	2.6
PEO-200	57	196	758	3.4
dextran	64	299	993	4.7

4.2.2 Determination of the temperature–composition phase diagram

To determine the temperature–composition phase diagram, mixtures of equal mass concentrations of PEO and dextran were made. For this purpose equal masses of dextran solutions and PEO solutions with the same mass percentage of polymer were mixed. Approximately 10 gram of this mixture was put into a plastic tube. To prevent evaporation of the solvent, a layer of paraffin oil was brought on the top of the meniscus. The tubes were held in a water bath for approximately 20 hours to reach equilibrium phase separation. Equilibrium was assumed to be reached when a sharp meniscus between

transparent PEO-rich and dextran-rich fluid layers was observed. Hereafter, the heights of the PEO-rich and dextran-rich phase were measured and related to the volume of the phases. The experimental error of these measurements was in the order of 1%. Samples of these phases were taken with a syringe with hypodermic needle.

To determine the concentration of PEO and dextran in the coexisting phases, samples taken from both phases were diluted 40 times. Optical rotation was measured at 365 nm and 80°C. Because dextran shows optical rotation and PEO does not, the concentration dextran was measured using

$$\alpha_{meas}(c_d, \lambda = 365\ nm) = [\alpha]_{d,\lambda=365\ nm} c_d \tag{4.1}$$

where subscripts 'meas' and 'd' denote measured and dextran and where $[\alpha]$ denotes the specific rotation (per % (w/w per dm)). The experimental error of this method is 1%.

The concentrations PEO in the coexisting phases were determined by measuring the density of the phases. By using the known concentration of dextran the concentration PEO (in % (w/w)) could be calculated using

$$\rho_{meas} = \rho_s + \Delta\rho_P c_P + \Delta\rho_d c_d \tag{4.2}$$

where ρ denotes the density and $\Delta\rho$ the density increment per unit concentration. The subscripts 'meas', 's', 'P' and 'd' denote measured, solvent, PEO and dextran respectively. $\Delta\rho$ was measured using a Mettler/Paar DMA 45 density meter. For this method, additivity of volumes was assumed. This method was tested by measuring the density of mixtures of PEO and dextran with various concentrations from which the concentration PEO was calculated again. The experimental error of this method turned out to be 10%.

4.2.3 Determination of the cloud point of PEO in water

Solutions of PEO in water were made in a concentration range between 5 and 10% (w/w). Glass tubes were almost completely filled with these solutions and sealed with a screw cap. These tubes were kept in a thermostatted oil bath. The cloud point was determined by eye (experimental error 0.5°C).

4.2.4 Determination of the phase separation temperatures

Mixtures of equal concentrations of PEO and dextran were made. From this mixture a series of mixtures with decreasing polymer concentration (steps of 0.02% (w/w) total

polymer concentration) was made. This series was held overnight in a water bath at a fixed temperature. After 20 hours the mixtures were checked on phase separation.

4.2.5 Determination of the molar mass distribution of PEO and dextran

SEC-MALLS equipped with and a refractive index (RI) detector was used to determine the molar mass distribution of PEO and dextran. For mixtures of PEO and dextran, an additional detector monitoring optical rotation (OR) at 365 nm was used. By combining the signals from the RI detector and the OR detector, the contribution from dextran and PEO to the two signals can be unravelled because both signals are different linear combinations of dextran and PEO contributions. This method was tested by measuring the native polymers and a mixture of these polymers. It turned out that the same molar mass distributions were found for the native polymers and the polymers in the mixture.

The conditions for determining the molar mass distributions were chosen the same as in ref. (5). A $LiNO_3/KH_2PO_4/K_2HPO_4$ (pH 6.7) buffer was used as eluent. The flow rate was 1 ml min^{-1}. The columns that were used were a combination of TSK guard + TSK G5000PW + TSK G3000PW (TosoHaas GmbH, Stuttgart, Germany). Typically 4 mg of dry material in 200 µl was injected, resulting in a concentration of 0.2% (w/w) in the detector cells.

Table 4.2: Overview of the samples analysed with SEC-MALLS. Values represent the concentration polymer (in [%(w/w]) in the samples before phase separation.

sample code	conc. PEO-100	conc. PEO-200	conc. dextran	T_{ps}[a]
100A	3.5		3.5	60.0
100B60	3.0		3.0	60.0
100C	2.5		2.5	60.0
100B40	3.0		3.0	40.0
100B80	3.0		3.0	80.0
200A		3.5	3.5	60.0
200B60		3.0	3.0	60.0
200C		2.5	2.5	60.0
200B40		3.0	3.0	40.0
200B80		3.0	3.0	80.0

[a] T_{ps} represents the temperature at which phase separation was made to take place.

For the determination of the molar mass distribution of PEO and dextran in coexisting phases, samples were taken from the PEO-rich and dextran-rich phases after equilibration during about 20 hours. Table 4.2 gives an overview of the samples from which the coexisting phases were analysed with SEC-MALLS. All samples from the coexisting phases were diluted 40 times in the eluent and put in vials. Besides these samples, also the starting material was analysed. The temperatures of the SEC column and MALLS detector cell were 50°C. The OR detector cell had a temperature of 40°C and RI detector cell was not temperature controlled. For the formulas used to calculate M_n, M_w, M_z and the polydispersity, see ref. (5).

4.3 Results

4.3.1 Phase behaviour

Figure 4.1 presents the temperature–composition phase diagram of mixtures of 3.5% (w/w) PEO and 3.5% (w/w) dextran for the two molar masses of PEO studied. It turns out that in the temperature range studied, no significant effect of PEO molar mass on the concentration of dextran and PEO in the coexisting phases could be detected.

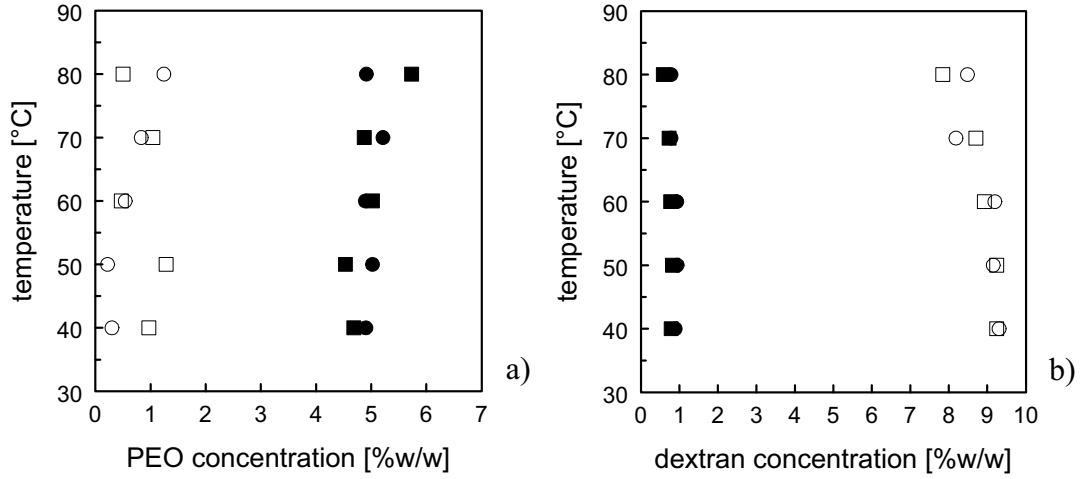

Figure 4.1: Coexisting phase compositions after full phase separation in 3.5% PEO/3.5% dextran/water. a) concentrations of PEO and b) concentrations of dextran. PEO-100 (●) and PEO-200; (■). Open symbols: dextran-rich phase; closed symbols: PEO-rich phase.

Figure 4.2 shows the influence of the initial polymer concentration on the concentrations of the two polymers in the temperature–composition phase diagram for the mixture of PEO-100 and dextran. It turns out that the higher the initial concentration, the larger the difference is between the concentrations of polymer in the two coexisting phases. Figure 4.1 and figure 4.2 also show that with increasing temperature the concentration of

dextran in the dextran-rich phase decreases, while the concentration PEO in the PEO-rich phase increases.

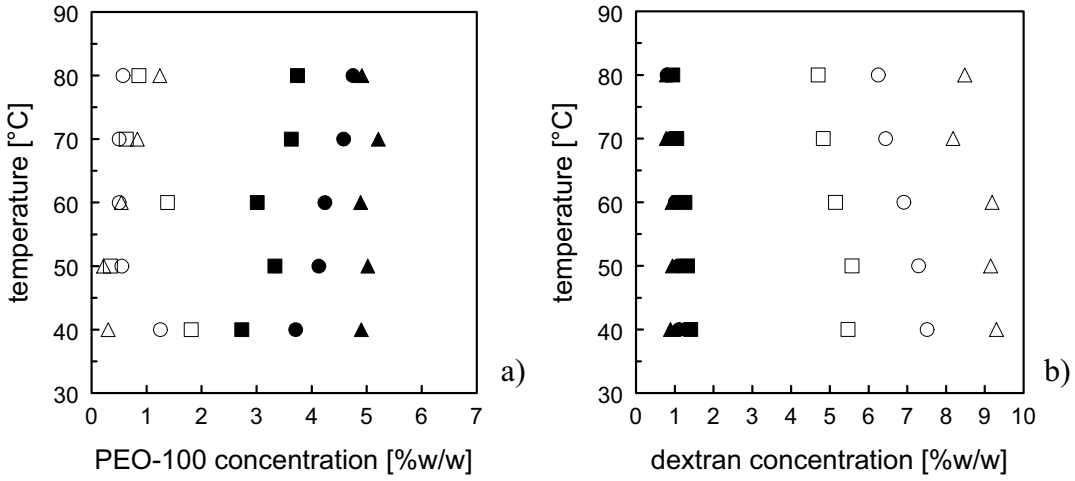

Figure 4.2: Coexisting phase compositions after full phase separation of the mixture PEO-100/dextran/water. a) concentrations of PEO-100 and b) concentrations of dextran. Initial compositions PEO-100/dextran: 3.5%/3.5% (▲), 3.0%/3.0% (●) and 2.5%/2.5% (■). Open symbols dextran-rich phase, closed symbols PEO-rich phase.

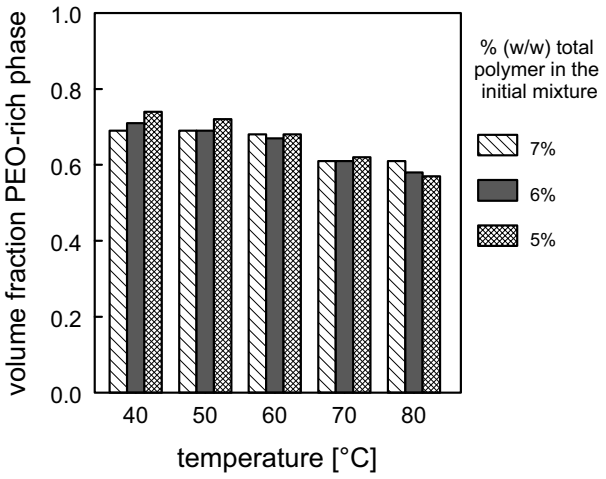

Figure 4.3: Volume fractions of the PEO-100-rich phase as function of the temperature at which phase separation was established. Solute composition: 50% dextran + 50% PEO-100.

