Applications of Polyelectrolyte Complexes for Separation Processes

J.J. van Lente

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Jéré Jacko van Lente

APPLICATIONS OF POLYELECTROLYTE COMPLEXES FOR SEPARATION PROCESSES

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Summary

Polyelectrolytes are polymers with potentially charged monomer units. Under the right conditions, oppositely charged polyelectrolytes in solution can interact and form polyelectrolyte complexes (PECs). These PECs have peculiar emergent properties. One of these properties is the ability to accumulate certain molecules in very high concentrations. A comparison can be made between PECs and membraneless-organelles (MLOs), which are droplet-like liquid-in-liquid phases present in our cells that aid the cell in organising its interior milieu through selective partitioning of specific molecules. Similar to PECs, MLOs are also composed op oppositely charged polyelectrolytes. In the case of MLOs, these are typically negatively charged RNA molecules and positively charged intrinsically disordered proteins. Taking inspiration from nature, in this dissertation we present the use of PECs for the partitioning of molecules. We investigated whether it was possible to use PECs to selectively extract a protein from a mixture. Followed by whether a PEC containing a protein could be used as a biocatalytic membrane.

Chapter 1 is a theoretical introduction to polyelectrolytes and PECs. It includes an explanation of the different types of polyelectrolyte classification, as well as the current (limited) applications in separation processes for both polyelectrolytes and PECs. The chapter also introduces polymer membranes with a focus on those consisting of polyelectrolytes and produced by aqueous phase separation (APS).

Similar to how MLOs help organise the cytosol by partitioning specific compounds, in **chapter 2** we explore if complex coacervates (which are liquid-like PECs) consisting of poly(ethylenimine) and poly(acrylic acid) can be used as extraction media. We tested this for lactic acid, butanol, and three varieties of lipase enzymes. Parameters such as polyelectrolyte (a.k.a. polyion) ratio, ionic strength, polyelectrolyte concentration, compound concentration, temperature, and the presence of other components in the complex coacervates very strongly influenced the compounds' partitioning properties. Distribution coefficients between the PEC and supernatant phases ranged from approximately 2 to 50 depending on the exact parameters. To demonstrate that PECs can be used as extraction media, we show that butanol can be extracted from an aqueous solution and can then be recovered from the PEC phase with an efficiency of 21.1 % by only varying the temperature.

In **chapter 3**, the potential use of PECs as an extraction medium is further explored. We used PECs consisting of poly(allylamine hydrochloride) and poly(acrylic acid) to selectively extract either the enzyme lysozyme or modified succinylated lysozyme from a mixture of both, by only varying the polyelectrolyte ratio. The extracted protein could then be recovered from the PEC by lowering the pH from 7 to 4. Lysozyme and succinylated lysozyme are structurally very similar, though succinylated lysozyme has an opposite but equal (net) charge, suggesting that the charge of a molecule is an important factor in its partitioning behaviour in PECs.

Having selectively extracted one protein from a mixture of two, we investigate the extraction of lysozyme from a more complex mixtures in **chapter 4**. PECs were used to extract lysozyme from lyophilised egg white (albumen). We showed that after extraction and recovery *via* the methods of chapter 3, the lysozyme remained enzymatically active. In addition, we observed that the partitioning behaviour of lysozyme in PECs as a function of varying polyelectrolyte ratio is largely independent of the chemistry of the polyelectrolytes.

In **chapter 5**, we look into the creation of biocatalytic polymer membranes produced *via* APS. We modified an existing APS method for the creation of poly(allylamine hydrochloride)/poly(styrene sulfonate) PEC membranes and were able to incorporate lysozyme in the membrane structure. The lysozyme remained enzymatically active within the membrane. As a result, the membrane gained the enzymatic properties of the lysozyme while retaining the same water permeability and selective properties relative to the absence of lysozyme. This proof-of-concept biocatalytic membrane demonstrates the potential of APS membranes for a future generation of functional membranes.

Finally, in **chapter 6** we look back at the research of the previous chapters to reflect on how the future of these research lines could look. Attention is given to the choice of polyelectrolytes, suggestions on how to gain a more comprehensive insight in the partitioning behaviour, and additional extraction- and APS biocatalytic membrane-systems of interest.

Samenvatting

Polyelectrolyten zijn polymeren die bestaan uit potentiaal geladen monomeren. Onder de juiste omstandigheden kunnen tegengesteld geladen polyelectrolyten in een oplossing met elkaar interacties aangaan en een polyelectrolyt complex (PEC) vormen. Deze PECs hebben eigenaardige eigenschappen die individuele polyelectrolyten niet hebben. Een van deze eigenschappen is de mogelijkheid om bepaalde moleculen zeer sterk te concentreren. PECs kunnen worden vergeleken met membraanloze organellen (MLOs); druppel-achtige vloeistof-in-vloeistof fases die aanwezig zijn in onze cellen en de cel helpen om het cytosol te organiseren door specifieke moleculen te partitioneren. Net als PECs bestaan MLOs ook uit tegengesteld geladen polyelectrolyten. Voor MLOs zijn dit typisch negatief geladen RNA-moleculen en positief geladen intrinsiek ongeordende eiwitten. Met deze inspiratie uit de natuur presenteren we in dit proefschrift het gebruik van PECs om moleculen te partitioneren. We hebben onderzocht of het mogelijk was om PECs te gebruiken om selectief een eiwit uit een mengsel te extraheren. Daarna keken we of een PEC die een eiwit bevat gebruikt zou kunnen worden als bio-katalytisch membraan.

Hoofdstuk 1 is een theoretische introductie over polyelectrolyten en PECs. Het legt uit wat voor classificaties van polyelectrolyten er zijn en kijkt naar enkele (beperkte) toepassingen van zowel polyelectrolyten als PECs in scheidingsprocessen. Het hoofdstuk introduceert ook polymeermembranen met de nadruk op membranen die gemaakt zijn via de waterige fasescheiding (APS) methode.

Soortgelijk hoe MLOs de cytosol helpt organiseren door specifieke moleculen te partitioneren, kijken we in **hoofdstuk 2** of complex coacervaten (dat zijnvloeistof-achtige PECs) bestaande uit poly(ethyleenimine) en poly(acrylzuur) gebruikt kunnen worden als extractiemedia. We hebben dit getest met melkzuur, butanol, en drie verschillende lipase enzymen. Parameters zoals polyelectrolyt (ook bekend als ‘polyion’)-verhouding, ionsterkte, polyelectrolytconcentratie, stofconcentratie, temperatuur, en de aanwezigheid van andere componenten in de complex coacervaten hadden een sterke invloed op de partitioneringeigenschappen. Verdelingsconstantes tussen de PECs en supernatant fases varieerde van ongeveer 2 tot 50 afhankelijk van de exacte systeemparameters. Om te demonstreren dat PECs als extractiemedia gebruikt kunnen worden, lieten we zien dat butanol ge-extraheerd kon worden uit een waterige oplossing en uit de PEC fase teruggewonnen kon worden met een efficiëntie van 21.1 % door enkel de temperatuur te veranderen.

In **hoofdstuk 3** wordt de mogelijkheid om PECs te gebruiken als een extractiemedium verder onderzocht. We gebruikten PECs bestaande uit poly(allylamine hydrochloride) en poly(acrylzuur) om selectief het enzyme lysozyme óf gemodificeerd gesuccinyleerd lysozyme te extraheren uit een mengsel van beide door alleen de polyelectrolytverhouding te veranderen. Het geëxtraheerde eiwit kon worden teruggewonnen uit het PEC door de pH te verlagen van 7 naar 4.

Lysozyme en gesuccinyleerd lysozyme lijken structureel op elkaar, maar gesuccinyleerd lysozyme heeft een (netto) gelijke maar tegengestelde lading. Dit suggereerd dat de lading van een molecuul een belangrijke factor is in het verdelingsgedrag in PECs.

Nu we één eiwit kunnen scheiden van een mengsel van twee, hebben we in **hoofdstuk 4** onderzocht of lysozyme ge-extraheerd kon worden uit een complex mengsel. We gebruikten PECs om lysozyme te extraheren uit gevriesdroogd ei-eiwit. We hebben laten zien dat na de extractie en terugwinning, volgens de methodes van hoofdstuk 3, de lysozyme nog steeds enzymatisch actief was. Ook zagen we dat de partitionering van lysozyme in PECs al seen functie van de polyelectrolytverhouding grotendeels onafhankelijk is van de chemie van de polyelectrolyten.

In **hoofdstuk 5** kijken we naar het maken van bio-katalytische polymeermembranen *via* de APS methode. We pasten een bestaande APS methode voor het maken van poly(allylamine hydrochloride)/poly(styrene sulfonaat) PEC membrane aan en konden lysozyme in de membraanstructuur incorporeren. De lysozyme bleef enzymatisch actief in het membraan. Hierdoor krijgt het membraan de enzymatische eigenschappen van lysozyme ondanks dat de permeabiliteit- en selectiviteit-eigenschappen onveranderd bleven vergeleken met membraan zonder lysozyme. Dit *proof-of-concept* bio-katalytisch membraan demonstreert de mogelijkheid voor APS om membranen te maken voor een toekomstige generatie van functionele membranen.

Tot slotte kijken we in **hoofdstuk 6** terug op het onderzoek van de voorgaande hoofdstukken en reflecteren we hoe de toekomst van onderzoek in deze richting er uit zou kunnen zien. Nadruk wordt gelegd op de selectie van polyelectrolyten, suggesties hoe we meer kunnen leren over het partitioneringsgedrag, en welke extracties en bio-katalytische membranen interessant zijn om verder te onderzoeken.

Chapter 1

Introduction

Separation Processes

The natural world is filled with mixtures, and pure materials or fluids are rare. Our modern society requires us to exert a great deal of control over these natural mixtures. We can't just drink most surface water; we need ways to separate the harmful pathogens and chemicals before consumption. For industry, we require varying degrees of purity in (raw) materials. For medicine, (near-) pure molecules are essential to effectively produce our medications. For science, we need to know exactly what chemicals are used to be able to say anything meaningful about our observations. [1] Enter the field of separation processes.

Fortunately for us, many clever minds have been hard at work over the millennia to develop separation processes for a wide variety of applications. At its foundation, a separation process exploits a difference in a physical or chemical property of molecules in a mixture to selectively extract or enrich one or more of the molecules. This is a somewhat vague description due to the existence of many different separation processes have been invented throughout history. One of the oldest technologies to separate different molecules is distillation, where differences in boiling points are used to selectively evaporate and condense molecules from a liquid. It is likely this separation technique was already known to the ancient Mesopotamians in the second millennium BCE, and the ancient Greek civilisations also made use of it. [2,3] Another commonly used separation method is filtration, where a liquid or gas is moved through some form of medium. The filtration medium can be low-tech; sand filters are surprisingly effective and are still used to prepare drinking water today. [4,5] Membranes, a more modern type of filtration medium, are essentially thin selective filters that retain some molecules while letting others permeate through. They will be discussed in more depth later in this chapter. Some other techniques that by no means form a comprehensive list are centrifugation, chromatography, precipitation, flocculation, or sedimentation.

A particular interesting type of separation technology is liquid-liquid extraction (LLX). In LLX a specific molecule that is dissolved or suspended in a liquid (the feed) is selectively extracted into an extraction medium. This extraction medium is a different liquid such as an organic solvent, ionic solvent, or deep eutectic solvent. Preferred solubility of the target molecule for the extraction medium relative to the feed solution results in the molecule partitioning in the extraction medium. For LLX to work it is important that the extraction medium does not mix with the feed solution (typically an aqueous solution).

This dissertation focuses on the use of polyelectrolyte complexes (PECs) for separation processes. PECs are complexes formed by the physical association of two oppositely charged polymers (polyelectrolytes). In chapters 2 to 4 of this dissertation we present PECs as a new type of extraction medium similar to LLX. When

polyelectrolytes are mixed with the feed solution they form a distinct PEC phase that is either solid- or liquid-like. The phase separation into PECs looks very much like a separation process found in cells. One of the strategies cells use to create environments that differ in molecular composition is by the formation of membraneless organelles (MLOs). MLOs typically consist of complexes of cationic and anionic biomacromolecules. These complexes essentially act as a compartmentalised solvent and can selectively extract molecules from the cytosol, not dissimilar from LLX. [6–10]

Chapter 5 discusses the use of PECs to create biocatalytic water filtration membranes. These PEC membranes are created *via* the process of aqueous phase separation (APS). APS is a recently developed technique that allows for the production of polymer membranes in aqueous solutions as a greener alternative to the traditional organic solvent-based approach of membrane production. The APS production process is modified to incorporate functional proteins in the membrane structure. The membranes can then fulfil a dual role; they are useful as selective filters and gain the functionality of the incorporated biomolecules.

A central theme throughout all the chapters is the connection between PECs and proteins. When PECs are presented as extraction media, this primarily concerns the extraction of proteins. The PEC membranes created *via* APS incorporate proteins to gain biocatalytic functionality. First, I will present general information on polyelectrolytes, polyelectrolyte complexation, and PECs. Then there will be an introduction to the interaction between polyelectrolytes and proteins. Finally, these subjects will be combined in polymer membranes in general, PEC membranes made *via* APS, and biocatalytic membranes.

Polyelectrolytes

The basic building block of both the PEC extraction media and the PEC membranes are the polyelectrolytes. Polyelectrolytes are polymers of which the monomer units possess an electrolyte group. When dissolved in a polar solvent, like water, the electrolyte can separate into a cation and anion. In the case of a polycation the group connected to the polymer receives a positive charge. The negative charge is then carried by a released counterion (Figure 1.1). In the case of a polyanion, the opposite applies. Due to this behaviour polyelectrolytes are also known as polyions or polysalts. Polyelectrolytes that carry both negative and positive charges are known as polyampholytes. In this section some categorisations of polyelectrolytes with specific examples will be described. There are many (possible) different types of polyelectrolytes, and they can be categorised in several ways such as via origin, charge behaviour, and/or structure. [11]

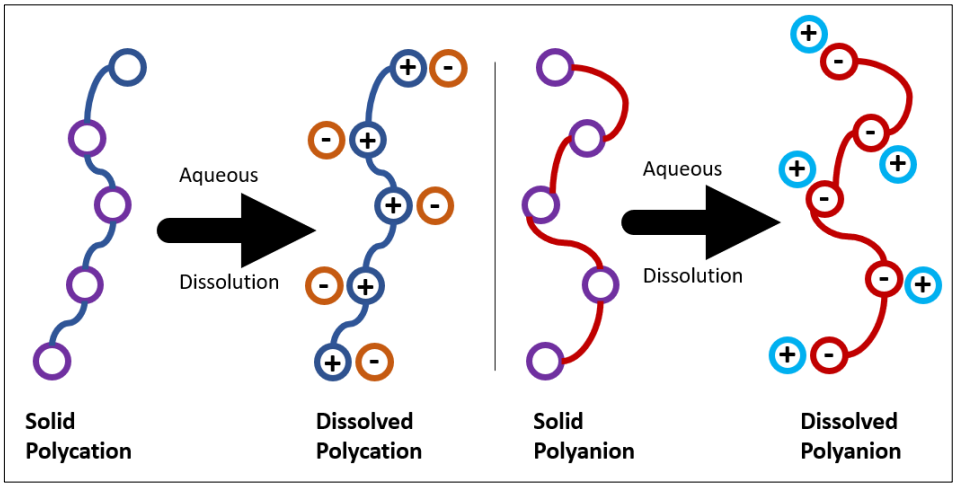

Figure 1.1. *Schematic depiction of a polycation and polyanion dissolving in water. A solid polyelectrolyte has its counterion strongly bound to the charged group on the polymer backbone. When dissolved, the counterions will continue to associate with the polyelectrolytes due to electrostatic attraction.* [12]

Synthetic polyelectrolytes

When categorised via their origin, we distinguish synthetic from natural polyelectrolytes. Synthetic polyelectrolytes are mostly derived from petrochemistry and are human-made via various chemical industrial processes. [13] Synthetic polyelectrolytes are highly customisable both in monomer structure as well as total polymer size. Examples of several synthetic polyelectrolytes that will make appearances in this dissertation are shown in Figure 1.2. It is not a requirement that synthetic polyelectrolytes are homogeneous in monomer composition, and various copolymer systems are used for broad applications. [14–16] In principle the realm of synthetic polyelectrolytes is only limited by the chemist's imagination and practical concerns regarding polymerisation conditions and feasibility of synthesis. Two of the most structurally simple synthetic polyelectrolytes are poly(acrylic acid) (PAA) and poly(allylamine) (PAH), a polyanion and polycation respectively.