From figure 4.3 it turns out that when the temperature at which phase separation is established increases, the phase volume of the PEO-rich phase decreases. It is known from literature [14,15] that the solubility of PEO in water decreases with increasing temperature and that the cloud point for PEO in water is near 100°C. For the PEO we used, the cloud point in a concentration range of 5 to 10% (w/w) PEO was determined. For all samples of PEO-100 in the concentration range measured, the cloud point was established at 106°C, while for the samples of PEO-200, the cloud point was determined to be 103°C.

The temperature dependence of the phase diagram was studied by determining the cloud point of the mixture PEO-100/dextran. In the temperature range between 40 and 70°C, it appears that the position of the cloud point is located between 4.06% and 4.08% (w/w) total polymer (see figure 4.4). At 80°C, the position of the binodal shifts to a lower total polymer concentration. This is probably due to the decrease of quality of water as a solvent for PEO.

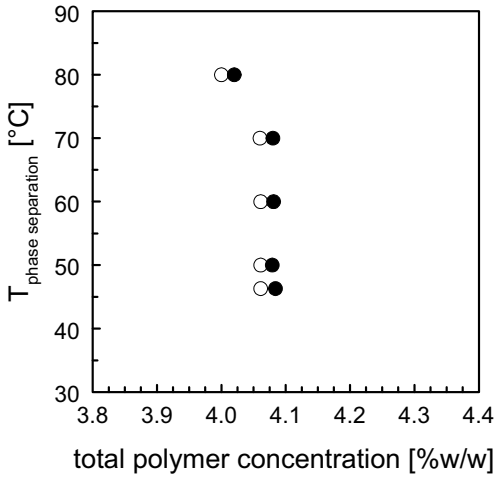

Figure 4.4: Phase separation temperatures vs. total polymer concentration. The solute consists of 50% PEO-100 + 50% dextran. Open symbols: no phase separation occurred; closed symbols: phase separation occurred.

4.3.2 Fractionation in molar mass

For dextran as well as for PEO in the coexisting phases, the molar mass distribution was determined. The molar mass of PEO, the initial polymer concentration and the temperature at which phase separation was established were varied. An overview of the analysed samples is given in table 4.2.

Concentration effects

Figure 4.5 and 4.6 show the influence of the initial polymer concentration on the molar mass distributions of PEO and dextran after phase separation at 60°C. The area under the curves represent the concentration in the coexisting phases (see also ref. (5)).

Figure 4.5 and 4.6 show that decreasing the total polymer concentration results, for the material in the depleted phases, in broader peaks. Table 4.3a and 4.3b show that the molar mass averages of the PEO as well as the dextran in their depleted phases increase when the initial polymer concentration is decreased. On the other hand, the molar mass averages of PEO and dextran in their enriched phase stay more or less constant with decreasing concentration. The same behaviour was found for the molar mass distributions of the samples containing PEO-200. For the three different averages of the molar mass given in

table 4.3a and 4.3b, the values of M_z are the most reliable. This is due to the fact that for PEO as well as for dextran, the lower part of the molar mass distribution could not be measured and this has the least effect on the value of M_z compared to the values of M_n and M_w.

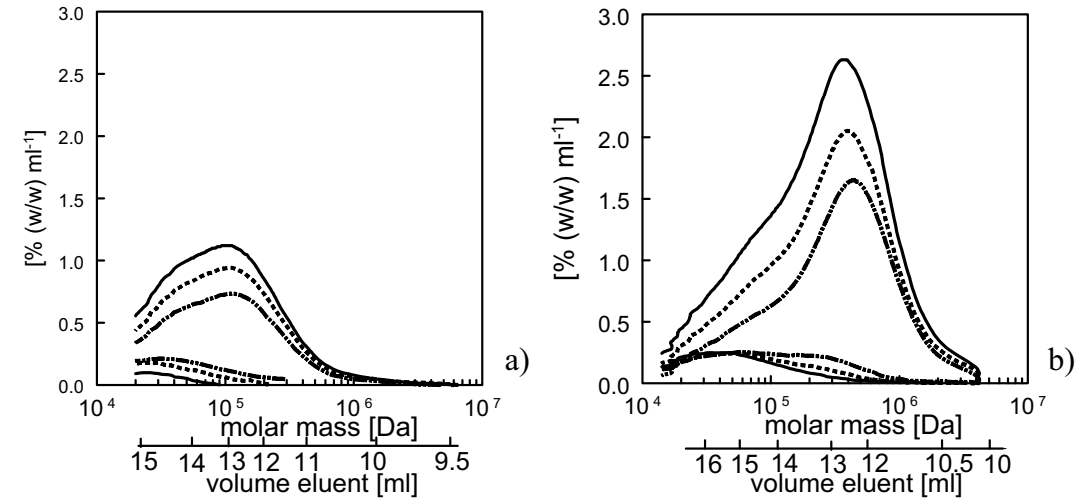

Figure 4.5: Molar mass distributions in coexisting phases at 60°C, for various total polymer concentrations. a) PEO-100 and b) dextran. Upper set of lines: rich phase, lower set of lines: poor phase. Sample 100A (—), sample 100B60 (- -) and sample 100C (— · ·).

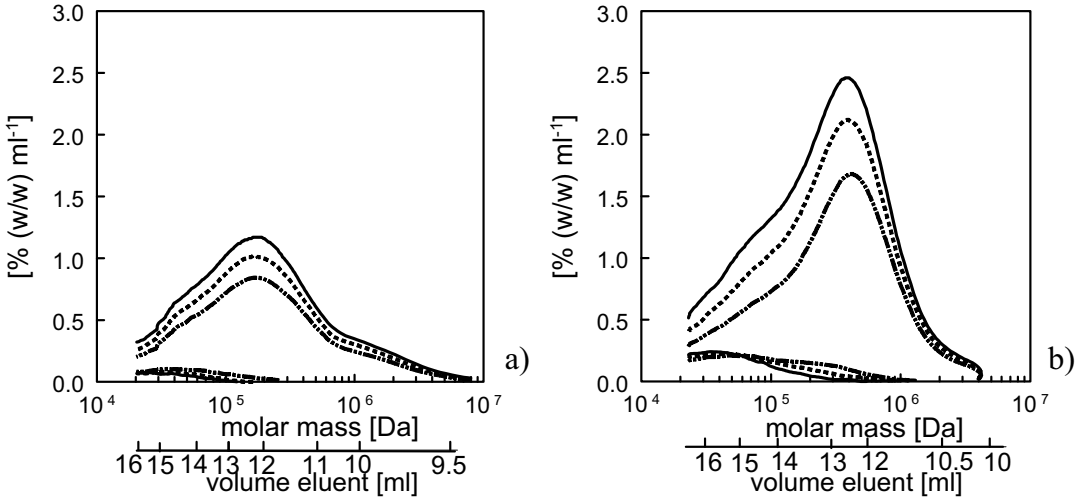

Figure 4.6: Molar mass distributions in coexisting phases at 60°C, for various total polymer concentrations. a) PEO-200 and b) dextran. Upper set of lines: rich phase, lower set of lines: poor phase. Sample 200A (—), sample 200B60 (- -) and sample 200C (— · ·).

Table 4.3: M_n, M_w and M_z and the polydispersity for (a) PEO and (b) dextran for the native material and the material in the different phases for the different mixtures (concentrations in [%(w/w)], molar mass averages in[kDa]).

(a) PEO

	PEO IN PEO-RICH PHASE					PEO IN DEXTRAN-RICH PHASE				
	conc. PEO	M_n	M_w	M_z	$\frac{M_w}{M_n}$	conc. PEO	M_n	M_w	M_z	$\frac{M_w}{M_n}$
native PEO-100		39	104	253	2.6		39	104	253	2.6
100A	5.0	55	120	281	2.2	0.3	27	32	38	1.2
100B60	4.1	56	124	287	2.2	0.7	32	43	63	1.4
100C	3.2	57	123	280	2.2	1.0	37	61	107	1.6
100B40	4.1	53	103	186	1.9	0.4	30	37	47	1.2
100B80	4.8	51	102	213	2.0	0.5	30	38	50	1.3
native PEO-200		57	196	758	3.4		57	196	758	3.4
200A	5.1	80	242	1010	3.0	0.3	32	38	47	1.2
200B60	4.3	81	243	982	3.0	0.3	33	42	53	1.3
200C	3.5	83	243	943	2.9	0.4	40	59	93	1.6
200B40	4.2	83	237	836	2.9	0.4	34	44	59	1.3
200B80	4.7	79	210	704	2.7	0.4	35	49	72	1.4

(b) dextran

	DEXTRAN IN PEO-RICH PHASE					DEXTRAN IN DEXTRAN-RICH PHASE				
	conc. dextran	M_n	M_w	M_z	$\frac{M_w}{M_n}$	conc. dextran	M_n	M_w	M_z	$\frac{M_w}{M_n}$
native dextran		64	229	993	4.7		64	229	993	4.7
100A	0.9	40	86	257	2.2	9.2	104	386	1012	3.7
100B60	1.0	46	109	274	2.4	6.9	111	400	1030	3.6
100C	1.3	56	160	391	2.9	5.2	135	439	937	3.3
100B40	1.1	47	113	264	2.4	7.5	122	428	1000	3.5
100B80	1.0	41	91	25	2.2	6.3	103	368	918	3.6
200A	0.8	36	69	234	1.9	8.9	108	381	945	3.5
200B60	0.9	42	69	266	2.3	7.4	114	399	965	3.5
200C	0.9	50	133	327	2.7	5.6	120	422	988	3.5
200B40	0.8	41	95	263	2.3	7.7	116	410	992	3.5
200B80	0.7	38	77	221	2.0	6.7	108	384	935	3.5

Temperature effects

Figure 4.7 and 4.8 show the influence of the temperature at which phase separation was established on the molar mass distributions of the polymers. The same behaviour can be observed for mixtures containing PEO-100 as for mixtures containing PEO-200. Figure 4.7 and 4.8 show the opposite behaviour for PEO and dextran; with increasing temperature, the peak height for PEO in its enriched phase increases, while that of dextran in its enriched phase decreases. This is in agreement with figure 4.1 and 4.2 where it is shown that with increasing temperature the concentration of PEO in its enriched phase increases whereas that of dextran in its enriched phase decreases. From table 4.3a and 4.3b it appears that the temperature has hardly any influence on the average molar mass values of PEO and dextran in their enriched as well as in their depleted phase.

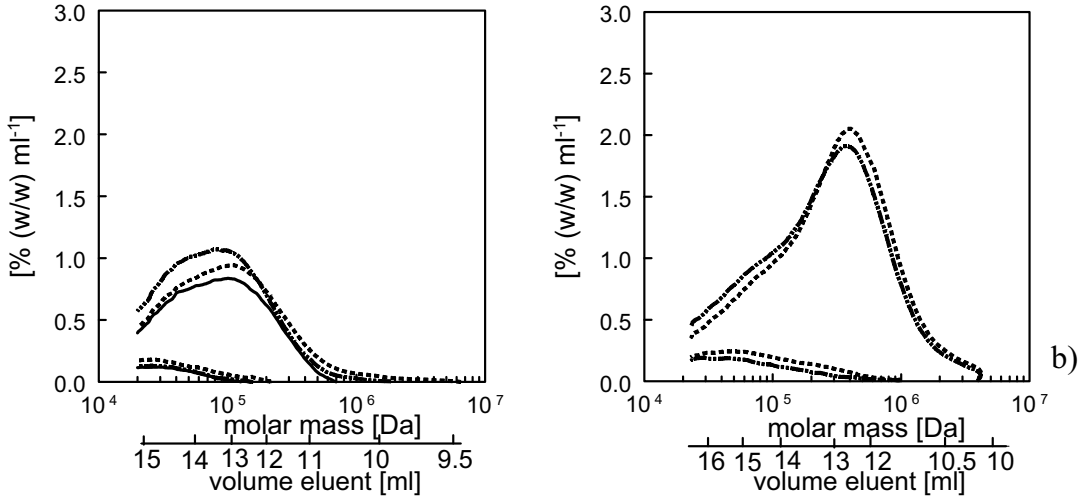

Figure 4.7: The effect of temperature on the molar mass distributions in coexisting phases. a) PEO-100 and b) dextran. Upper set of lines: rich phase, lower set of lines: poor phase. Sample 100B40 (—), sample 100B60 (- -) and sample 100B80 (— · ·).