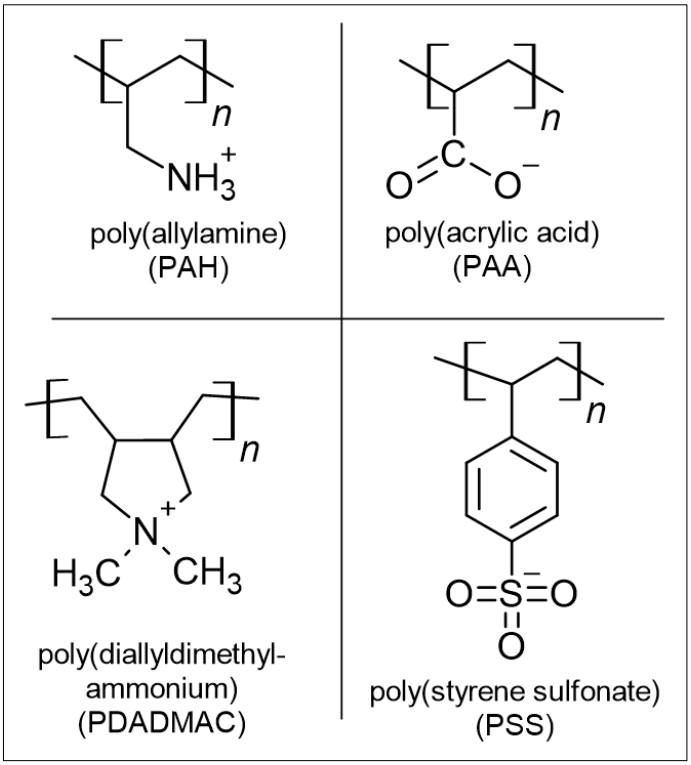

***Figure 1.2**. Examples of various synthetic polyelectrolytes that are directly relevant to this dissertation. The top structures are weak polyelectrolytes, the bottom structures are strong polyelectrolytes. The left structures are polycations, the right structures are polyanions. Counterions are not shown.*

Natural polyelectrolytes

Natural polyelectrolytes are large biomolecules often found in living organisms. In contrast to their synthetic counterparts, natural polyelectrolytes typically have more structural variety in monomer units. Several natural polyelectrolytes are shown in Figure 1.3. A well-known biomolecule that is not often called a polyelectrolyte but does meet the requirements in both structure and behaviour is deoxyribonucleic acid (DNA). DNA is a polymer with nucleotide monomer units. Usually, we are interested in whether the monomers contain cytosine, guanine, adenine, or thymine nucleobases as these encode for the amino acid sequence of proteins and hence the biological function of proteins. However, every nucleotide monomer unit also carries a negative charge via the phosphate group on the DNA backbone regardless of the nucleobase. It is this negatively charged phosphate group that makes DNA a polyelectrolyte.

Proteins can also be considered polyelectrolytes. Proteins are mostly known as the primary molecular workforce within organisms. In their primary structure they are linear polymers with amino acids acting as the monomeric unit. Some amino acids can carry a charge. At physiological conditions (pH ~7-7.4) the amino acids glutamate and aspartate are typically negatively charged, arginine and lysine are typically positively charged, and histidine is typically partially positively charged depending on the exact local environment. In addition, proteins can undergo post-translational modifications such as phosphorylation or acetylation that can further affect the amino acids' charge. [17] As typical proteins consist of dozens to hundreds of amino acids, they are in practice always polyampholytes.

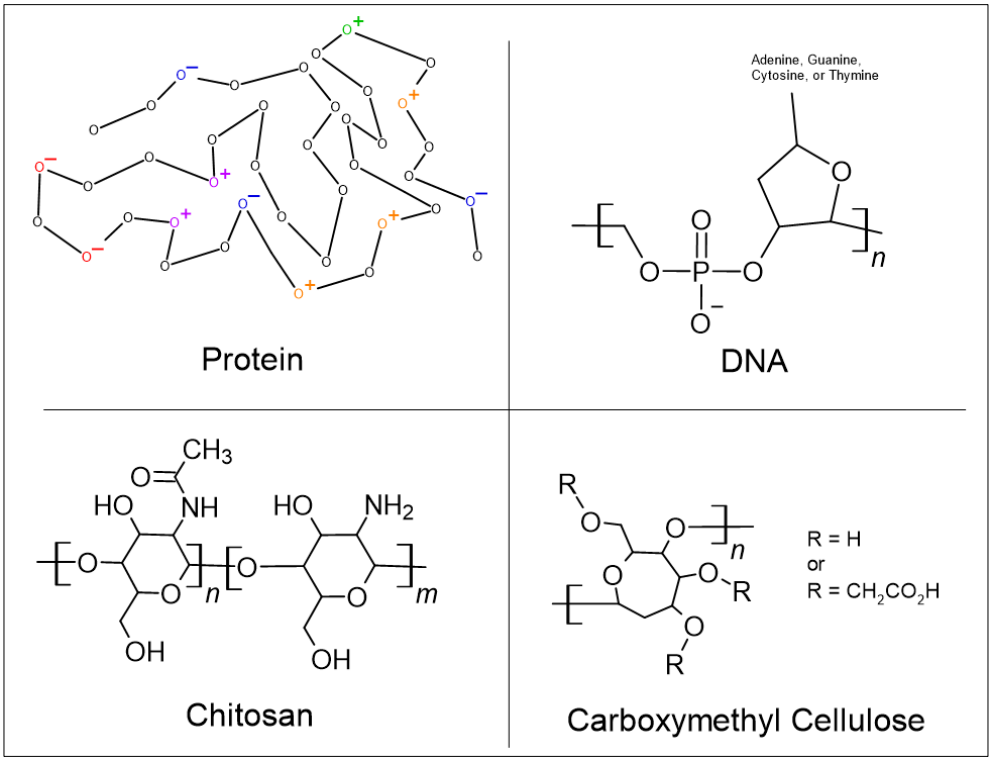

Figure 1.3. *Example of several natural polyelectrolytes. Top left; a fictional (small) protein. Circles represent uncharged, positive, or negative amino acids. Top right; a nucleotide, the monomeric unit of DNA. Bottom left; chitosan. Chitosan consists of a mixture of the left and right structures. The ratio of this mixture affects the chitosan properties. Bottom right; carboxymethyl cellulose. The degree of Rs that consist of carboxymethyl groups affects the properties.*

The final common type of natural polyelectrolytes are polycarbohydrates (also known as polysaccharides), with either mono- or disaccharides (single- or double sugar rings) as their monomeric unit. Not all polycarbohydrates are charged in solution or even dissolve in water under physiological conditions. For example, cellulose is insoluble in water but modified carboxymethyl cellulose (CMC) is negatively charged in water and is readily soluble. Typically, polycarbohydrates or other biological polyelectrolytes with such minor modifications are still considered natural polyelectrolytes. Polycarbohydrates can be either polycationic or polyanionic. Another popular polycarbohydrate is chitosan which consists of deacetylated chitin. Chitin is the main component of arthropod exoskeletons and fungi cell walls. Much like cellulose and CMC, chitin is poorly soluble in water and not particularly useful, but chitosan is immensely useful and one of the more commonly used natural polyelectrolytes.

Weak and strong polyelectrolytes

Another important distinction is between strong and weak polyelectrolytes. Strong polyelectrolytes are charged regardless of the solution pH while the charge of weak polyelectrolytes is dependent on the solution pH. [18] A weak polyanion will typically be neutral at lower pH values, then increase in charge as pH increases until it reaches full protonation. A weak polycation will follow the opposite pattern; neutral at high pH followed by an increase in charge with a decrease in pH until it reaches full ionisation. The exact pH threshold where ionisation begins and when full ionisation is reached depends on the chemistry of the individual polyelectrolyte. In Figure 1.1, PAA and PAH are both weak polyelectrolytes as the ionisation of respectively the carboxylic acid- and amine-groups depends on the solution pH. In contrast, poly(styrene sulfonate) (PSS) and poly(diallyldimethylammonium) (PDADMAC) are strong polyelectrolytes. Their specific molecular structure allows them to maintain a charged group largely independent of environmental pH, barring extremes.

Both strong and weak polyelectrolytes are also present in the subset of natural polyelectrolytes. Proteins are weak polyelectrolytes, as the charge of the amino acids is dependent on the pH. [19] Polycarbohydrates are also typically weak polyelectrolytes, as their charge is usually the result of a carboxylic acid-, amine-, or alcohol functional group. DNA is a notable strong polyelectrolyte, as the phosphate group on the molecular backbone is a resilient carrier for a negative charge.

We use both weak and strong polyelectrolytes in this dissertation. By choosing to use weak polyelectrolytes, their charges and behaviour as well as the behaviour of any complexes that consist of at least one weak polyelectrolyte can be manipulated

by changing the pH to achieve a wide variety of effects which will be explored further in chapters 3 and 4.

Finally, when categorised via their structure, we use the same nomenclature that is typically used in (polymer) chemistry. There are linear polyelectrolytes, branched polyelectrolytes, co- and homopolymer structures, polyamides, polyesters, polyethers, *etc.*

Separation processes with polyelectrolytes

With the description of polyelectrolytes out of the way, let's look at some of their applications. Polyelectrolytes are already used in separation technology, typically as flocculants. [13,20,21] For example, a dispersion of nanometre-sized anionic particles may precipitate by the addition of a polycation. The polycation can bridge multiple anionic particles (which in turn can connect to multiple polycations) until either the size of the conglomerate particle is large enough to precipitate or due to the interaction of charges the conglomerate particle is no longer soluble and precipitates.

The ability to induce flocculation is a useful property of polyelectrolytes in the treatment of wastewater. [21] Out of many flocculation agents, polymers (and by extension polyelectrolytes) have advantages such as a lower required dose, a smaller final volume of the precipitate, and reduced costs. [21–24] Polyelectrolyte flocculants are used for the treatment of a wide variety of wastewater streams, such as those of paper mills, dye industries, oil refineries, ceramic industries, food industries, sewage, and polymer manufacturers. [25,26] Efficiencies are somewhat varied, ranging from as low as ~12% to effectively 100% target particle removal. [26] Similar to wastewater treatment, drinking water treatment is possible with polyelectrolytes (typically polycations) to remove comparatively large contaminants such as bacteria, algae, and viruses. [25]

Interestingly, polyelectrolytes can also do the opposite of flocculation: dispersion. By polyelectrolytes adsorbing to particles, an electrostatic repulsive force between the polyelectrolytes prevents these particles from coagulating. [27] In manufacturing, CMC is used as a dispersant to create thin homogeneous films by preventing the film components from coagulating. [27–29] Silver nanoparticles can be stabilised with polyelectrolytes and applied as an antimicrobial agent. [30] Similarly, gold nanorods can be coated with different polyelectrolytes to prevent aggregation in different organic solvents. [31] Various suspensions can be kept stable by adding polyelectrolytes. [32–35]

Polyelectrolytes may also be prepared into hydrogels, typically by crosslinking. The resulting polyelectrolyte gels have high metal chelating properties and can be used

to remove (heavy) metals from water. [36,37] They can remove up to 99 % of metals from aqueous solutions under lab conditions and up to 95 % of studied metals from natural water sources. [38] Polyelectrolyte gels can be made from either synthetic or natural polyelectrolytes. Recently, alginate has been used to remove copper and zinc from contaminated soil. [39] DNA-chitosan hydrogels have been suggested with the additional capability of adsorbing small molecule pharmaceuticals by using DNA's double helix structure for binding to organic contaminants. [40]

Polyelectrolyte complexation

Polyelectrolytes form the building blocks of polyelectrolyte complexes. When oppositely charged polyelectrolyte solutions are mixed a PEC can form. The formation of PECs compared to free polyelectrolytes in solution is entropically favourable. [12,18,41] Two polyelectrolytes associated with their counterions have a lower entropy compared to a polyelectrolyte pair and many counterions associating with one another (Figure 1.4). [18] In addition, oppositely charged polyelectrolytes in solution can feel a coulombic charge attraction. [18,42] The creation of PECs is not a given and depends on various system parameters.

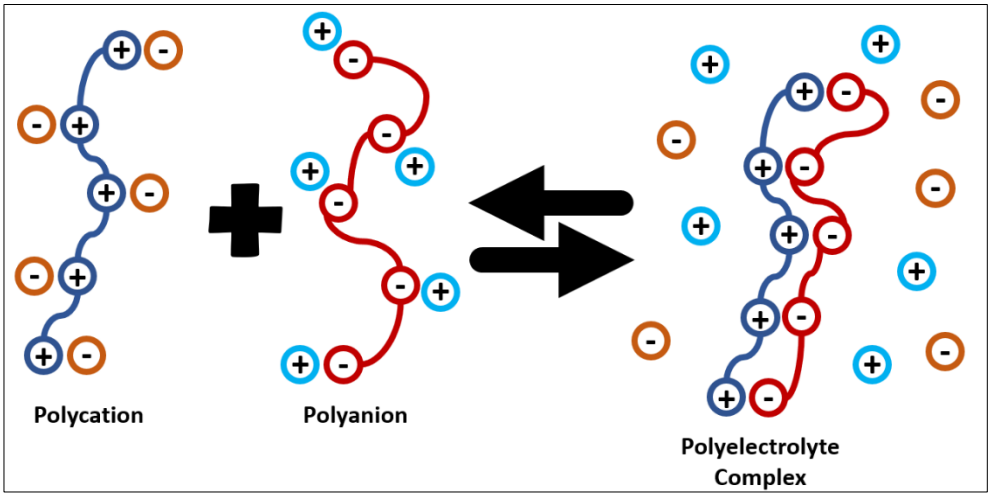

***Figure 1.4**. Schematic depiction of polyelectrolytes forming a polyelectrolyte complex in solution. The complex to the right of the equilibrium arrows is more entropically favourable than the separate polyelectrolytes to the left of the arrows.*

Polyelectrolyte complex properties

PECs have different properties. The subset of PECs that are liquid-like are known as complex coacervates (CCs). Within this dissertation, solid-like PEC precipitates are used in chapters 3 to 5. CCs are used in chapter 2. In addition, soluble complexes may form, which are small PECs that do not phase separate on a macro-scale. Whether a PEC, a CC, soluble complexes, or no complex forms after mixing oppositely charged polyelectrolytes and which properties and characteristics these complexes have depends on factors such as polyelectrolyte mixing ratios, polyelectrolyte concentration, polyelectrolyte structure, ionic strength of the solution, pH, and temperature. [11,12,18,41–43]

Maximum PEC formation would typically be expected at charge stoichiometry, where an equal number of positive and negative charges are brought in by the polyelectrolyte monomers. This intuition is not necessarily true, and we find in chapter 2 that maximum CC formation can occur at other charge ratios. The polyelectrolyte concentration influences the size of the PEC particles, with higher concentrations leading to larger sizes. [44] The exact polyelectrolyte structure and size are very important factors in the complexation outcome, though not all the processes involved in complexation are thoroughly understood. The structure influences the physical spacing between charges, polyelectrolyte chain flexibility, hydrophobicity, and the nature of the charged functional group (and of the counterions), amongst others. In general, it can be said that weak polyelectrolytes tend more towards the formation of CCs and strong polyelectrolytes towards the formation of precipitates. However, one should be careful not to overgeneralise. [18] As an immediate exception; chapters 3 and 4 of this dissertation deal with complexes of weak polyelectrolytes that form PEC precipitates and not CCs.