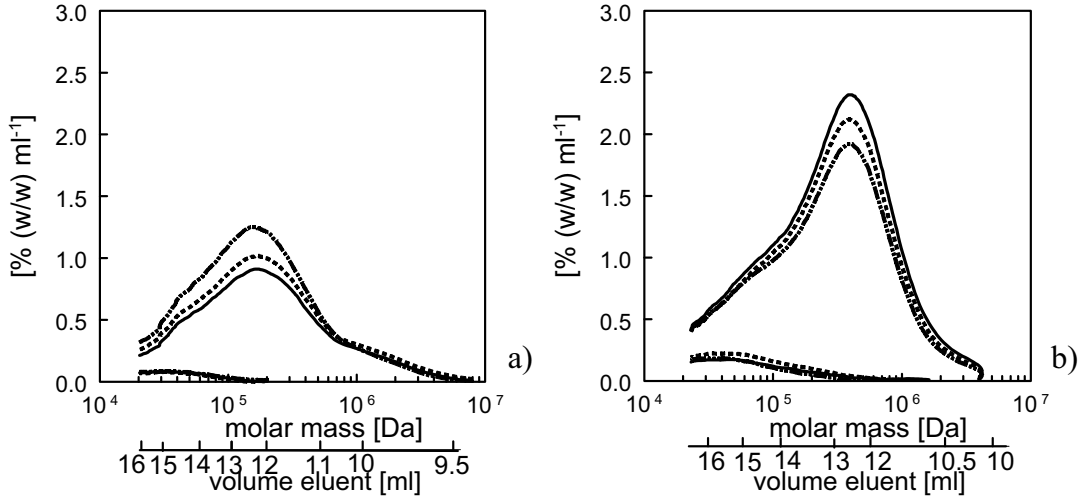

Figure 4.8: The effect of temperature on the molar mass distributions in coexisting phases. a) PEO-200 and b) dextran. Upper set of lines: rich phase, lower set of lines: poor phase. Sample 200B40 (—), sample 200B60 (- -) and sample 200B80 (— · ·).

4.4 Discussion

4.4.1 Phase behaviour of PEO/dextran

This paper gives an overview of the phase behaviour of the aqueous mixture of PEO and dextran as function of the temperature at which phase separation is established, of the total polymer concentration and of the molar mass of PEO. It appears that the phase separation between PEO and dextran is affected by temperature and is accompanied by a fractionation in molar mass of both polymers.

It turns out that the concentration of PEO and dextran in the coexisting phases is not affected by the molar mass of PEO. This is in line with Forciniti *et al*. [13] who reported that, if the molar mass of the polymers is high enough, an increase of molar mass has no effect on the polymer concentrations in the coexisting phases.

Figure 4.4 shows that the position of the cloud point as function of the total polymer concentration is hardly affected by the temperature for temperatures below 80°C, which means that phase separation can only be established by increasing the concentration and not by decreasing the temperature. This temperature independence can be interpreted in two ways. Either the phase separation is caused by sterical interactions between the polymers, or the enthalpic and entropic contributions of the interaction parameters cancel. PEO is a relatively hydrophobic polymer. The interaction between PEO and water therefore becomes less favourable with increasing temperature, which has a negative influence on the stability of the dissolved state [14]. The solubility of dextran, on the other hand, increases with increasing temperature. In principle, the temperature dependence of phase separation behaviour could vanish if solvent quality differences would cause phase separation and if these opposed tendencies in solvent qualities with temperature would cancel each other. However, since the temperature independence of the phase separation of aqueous systems of PEO and dextran is observed in systems for various molar masses of PEO and polymer concentrations, an explanation of the temperature independence in terms of solvent quality of the phase separation is not very plausible and the phase separation being driven by sterical interactions between dissimilar polymers appears more plausible. We therefore interpret the mechanism of phase separation as phase separation induced by sterical interactions between the PEO and dextran molecules. However, at 80°C, the total concentration at phase separation does change (it decreases upon increasing temperature). Increasing the temperature also results in an increase of the PEO concentration in the PEO-rich phase, and a slight decrease of the dextran concentration in the dextran-rich phase. From literature [14,15] and from our own observations we know that the solubility of PEO in water decreases with increasing temperature and that the cloud point of PEO lies at temperatures above 100°C. This lower solubility of PEO in water with increasing temperature might dominate the temperature dependence observed in the temperature–

composition phase diagram. Indeed, since we observed that the phase volume of the PEO-rich phase decreases at higher temperatures (due to the decreased solubility of PEO at these higher temperatures), we conclude that water is expelled from the PEO-rich phase towards the dextran-rich phase. As a consequence, the concentration of dextran in the dextran-rich phase will decrease as is shown in figure 4.1 and 4.2.

The figures of the molar mass distributions of PEO and dextran after phase separation (figure 4.5 to 4.8), as well as the average molar mass values in table 4.3a and 4.3b show that for both polymers phase separation results in a fractionation in molar mass. The reason for the fractionation during phase separation of polydisperse polymers is discussed by several authors [5,16-23]. Analogous to the results of Forciniti *et al*. [24], we found that the molar mass of dextran in the dextran-rich phase is independent on the molar mass of PEO.

If the degree of fractionation ($c_{x,poor,m}/c_{x,rich,m}$) is calculated and plotted versus the number of monomers, an exponential dependence on the molar mass is expected [5,16-19] when only sterical interactions (packing effects) between polymers govern the phase separation. However, figure 4.9 and 4.10 show that this is not the case for dextran in PEO/dextran mixtures: the degree of fractionation shows a deviation from this exponential dependence. Considering the fact that the solvent quality is involved in the phase separation between PEO and dextran, it is suggested that this also plays a role in the fractionation. If this is the case, the largest effect of the solvent quality is expected in the PEO-rich phase (which is the dextran-poor phase). Presumably, the dextran molecules in this phase cannot realise the same conformation as in the dextran-rich phase. In other words, the relation between molar mass and molecular size is different for the two phases. In the case that sterical interactions between the polymers (packing effects) rule the phase separation, a non-trivial dependence of the degree of fractionation on molar mass can be expected. The involvement of solvent in the polymer conformation will be the largest at higher molar mass. This is in agreement with computer simulations that show that the dependence of the degree of fractionation on the degree of polymerisation shows a similar concave deviation, if the radius of gyration in the poor phase is smaller than in the rich phase [25]. The result of the computer simulation would then imply that at increasing total polymer concentration or increasing temperature, the deviation from exponential behaviour would start at lower values of *m*. As can be seen in figure 4.9 and 4.10, this is indeed the case. Note that the fractionation of PEO does not show a deviation of the exponential dependence. The explanation of this appearance might be found in the assumption that PEO molecules do not make a distinction between water and dextran, since the surface of dextran is very hydrophilic.

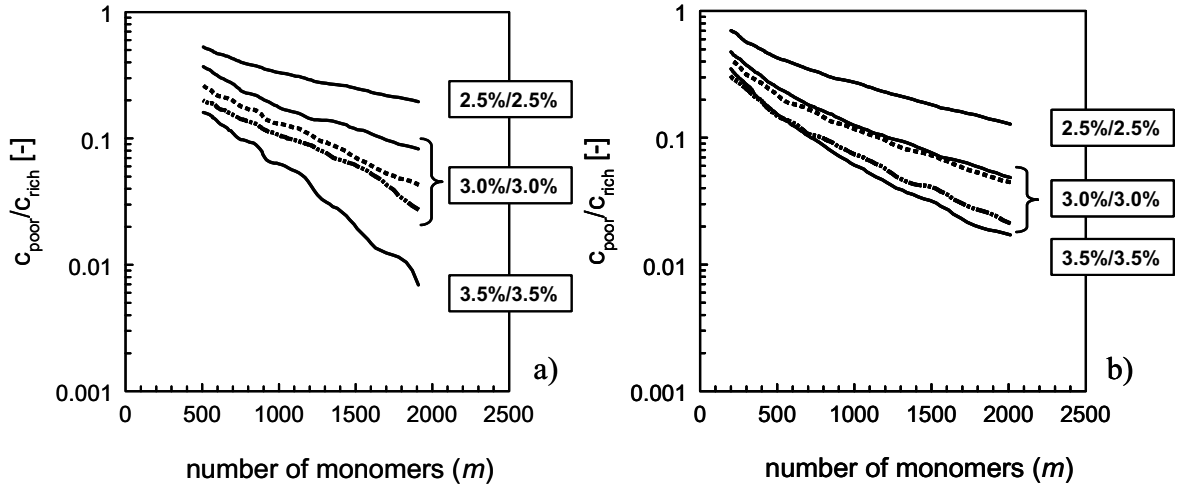

Figure 4.9: $c_{x,poor,m}/c_{x,rich,m}$ as function of the number of monomers (m) in a polymer chain. a) for PEO-100 and b) for dextran. Percentages (w/w) are those of PEO and dextran in the mixture before phase separation. For 3.0%/3.0% results for three temperatures are given: T=40°C (- -), T=60°C (—) and T=80°C (— · ·). At 2.5%/2.5% and 3.5%/3.5%, T was 60°C.

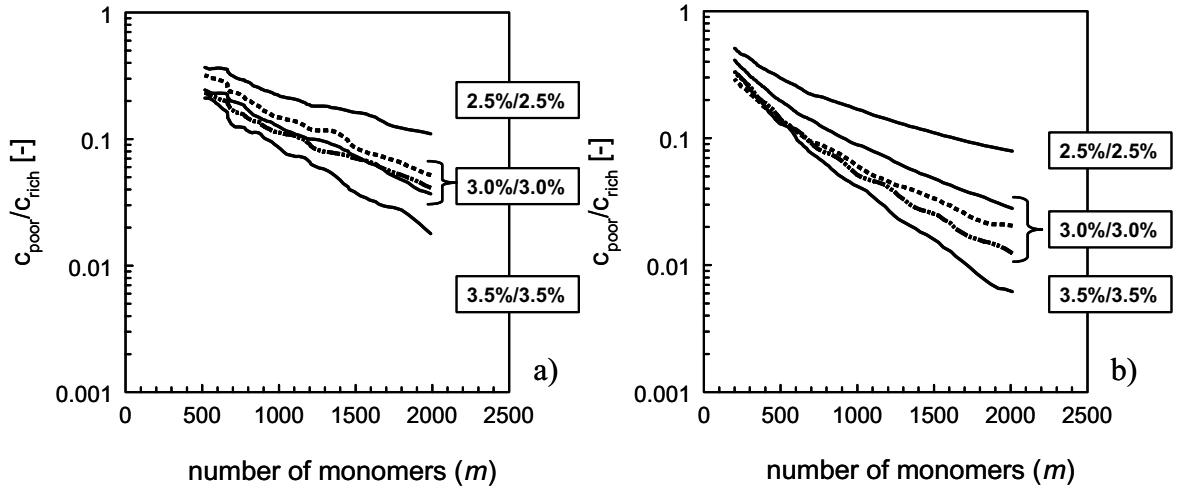

Figure 4.10: $c_{x,poor,m}/c_{x,rich,m}$ as function of the number of monomers (m) in a polymer chain. a) for PEO-200 and b) for dextran. Percentages (w/w) are those of PEO and dextran in the mixture before phase separation. For 3.0%/3.0% results for three temperatures are given: T=40°C (- -), T=60°C (—), and T=80°C (— · ·). At 2.5%/2.5% and 3.5%/3.5%, T was 60°C.

In polymer science in general, one is also interested in the phase behaviour of polymers and the dependence of the degree of fractionation on the molar mass in particular. This behaviour is studied experimentally [5,26], theoretically [17-23,25,27-30] as well as with use of computer simulations [25,30]. All theoretical models use a mean field approach. However, a simple mean field approach predicts a exponential dependence of the degree of fractionation on the molar mass, where in the experimental case this dependence is not always exponential [26]. An other difference between the mean field

predictions and the experimental work is that the intercept at m=0 is expected to be at $c_{x,poor,m}/c_{x,rich,m}$ =1, whereas the experimental results do not show this value of the intercept when the data are extrapolated towards m=0. These contradictions between the theoretical predictions and the results of experimental work show that more research is needed on this subject and that a simple mean field approach is inadequate for the prediction of the degree of fractionation.

4.4.2 Comparison with the system gelatine/dextran

As mentioned in the introduction, the phase behaviour of PEO and dextran was studied in order to compare it with the phase behaviour of gelatine and dextran which we studied before [2,5]. We found that the phase separation between gelatine and dextran is driven by sterical interactions between dissimilar polymers and can be described with use of a depletion theory [2-4]. From the results of the research on the system PEO/dextran, it cannot been concluded that the phase separation mechanism is the same as for the system gelatine/dextran, although there are some similarities. These similarities concern the way the systems react on changing the polymer concentration, changing the molar mass of one of the polymers and the temperature dependence [2,5].

It appears that differences between the phase behaviour of gelatine/dextran and PEO/dextran are caused by the effect of solvent quality. The first difference is that for the phase separation of gelatine and dextran, the temperature has no effect on the composition of the coexisting phases [2,5], whereas the temperature does affect the composition of the coexisting phases of phase separated mixtures of PEO and dextran. Comparing the phase volumes of the coexisting phases, it turns out that for the mixture of gelatine and dextran the phase volumes after phase separation are the same for each temperature in the temperature range probed, whereas for the system PEO/dextran the phase volume of the PEO-rich phase decreases with increasing temperature as a result of the decreasing solvent quality of water for PEO. This change in the phase volumes might also be the reason why the temperature–composition phase diagram of the system PEO/dextran is slightly dependent on the temperature.

The second difference between the phase behaviour of the system gelatine/dextran and PEO/dextran which might be caused by the effect of the solvent quality, is the dependence of the degree of fractionation on the molar mass. If the degree of fractionation in the system PEO/dextran is considered to be exponential with the molar mass, and the slope at low m is interpreted as the slope in the (hypothetical) absence of solvent quality influence, a comparison can be made between the system PEO/dextran and gelatine/dextran. The solvent quality dependence of molecular conformation, and therefore on fractionation, is expected to be the strongest for molecules with a higher degree of polymerisation. First,

consider the degree of fractionation for a dextran molecule consisting of 1000 monomers. These values are summarised in table 4.4. It must be taken into account that the various systems are not on the same distance from their critical point and that the data of the PEO-containing systems are the result of an extrapolation. In spite of the differences between the two types of systems, the degree of fractionation is of the same order of magnitude.

Table 4.4: Overview of the values for the degree of fractionation of dextran for m=1000, for the system gelatine/dextran as well as the system PEO/dextran. The temperature at which phase separation was established was 60°C.

system	concentration [%(w/w)]					
	5.0/5.0	4.5/4.5	4.0/4.0	3.5/3.5	3.0/3.0	2.5/2.5
gelatine/dextran[a]	0.04	0.11	0.25			
PEO-100/dextran				0.03	0.08	0.19
PEO-200/dextran				0.02	0.05	0.14

[a] see ref. (5).

Apart to this, the values for the free energy needed for the transfer of a polymer from its enriched to its depleted phase can be compared (see table 4.5). These data are calculated with use of the equations mentioned in ref. (5). In essence, the degree of fractionation is interpreted as a Boltzmann factor, with the exponent equal to $\Delta G/RT$, where ΔG is the free energy involved in transferring from the rich phase to the poor phase, a mole of a polymer with a certain molar mass m. This table shows that the value for the free energy for the system gelatine/dextran is of the same order of magnitude as that for the system PEO/dextran. This similarity between the two systems supports the view that when it is found that the degree of fractionation is exponential in the molar mass, entropy is governing the phase separation. An entropic ΔG of mixing of dextran with a linear polymer, is not expected to depend strongly on the chemical structure of the linear polymer.

Table 4.5: Overview of the values for free energy ΔG [J mol^{-1}] involved in transferring a mole dextran molecules of 1000 monomers from the enriched to the depleted phase, for the system gelatine/dextran and the system PEO/dextran. The temperature at which phase separation was established was 60°C.

system	concentration [%(w/w)]					
	5.0/5.0	4.5/4.5	4.0/4.0	3.5/3.5	3.0/3.0	2.5/2.5
gelatine/dextran[a]	$10.05\cdot10^3$	$6.73\cdot10^3$	$4.85\cdot10^3$			
PEO-100/dextran				$7.06\cdot10^3$	$5.01\cdot10^3$	$2.72\cdot10^3$
PEO-200/dextran				$8.30\cdot10^3$	$5.84\cdot10^3$	$3.93\cdot10^3$

[a] data from ref. (5).

Summarising the similarities and differences between the two different systems, it appears that the phase separation of aqueous (bio)polymer mixtures can only be established by increasing the polymer concentration and not by decreasing the temperature. This temperature independence of the phase separation suggests that this phase separation is caused by sterical interactions between the dissimilar polymers. The solvent quality difference does not appear to be the driving force of the phase separation. Because if the solvent quality would influence the mechanism of the phase separation, a stronger influence of the temperature was expected. In this case also larger differences would be expected between the values of ΔG of the systems containing PEO or gelatine.

4.5 Conclusions

From the temperature dependence of the cloud points, it can be concluded that the phase separation between PEO and dextran below 80°C, is mainly caused by sterical interactions between dissimilar polymers. Due to the fact that, with increasing temperature, the solvent quality of water for PEO decreases, the phase volume of the PEO-rich phase decreases with increasing temperature. This decreasing phase volume of the PEO-rich phase affects the concentration PEO as well as dextran in the coexisting phases.

Summarising the similarities and differences between the phase behaviour of the systems PEO/dextran and another well studied system gelatine/dextran, it can be concluded that for both systems the main driving force is of entropic nature. It is supposed that this can be interpreted as that the phase separation is the result of the sterical repulsion between the disparate polymer chains. However, the equilibrium state of the PEO/dextran mixture is also influenced by the solvent quality of water for PEO. With respect to the mass fractionation of the polymers, it appears that for both systems the effect of the molar mass and the concentration are the same. There are differences as well between the systems, with respect to the degree of fractionation. For the system gelatine/dextran the degree of fractionation shows an exponential dependence on the molar mass for both gelatine and dextran, whereas this dependence in the system PEO/dextran shows a deviation from exponentiality for dextran at high molar mass. The degree of fractionation of dextran as well as the free energy of transfer of a dextran molecule from the enriched to the depleted phase are in the same order of magnitude for both systems.

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Chapter 5

Interfacial adsorption of high molar mass dextran in phase separating aqueous gelatine/dextran mixtures*

ABSTRACT

Adsorption of high molar mass dextran at the interface between gelatine and dextran during the phase separation of aqueous mixtures of these two polymers is studied. This adsorption is observed after macroscopic phase separation as well as during the phase separation. In the last case, the system is studied with Confocal Scanning Light Microscopy. For this purpose, the dextran is covalently labelled with fluorescein 5-isothiocyanate (FITC). It turns out that the adsorption of high molar mass dextran is highly affected by the labelling. The adsorption of dextran at the interface was studied as function of the molar mass of the dextran, the degree of labelling of the dextran and the pH of the system. It turns out that only the labelled high molar mass dextran adsorbs at the interface, provided that the amount of FITC groups on the dextran backbone is sufficient. The adsorption of labelled dextran leads to a stable film between the two coexisting phases, thus preventing dextran droplets to coalesce. If the degree of labelling increases above a certain level, the labelled dextran does not lead to a stable film, but is preferentially present in the gelatine-rich phase.

* *submitted for publication*

5.1 Introduction

When gelatine and dextran are mixed in the appropriate concentration (typically above 3% (w/w) of each component) in an aqueous solvent, segregative phase separation takes place. Directly after mixing of the two polymer solutions, phase separation sets in. The early stage of this phase separation process is spinodal decomposition [1] and will, in the absence of gelation, be followed by a coarsening and break up of one of the phases from which droplets will be formed. These droplets will coalesce and due to the gravity two macroscopic phases will be formed. One of these phases is rich in dextran and the other is rich in gelatine.

The formation of the two phases implies an interfacial tension between the two coexisting phases. The magnitude of this interfacial tension is known to be in the order of 1-100 $\mu N\ m^{-1}$ [2-4]. Scholten *et al*. [2] reported that, for the aqueous mixture of gelatine and dextran, the surface tension between the two coexisting phases can be estimated using the relation

$$\gamma \sim \frac{kT}{\xi^2} \tag{5.1}$$

where γ denotes the interfacial tension and ξ is the width of the region in which the concentration of the components differ from their bulk concentration in the coexisting phases [5,6]. The value of ξ increases with increasing radius of gyration of the molecules.

Gelatine and dextran are both polydisperse in their molar mass [7]. During the phase separation of gelatine and dextran, fractionation in molar mass takes place, which results in a different molar mass distribution of both polymers in both phases [7-12].

In this paper the behavior of dextran at the interface between gelatine- and dextran-rich coexisting phases is studied. We assumed that the high molar mass fraction of the dextran adsorbs at the interface. However, to make dextran visible using Confocal Scanning Light Microscopy (CSLM), the dextran had to be covalently labelled with a fluorescent label. For this purpose we used dextran which was covalently labelled with fluorescein 5-isothiocyanate (FITC). It turned out that the use of this fluorescent label affects the adsorption of the labelled dextran at the interface significantly. Particularly the hydrophobicity of FITC turned out to play an important role.

With the use of CSLM, the adsorption behavior of dextran at the interface is studied during the early stages of the phase separation with gelatine. This behavior is studied as function of the molar mass, degree of labelling, and pH. The microscopic phase separation was also compared with macroscopic phase separation.

5.2 Experimental

5.2.1 Materials

High molar mass fish gelatine (IEP ± 7.8) was obtained from Multi Products (Amersfoort, The Netherlands). Dextran (148, 282, 464, and 2000 kDa, these values verified by SEC-MALLS) and fluorescein 5-isothiocyanate (FITC) were purchased from Sigma Chemicals. All materials were used without further purification.