The influence of the ionic strength of the solution on complexation is a well-researched topic within the context of polyelectrolyte complexation. [11,12,18,41–43] The ionic strength can be manipulated by adding ions, usually in the form of monovalent inorganic salts. With a sufficiently high ionic strength the entropy gain by counterion release becomes negligible, and the electrostatic interaction between polyelectrolytes is screened by the presence of the additional ions. [12] With a higher ionic strength, less interaction between polyelectrolytes can be expected including the possibility of changing from a PEC precipitate to a CC. [45] At high enough ionic strength complexation can be prevented entirely.

The solution pH will primarily affect PECs composed of at least one weak polyelectrolyte. Different pH values lead to different degrees of polyelectrolyte protonation and influences the charge stoichiometry and charge density, which in turn changes the properties of the PECs. [11,41,42] These changing properties are used in chapters 3 to 4 to achieve a variety of effects. In these chapters, lowering the pH

results in a dramatically different partitioning of lysozyme in PECs. In chapter 5, controlling the interaction between polyelectrolytes by changing the pH is central to the creation of PEC membranes via APS, though this will be discussed in more detail in the section on PEC membranes later in the current chapter.

Temperature can have an effect on the PEC properties as well. We observed that increasing the temperature of PAH/PAA PECs resulted in the precipitates taking a CC form (Figure 1.5). Other studies found that temperature can enhance the complexation between lignosulfonate and chitosan as opposed to disrupt [12], and that the temperature can influence the microstructure conformation of PECs. [46] The variation in PEC properties by temperature is especially interesting to the application of PECs for drug delivery, which will be discussed in the next section. Temperature variations also play an important role in chapter 2, where the temperature increases the partitioning of butanol between the CC and supernatant phases.

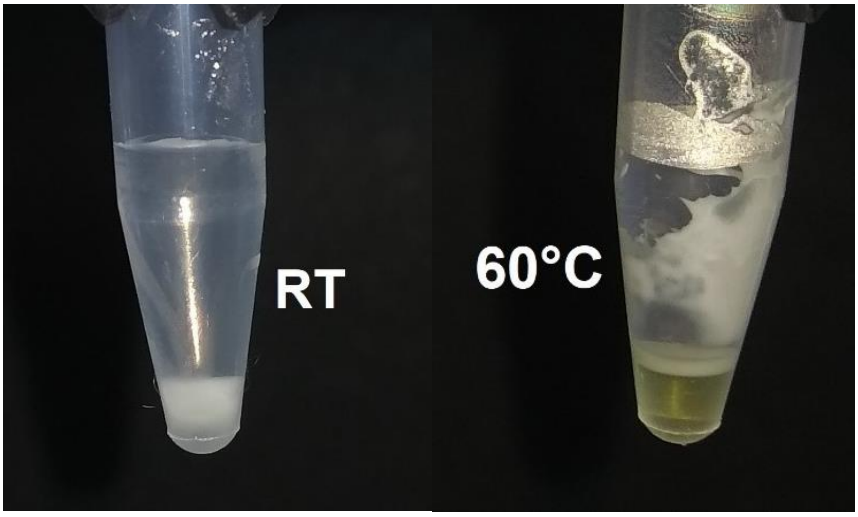

***Figure 1.5**. PAH/PAA PECs stored at different temperatures. Room temperature (RT) PECs are in an opaque white solid-like precipitate form (left), while PECs stored at 60 °C are in a translucent yellow liquid-like form (right). Both sit at the bottom of a tube and form a distinct polyelectrolyte-rich phase in contrast to the polyelectrolyte-poor supernatant phase.*

In this dissertation PECs are presented for separation processes in the form of extraction media and as building blocks for membranes. However, like individual polyelectrolytes, PECs have already been used for separation processes as binders and flocculants in for example wastewater treatment and paper mills. [20,47–49] The exact formulation and preparation of the flocculant PECs provides more variables

than single polyelectrolytes that can be used to tune their flocculation behaviour. [47] In addition, PECs have been used as soil binders to prevent erosion. [50]

Polyelectrolytes and proteins

In all subsequent chapters, proteins feature prominently. In chapters 2 to 4 the PEC acts as a separate phase into which proteins are partitioned. In chapter 5 the PEC membrane as a whole acts as a matrix for the proteins. In both cases, the interaction between polyelectrolytes and proteins is crucial for their application in separation processes.

Proteins and enzymes

Proteins are (long) polypeptides consisting of polymerised amino acids. From a biological perspective, proteins are one of the primary drivers of biochemical processes that take place within organisms. As proteins are often the result of millions if not billions of years of evolution, they can have extreme efficiencies and properties compared to artificial human-made processes and materials. Proteins that catalyse a reaction are called enzymes. Enzymes can greatly enhance conventional reactions or perform reactions not currently achievable by conventional chemistry. In addition, unwanted by-products are less common in enzymatic reactions compared to typical organic synthesis. [51] These properties make enzymes interesting for industrial and research applications. Enzymes are used in the textile industry, laundry detergents, research, medical industry, food industry, production, cosmetic industry, as well as more futurist applications such as biofuel production. [52–54] There are several ways in which enzymes are used in these applications. Lipases are one of the most widely used enzymes and are used for the breakdown and removal of fats, as well as catalysis to produce (intermediates for) pharmaceuticals. [55] Amylases are used to catalyse the reaction of starches into sugars such as high-fructose corn syrup, an incredibly common food additive. [56]

The polymerase chain reaction (PCR) test primarily responsible for detecting SARS-CoV-2 infections is only possible thanks due to a specific polymerase enzyme which had been isolated from extremophile bacteria. PCRs are ubiquitous in (biological) research. [57,58]

Protein-polyelectrolyte interaction

That proteins can be considered polyelectrolytes (polyampholytes) themselves has been touched upon in an earlier section of this introduction. It stands to reason then, that proteins can interact with (other) polyelectrolytes. Like PECs, proteins can associate with polyelectrolytes to form protein-polyelectrolyte complexes (P-PECs). A study on the P-PEC complexation behaviour of the protein bovine serum albumin (BSA) showed significantly different properties and complexation conditions between chitosan/BSA and PDADMAC/BSA P-PECs, indicating that like with the formation of PECs, the nature of both the polyelectrolyte and the protein are essential to the P-PEC properties. [59] In an attempt to find patterns to explain P-PEC behaviour, it has been hypothesised that charge distributions expressed as charge anisotropy of the protein could be an important factor. [60,61] To try to demonstrate this principle, protein mutants were used that exhibited different levels of charge 'patchiness' (as determined by an algorithm) and reported results showed correlation between patchiness and 'strength' of complexation. [60] This study found differences between the complexation behaviour of weak- and strong polyelectrolytes and the protein. Weak polyelectrolytes were found to exhibit better complexation behaviour, hypothesised as a result of the ability of weak polyelectrolytes to charge-regulate. [60] Another study on supercharged proteins found that the ratio of positive to negative charge residues on the protein is another factor that determines a system's tendency to form P-PECs. [62] Unfortunately, it is very difficult to achieve a thorough understanding of P-PEC systems when investigating local charge distributions on proteins, as the protein confirmation itself (and thus the location of the charges and the charge density) tends to change when in contact with a polyelectrolyte. [63] In short, the complexation behaviour of P-PECs is opaque and complicated but some trends regarding the protein charge and charge distribution have been identified.

Besides P-PECs consisting of one species of polyelectrolyte interacting with a protein, there is also the situation where proteins interact with a (pre-formed) PEC. Naturally, adding another species of molecule does not simplify the situation and substantially less research is done on protein-PEC systems. Existing literature primarily focuses on the partitioning of proteins into PECs either for its own sake or to develop a protein delivery system for medical purposes. [61,64–66] Perhaps the most widespread and well-known example of a therapeutic protein is insulin, used for the treatment of type-1 diabetes. Insulin can be encapsulated in PECs of chitosan and dextran sulfate and is released over time after oral delivery. [67] In a two-step process, another study first bound vascular endothelial growth factor protein to the polyanion dextran sulfate and then added polycations. The resulting complex released the protein over a period of more than 10 days. [68]

Considering the current pandemic, it is also interesting to note that PECs have been suggested for vaccine delivery. A model antigen was loaded in a trimethyl

chitosan/thiolated hyaluronic acid PEC with a poly(ethylene glycol) coating and showed an increased immunogenic response as measured by antibody titres for PEC-antigen systems compared to antigen-only systems. [69] A more recent study tested five different PEC compositions for antigen delivery and found that the immune responses were dependent on the composite polyelectrolytes. [70] Other studies have confirmed the feasibility of PECs as vaccine delivery agents. [70,71]

Nearly all the medical PEC delivery systems use natural polyelectrolytes. Natural polyelectrolytes are preferred in biomedical drug delivery applications due a decreased immune reaction or their ability to eventually be degraded into harmless waste products, eventually leaving no trace of the delivery system. [72] In general, these PEC delivery systems are simple and relatively cheap to produce by preparing the PECs in a solution already containing the to-be-delivered drug. As a side effect of this production technique, the loading density of the drugs is not particularly high.

Polyelectrolyte complex membranes

Polymer membranes are commonly used for treatment of drinking water or wastewater. PECs contribute to the world of water filtration membranes in two ways; with polyelectrolyte multilayer (PEM) membranes and freestanding PEC membranes produced *via* aqueous phase separation (APS). PEM membranes are produced by alternating oppositely charged polyelectrolytes one layer at a time resulting in a slowly growing membrane, while APS membranes consist of bulk structures that are formed *via* a phase inversion process (Figure 1.6).

Chapter 4 of this dissertation further demonstrates an example of APS membranes in which a protein is incorporated.

Membranes

Membranes are essentially filters. The most conceptually simple polymer membrane is a flat polymer sheet with homogenous pores equidistant from each other. This membrane would allow any molecule in a feed solution below a certain size to pass through the pores and it would retain any larger molecules. Additional selectivity is possible by for example varying pore size and shape, membrane charge, membrane affinity for certain molecules, or doping the membranes with additional compounds. The smaller the pores, the more important charge- and affinity-interactions become. Together, these factors determine the membrane's properties and function. Depending on the desired purpose there are various possible definitions of what 'function' means for a given membrane.

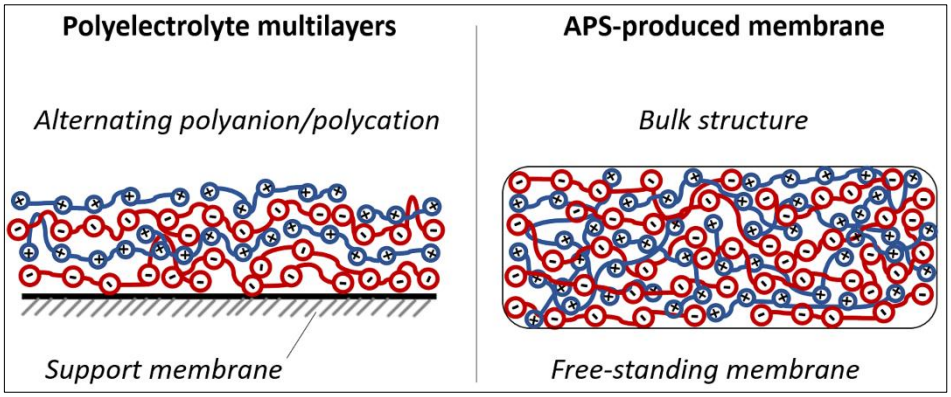

***Figure 1.6**. Schematic difference in the structures between PEM and APS membranes. (Left) PEM membranes are formed by alternating layers of oppositely charged polyelectrolytes deposited on a support membrane. (Right) Membranes made via APS are freestanding with both polyelectrolytes spread throughout the structure.*

Typical characteristics to determine membrane functionality are looking at the molecular weight cut-off (MWCO), salt retention, specific compound retention, and pure water permeability (PWP). MWCO is determined by subjecting the membrane to increasingly large (inert) molecules and observing what size molecules are retained (the retentate) and which can pass through (the permeate). Salt retention refers to the specific salt ions retained by the membrane. This can vary based on the ion's valence as well as the ion's elemental makeup. When a membrane is designed to filter out (a) specific compound(s) from the feed solution, it is important to measure the retention of those specific compound(s). Finally, PWP is a key property of membranes. PWP is determined by measuring the flux of pure water through a membrane as a function of the applied pressure. Generally, the more selective a membrane becomes (that is to say it has a high retention and/or low MWCO) the lower the PWP. A lower PWP means that more energy is required to push water through the membrane, which translates to increased operating costs. If a membrane is designed to work with solvents other than water, PWP can be substituted by a similar test with the desired solvent.

Biocatalytic membranes

The polymer membranes as described so far act as passive filters; there is some property of the membrane that results in separation, but no chemical reaction takes place. A biocatalytic membrane is a polymer membrane that has been functionalised by a biological agent (often an enzyme). The typical way to produce biocatalytic

membranes is to produce the polymer membrane as normal and attach the enzymes to the surface in post-production processing steps. This is done variably by physical adsorption or by chemical crosslinking. [73,74] In theory the membrane would then gain the enzymatic properties of the attached enzyme in addition to its original membrane properties. In practice the enzymes often result in reduced membrane functionality; the enzymes may not remain on the membrane, or the membrane properties are affected negatively by the enzymes' presence. [75]

While the practical application of biocatalytic membranes in industrial applications still has its difficulties, the potential benefits are great. We have increasing amount of micropollutants in our (drinking) water that are difficult to remove with most commercial membranes. Biocatalytic membranes functionalised with oxidoreductase enzymes have been suggested as a way to remove these micropollutants. [76,77] Digestive enzymes could be used to prevent the build-up of bacteria or proteins from clogging up the membrane (known as 'fouling') by actively destroying the deposited biological matter. [78] A cheap, effective, and reliable way to produce biocatalytic membranes could be an important next step for membrane science.