5.2.2 Preparation of the samples

Separate solutions of gelatine and dextran were made by gravimetrically adding the solvent (Reversed Osmosis (RO) water containing 0.02% sodium azide to prevent bacterial growth) to the proper amount of solute. Dextran dissolves readily at room temperature, while gelatine was stirred over night at room temperature on a magnetic stirrer. Mixtures containing 10% (w/w) gelatine and 10% (w/w) dextran were prepared by gravimetrically adding the solutions together. The dextran in the final mixture contained both low molar mass dextran (i.e. M_w 148 or 282 kDa) and high molar mass dextran (i.e. M_w 464 or 2000 kDa). To make a distinction with the CSLM between the two different molar mass fractions, either the high molar mass dextran or the low molar mass dextran was covalently labelled with FITC.

5.2.3 Labelling

To make the dextran visible with CSLM, it was covalently labelled with FITC, following the procedure described by de Belder and Granath [13] and Tromp *et al.* [14]. To vary the degree of labelling, the amount of FITC used was varied.

The degree of the labelling is given as the number of FITC molecules per 1000 monomers of dextran. A dextran monomer was taken to be a glucose unit. The amount of FITC molecules present on the dextran backbone was determined by measuring the absorption at 492 nm of a solution with a known concentration labelled dextran. With use of a calibration line, the concentration FITC present in the dextran sample could be calculated. For the determination of the amount of FITC present in the sample, the pH was set at a value higher than 6.4 (i.e. the pK of FITC), because the absorbance at 492 nm is dependent on the presence of a negative charge at the FITC molecules.

5.2.4 Observations with CSLM

For the observations with CSLM, the molar mass of dextran, the degree of labelling, the pH and the fraction of labelled dextran were varied. For the series of samples in which the pH was varied, a 0.1 M NaOH or a 0.1 M HCl solution was used to adjust the pH of the sample.

The observations were carried out on a LEICA TCS SP Confocal Scanning Light Microscope, in single photon mode, configured with an inverted microscope (model LEICA DM IRBE) and using an Ar/Kr laser. The excitation wave length of FITC was 488 nm and the emission maximum was at 518 nm.

5.2.5 Macroscopic phase separation

In order to investigate the influence of the labelling on the macroscopic phase separation, mixtures of gelatine and dextran were made in which the concentration labelled dextran and de degree of labelling were varied (see table 5.1). The samples were prepared in a glass jar and covered with a layer of paraffin oil to prevent evaporation. The samples were first heated for 24 hours in a water bath at 50°C, after which they were stored for three days at room temperature. Finally, samples of both phases were taken with a syringe with a hypodermic needle.

The concentration gelatine and dextran was determined by measuring the optical rotation (OR) at 365 nm and 578 nm (see [15] for details of the method). This method could be used because FITC does not cause optical rotation. Attention was also paid to the influence of the FITC on the intensity of the OR signal, since FITC absorbs light at 365 nm. However, due to the fact that the samples were diluted 40 times before measuring the optical rotation, the concentration FITC was too low to disturb the measurements. The concentration FITC in the coexisting phases was measured by determining the absorption of the samples at 492 nm.

Table 5.1: Overview of the samples which were used to study macroscopic phase separation. Concentrations are given in [%(w/w]).

degree of labelling	conc. gelatine	conc. dextran 282 kDa	conc. dextran 2000 kDa	conc. FITC-labelled dextran 2000 kDa
6	10	9.5	-	0.5
6	10	9.5	0.2	0.3
6	10	9.5	0.4	0.1
19	10	9.5	-	0.5
19	10	9.5	0.2	0.3
19	10	9.5	0.4	0.1
30	10	9.5	-	0.5
30	10	9.5	0.2	0.3
30	10	9.5	0.4	0.1

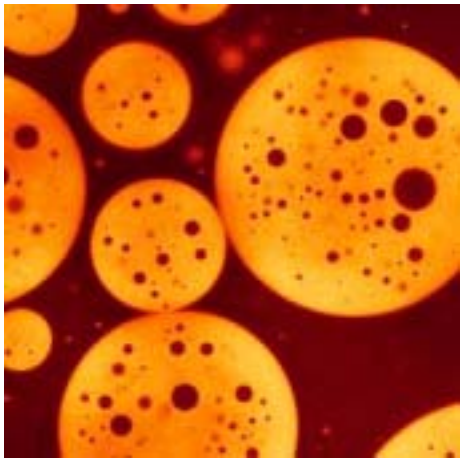


Figure 5.1: CSLM-images of phase a phase separating mixture of 10% (w/w) gelatine and 10% (w/w) dextran. The dextran fraction consists of 95% dextran 2000 kDa and 5% labelled dextran 282 kDa. The degree of labelling is 30. The size of the image is 160 × 160 µm.

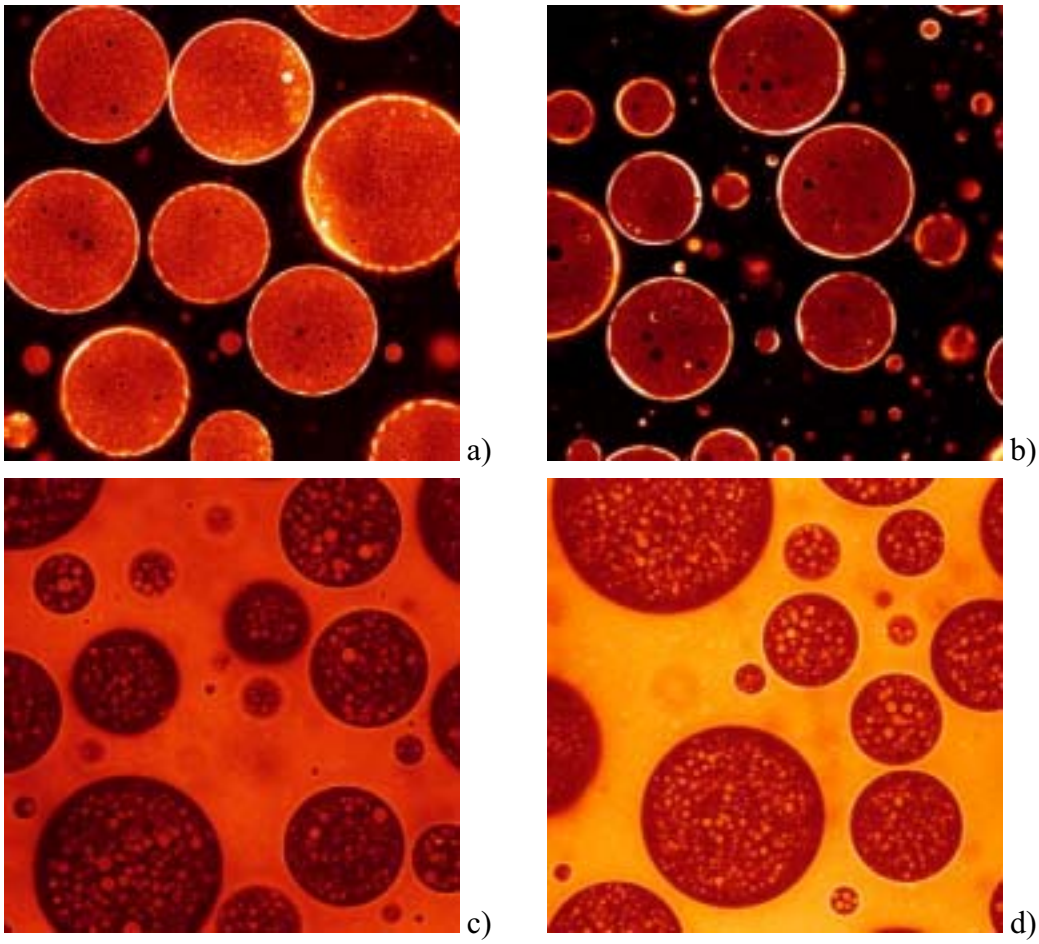


Figure 5.2: CSLM-images of phase separating mixtures of gelatine and dextran. All mixtures contain 10% (w/w) gelatine and 10% (w/w) dextran. The dextran fraction consists of a) 95% dextran 148 kDa + 5% labelled dextran 2000 kDa, b) 95% dextran 282 kDa + 5% labelled dextran 2000 kDa, c) 95% dextran 148 kDa + 5% labelled dextran 464 kDa and d) 95% dextran 282 kDa + 5% labelled dextran 464 kDa. The degree of labelling is 30. The size of the images is 160 × 160 µm.

5.3 Results

5.3.1 CSLM observations

When the mixtures of gelatine and dextran are observed with CSLM, droplets of gelatine in a dextran continuous phase or droplets of dextran in a gelatine continuous phase are seen. If the high molar mass fraction of the dextran is labelled with FITC and the ratio between the high- and low molar mass fractions is appropriately chosen, the dextran droplets are surrounded by a bright 'ring' (in 2D). Since only the labelled dextran is visible with CSLM, these rings have to consist of the labelled fraction (i.e. the high molar mass fraction) of dextran. However, it turns out that the appearance of these rings depends on various conditions. The results of changing these conditions are described below.

Effect of molar mass

Figure 5.2 shows that if the high molar mass fraction of dextran is labelled, rings are visible, whereas if the low molar mass fraction of dextran is labelled, the rings do not appear (see figure 5.1) The degree of labelling is the same for all samples, namely 30.

Figure 5.3 shows that if dextran only consists of high molar mass dextran, from which 5% is labelled with FITC, rings are also visible.

Effect of degree of labelling

Figure 5.4 shows to what extend the degree of labelling influences the appearance of the rings. If the amount of FITC molecules on the dextran chains decreases, the rings around the droplets become less thick and finally disappear. It also appears that the droplets in figure 5.4b (degree of labelling is 19) are smaller than those shown in figure 5.4a and 5.4c (degree of labelling is 6 and 30 respectively).

Effect of the pH

Figure 5.5 shows the effect of the pH on the appearance of the rings. Below the iso-electric point of gelatine, rings are visible. If the pH increases above the iso-electric point of gelatine, the rings do not exist. The images shown are made with dextran with a degree of labelling of 17. If the degree of labelling is increased to 30, it turns out that also at a pH above the iso-electric point of gelatine, rings appear after some time (images not shown).

5.3.2 Macroscopic phase separation

Observations by eye

After macroscopic phase separation, differences were observed with different degrees of labelling, cf. figure 5.6. This figure shows that the samples in which the degree of labelling was 6 (A) or 30 (C) undergo macroscopic phase separation resulting in a sharp

meniscus between the two coexisting phases. For a degree of labelling of 19 molecules of FITC per 1000 monomers dextran (B), one obtains a stable emulsion, and no macroscopic phase separation occurs. When this sample was studied with CSLM, it resembles a structure as shown in figure 5.4b. When the amount of labelled dextran was decreased to 0.1% (w/w) (keeping the total dextran concentration constant) this sample also showed macroscopic phase separation. However, the interface between the phases was not very sharp and the gelatine-rich phase was much smaller than the dextran-rich phase.

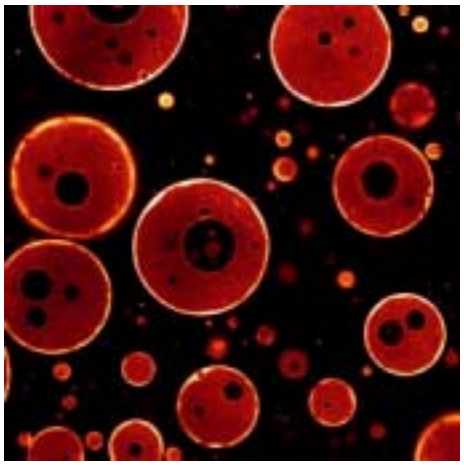
When the upper and lower phases of samples A and C are compared it appears that in sample A (degree of labelling is 6) the FITC labelled dextran is mainly in the lower, dextran-rich phase, while in sample C (degree of labelling is 30) the FITC labelled dextran is mainly in the upper, gelatine-rich phase.

Analysis of the phases

Both phases of the macroscopic phase separated mixtures were analysed with respect to concentration gelatine, dextran and FITC. The results are given in table 5.2. The value for the concentration FITC-labelled dextran after phase separation is made with the assumption that the dextran chains have an average distribution of FITC molecules. Since the mixtures in which dextran had a degree of labelling of 19 were not separated into two clear macroscopic phases, no samples were taken from these mixtures.

Table 5.2: Concentration gelatine, dextran and FITC in the upper and lower phase of the mixtures containing dextran with a degree of labelling of 6 and 30. The concentrations of gelatine and dextran are given in [%(w/w)] and the concentration FITC is given in [mmol kg^{-1}].

degree of labelling	phase	conc. FITC-labelled dextran before phase separation	conc. gelatine	conc. dextran	conc. FITC-labelled dextran after phase separation	conc. FITC
6	upper	0.5	20.0	2.1	0.22	0.08
6	upper	0.3	17.4	1.8	0.11	0.04
6	upper	0.1	17.2	3.7	0.08	0.03
6	lower	0.5	0.5	17.7	0.73	0.27
6	lower	0.3	0.8	17.9	0.46	0.17
6	lower	0.1	1.2	18.4	0.14	0.05
30	upper	0.5	15.7	3.1	0.70	1.30
30	upper	0.3	18.0	3.8	0.43	0.80
30	upper	0.1	15.4	3.4	0.13	0.24
30	lower	0.5	0.8	17.5	0.06	0.11
30	lower	0.3	0.5	17.3	0.13	0.24
30	lower	0.1	0.7	17.5	0.06	0.12


a)

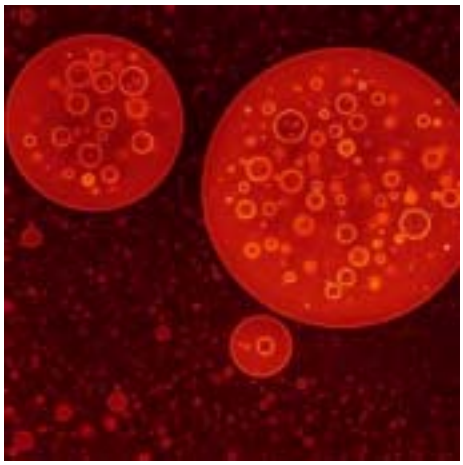
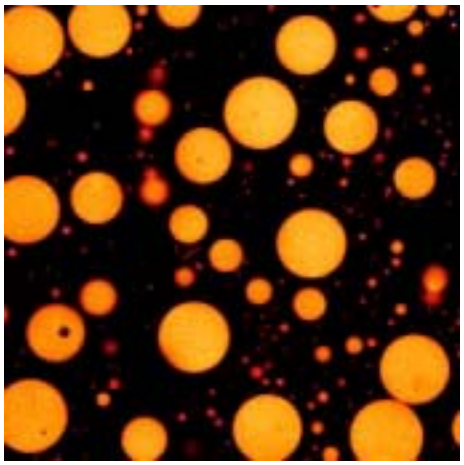

b)

Figure 5.3:CSLM-images of phase separating mixtures of 10% (w/w) gelatine and 10% (w/w) dextran. The molar mass of dextran is in figure a) 2000 kDa and in figure b) 464 kDa. In both figures 5% of the dextran fraction is covalently labelled with FITC. The degree of labelling is 30. The size of the images is 160 × 160 μm.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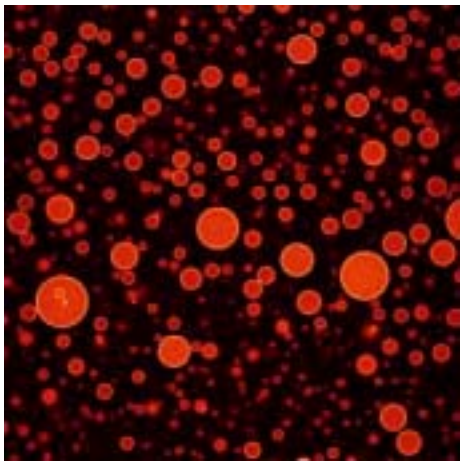
b)

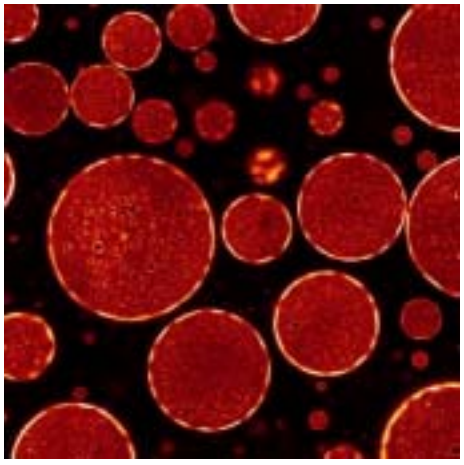
c)

Figure 5.4: CSLM-images of phase separating mixtures of 10% (w/w) gelatine and 10% (w/w) dextran. The dextran fraction consists of 95% dextran 282 kDa and 5% labelled dextran 2000 kDa. The degree of labelling is in a) 6, in b) 19 and in c) 30 FITC molecules per 1000 monomers dextran. The size of the images is 160 × 160 μm.

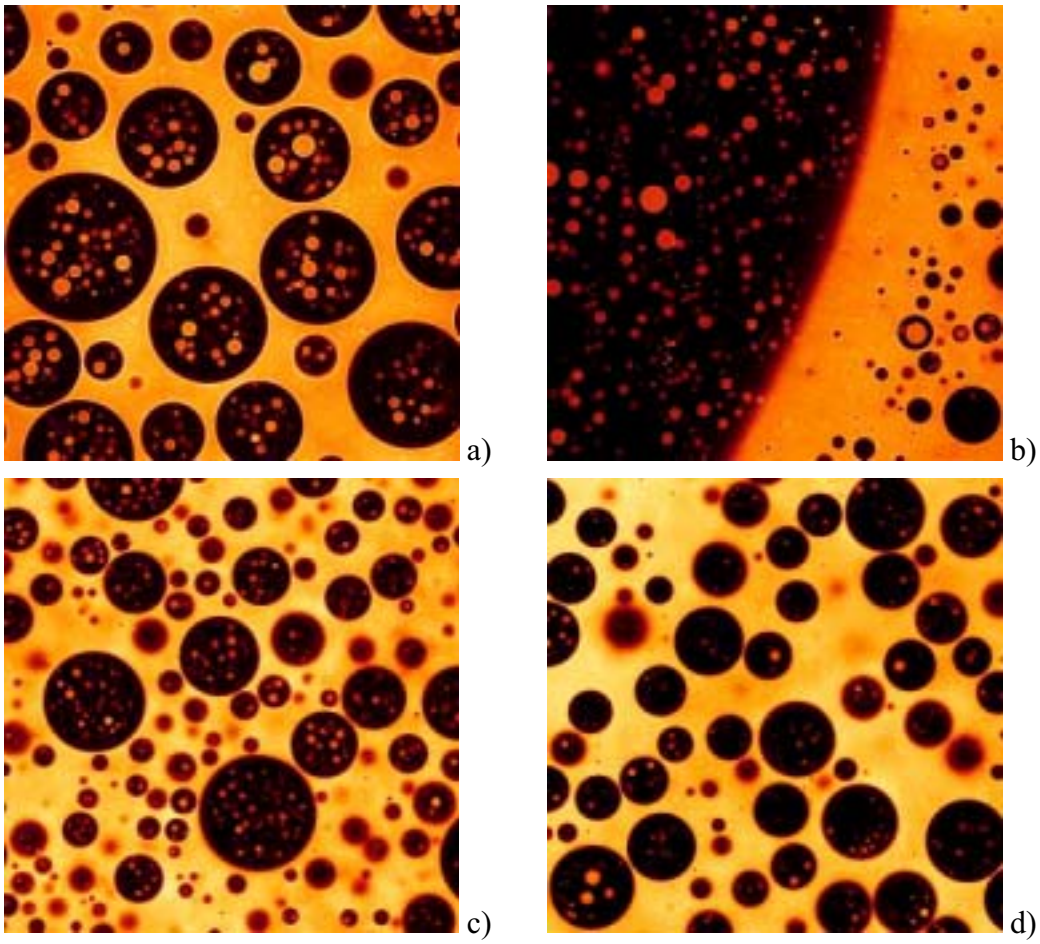

Figure 5.5: CSLM-images of phase separating mixtures of 10% (w/w) gelatine and 10% (w/w) dextran. The dextran fraction consists of 95% dextran 282 kDa and 5% labelled dextran 2000 kDa. The degree of labelling is 17. The pH has a value of a) 6.0, b) 6.8, c) 8.5 and d) 9.3. The size of the images is 160 × 160 µm.

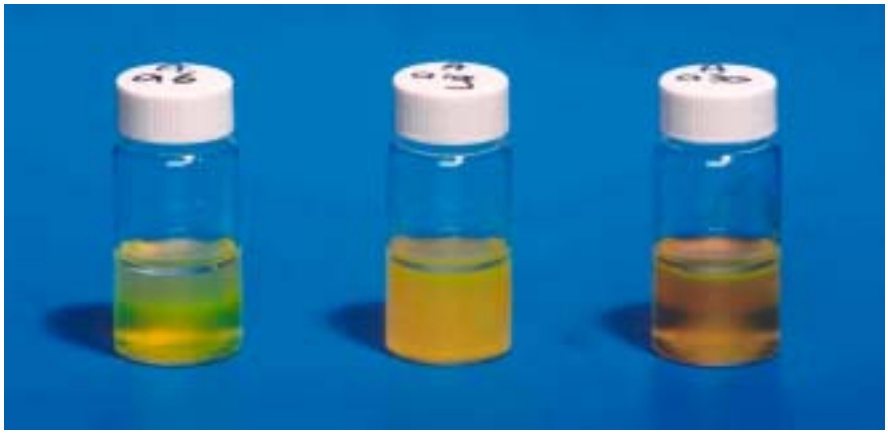

Figure 5.6: Macroscopic phase separated mixtures of 10% gelatine, 9.5% dextran 282 kDa and 0.5% labelled dextran 2000 kDa. The degree of labelling is A (left): 6, B (middle): 19 and C (right): 30 FITC molecules per 1000 monomers dextran. The transparent layer on top of the samples is paraffin oil which was added to prevent evaporation of the sample.

The results show that for all mixtures, the upper phase is the gelatine-rich phase whereas the lower phase is rich in dextran. The other observation is that in the mixtures containing dextran with a degree of labelling of 6, the concentration FITC is the highest in the dextran-rich phase, whereas for the mixtures containing the dextran with a degree of labelling of 30, the FITC favours the gelatine-rich phase.