Polyelectrolyte multilayer membranes

The most common type of PEC membrane are PEM membranes prepared via the layer-by-layer (LbL) method. As the name implies, the LbL method consists of alternating layers of polycation and polyanions, typically on a support substrate. PEM membranes are produced by first submerging the support substrate in a polyelectrolyte solution and attaching the polyelectrolyte to the support. This initial attachment does not need to be a covalent bond but can be via adsorption to the support substrate or via some other physical process. Subsequently the entire structure is rinsed to remove any excess non- or weakly-attached polyelectrolytes and it is then submerged in a separate solution containing oppositely charged polyelectrolytes. It is then once again rinsed and submerged in a new polyelectrolyte solution and this process is repeated until the desired number of layers is achieved. An advantage is that the manufacturer has a great degree of control over the PEM thickness. Extremely thin PEM layers in the order of a few nanometres have been produced that still have desirable membrane properties. [79–81] As a downside, the production of PEM membranes requires many separate fabrication steps and can be time-consuming and tedious, especially as polyelectrolyte solutions may need to be prepared at varying ionic strengths or pH. [82,83] Some alternative fabrication methods have been suggested employing automation of the rinsing and immersion or using spraying of solution as opposed to total submersion. [84]

While fewer layers would be preferred as a thinner PEM membrane typically means a higher PWP, too few layers can result in an incomplete coating of the support substrate effectively creating holes in the PEM. The support substrate is typically a porous membrane with pore sizes and permeabilities much higher than what is expected from the PEM. Studies have shown the ability to get PEMs within the order of magnitude of around 10 nm with permeabilities in some cases higher than that of commercially available alternatives. [85,86] The current crown goes to a PEM system consisting of PAH/PSS layers, with a PAH/PAA layer on top. While this entire PEM has a thickness of approximately 30 nm, the selective PAH/PAA layer is estimated at only 4 nm with the ability to retain 98% of the target compound(s) while maintaining a reasonable PWP. [79] The long-term stability of PEMs appears to be a function of the local environment as well as the exact polyelectrolytes used, with strong polyelectrolyte pairs unsurprisingly showing better stability at high and low pH environments compared to a weak polyelectrolyte pair. [87] Some LbL methods incorporate DNA or proteins as a layer to create highly specific biosensors. [88] The success of LbL-produced PEM membranes over the last decade has resulted in their successful commercialisation. [89]

Aqueous phase separation for membrane production

APS has only recently been developed as a method to produce bulk PEC membranes, [90-95] but it is quickly gaining appreciation. [96,97] It shows similarities to non-solvent induced phase separation (NIPS), which is one of the standard polymer membrane production techniques. With NIPS, a polymer is dissolved in an organic solvent resulting in a homogenous viscous polymer solution. This viscous solution is shaped in the desired form (‘casted’) of the membrane, after which the shaped solution is submerged in a non-solvent. This process leads to the removal of the organic solvent from the polymer solution and the precipitation of the polymer into the cast shape. The resulting polymer can be a flat membrane, patterned flat membrane, or hollow fibre. [98–100] With APS, instead of having the phase inversion be from a(n organic) polymer solvent to non-solvent, the phase inversion is from a condition in which polyelectrolytes do not complexate to a condition in which polyelectrolytes form a PEC precipitate. An example of such a process is schematically depicted in Figure 1.7. As the name suggests, one of the strengths is that both phases of APS can be aqueous. For example, the phase inversion can be from a high pH to a low pH [91] or from high salinity to low salinity. [93]

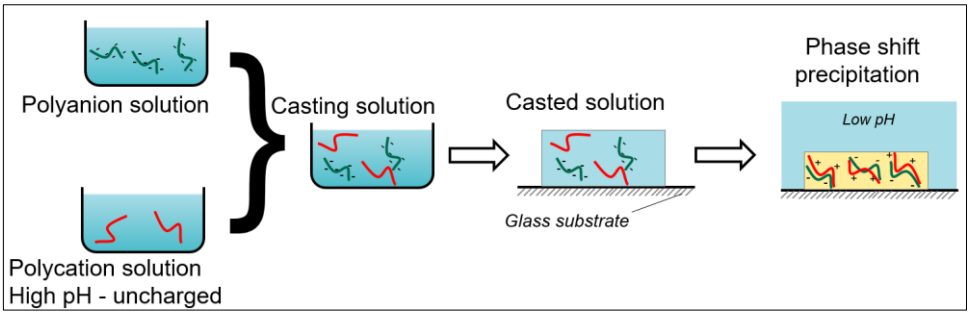

***Figure 1.7**. Schematic diagram of an example of APS. A solution is prepared under conditions that prevent polyelectrolyte complexation. This viscous solution is cast in the desired membrane shape on a (glass) substrate and finally submerged in a precipitation solution under conditions which the complexation can takes place. The figure here depicts a pH-phase shift.* [91] *It is adapted from chapter 5.*

The first publication of the APS principle in 2019 used a KBr salt-induced phase inversion to create a PEC membrane of poly(N-ethyl-4-vinylpyridinium) and PSS. Later, a study showed a NaCl salt-induced phase inversion using PDADMAC and PSS demonstrating that the APS method is also possible with a simpler salt and different polyelectrolytes. [93,94] APS is not limited to salt-induced phase inversion; other studies have shown that it is feasible to use pH-induced phase inversion as well. [91,92]

Tuning parameters like the polyelectrolyte concentration and NaCl concentration of the precipitation solution allow for control over pore sizes, retention properties, and PWP. Research into the exact effect of the precipitation solution has already shown that the type of salt ion affects membrane properties even at equal ionic strengths. [95] In chapter 5 of this dissertation one of the reported APS methods [91] is adapted to include an enzyme within the PEC structure with the idea of producing a biocatalytic membrane. This is made possible because the enzyme is able to survive the aqueous conditions of the APS process, while it would typically not survive the organic solvents of NIPS.

Topics of this dissertation

In the research described in this dissertation, PECs are applied in separation processes in two specific ways. First, PECs are used as (selective) extraction media in chapters 2 to 4. Second, a PEC water filtration membrane produced via APS that incorporates a functional enzyme is presented in chapter 5.

In chapter 2, a new type of PEC application is introduced; we show that CCs can be used as extraction media. We show that lactic acid, butanol, and three lipases can be partitioned from an aqueous solution. Butanol could be extracted and back-extracted using a change in temperature. In chapter 3, selective extraction is demonstrated by extracting one protein from a mixture of two proteins using a PEC. The selectivity is a function of the PEC composition, specifically the ratio of polyanions to polycations. Selectivity is rather important for extraction media as an extraction process typically is not used on a homogenous feed solution. Chapter 4 goes even further and selectively extracts one protein (lysozyme) from a chicken albumen (egg white) solution. Albumen is a natural mixture consisting mostly of proteins. After extracting lysozyme, we show that the enzyme retains its original enzymatic function. These three chapters demonstrate the potential of PECs as selective extraction media. They show that PECs can be versatile and used for the extraction and back-extraction of a variety of compounds. Furthermore, that proteins (back-) extracted with PECs can retain their original function. All of these are important factors for developing PECs as extraction media in the future.

Chapter 5 demonstrates a biocatalytic membrane incorporating lysozyme made *via* APS. The functionalisation of these membranes with lysozyme was a single-step modification to the APS production method. The biocatalytic membrane demonstrated enzymatic activity for (at least) one week. Membranes functionalised with enzymes and other proteins have broad applications in water filtration and biomedical engineering.

Finally, the findings are discussed in chapter 6 and an outlook is given that contemplates further applications of PECs for separation processes as well as suggestions for further research.

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Chapter 2

Complex Coacervates as Extraction Media

This chapter was adapted from its publication as:
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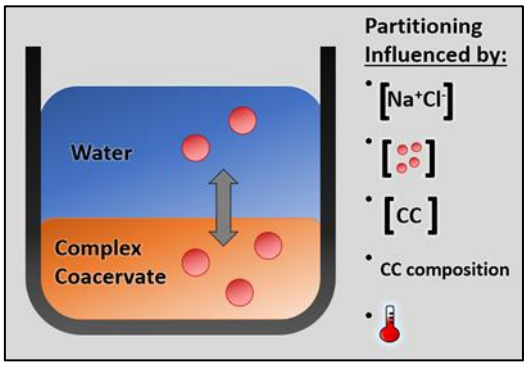
Abstract

Various solvents such as ionic liquids, deep eutectic solvents, and aqueous two phase systems have been suggested as greener alternatives to existing extraction processes. We propose to add macroscopic complex coacervates to this list. Complex coacervates are liquid-like forms of polyion condensates and consist of a complex of oppositely charged polyions and water. Previous research focussing on the biological significance of these polyion-rich phases has shown that polyion condensates have the ability to extract certain solutes from water and back-extract them by changing parameters such as ionic strength and pH. In this study, we present the distribution coefficients of five commonly used industrial chemicals, namely lactic acid, butanol, and three types of lipase enzymes in poly(ethylenimine)/poly(acrylic acid) complex coacervates. It was found that the distribution coefficients can vary strongly upon variation of tunable parameters such as polyion ratio, ionic strength, polyion and compound concentrations, and temperature. Distribution coefficients ranged from approximately 2 to 50 depending on the tuning of the system parameters. It was also demonstrated that a temperature-swing extraction is possible, with back-extraction of butanol from complex coacervates with a recovery of 21.1 %, demonstrating their potential as extraction media.



Introduction

Solvent extractions are important processes in many industrial separation processes ranging from the chemical industry, the food industry, to the pharmaceutical industry. An application of liquid extraction that has been receiving increasing attention is in the field of bio-based chemical production. There are many different categories of bio-based chemicals and the feature that they often have in common is that typically large amounts of water are present. Removing water by evaporation is among the costliest operations in industry, and therefore when aqueous solutions are present, liquid–liquid extraction (LLX) may be applied. In LLX an additional liquid phase, typically an organic solvent exhibiting preferential solubility for a specific solute, is used to selectively extract the solute from the initial liquid phase. Unfortunately, organic solvents that have been proven to be effective for extraction can be toxic for individual organisms and/or the environment. [1,2] There is great interest in the design of 'green solvents' that are more environmentally friendly in terms of production, usage, and disposal. For extraction from aqueous solutions, several alternatives to conventional organic solvents have been proposed in the past years such as ionic liquids (ILs), [3,4] deep eutectic solvents (DESs), [5] and aqueous two phase systems (ATPSs). [6–8]

ILs are essentially molten salts with a relatively low melting point (per definition, ≤ 100 °C). [9] ILs have shown a broad range of applications in part due to the customisation possible as a result of the large variety of composite components. [3,10] They are generally less volatile in nature compared to organic solvents and the negligible vapour pressure eliminates solvent losses through evaporation. [11] Unfortunately, many ILs are potentially toxic and not biodegradable. [12]

DESs are mixtures of hydrogen bond donors and hydrogen bond acceptors that form liquids on mixing and exhibit eutectic behaviour by having melting points lower than that those of their constituent components. They have been proposed as new extraction solvents and share many advantageous characteristics with ILs. [5,13] The toxicity of DESs varies, and in some cases the DES is even more toxic than its constituent components, [14,15] which is a factor to be taken into consideration when formulating DESs for sustainable extraction. Additionally, due to the fact that DESs are composite solvents, the molar ratio between the hydrogen bond acceptor and donor may change during the extraction. [16] This can result in solidification of the DES components and affect the subsequent extraction steps.

ATPSs function *via* segregative phase separation and consist of two (partially) immiscible aqueous phases. The most common ATPSs are formed when two constituents (often polymer–polymer or polymer–salt (or even ILs [7,8])) are mixed in an aqueous solution, resulting in two distinct segregated phases. Each of the segregated phases is rich in one of the two constituents. When used for the separation

of molecules, one of these phases will be the preferred phase for the compound of interest, while the remaining impurities hopefully concentrate in the other phase. [17-20] ATPSs are currently extensively used for the isolation and extraction of various biological compounds ranging from small molecules, hormones, up to the isolation of entire cells. [20–22] Also, micellar systems have been proposed as the foundation for new greener extraction methods with extraction principles similar to those of ATPSs. [23]

Similar to segregative phase separation, two phase systems can also be formed *via* associative phase separation such as complex coacervation (Figure 2.1). This process occurs when oppositely charged polyions (a.k.a. polyelectrolytes) are mixed under conditions that allow them to associate. The formed complex coacervates (CCs) are macroscopic liquid-like aqueous polyion-rich condensates, which are in equilibrium with an aqueous polyion-poor phase, also called the supernatant. Depending on the chemistry of the polyions and the environmental conditions, solid-like condensates can also form, called polyelectrolyte complexes (PECs). In this study, we will make use of complex coacervates.

In previous studies, CCs and PECs have been reported with the property of partitioning certain proteins into the complex phase over the supernatant phase. [24-26] The ability to isolate proteins using single polyions is already well established, but a previous study has shown that in some cases the addition of a mixture of both polycations and polyanions can lead to better partitioning than the addition of only one species of polyions. For example, the addition of the polyanion poly(acrylic acid) (PAA) alone is not enough to extract the positively charged protein lysozyme from an aqueous solution, but with the addition of a polycation (and thus the formation of a PEC), the lysozyme could be extracted completely. [24] CCs therefore show emergent properties that their constituent components do not.

A potential advantage of associative phase separation of CCs and PECs over segregative phase separation of ATPSs is that the distribution coefficients of CCs can be dependent on the composition of the CCs, resulting in different partitioning behaviours for the same constituent polyions present in different ratios. [24,25] There are a handful of studies that show that PECs have the ability to partition certain proteins [24–28] as well as certain small molecule dyes [29,30] from an aqueous solution. In some cases, the distribution coefficients reported were in the order of 10^4 in favour of distribution in the PEC for a specific protein and polyion pair. [24] These studies hint at the potential of CCs and PECs as extraction media, though they are typically concerned with biomedical applications such as intracellular drug delivery. We have previously achieved success in using structurally simple polyions in order to selectively extract lysozyme from an aqueous solution in the presence of another protein. [24] Beyond varying the ratio of the polycation to the polyanion, there are other factors that influence the CC properties such as solution ionic strength,

temperature, and varying concentrations of the system's constituents. There are no systematic studies that go into the details of the effect of such system parameters on the partitioning behaviour of the solutes.

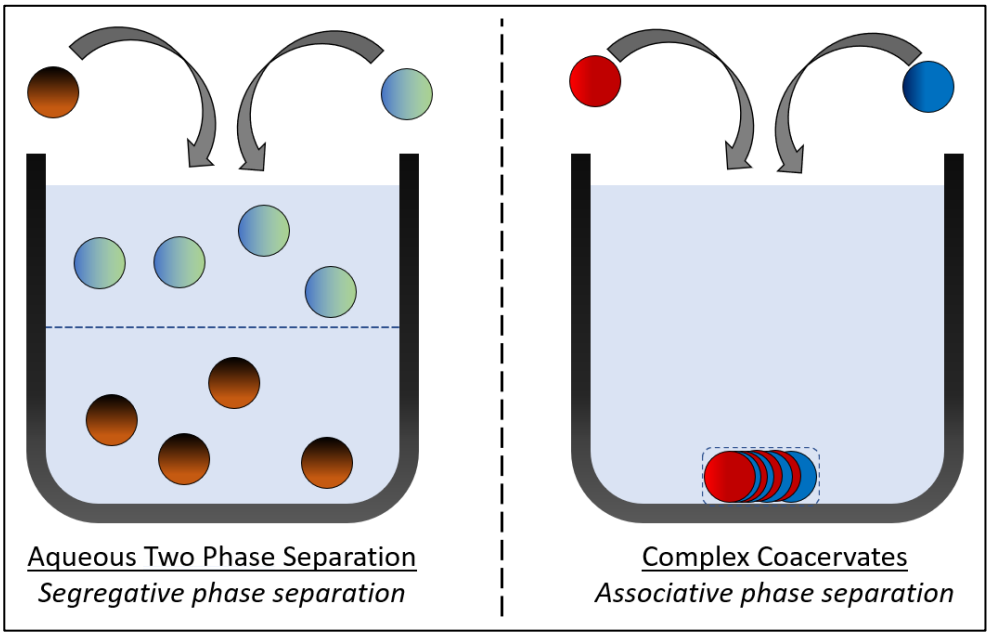


Figure 2.1. *A schematic representation of the difference between ATPS and CC phase separation. In ATPS (left), the added constituents form separate aqueous phases. In CC (right), two oppositely charged polyions form a polyion-rich aqueous phase.*

The inspiration for CCs as extraction media comes from the partitioning behaviour of solutes between cellular fluids and membraneless organelle (MLO) compartments within living cells. MLOs consist of both negatively and positively charged biomacromolecules such as negatively charged RNA and positively charged intrinsically disordered proteins. [31] The MLO phase behaviour strongly resembles the phase behaviour of CCs. Our cells use MLO droplets to perform very specific biological functions, including the partitioning and release of specific targeted compounds in response to changes in the stimuli in the cellular environment. [32–35] While nature undoubtedly has a head start regarding the design of MLOs, their functionality in cells shows that there is currently untapped potential for CCs as media for extraction processes. Developing CCs with distribution coefficients that

are strongly dependent on tunable stimuli and environmental parameters would be of great benefit to the development of extraction processes.