5.4 Discussion

In this paper, observations, on microscopic as well as on macroscopic scale, are presented on the behaviour of dextran which is covalently labelled with fluorescein 5-isothiocyanate, during the phase separation of gelatine and dextran. These observations were performed on systems in which the molar mass of dextran, the degree of labelling, the pH and the amount of labelled dextran were varied.

It turns out that only for the high molar mass dextran (2000 or 464 kDa) there is a driving force to adsorb at the interface between gelatine and dextran. This is apparent from the fact that rings are observed using CSLM. The rings are more clear in the case of a dextran molar mass of 2000 kDa than in the case where dextran with a lower molar mass (464 kDa) is used.

The CSLM images as well as the observations on the macroscopic phase separation show that if the degree of labelling is changed, the behaviour of the labelled dextran also changes. The CSLM images of figure 5.4 show that if the degree of labelling increases, the preference of the labelled dextran for the interface also increases. The effect of the degree of labelling on the macroscopic phase separation shows that when the degree of labelling increases above 6, the system first does not show macroscopic phase separation and, when the amount of FITC on the dextran backbone is high enough, the FITC-labelled dextran is mainly found in the gelatine-rich phase. FITC is a large hydrophobic molecule (see figure 5.7) and negatively charged at pH values above 6.4. Gelatine has hydrophobic groups and is, at pH values beneath the iso-electric point (~7.8), positively charged. This combination of properties and the fact that the pH is kept between 6.4 and 7.8 might explain why the FITC-labelled dextran fraction shows a preference for the gelatine-rich phase, as observed for the samples in which the degree of labelling was 30.

In this paper, the effect of three different degrees of labelling is studied. In the first case, the degree of labelling is 6 which means that only a few FITC molecules are present on an average dextran chain. In this case no influence of FITC is shown on the behaviour of the dextran: no rings are visible on the CSLM images and the dextran behaves like unlabelled dextran in the macroscopic phase separation. This suggests that the molar mass is not the main driving force for the adsorption of dextran at the interface.

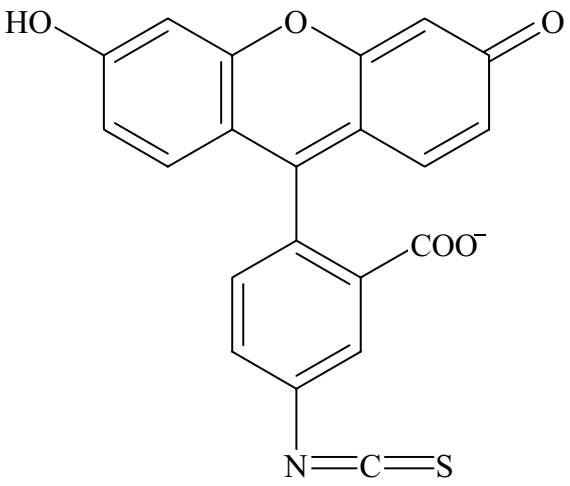

Figure 5.7: Structure of fluorescein 5-isothiocyanate.

In the second case, where the degree of labelling is 19, dextran becomes effectively hydrophobically modified and preferentially resides at the interfacial layer between the dextran-rich and the gelatine-rich phases. In this case, the modified dextran is able to stabilise the droplets against coalescence, as can be concluded from the observation that the droplets remain small and that no macroscopic phase separation occurs. However, if the fraction of labelled dextran is decreased while the total fraction of high molar mass dextran is kept constant, macroscopic phase separation does take place. Apparently, in this case the amount of labelled dextran at the interface is too low to prevent coalescence. This is once again an indication that the molar mass is not the only factor which causes the adsorption of high molar mass dextran at the interface.

In the last case, with of a degree of labelling of 30, the labelled dextran even appears to prefer the gelatine-rich phase. The CSLM images also show that the rings break up.

The role of FITC in the formation of the rings is also shown in the experiment in which the pH is varied. As long as the pH is below the iso-electric point of gelatine, the gelatine is positively charged, so the labelled dextran and the gelatine have opposite charges. In this situation, rings are visible around the droplets shown in the CSLM images. However, as soon as the pH is higher than the iso-electric point of gelatine, both the labelled dextran and the gelatine are negatively charged and no rings are formed. These observations suggest that the interaction between the labelled dextran and gelatine are, among others, of electrostatic nature. If the degree of labelling has a value of 30, even at a pH above the iso-electric point of gelatine, rings do appear. We propose that this is due to hydrophobic effects and/or due to the fact that gelatine might have a distribution of iso-electric points, thereby leaving some positively charged spots on the gelatine above a pH of 7.8.

On basis of all observations, we propose that the rings are formed due to complexation between gelatine and FITC-labelled dextran. These complexes may be interfacially active between the gelatine and dextran phases. In other words, the complex of FITC-labelled dextran and gelatine may be seen as a surfactant for mixtures of gelatine and dextran.

During coalescence of two droplets, most of the time the film around the droplets disappears and a new film is formed around the newly formed droplet. This disappearance and new formation of the film happens within the time scale of the observation (a few minutes). This suggests that the film is liquid. In some occasions it is observed that the film does not break up in the time scale of the experiment (see figure 5.8), suggesting a relatively high viscosity of the liquid film. This would be in accordance with the idea that the complexes between gelatine and FITC-labelled dextran form a coacervate around the droplets [16]. This coacervate phase would also explain the fact that coalescence between droplets does not occur as soon as the droplets have rings around them.

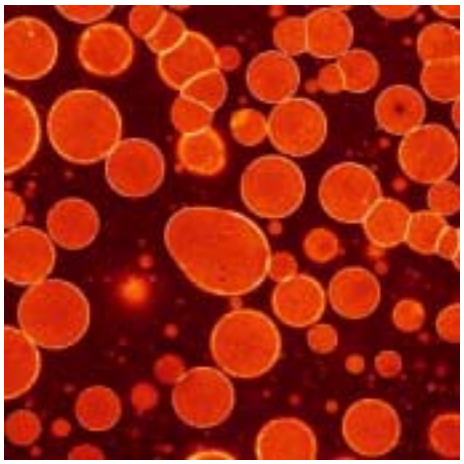

Figure 5.8: CSLM image of a phase separating mixture of 10% (w/w) gelatine and 10% (w/w) dextran. The dextran fraction consists of 95% dextran 282 kDa and 5% labelled dextran 2000 kDa. The degree of labelling is 30. The size of the image is 450 × 450 μm

Some CSLM images (particularly figure 5.4c) show that the rings can break up into small curved and interrupted lines remaining at the interface. Since the space between the interruptions is rather regular and the film is liquid, it is assumed here that the break-up of the film is caused by Rayleigh instabilities [17,18].

5.5 Conclusions

In phase separating mixtures of polydisperse gelatine and dextran, in which the high or the low molar mass fraction of dextran is covalently labelled with FITC, the high molar mass fraction of the labelled dextran adsorbs at the interface between the gelatine-rich and

the dextran-rich phase. The adsorption is strongly affected by the presence of FITC-labels on the dextran molecules. FITC changes the interfacial activity of dextran. Due to the charged groups on the labelled dextran backbone, complexes between the labelled dextran and the gelatine can be formed. If the dextran backbone has a degree of labelling of 19 and the amount of labelled dextran present is high enough (i.e. 0.5% (w/w)), the labelled dextran is able to form a stable interfacial film, which prevents the droplets to coalesce. This interfacial film is supposedly existing of complexes between the FITC-labelled dextran and the gelatine which might be surface active. In conclusion, the work suggests that FITC-labelled dextran is a surfactant for phase separating mixtures of gelatine and dextran. If the degree of labelling increases to a value of 30, the labelled dextran is preferentially present in the gelatine-rich phase.

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Summary & Samenvatting

Summary

The work in this thesis focuses on segregative phase separation in aqueous polymer mixtures. This phase separation results in the appearance of separate macroscopic layers, each of them rich in one of the polymers. To understand the mechanism of the phase separation of polymer mixtures, the phase separation of two polymer mixtures is experimentally investigated and the results are related to theoretical models known from the literature. Experimental parameters that are varied are the temperature, the molar mass of the polymers and the polymer concentration. Next to the mechanism of the phase separation, also the fractionation, which is the result of the phase separation, is studied.

In particular, the phase separation of aqueous mixtures of gelatine and dextran (chapter 2, 3 and 5), as well as of poly(etylene oxide) (PEO) and dextran (chapter 4) is discussed. Most of the work is performed on the equilibrium state of the phase separation, but chapter 5 deals with some non-equilibrium aspects.

In chapter 2, the phase separation of aqueous mixtures of gelatine and dextran above the gelation temperature of gelatine is discussed. First, the temperature at which phase separation takes place as function of the total polymer concentration was determined. The results show that the phase separation only takes place by increasing the polymer concentration, whereas decreasing the temperature has hardly any effect. The results also show that the higher the molar mass of dextran, the lower the total polymer concentration at which phase separation occurs. Apart from this, temperature-composition phase diagrams of both polymers were obtained. It turns out that the composition of the coexisting phases is only affected by the total polymer concentration; the lower the polymer concentration in the starting mixture, the more similar the concentrations of the polymers in the coexisting phases. The molar mass of dextran and the temperature at which phase separation is established does not show any influence on the concentration in the coexisting phases. Since the temperature has no influence on the phase separation, and the polymer concentration is determining the phase separation, it is concluded that the phase separation is mainly one of entropic nature. Indeed, by relating the experimental data to the theoretical models which were available in the literature, it turned out that the data were in good agreement with a recently developed depletion model.

Gelatine as well as dextran are polydisperse in their molar mass. In chapter 2 it is shown that the phase separation induces a strong fractionation of the molar mass of gelatine as well as dextran. In chapter 3, a more detailed study to this fractionation is presented. In this chapter, the molar mass distributions of gelatine as well as dextran in the coexisting phases were determined. The results show that after phase separation molecules of the high molar mass part of the distribution are found in the enriched phase whereas the depleted phase only contains molecules of the low molar mass part of the distribution. It

turns out that the fractionation is affected by the polymer concentration and the average molar mass of the polymers. The temperature at which phase separation is established does not show an influence on the molar mass distribution after phase separation. A measure for the fractionation can be given by dividing the concentration of polymer x with a molar mass, m, in its poor phase ($c_{x,poor,m}$) by the concentration of the same polymer with the same molar mass in its rich phase ($c_{x,rich,m}$). The fractionation is exponentially dependent on the molar mass. From this, the energy for transferring a polymer with a molar mass m from its enriched to its depleted phase is deduced and related to the interfacial tension between the coexisting phases. The value of the interfacial tension thus obtained is in good agreement with the values of experimentally measured interfacial tensions reported in the literature. Another aspect of the fractionation is a possible chemical difference between gelatine in the two coexisting phases which results, together with the molar mass fractionation, in other functional properties for the material in the different phases. The gelatine in its rich phase has other gelling properties compared to the gelatine in its poor phase and compared to the native material. Finally it was found that the low molar mass part of the distribution in the poor phase contributes to the osmotic pressure in the same way whether it is in the poor phase of a system in which the molecules can move freely or in the rich phase of a system which is forcibly separated with a dialysis membrane. From this it is concluded that water affinity is no driving force for the phase separation.

To obtain a more general view on the phase separation of polymers, in chapter 4 results are shown of the phase separation of aqueous mixtures of poly(ethylene oxide) (PEO) and dextran. For this purpose, the same experiments were carried out as already described in chapter 2 and 3 for mixtures of gelatine and dextran. The results of the phase separation of mixtures of PEO and dextran are, for temperatures below 80°C, similar to the results of the phase separation of mixtures of gelatine and dextran. E.g. it turns out that the polymer concentration is the only factor that affects the occurence of the phase separation. From this it is concluded that the phase separation of PEO and dextran below 80°C is, like that of gelatine and dextran, mainly caused by sterical interactions between the dissimilar polymers. At 80° the solvent quality does get involved and becomes determinant for the occurrence of phase separation. At increasing temperature, the phase volume of the PEO-rich phase decreases. This affects both the concentration of PEO as well as of dextran in the coexisting phases (mainly the rich phases). The fractionation of dextran in this case is not exponentially dependent on the molar mass, in contrast to the gelatine/dextran system.

In chapter 5, the effect of the polydispersity of dextran during the phase separation is discussed. In this chapter the phase separation of gelatine and dextran was studied with use of Confocal Scanning Light Microscopy (CSLM). To visualise the two disparate phases, the dextran was covalently labelled with fluorescein 5-isothiocyanate (FITC). During the phase separation process, droplets of either the gelatine-rich phase or the dextran-rich

phase are formed. If the high molar mass fraction of dextran is labelled, a bright ring around the dextran-rich droplets becomes visible. The appearance of the rings is strongly affected by the degree of labelling of the dextran molecules and by the pH of the system. It is proposed that labelled dextran forms complex coacervates with the gelatine in the gelatine-rich phase and that these complexes are also situated at the interface. In this case a stable interfacial layer is formed which prevents the dextran droplets to coalesce, thus forming a stable emulsion.

Samenvatting

Het doel van het in dit proefschrift beschreven onderzoek is het geven van een kwantitatieve beschrijving van de segregatieve fasescheiding van biopolymeermengsels in water. Deze vorm van fasescheiding resulteert in afzondelijke, macroscopische fases die elk rijk zijn aan één van de biopolymeren. Om het mechanisme van deze fasescheiding te kunnen begrijpen is de fasescheiding van twee verschillende polymeermengsels experimenteel bestudeerd en zijn de resultaten van de uitgevoerde experimenten vergeleken met theoretische modellen die in de literatuur beschreven zijn. De experimentele parameters die in dit onderzoek zijn gevarieerd, zijn: 1. de temperatuur, 2. het molecuulgewicht van de polymeren en 3. de polymeerconcentratie. Naast het mechanisme van de fasescheiding is ook de fractionering van het molecuulgewicht als gevolg van de fasescheiding bestudeerd.

In dit proefschrift zijn met name mengsels van gelatine en dextraan (hoofdstuk 2, 3 en 5) en polyethyleenoxide (PEO) en dextraan (hoofdstuk 4) bestudeerd. Het meeste onderzoek is gedaan aan systemen in evenwicht, maar in hoofdstuk 5 worden ook enkele niet-evenwichts verschijnselen beschreven.

In hoofdstuk 2 wordt de fasescheiding van gelatine en dextraan beschreven. De experimenten zijn uitgevoerd boven de geleringstemperatuur van gelatine. In de eerste plaats is de temperatuur waarbij fasescheiding optreedt als functie van de polymeerconcentratie bepaald. Het blijkt dat fasescheiding met name veroorzaakt wordt door het verhogen van de polymeerconcentratie, terwijl het verlagen van de temperatuur nauwelijks een effect heeft op het al dan niet optreden van fasescheiding. Ook blijkt dat als het molecuulgewicht van de polymeren toeneemt, de concentratie waarbij fasescheiding optreedt afneemt. Vervolgens zijn er temperatuur-compositie fasediagrammen gemaakt. Hieruit is op te maken dat alleen de polymeerconcentratie van invloed is op de samenstelling van de beide fases; hoe hoger de totale polymeerconcentratie, des te groter is het verschil in samenstelling van de beide fases. Omdat de temperatuur niet, en de polymeerconcentratie juist wel van invloed blijkt te zijn op de fasescheiding, is de conclusie getrokken dat de fasescheiding van gelatine en dextraan entropisch gedreven is. Bij toetsing van de experimentele resultaten aan theoretische modellen, blijkt dat ze goed passen in een onlangs ontwikkeld depletiemodel.

Zowel gelatine als dextraan zijn polydispers in hun molecuulgewicht. In hoofdstuk 2 is al laten zien dat zowel in het geval van gelatine als dextraan na fasescheiding de molecuulgewichtsverdeling in de beide fases van elkaar verschilt. In hoofdstuk 3 is deze fractionering verder onderzocht. In dit hoofdstuk zijn de molecuulgewichtsverdeling van zowel gelatine als dextraan vóór en na fasescheiding beschreven. Het blijkt dat de moleculen met een hoog molecuulgewicht met name in hun geconcentreerde fase zitten,

terwijl de moleculen met een laag molecuulgewicht vooral in hun verdunde fase te vinden zijn. Ook blijkt dat de fractionering van het molecuulgewicht beïnvloed wordt door de polymeerconcentratie en het gemiddelde molecuulgewicht van de polymeren en dat de temperatuur geen invloed heeft op de fractionering. Een maat voor de fractionering kan worden gegeven door de concentratie van polymeer *x* met molecuulgewicht *m* in zijn verdunde (arme) fase ($c_{x,arm,m}$) te delen door de concentratie van hetzelfde polymeer met hetzelfde molecuulgewicht in zijn geconcentreerde (rijke) fase ($c_{x,rijk,m}$). Het blijkt dat fractionering exponentieel afhangt van het molecuulgewicht. Uit deze afhankelijkheid kan de vrije energie berekend worden die nodig is om een polymeer met molecuulgewicht *m* te verplaatsen van zijn geconcentreerde naar zijn verdunde fase. Aan de waarde voor deze vrije energie kan vervolgens een waarde voor de oppervlaktespanning tussen de twee fases gerelateerd worden. Het blijkt dat de waarde van deze oppervlaktespanning goed overeen komt met waardes die experimenteel bepaald zijn en in de literatuur vermeld worden. Een gevolg van de fractionering is ook dat de geleringseigenschappen van de gelatine uit de twee verschillende fases van elkaar verschillen en bovendien verschillen van de geleringseigenschappen van de gelatine vóór faseschеiding. Tenslotte blijkt dat de polymeermoleculen met een laag molecuulgewicht die zich in de arme fase bevinden op dezelfde manier bijdragen aan de osmotische druk als dezelfde moleculen die zich in de rijke fase bevinden van een systeem dat gedwongen fasegescheiden is door middel van een dialysemembraan. Hieruit wordt volgt de conclusie dat wateraffiniteit geen drijvende kracht voor de faseschеiding is.

Om een generiek beeld te krijgen van de faseschеiding van oplossingen van polymeermengsels, wordt in hoofdstuk 4 de faseschеiding van oplossingen van polyethyleenoxide (PEO) en dextraan bestudeerd. Met dit mengsel zijn dezelfde experimenten uitgevoerd als met het mengsel van gelatine en dextraan zoals die in hoofdstuk 2 en 3 beschreven zijn. Het blijkt dat als de temperatuur lager dan 80°C is, alleen de polymeerconcentratie bepalend is voor het optreden van faseschеiding en de samenstelling van de verschillende fases. Dit betekent dat de faseschеiding van PEO en dextraan in water hetzelfde gedrag vertoont als dat van de faseschеiding van gelatine en dextraan in water. Hieruit wordt geconcludeerd dat de faseschеiding van PEO en dextraan beneden 80°C, net als die van gelatine en dextraan, met name veroorzaakt wordt door sterische interacties tussen de verschillende polymeren. Bij 80°C blijkt echter de oplosmiddelkwaliteit te bepalen of er faseschеiding optreedt. Het blijkt dat over het hele temperatuurgebied waar metingen zijn gedaan, bij toenemende temperauur het fasevolume van de PEO-rijke fase afneemt. Dit beïnvloedt zowel de concentratie van PEO als van dextraan, met name in hun rijke fase. Ook blijkt dat de fractionering van dextraan niet langer exponentieel afhangt van het molecuulgewicht.

In hoofdstuk 5 wordt gekeken naar het effect van de polydispersiteit van dextraan tijdens de fasescheiding. In dit hoofdstuk wordt de fasescheiding van gelatine en dextraan bestudeerd met behulp van 'Confocal Scanning Light Microscopy' (CSLM). Om de twee fases zichtbaar te maken zijn de dextraanmoleculen covalent gelabeld met fluorescine 5-isothiocyanaat (FITC). Tijdens de fasescheiding ontstaan er druppels van de gelatine-rijke fase in een dextraanmatrix ofwel van de dextraan-rijke fase in een gelatinematrix. Als de fractie van dextraan met een hoog molecuulgewicht gelabeld wordt, is er een heldere ring om de druppels van de dextraan-rijke fase zichtbaar. Het al of niet verschijnen van deze ringen wordt sterk beïnvloed door de aanwezigheid van de FITC-moleculen op de dextraanketen en de pH van het systeem. Er wordt gesuggereerd dat de gelabelde dextraanmoleculen een complex vormen met de gelatine in de gelatine-rijke fase. Deze complexen bevinden zich ook aan het grensvlak van de gelatine- en dextraan-rijke fase en voorkomen op deze manier dat de druppels kunnen coalesceren. In dit geval wordt er een stabiele emulsie gevormd.

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Curriculum Vitae

Marijke Willemien Edelman werd op 11 oktober 1974 te Rotterdam geboren. In 1993 behaalde zij haar Gymnasium-β diploma aan het Rotterdams Montessori Lyceum. In datzelfde jaar begon zij aan de studie levensmiddelentechnologie aan de Landbouwuniversiteit Wageningen. Zij volgde de specialisatie levensmiddelennatuurkunde en tijdens haar studie heeft zij afstudeervakken uitgevoerd bij de toenmalige secties Levensmiddelennatuurkunde en Levensmiddelenchemie. Haar stage heeft zij gelopen bij Quest International in Naarden. In 1999 studeerde zij af. Van juli 1999 tot en met juli 2003 was zij als Assistent in Opleiding in dienst van (inmiddels) Wageningen Universiteit bij de leerstoelgroep 'Fysica en Fysische Chemie van Levensmiddelen'. Gedurende deze vier jaar was zij gedetacheerd bij het Wageningen Centre for Food Sciences en verrichtte zij het in dit proefschrift beschreven onderzoek op de afdeling Product Technology van NIZO food research te Ede.

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Het is een beetje een cliché, maar wel een waarheid als een koe: onderzoek doe je niet alleen. Terugkijkend op de afgelopen vier jaar moet ik dan ook concluderen dat dit ook voor mijn promotieonderzoek geldt en na vier jaar werken als WCFS-AIO komt er dan nu een einde aan een periode waarin ik een raar soort driehoeksverhouding heb onderhouden tussen Wageningen Universiteit, NIZO food research en WCFS. Gedurende deze vier jaar heb ik dan ook samengewerkt met mensen op al deze drie instituten en op deze plaats wil ik iedereen die mij op welke wijze dan ook geholpen heeft hiervoor hartelijk bedanken.

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Cover: CSLM image of gelatine droplets, phase separating from the dextran-continuous phase. Image by Jan van Riel.
Cover design: Pieter Edelman with kind help of Michel Renders