In this study, we investigate the extraction of several compounds from aqueous solutions using complex coacervates formed by branched poly(ethylenimine) (PEI) and poly(acrylic acid) (PAA). PEI-based nanocrystals have been used as extraction media for rare earth element recovery and are increasingly used as vehicles for drug delivery. [36,37] Higher molecular weight PEI is typically considered cytotoxic, though this effect can be decreased by using the low molecular weight (1.8 kDa) variant that is used in this study. [38] PAA is commercially used as a thickening agent and water absorber in the hygiene, cosmetic, agricultural, and food industries. In these contexts, PAA is usually known as sodium polyacrylate or waterlock.

We consider lactic acid (LA), butanol, and three varieties of industrial lipase enzymes as model compounds for the extraction from the aqueous supernatant into the PEI/PAA CC. These industrially relevant lipases are widely used in food, detergents, and pharmaceuticals [39,40] and represent up to 10% of the total global enzyme market. [41] LA extraction from an aqueous fermentation broth has received increased attention in the last few years amongst others due to the possibility of poly(lactic acid) being a sustainable alternative to many commonly used plastics. [42] The use of poly(lactic acid) as a competitor to modern plastics is currently restricted to application areas where the higher costs associated with purification and extraction from the fermentation broth can be tolerated. Several techniques have been in development for the recovery of LA from the fermentation broth aiming to reduce the production cost and decrease the impact of by-product formation during lactic acid production on the environment, and CCs may be a new technique to address the LLX of LA. [43,44] Butanol, being a popular solvent and a popular candidate for biofuels, can also be extracted from fermentation broths. [45]

In this study, we create macroscopic CCs *via* associative phase separation of PEI and PAA. We investigate the effect of several parameters such as CC composition, reagent concentrations, and temperature on the partitioning of lipases, lactic acid, and butanol to demonstrate a proof of concept to draw attention to the use of CCs for extraction purposes.

Materials and methods

Materials

Poly(acrylic acid) (PAA) sodium salt powder with a molecular weight of 6.0 kDa and branched poly(ethylenimine) (PEI) with a molecular weight of 1.8 kDa were

purchased from Polysciences, Inc. Sodium chloride (NaCl, > 99 %), sodium hydroxide (NaOH, > 98 %), fuming hydrogen chloride (HCl, 37 ± 1 wt %), n-butanol (> 99 %), and lipase from porcine pancreas (PPL) were purchased from Sigma-Aldrich/Merck. NovoCor AD L lipase (CALA) and Novozyme CALB lipase (CALB) were donated by Novozymes A/S. Crystalline L-lactic acid was donated by Corbion N.V. Unless otherwise specified, water used for the solutions and dilutions was ultrapure Milli-Q water dispensed from a PURELAB flex system at a resistivity of 18.2 MΩ.

Experimental methods

Complex coacervates were prepared by mixing prepared aqueous polyion solutions (PAA and PEI) for a total polyion concentration of up to 20 g/L in the presence of up to 400 mM NaCl. All solutions are set to pH 7 before mixing. In the case of lipases, they are added to the solution with the polyions at a lipase concentration of 67 µM, consistent with earlier studies. [25,26,33] Unless otherwise specified, butanol was added at 400 mM and lactic acid at 100 mM. In the case of butanol and lactic acid, the mixed polyion solution is first left to equilibrate overnight into a CC. Then it is centrifuged at 12,500 g for 30 minutes using a Centrifuge 5425 (Eppendorf) to expedite the separation of polyion-rich complex coacervates from polyion-poor aqueous supernatant phases. The supernatant is then replaced with a new solution containing either lactic acid or butanol in an aqueous NaCl solution with the same NaCl concentration as during the preparation of the CC. Total volumes for each experiment were fixed at 500 µl unless otherwise specified.

The composition of the CC is defined *via* F^-:

$$F^- = \frac{[n^-]}{[n^-] + [n^+]} \quad (1)$$

where $[n]^-$ and $[n]^+$ are the concentrations of PAA and PEI monomers, respectively, which are mixed in solution. For example, at $F^- = 0.50$, there is an equal molar amount of PEI and PAA monomers present, and at $F^- = 0.75$, there are 3 PAA monomers for every 1 PEI monomer. The assumption being that at pH = 7 both polyions are fully charged due to the interaction between the two polyions. [24,25,46,47] Under this assumption, PAA has a mass of 76.7 g/mol of negative charge and PEI has a mass of 43.0 g/mol of positive charge.

Analytical methods

The total mass of the complex coacervates was determined by comparing the mass of the sample tubes when empty to that of those containing only the complex coacervates. The volume was determined under the assumption that the density of the CCs was approximately equivalent to that of water. [24] This assumption is based on the densities of PEI (1.03 g/ml) and 50% PAA solution (1.15 g/ml) reported by the manufacturer. Considering that the majority of the CC consists of water, total CC density is within a few percent of water, in the calculated range of 1.02–1.04 g/ml.

The water content of the PEI/PAA complex coacervates was determined *via* thermogravimetric analysis (TGA) using a STA 449 F3 Jupiter (Netzsch) thermal analyser on CCs formed at 10 g/L total polyion concentration. The temperature was increased from 30 to 120 °C at a rate of 5 °C/min and then kept constant at 120° for 40 minutes to evaporate the water present in the complex coacervates. The mass of the samples is recorded to obtain the mass loss corresponding with the evaporated water.

Prior to the determination of the concentration of the solute present in the supernatant, the systems were centrifuged for 30 minutes at 12,500 g in an Eppendorf Centrifuge 5425. Enzyme concentration from the supernatant was determined by evaluating the absorbance at 280 nm using a Shimadzu UV-2401PC spectrophotometer. Extinction coefficients for PPL and CALA were calculated to be 68 kM/cm and 54 kM/cm based on the peptide sequence. The extinction coefficient for CALB has been reported in literature as 41 kM/cm. [48]

Butanol concentration was determined using a Thermo Scientific Trace 1300 gas chromatograph with two parallel ovens, an auto sampler TriPlus 100 Liquid Samples and an Agilent DB-1MS column (60 m × 0.25 mm × 0.25 μm) with an injection volume of 1 μL diluted in analytical acetone. A ramped temperature profile was used, in which the initial temperature was 30 °C, followed by a ramp of 10 °C/min to 140 °C. The second ramp of 50 °C/min to 340 °C finished the program, which lasted for 15 minutes. The flame ionisation detector temperature was 440 °C. A column flow of 2 mL/min with a split ratio of 25, an airflow of 350 mL/min, a helium make-up flow of 40 mL/min and a hydrogen flow of 50 mL/min was used.

Lactic acid concentration was determined using a Grom Resin H + IEX column on a Metrohm 850 Professional ion chromatograph. The mobile phase was 1 mM H_2SO_4 solution with a flow rate of 0.6 mL/min. The column temperature was 45 °C.

As the total amount of the added compound is known and the concentration of the compound in the supernatant is measured, the compound concentration in the complex coacervate can be calculated. The distribution coefficient is then determined *via*:

$$K_D = \frac{[X]_{CC}}{[X]_{SN}} \quad (2)$$

where $[X]_{CC}$ and $[X]_{SN}$ are the concentrations of the compound in the complex coacervate and supernatant, respectively. The distribution coefficient changes depending on the varied parameter, resulting in a distribution profile.

Butanol extraction and back-extraction

PEI/PAA CC systems were prepared with a total polyion concentration of 50 g/L in 1 ml with a composition of F^- = 0.26. The increased polyion concentration was chosen to produce more CC as a simulation of upscaling compared to the previous experiments. This mixture was centrifuged for 30 minutes at 1000 g. The aqueous supernatant was then replaced with 650 µl of 5.7% butanol and 10 mM NaCl solution. The samples were collected to determine the butanol concentration after 24 h of incubation at room temperature (RT), and again after 24 h of incubation at 70 °C. The supernatant was then decanted, and any excess supernatant drops were removed using pressured nitrogen gas. 600 µl of fresh 10 mM NaCl solution was added to the CC as a back-extraction phase, and the samples were collected from the back-extract after 24 h of incubation at 60 °C. Then, the samples were collected after another 24 h of equilibration at 40 °C, and once more after another 24 h at RT.

The butanol concentration of all the samples was determined as described previously and the amount of butanol present in the CC was calculated taking into account the varying volumes of the supernatant due to sample extraction.

Results and discussion

PEI/PAA complex coacervate formation and water content

Complex coacervates are formed due to the interactions between the oppositely charged polymer chains, with the driving force being both entropy gain due to the

release of counterions and electrostatic interaction. The fraction of the negative and positive charges is important for the total extent of CC formation. To narrow down the region of interest for evaluating the partitioning, we first evaluated the total CC formed as a function of the composition F^- and looked at the water content for two CC compositions of interest.

In Figure 2.2A, it is shown that the largest amount of CC was formed around $F^- = 0.25$ to 0.50, with the highest values found at 0.26 and 0.36 with 23.1 ± 3.3 mg and 22.4 ± 3.3 mg, respectively. Figure 2.2B shows the photographs of the relative quantities of CC as a function of F^-.

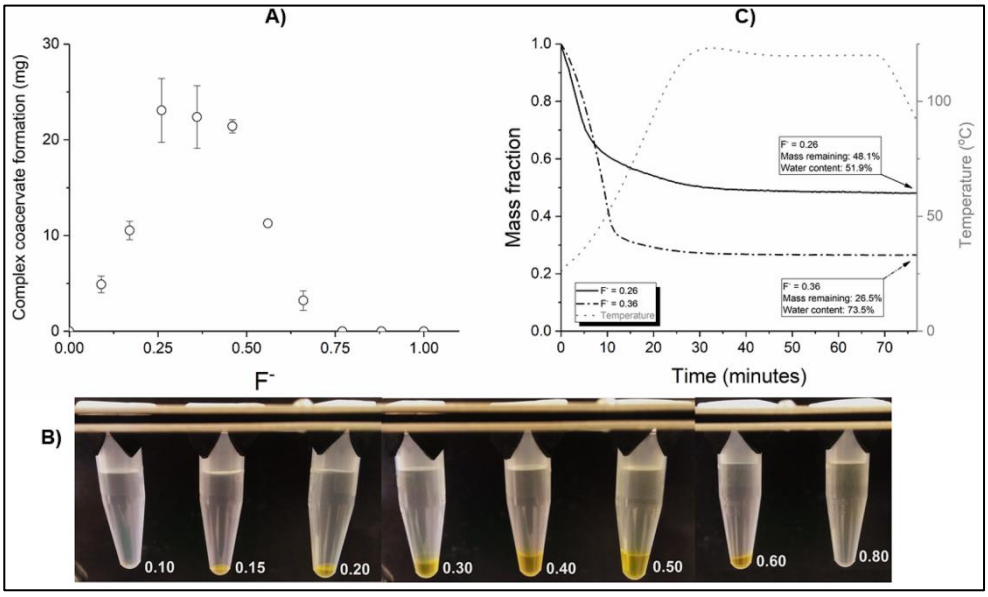


Figure 2.2. *Analysis of CC formation properties. (A) Total CC formed as a function of F^- at a polyion concentration of 20 g/L and a NaCl concentration of 10 mM. Values are represented as average with standard deviations from triplicate experiments. (B) Photographs of different F^- ratios with consistent amounts of total polyions. (C) The water content of the formed CCs was determined using TGA for $F^- = 0.26$ and 0.36 at a polyion concentration of 10 g/L and a NaCl concentration of 10 mM. The left Y-axis shows the remaining mass fraction of the CC as the temperature presented on the right Y-axis is increased and water is vaporised.*

As shown in Figure 2.2C, we evaluated the water content of the two F^- values with the highest CC formation as seen from Figure 2.2A and found that for PEI/PAA CCs the water content varies drastically based on CC composition, with the water content for $F^- = 0.36$ being 73.5%, and for $F^- = 0.26$ being 51.9%. Comparing the remaining

mass of the polyions in the CC to the total polyions added, it appears that for F^- = 0.26 all the polyions form the CC mass, while for F^- = 0.36 only approximately 60 % of the polyions form the CC, with the rest presumably remaining in solution.

Intuitively, it might be expected that the largest volume of CC formation occurs at the composition F^- = 0.50, where an equal amount of positive and negative monomers is present. However, this is not necessarily the case as demonstrated by the PEI/PAA CC system. One explanation for this discrepancy is that the interactions between polyions, water, and salts can affect the degree of ionisation of the monomers.

Water content of CCs and PECs is typically reported to be between 60 and 80 %. [49-51] We found using TGA that for PEI/PAA CCs at a composition of F^- = 0.36 the CCs fall within the reported range, though the water content at F^- = 0.26 is approximately 10 % lower than expected. The water content of CCs can impact the partitioning behaviour of solutes based on their preferential association with water. For example, lipases in general are known to prefer oil–water interfaces over fully aqueous environments. [52] Both PEI and PAA are not expected to decompose at the given conditions, temperature, and timescale. [53,54]

Lipase enzyme distribution

In this section, the partitioning of several types of lipases in the PEI/PAA complex coacervates is described. In Figure 2.3, the distribution coefficients (K_D) of three commonly used lipases PPL, CALB, and CALA as a function of the CC composition, the NaCl concentration, and the total polyion concentration are shown.

We found that the K_D of all lipase types varies greatly as a result of the adjusted parameters. The charge ratio F^- has the most significant consistent effect (Figures 2.3A–C), showing distinct K_D maxima at a composition of F^- = 0.36 for CALB (K_D maximum of 11.0 ± 0.9) and F^- = 0.26 for CALA (K_D maximum of 23.0 ± 0.5) and PPL (K_D maximum of 19.2 ± 1.9). These maxima are partially consistent with the maximum values of CC formed (Figure 2.2A); however, a small deviation in the composition results in a larger change in K_D than that can be solely attributed to a difference in the CC quantity: for the region with the highest constant CC formation (F^- ranging from 0.25 to 0.50), there are variations in the K_D of up to a factor 4 for CALA (Figure 2.3A).

The distribution profiles were found to be dependent on the specific protein investigated. The results for these lipases corroborate the earlier studies that report similar nearly symmetrical distribution profiles (though centred around different F^-

values) for three proteins with poly(lysine)/poly(glutamate) CCs. [26] Other proteins with different polyion pairs show completely different distribution profiles altogether that are not necessarily symmetrical. [24,25] For now, there are no reliable methods to predict the distribution profile in advance as a result of the parameters. Many studies that look into the partitioning of proteins assume F^- = 0.50 is the optimal composition for both PEC formation and partitioning and do not investigate the other charge ratios. [27,28,30] Based on the results presented here, there might be opportunities for working at other compositions that result in more desired K_D values.

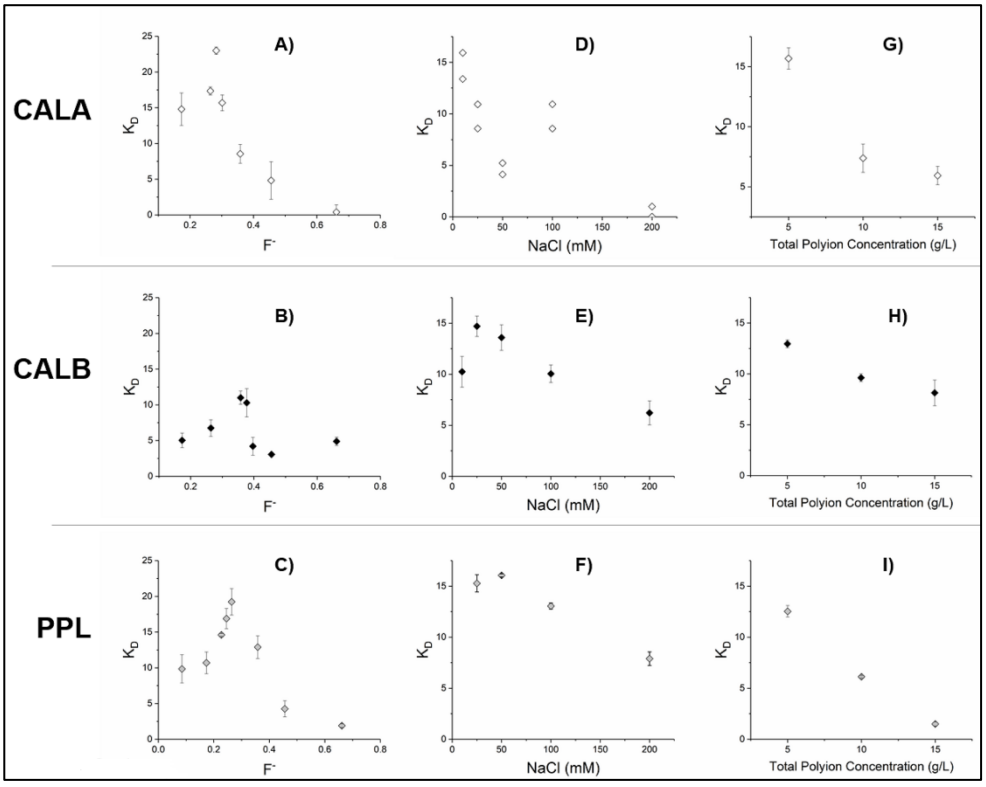


***Figure 2.3**. Distribution coefficients K_D for CALA (A, D, and G), CALB (B, E, and H), and PPL (C, F, and I) as a function of CC composition (A–C), NaCl concentration (D–F), and polyion concentration (G–I). Unless otherwise specified, total polyion concentration is 5 g/L, enzyme concentration is 67 µM, NaCl concentration is 10 mM, and F^- is 0.36 for CALB and 0.26 for CALA and PPL. Measurements are shown as average with standard deviation for n = 3, except (D), which is shown as individual measurements.*

Both CALB and PPL show a similar distribution profile as a function of the NaCl concentration (Figures 2.3E and F) with a slight K_D increase initially, followed by a decrease. CALA however shows an immediate decrease, followed by a local maximum (Figure 2.3D) at a comparatively high salt concentration. By varying the NaCl concentration, the K_D varies between approximately 5 and 15–20 for the investigated enzymes. We hypothesise that for CALB and PPL a partial screening of the polyion charges by the salt ions results in the CC being less densely packed, essentially increasing the distance between polyion chains and allowing the proteins (or other solutes) to enter the CC more easily. Polyion condensation in the presence of other ions (such as salt ions) results in ion association with charged monomer subunits of the polyion. This effectively screens the electrostatic interaction between the oppositely charged monomers of each polyion. Indeed, if the ionic strength of the solution becomes too high, the polyion structures dissolve completely as the degree of screening prevents the complex formation between polyions. [46] Between complete complex dissolution and the absence of additional ions beyond the counterions brought in by the polyions, there is a concentration region where the salt ions prevent part of the oppositely charged polyions from associating. Subsequently, this can influence the behaviour of the condensates.

All three enzymes showed a similar trend of K_D decrease as the total polyion concentration increased. A possible explanation is that as the total mass of CC increases, this does not result in a proportional increase of the CC–water interface, limiting the penetration of the solutes into the CC.

It is worth mentioning that there are other advantages of concentrating enzymes in CCs or PECs beyond extraction purposes. It has been reported that the activity of proteins may be enhanced in CCs compared to the same proteins in regular aqueous solutions. [27,47] In addition, the polyions may protect the proteins from degradation, increasing the shelf life of (extracted) proteins. [55] The mechanism for this is unknown, though the ability to both highly concentrate the enzymes and increase their activity is particularly interesting for industrial applications.

Lactic acid distribution

The partitioning of lactic acid into CCs was studied, as LA is an industrially relevant small molecule. The effects of the CC composition, the NaCl concentration, the total polyion concentration, the initial LA concentration, and the temperature on the LA K_D were studied, and the results can be observed in Figure 2.4.

Unlike the distribution profiles for the lipase enzymes, we found only very little effect of the composition on the K_D (Figure 2.4A), which remained between 2 and 4. In contrast, the effect of NaCl on K_D (Figure 2.4B) of LA was more pronounced than

those of CALB and PPL while following a similar distribution profile. Within our hypothesis of salt ions influencing the distance between polyion chains, the effect of the salt NaCl concentration may be more pronounced for LA, as it is substantially smaller than any of the lipases. By varying the NaCl concentration, we found the highest K_D for LA at 7.4 ± 0.5 for 100 mM NaCl.

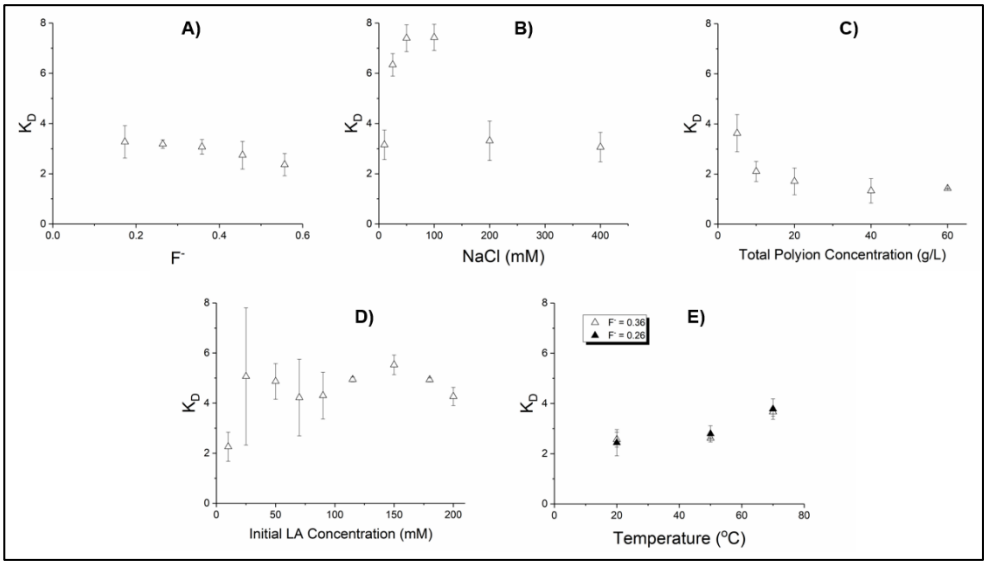


***Figure 2.4**. Lactic acid partitioning in PEI/PAA CCs as a function of (A) CC composition, (B) NaCl concentration, (C) polyion concentration, (D) initial LA concentration, and (E) temperature. Unless otherwise specified, the experiments took place at approximately 20 °C, a polyion concentration of 5 g/L, a LA concentration of 100 mM, a NaCl concentration of 10 mM, pH = 7, and F^- = 0.26. Results are shown as average with standard deviations for n = 3.*

Similar to the trend with lipases, increasing polyion concentration had an adverse effect on the partitioning (Figure 2.4C). However, altering the initial concentration of lactic acid only slightly affects the partitioning in the evaluated range (Figure 2.4D), suggesting that the saturation point for the CC has not yet been reached as this would result in an expected decrease in K_D at higher LA concentrations. [24] Figure 2.4E shows that an increase in temperature has a small but consistent positive effect on the K_D in the investigated range. This suggests that the extraction process is endothermic and that the driving force behind the partitioning is an increase in total entropy, perhaps similar to how an increase in entropy is the primary driving force for polyion–polyion association in the first place. [23]

The optimal K_D for LA in our PEI/PAA CC system at approximately 100 mM NaCl is comparable to or greater than many other liquid–liquid extraction systems. [44,56-58]

A disadvantage of some of these reported systems is their reliance on low pH [59] or the toxicity of the solvents. [58] While some established extraction methods, such as tri-n-octylamine in 1-octanol, [60] outperform CC systems with regard to LA for now, we show that the effects of system parameters for CC systems can substantially alter the K_D. Interestingly, where the common method using tri-n-octylamine appears to decrease the distribution coefficient at higher temperatures, the opposite is true for PEI/PAA CCs. [56] There are many additional parameters that can be further fine-tuned, suggesting the ability to achieve much higher K_D values.

Butanol distribution, extraction, and back-extraction

We investigated the K_D of butanol as a function of the CC composition (Figure 2.5A) as well as the temperature (Figure 2.5B). As butanol partitioning showed a remarkable temperature sensitivity, we evaluated the possibility of extraction and back-extraction of butanol using CCs by alternating between room temperature and 70 °C (Figure 2.5D).

Contrary to the lipases, we observe the highest K_D for butanol as a function of CC composition quite distant from the optimal CC formation, resulting in the highest value of K_D of 22.7 ± 0.7 at $F^- = 0.56$. This K_D is very similar to that of a reported task-specific IL and substantially higher than the standard of oleyl alcohol, which are K_D = 21 and 3.4, respectively. [61,62]

Whereas LA demonstrated only a minor temperature dependence of the K_D (Figure 2.4E), the butanol distribution shows a large difference between RT and 70 °C, roughly at a factor of 4–5. Out of the evaluated parameters, temperature is the most practical to change for the existing systems as it does not require the addition or removal of chemicals and is straightforward to implement. For this reason, we envisioned a PEI/PAA CC system that was able to partition butanol within the CC to a greater degree at high temperatures and could then be coaxed to release butanol into a separate aqueous environment at lower temperatures such as RT. To evaluate such a system for extraction and back-extraction of butanol, we prepared PEI/PAA CCs at higher concentrations of polyions (Figure 2.5C). The resulting CCs had a mass of 62.2 ± 1.7 mg (average ± standard deviation, n = 4). A supernatant containing butanol was added to the CCs, and the temperature was increased from RT to 70 °C for butanol extraction. For back-extraction, the supernatant was replaced with fresh supernatant containing no butanol, and the temperature was decreased first to 60 °C, then to 40 °C, and finally to RT (Figure 2.5D).

Consistent with the observations of Figure 2.5B, increasing the temperature to 70 °C substantially increases the butanol content in the CC. Figure 2.5B shows an approximate quadrupling of the K_D, whereas Figure 2.5D only shows a CC butanol

increase from 8.80 ± 0.03 to 20.39 ± 0.80 mg, corresponding with a decrease of the supernatant butanol concentration from 4.39 ± 0.00% at RT to 2.28 ± 0.14% at 70 °C. A possible explanation for this discrepancy is the difference in the total polyion concentration, as Figures 2.3G/H/I and 2.4C show that increased polyion concentrations do not necessarily lead to an increase in partitioning.

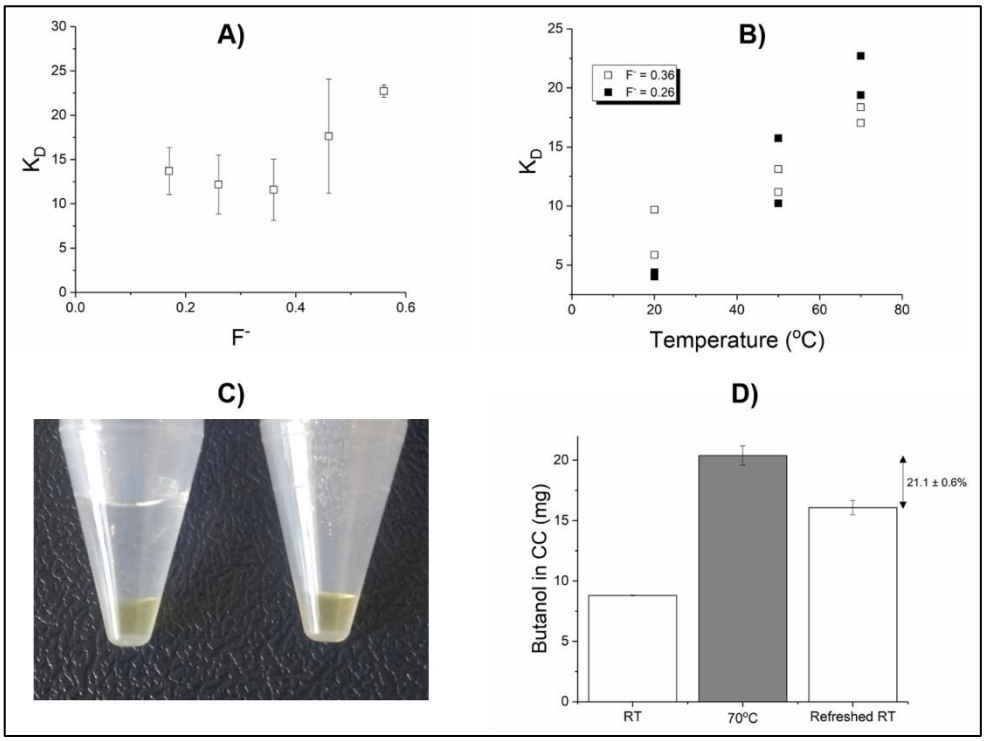


***Figure 2.5**. Interaction between butanol and PEI/PAA CCs. (A) Distribution coefficients of butanol in PEI/PAA CCs as a function of CC composition shown as an average with standard deviation with n = 2. (B) Distribution of butanol as a function of temperature for F^- = 0.26 and 0.36. For (A) and (B), the total polyion concentration was 5 g/L, the NaCl concentration was 10 mM, and the butanol concentration was 400 mM. (C) Image of PEI/PAA CC at 50 g/L with (left) and without (right) the aqueous supernatant. (D) Butanol remaining in the CC at F^- = 0.26 during RT to 70 °C extraction and 70 °C to RT back-extraction. Data is shown as an average with standard deviation with n = 4.*

By replacing the supernatant and lowering the temperature in steps from 70 °C back to RT, 21.1 ± 0.6% of the butanol extracted into the CC could be back-extracted into a new aqueous solution. Interestingly, reverting the temperature back to RT did not

completely revert the butanol equilibrium and a fraction of butanol remains within the CC.

Considering the large number of tunable parameters, it is likely that with alterations a back-extraction higher than 21.1% is achievable. For example, increasing the salt concentration has been used to back-extract proteins from polyion micelles and polyion precipitates by disrupting the polyion complex, [46] while varying the pH has been used to back-extract proteins, keeping the polyion precipitates intact. [24] Other experimental parameters such as increasing the number of temperature steps or increasing the equilibration time may also prove to be beneficial. Further research should find improved recovery methods as well as better understanding of the physicochemical mechanisms allowing for a larger fraction of the CC-extracted butanol to be recovered.

Lactic acid and butanol distribution in the enzyme-filled complex coacervates

We hypothesised that the presence of additional components in the CCs can influence the partitioning behaviour of LA and butanol in those CCs. For this reason, we investigated the distribution of LA and butanol in PEI/PAA CCs that already contained PPL, CALB, or CALA enzymes. Similar to the presence of salt ions, the presence of relatively large enzymes in the CCs may change the structure of the polyion complex by altering the distance between polyions and the properties of the CC–water interface. We fixed the compositions of the systems to the F^- at which the maximum K_D was found; $F^- = 0.36$ for PPL and CALB, and 0.26 for CALA. Then, LA (Figure 2.6) and butanol (Figure 2.7) partitioning was studied as a function of the ionic strength at 25 and 50 °C.

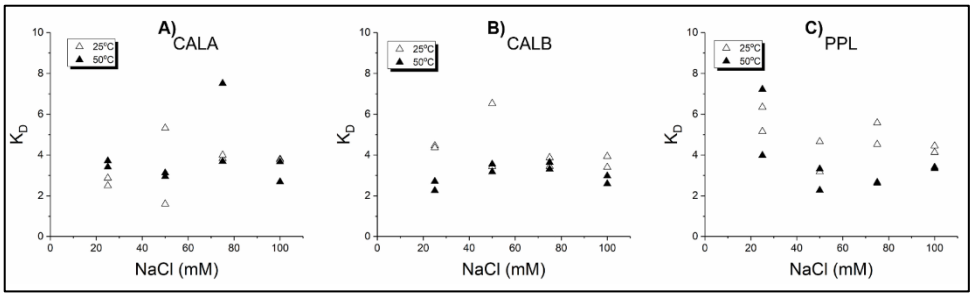


***Figure 2.6**. Distribution of LA as a function of NaCl concentration for PEI/PAA CCs containing (A) CALA, (B) CALB, and (C) PPL lipase enzymes. Initial LA concentration was 100 mM, polyion concentration was 5 g/L, and $F^- = 0.26$ for CALA, and 0.36 for CALB and PPL. Results are shown as individual independent experiments.*

In Figure 2.6, we can see a stabilizing effect of the lipases on the LA distribution coefficients as they no longer strongly increase between 10 and 100 mM NaCl compared to the PEI/PAA CCs without the lipases shown in Figure 2.4B. In addition, the presence of PPL slightly increases the 'stable' K_D to approximately 5 compared to 3 without PPL. CALB increases the K_D to approximately 4. For PL and CALB, a higher temperature resulted in a slightly lower K_D, comparable to values where the lipases were not present at all. Similar to Figure 2.4E, there is no strongly noticeable difference between the investigated temperatures.

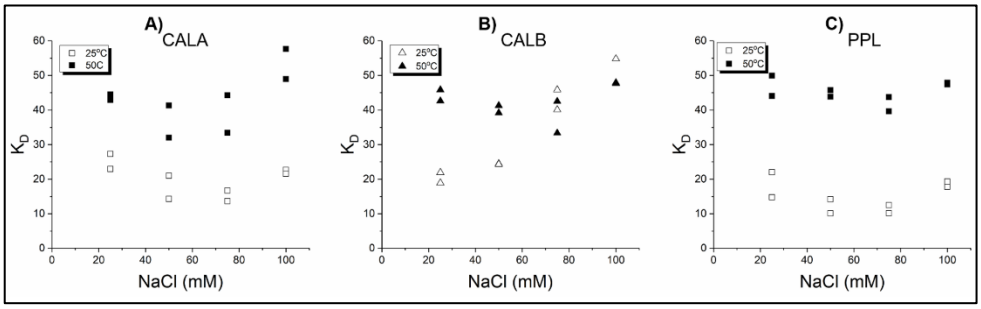


***Figure 2.7**. Distribution of butanol as a function of NaCl concentration for PEI/PAA CCs containing (A) CALA, (B) CALB, and (C) PPL lipase enzymes. Initial butanol concentration was 400 mM, polyion concentration was 5 g/L, and $F^- = 0.26$ for CALA, and 0.36 for CALB and PPL. Results are shown as individual independent samples.*

A much stronger effect is observed for the distribution of butanol shown in Figure 2.7. For PPL and CALA, the KD values are comparable to CCs without the lipases at K_D = 10–20, but with increased temperature the KD values increase to 40-50 for PPL and 30–50 for CALA. Interestingly, in the presence of CALB, the K_D for butanol increases linearly with the NaCl concentration (Figure 2.7B) at RT, but not at higher temperatures.

These 'doped' CCs show different distribution profiles than 'empty' CCs. Doped CCs may shield against the effect of increased NaCl concentration or simply increase the distribution coefficient by up to a factor of 3 compared to empty CCs. All in all, the concept of pre-filled CCs gives another parameter to tune and optimise the extraction potential of complex coacervates.

Conclusion and outlook

We present an exploratory study on new applications of complex coacervates. While the partitioning behaviour of CCs has been noted before, the step to develop them as an extraction medium has been absent. From previous studies as well as the results shown in this study, it has become clear that the partitioning behaviour of the compounds in CCs is a complex subject involving many tunable parameters that individually greatly influence the distribution coefficient between the aqueous environment and the CC.

In our study, we showed that the distributions of lipase enzymes, lactic acid, and butanol in PEI/PAA CCs are strongly affected by the CC composition, ionic strength as determined by the NaCl concentration, polyion concentration, temperature, and presence of other compounds in the CC. However, the effect of any of these parameters depends on the partitioned compound examined.

For example, we found that the CC composition has a great influence on the K_D of lipases (Figure 2.3A–C), while it has only a minimal effect on the K_D of LA (Figure 2.4A). Even within the category of lipases, the effect of the NaCl concentration on the K_D of CALA is much stronger than on the K_D of PPL (Figures 2.3D and F, respectively). The only consistent influences of the parameters found were that higher concentrations of polyions above 5 g/L or high concentrations of NaCl led to lower K_D values, though a small amount of NaCl was often (but not always) beneficial. The highest K_D experimentally found and the corresponding parameters for the 5 compounds are presented in Table 2.1.

We demonstrated that several relatively simple and tunable parameters can change the K_D by a factor of 4 for lipases and butanol as a result of the changes in the CC composition (Figures 2.3A–C) and temperature (Figure 2.5B), respectively. It is unfortunate that many studies investigating the partitioning behaviour of solutes in CCs do not investigate different compositions, and instead fix it at $F^- = 0.50$ where they might miss either compositions with greater partitioning or with greater PEC formation. [27,28,30] As is demonstrated with the PEI/PAA system, we have shown that it is far from a safe assumption that the optimal polyion complex formation takes place at $F^- = 0.50$, let alone the assumption that the desired partitioning properties are optimal at this composition.

Special emphasis has been laid on temperature as a parameter that is easily physically tunable without adding or removing chemicals to or from the system. Using temperature, we created a PEI/PAA CC temperature-swing extraction system that can extract approximately half the butanol from an aqueous supernatant at 70 °C, and then back-extract 21.1% of the extracted butanol back into a new aqueous phase at RT in a single-step system. In this way, CC extraction media can be considered

analogous to, for example, cyclic CO_2 absorption, where typical cyclic capacities are in the order of 5–15%. [63]

***Table 2.1**. Summary of the highest K_D for all the compounds and their corresponding parameters as experimentally found in this study.*

Compound	Highest K_D found	Figure	Composition (F^-)	Compound concentration	NaCl concentration	Polyion concentration	Temperature
CALA	23.0 ± 0.5	2.3A	0.26	67 µM	10 mM	5 g/L	20 °C
CALB	14.7 ± 1.0	2.3E	0.36	67 µM	25 mM	5 g/L	20 °C
PPL	16.1 ± 0.2	2.3F	0.36	67 µM	50 mM	5 g/L	20 °C
Lactic Acid	7.4 ± 0.5	2.4B	0.26	100 mM	100 mM	5 g/L	20 °C
Butanol	53.3 ± 6.2	2.7A	0.26 + CALA	100 mM	10 mM	5 g/L	50 °C

However, considering the number of tunable parameters, it is almost certain that the cyclic capacity can be made much more efficient, and that extraction/back-extraction of a variety of small molecules as well as proteins is possible. While the results for our butanol extraction were not directly comparable in efficiency to some of the results shown by ATPS systems, [64] where up to 95% of a protein was purified in a single step, such high extraction numbers have been shown with different PECs for different proteins, [24] suggesting that a similar potential for CCs exists.

There are several limitations of this study. Some of the experimental protocols in these experiments, such as centrifuging at 12,500 g for 30 minutes, are impractical for industrial applications. These protocols were based on earlier fundamental research [24,25] and it is likely (but not verified) that centrifuging at far lower speeds and durations is sufficient. Indeed, the butanol (back-)extraction was performed without additional centrifugation steps after the addition of butanol to the system.

The reasons for the variation in K_D values and the mechanisms determining the distributions in CCs or other PECs are not well understood. The partitioning behaviour is currently not well understood and cannot yet be accurately predicted. Currently, this means that extensive testing for the individual compound, polyion pair, and tunable parameters is required in order to learn how the parameters influence partitioning. It would be extremely beneficial for the development of CCs as extraction media if the fundamental mechanisms of partitioning in CCs were better understood. The ability to predict the influence of (combinations of) parameters on partitioning prevents the necessity of high-throughput testing to optimise the parameters for the extraction of a particular desired compound. With a greater understanding of the underlying mechanisms, CCs show promise as extraction media for a wide variety of compounds. The partitioning of solutes in CCs and PECs is the result of a complex interplay of at least 6 different compounds (polyanion, polycation, water, two salt ions, and the solute of interest), and the temperature will affect the interactions between all these compounds, making it difficult to predict the partitioning behaviour. For proteins, it is expected that the charge and charge distribution are important, and hydrophobic interactions will also

play a role. The temperature-dependent partitioning of butanol is promising, but systematic studies are required to unravel the detailed molecular mechanism.

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Chapter 3

Charge-Based Separation of Proteins Using Polyelectrolyte Complexes as Models for Membraneless Organelles

This chapter was adapted from its publication as:
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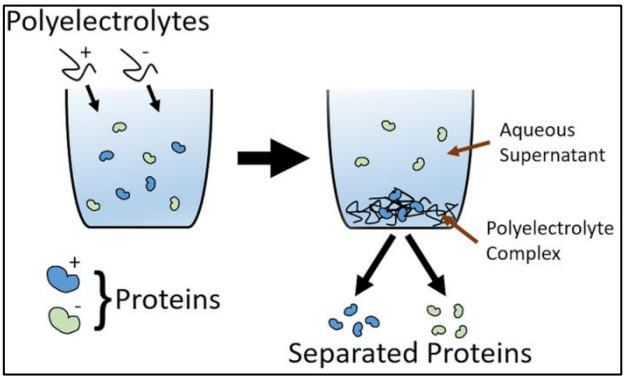
Abstract

Membraneless organelles are liquid compartments within cells with different solvent properties than the surrounding environment. This difference in solvent properties is thought to result in function-related selective partitioning of proteins. Proteins have also been shown to accumulate in polyelectrolyte complexes, but whether the uptake in these complexes is selective has not been ascertained yet. Here, we show the selective partitioning of two structurally similar but oppositely charged proteins into polyelectrolyte complexes. We demonstrate that these proteins can be separated from a mixture by altering the polyelectrolyte complex composition and released from the complex by lowering the pH. Combined, we demonstrate that polyelectrolyte complexes can separate proteins from a mixture based on protein charge. Besides providing deeper insight into the selective partitioning in membraneless organelles, potential applications for selective biomolecule partitioning in polyelectrolyte complexes include drug delivery or extraction processes.



Introduction

In cells, many (bio)chemical reactions and processes necessary for functioning require environmental conditions that deviate from those in the cytosol. These processes are often performed in specialised compartments called organelles. In some organelles, such as the nucleus and mitochondria, compartmentalisation is achieved by membrane encapsulation. Alternatively, cells create microenvironments by inducing liquid-liquid phase separation resulting in the formation of membraneless organelles (MLOs). For several MLOs, the presence of RNA and specific intrinsically disordered RNA-binding proteins (RBPs) with intrinsically disordered regions has been reported to drive phase separation and the formation of MLOs. [1-6] Both RNA and RBPs are natural polyelectrolytes; polymeric macromolecules consisting of charged monomeric subunits. RNA is a strong polyanion, while RBPs are typically weak polycations. [7] For these MLOs, phase separation is driven by complex coacervation. [8-10]

Although the exact function is not known for all MLOs, specific biological functions typically require the controlled accumulation and release of (bio)molecules. [3,11-14] Additionally, MLOs need to partition specific compounds with a high degree of specificity as the cytosol contains a large variety of different compounds, many of which share structural and physicochemical similarities. MLO misfunction may lead to undesired biological consequences. [15] For example, the hyperphosphorylation of tau observed in several neurodegenerative diseases has been reported to drive liquid-liquid phase separation by coacervation. These tau droplets can serve as an intermediate toward the formation of amyloid deposits of tau found in neurodegenerative diseases. [16]

Since the ability to specifically and dynamically accumulate and release compounds is an emergent property of MLOs, it may also be possible to induce this behaviour in alternative systems that phase-separate *via* polyelectrolyte complexation. Oppositely charged polyelectrolytes can phase-separate into polyelectrolyte complexes (PECs) in aqueous solutions. The properties of PECs consisting of synthetic polyelectrolytes resemble those of MLOs. Several studies have reported that proteins can accumulate in PECs. [17-23] However, it is unclear if more complex behaviour such as the selective accumulation of compounds also emerges in PECs.

In this study, we investigated the ability of PECs composed of the weak polyelectrolytes poly(acrylic acid) (PAA) and poly(allylamine hydrochloride) (PAH) to dynamically discriminate between two oppositely charged protein species; lysozyme and succinylated lysozyme. Previous research has focused on two-component systems containing a protein and an (oppositely charged) polyelectrolyte. [24-29] Such systems have been shown to be able to separate proteins by selective interaction with a polyelectrolyte. [29-32] In these works, a specific protein

in a mixture has a higher affinity to the added polyelectrolyte, allowing the specific protein to complexate with the polyelectrolyte into a coacervate, leaving the other proteins in solution. In our system, the polyelectrolyte complex is formed by two oppositely charged polyelectrolytes, which both interact with the protein, resulting in a three-component system. This allows us to change the ratio between the polyelectrolytes and thus gives us an additional parameter by which we can tune partitioning of proteins into the PECs.

Lysozyme is a common antimicrobial enzyme that has been reported to partition in a PEC system. [17] Succinylated lysozyme is chemically modified to hold an equal but opposite charge at physiological pH with a very similar structure [33] to native lysozyme.

PAH and PAA are commonly used polyelectrolytes with known phase behaviour. PECs of these polyelectrolytes have been observed previously to enrich proteins. [21,34] PEC model systems are less complex compared to MLOs and may help provide a better physicochemical understanding of how complex coacervation contributes to intracellular organisation. In this study, we find that the partitioning of both lysozyme and succinylated lysozyme strongly depends on the PEC composition with maximal protein partitioning into PECs observed at distinct but different charge ratios. At the charge ratio where maximal partitioning is observed, the partitioning coefficient remains constant for a range of protein concentrations indicating that the PECs behave as a solvent for the protein. Sharp transitions were observed between complete and no protein partitioning, both as functions of the PEC composition and solution pH. We demonstrate that the sharp transitions and difference in PEC composition at which maximal partitioning is observed can be exploited to separate structurally similar proteins of opposite charge from a mixture. We suggest that the mechanism responsible for the composition- and pH-dependent partitioning behaviour may be exploited by MLOs.

Materials and Methods

Materials

Commercially available materials used were poly-(acrylic acid) (PAA) (Polysciences, Cat# 06567, MW = ±6000), poly(allylamine hydrochloride) (PAH) (Sigma-Aldrich, 283215, MW = ±17,500 Da), and lysozyme (Sigma-Aldrich, L6876). Succinylated lysozyme was made as previously described. [33] Stock solutions were adjusted to pH 7 - 7.4 with HCl (Merck, 1.00317.1000) or NaOH (Merck, 1.06462.1000). Protein concentrations were determined using UV-vis at

281.5 nm on a Shimadzu UV-2401PC spectrophotometer, using a molecular extinction coefficient of 2.635 $\frac{g}{L*cm}$ for both proteins. [35]

Charge Concentration and Ratio

To determine the charge ratio, both polyelectrolytes were assumed to be fully charged at pH 7. Under this assumption, the charge of any amount of polyelectrolyte is a function of the molecular weight of the composite monomers. Lysozyme and succinylated lysozyme have charges of +7 and -7 at pH 7 - 7.4, respectively. [17,33,36,37] The charge ratio F^- was defined as:

$$F^- = \frac{[n^-]}{[n^-] + [n^+]} \quad (1)$$

where $[n^-]$ and $[n^+]$ are the negative (PAA) and positive (PAH) charge concentrations, respectively. [17,28,38] Different ratios of polyelectrolyte are mixed to result in different F^- charge ratios. The number of charges per polyelectrolyte molecule is a function of monomer weight and remained constant. To change F^-, the concentration of PAA was kept constant while the concentration of PAH was varied. Variation in the order of addition of the polyelectrolytes did not give different results. Lysozyme partitioning into PECs was evaluated for a range of polyelectrolyte and protein concentrations (Supplementary Figure S3.2). From these experiments, we decided to continue experimentation with concentrations of 1 g/L PAA and 1 g/L protein.

The optimal charge ratio F_{opt}^- was defined as the F^- corresponding to the lowest concentration of protein in the supernatant.

Protein Supernatant Measurements

Compounds are mixed as follows: first, mixtures of the like-charged molecule were prepared, and then these mixtures were combined, thoroughly vortexed, and left to equilibrate for 2 days. Protein concentration was set at 0.8 - 1 g/L unless otherwise specified. Samples were then centrifuged at 12,500 g for 30 min. Protein concentration in the supernatant was then determined by measuring the absorbance spectra of appropriately diluted supernatant on a Shimadzu UV-2401PC spectrophotometer as previously described. If supernatant samples showed an absorbance of over 0.01 AU at 400 nm, this was taken as indicative of the presence of dissolved complexes and the sample was discarded as the presence of dissolved

complexes interferes with the protein concentration determination. Protein concentration in the supernatant sample was compared to a control containing only protein, similar to other studies. [19] The presence of PAH or PAA had a negligible influence on the protein concentration measurements (Supplementary Figure S3.1).

For experiments investigating the supernatant protein as a function of pH, a pH-sensitive electrode (Mettler Toledo, InLab Flex-Micro) was used. Diluted (10 mM) HCl and NaOH were used to adjust the pH to the desired values.

Determination of Partition Coefficient and Partition Free Energy

To determine the partition coefficient and free energies, the supernatant protein concentration was measured as described previously. Additionally, the complex mass was calculated by measuring empty sample tubes and sample tubes with the dilute supernatant phase removed. As an approximation, the PEC density was taken as equal to that of water. From this data, the protein concentration in the complex was calculated, and the partition coefficient and partition free energies for the systems when equilibrated were calculated *via*:

$$K_{partition} = \frac{[protein]_{complex}}{[protein]_{supernatant}} \quad (2)$$

$$\Delta G_{partition} = -RT \ln\left(K_{partition}\right) \quad (3)$$

Protein Release from PEC

To evaluate whether protein partitioning was reversible, the ability of the PECs to release proteins was investigated using a pH change. First, proteins were partitioned at their optimal charge ratio $F_{opt}^{-} = 0.65$ for lysozyme or $F_{opt}^{-} = 0.55$ for succinylated lysozyme. The supernatant protein concentration was then measured as previously described, and 1 µL of 1 M HCl was added (resulting in a measured pH of approximately 4) to lower the pH. After 2 more days to equilibrate, supernatant protein concentration was measured again. Supernatant protein concentrations were compared to control samples not containing polyelectrolytes.

Protein Analysis on Polyacrylamide Gel

Polyacrylamide gel electrophoresis was used to qualitatively distinguish between lysozyme and succinylated lysozyme. For the different steps (A-D) of the protocol

shown in Figure 3.3A, supernatant samples were frozen at -80 °C until evaluation. A polyacrylamide gel solution consisting of 65 % 0.3 M tris(hydroxymethyl)aminomethane (Tris) (Merck, 1.08382.0500) adjusted to pH 8.5, 10 % acrylamide (Merck, 1.00639.1000), 0.1 % ammonium persulfate (Bio-Rad, 1610700), and 0.1 % tetramethylethylenediamine (Sigma-Aldrich, T7024) in MilliQ water was prepared. A comb was inserted approximately halfway the gel to create sample slots. The solution was left to polymerise for 45 minutes under a layer of isopropanol (Merck, 1.09634.1000). Afterward, the isopropanol was decanted, and leftovers were removed by rinsing the gel with demineralised water.

The undiluted supernatant was thawed and mixed 1:1 with sample-buffer consisting of 0.12 M Tris, 20 % glycerol (Merck, 356350), and 0.02 % bromophenol blue (Bio-Rad, 161-0404). Of the sample/sample-buffer mixture, 30 µL was transferred to the individual sample slots on the gel. The electrophoresis was done at 90 V for 3 h in running buffer consisting of 26 mM Tris and 192 mM glycine (Sigma, G8898) in MilliQ water.

After electrophoresis, the gel was fixed for 1 hour in a 30 % methanol (ATLAS & ASSINK CHEMIE, 0360.01.210.5) and 10 % acetic acid (Merck, 1.00063.1000) solution and then washed with MilliQ water for 30 minutes and 1 hour. The gel was left to stain in Imperial Protein Stain (Thermo Scientific, Prod# 24615) overnight before destaining with MilliQ water twice for 1 hour. The gel was imaged with a ProteinSimple Fluorchem M, and ImageJ was used to evenly remove the background intensity from the images.

Results

Protein Partitioning Depends on PEC Composition

Intracellular membraneless organelles are able to partition proteins from the cytosol. [12] Polyelectrolyte complexes have been reported to do the same. [17-21] We previously reported that lysozyme enrichment in PDMAEMA/PAA PECs is a function of the composition of the PEC F^- (equation 1), with maximal partitioning into the PEC at $F^- = \sim 0.63$. [17] To investigate whether enrichment in PECs depends on the protein properties such as the charge of the protein, we investigated the accumulation of lysozyme and chemically modified succinylated lysozyme as functions of F^-. Both proteins are structurally nearly identical but carry a net opposite charge at neutral pH. [33] To investigate the enrichment of both proteins in PAH/PAA PECs, F^- was varied and the amount of protein in the supernatant was measured. In Figure 3.1A, we show images of the PEC-containing samples after centrifugation within sample tubes. The polyelectrolytes have formed a viscoelastic dense white

solid-like precipitate. In a total volume of 250 µL, the PEC volume makes up around 5 µL (2 %) with the remaining volume consisting of the dilute supernatant aqueous phase.

Figure 3.1B shows distinct partitioning profiles for lysozyme and succinylated lysozyme between the PEC and dilute supernatant phase. Both proteins show a minimum in the supernatant protein concentration as a function of F^-. At this minimum, the protein has maximally accumulated in the PEC. For both proteins, we also observe an F^- region where no partitioning takes place and nearly all protein is found in the supernatant.

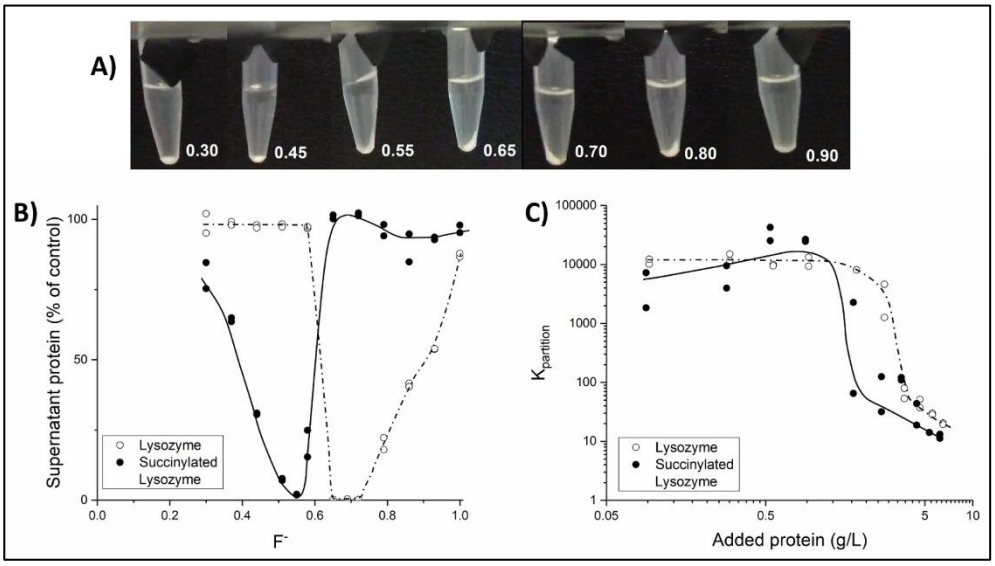


***Figure 3.1**. Partitioning of lysozyme (○) and succinylated lysozyme (●) in PAH/PAA PECs. Individual measurements are shown as dots, the lines are drawn to guide the eye. (A) Images of samples after centrifugation. The numbers in the images corresponding to the F^- values at which the samples were prepared, indicated by the white numbers. (B) Protein in the supernatant as a function of F^- at a protein concentration of 0.8 - 1 g/L. Protein concentration in the supernatant is expressed as a percentage of the control system without polyelectrolytes. (C) Partition coefficient of the proteins into the PECs at their F_{opt}^- as a function of added protein.*

Interestingly, the partitioning of lysozyme and succinylated lysozyme follows a mirrored pattern. We defined the optimal partitioning charge ratio F_{opt}^- as the charge ratio with maximum protein partitioning into the PEC. F_{opt}^- was determined to be $F_{opt}^- = F^- = 0.65$ for lysozyme and $F_{opt}^- = F^- = 0.55$ for succinylated lysozyme. Note that the optimal partitioning ratio F_{opt}^- for neither lysozyme nor succinylated

lysozyme is found at the (calculated) equal net charge of $F^- = 0.50$. The deviation of F_{opt}^- from $F^- = 0.50$ is not explained by the additional charges brought in by the proteins, which, when included, would shift the F_{opt}^- of lysozyme to 0.63 and not affect the F_{opt}^- of succinylated lysozyme. If the partitioning was a solely charge-driven process, we would expect maximum protein partitioning at $F^- = 0.50$. The fact that F_{opt}^- of both proteins deviates from 0.50 indicates that, regardless of the charge of the protein, both polyanions and polycations are required for proteins to accumulate in PECs. This may indicate that the selective partitioning of proteins into PECs is an emergent property of PECs. The necessity for an excess of positive or negative charges compared to positive charges (i.e., F_{opt}^- not equal to 0.5) has been observed previously for protein-polyelectrolyte systems, [28,38] although no clear mechanism has been established. Charge patchiness of the protein and charge regulation phenomena have been suggested as possible reasons. [22]

If the protein enrichment in PECs was solely governed by charge-charge interactions, one would expect the partitioning of lysozyme in PECs to increase with higher values of F^-. However, we observe that the supernatant lysozyme increases at F^- values higher than F_{opt}^-. The total PEC mass decreases at high F^-, as PAA has less PAH available to form PECs. At high F^-, it is likely that smaller soluble PAA-lysozyme complexes form instead. At low F^-, the same happens for soluble succinylated lysozyme-PAH complexes.

In a previous study, we have enriched lysozyme in a PDMAEMA/PAA complex coacervate system and observed a 90-95 % decrease of the protein in the supernatant phase and concomitant accumulation in the PEC phase. [17] For the PAH/PAA system investigated here, we report a decrease of 99.8 % of lysozyme in the supernatant at a comparable F^- (0.65 vs 0.63). Interestingly, Zhao and Zacharia used a similar PAH/PAA system to partition bovine serum albumin (BSA) but only saw a decrease of 50 % of the supernatant protein concentration. [21] Our experimental findings and the literature combined suggests that the partitioning behaviour of proteins in polyelectrolyte complexes is likely dependent on the structural and physicochemical properties of the polyelectrolytes and the partitioned protein. Future research in which multiple polyelectrolyte and protein systems with distinctly different properties are evaluated is necessary to elucidate the exact nature of the responsible interactions and mechanisms.

Protein Partitioning Coefficient Are Protein Concentration-Dependent

The PAH/PAA PECs studied here form a separate aqueous phase in which proteins can be localised. The partitioning between the dilute phase and the PEC phase can be quantified by the partitioning coefficient $K_{partition}$ (Equation 2), which we show as a function of the protein concentration ($c_{protein}$) in Figure 3.1C. In this figure, two

regimes of $K_{partition}$ as a function of $c_{protein}$ are visible. For low $c_{protein}$ up to 2 - 3 g/L, $K_{partition}$ > 1000 was found. At higher $c_{protein}$, the $K_{partition}$ decreases presumably because the PEC becomes saturated with proteins. In this regard, PAH/PAA PECs behave as normal solvents despite being in a solid-like phase.

The $K_{partition}$ values for (succinylated) lysozyme in PEC/water systems are within the range of reported $K_{partition}$ values for small molecules such as heptane in octanol/water systems. [39-41] Comparable or lower $K_{partition}$ values are reported for proteins in polypeptide coacervates [18,19,22] or in other synthetic polyelectrolyte systems. [23] Interestingly, BSA completely partitioned into polypeptide coacervates, [19] whereas only half of BSA was partitioned in PAH/PAA PECs. [21] In one study where multiple proteins were evaluated in the polypeptide coacervate system, lysozyme was found to have a noticeable higher maximum $K_{partition}$ (~1000) compared to other proteins, [22] although this $K_{partition}$ was still lower than that for lysozyme in the PAH/PAA PECs. It is important to note that different quantities of PECs and protein concentrations can give an inaccurate partition coefficient if the experimental conditions are not below that of the saturation of the PEC.

Like polyelectrolytes, the intrinsically disordered regions of some proteins have been shown to undergo liquid-liquid phase separation. [42,43] Schuster *et al*. prepared model MLOs from such proteins and investigated the partitioning of fluorescent proteins into the protein-rich phase. In these phases, partition coefficients up to 27 were found, depending on the type of fluorescent protein and any additional protein modification. [20] The differences in partitioning of proteins between the dilute and coacervate phases of different polyelectrolytes suggest that the exact partitioning properties of systems depend on the polyelectrolyte and protein species.

The protein partitioning between the PAH/PAA PECs and the dilute supernatant is a passive equilibration process; no active energy-consuming biological mechanism is required to enrich the proteins in the PECs. As such, the accumulation of protein in the PECs is associated with a gain in free energy. At their F_{opt}^-, we report a partition free energy of -20.2 ± 0.3 kJ/mol (mean ± standard deviation, n = 4) for lysozyme and -19.5 ± 0.5 kJ/mol (n = 5) for succinylated lysozyme at a protein concentration of 0.8-1 g/L (eq 3). In comparison, for a system of phase-separated complexes consisting of disordered regions of proteins, partition free energies of -8 kJ/mol for single-stranded DNA and 2 kJ/mol for double-stranded DNA were reported. [44]

Protein Partitioning is pH-Dependent

In Figure 3.1B, we modulated the partitioning of lysozyme and succinylated lysozyme in the PECs by changing the composition in terms of F^-. An alternative method to effectively alter F^- is by changing the pH of the solution. At low pH values,

polyanionic PAA will become less negatively charged while the charge of the polycationic PAH remains unaffected. At high pH values, PAA charge remains unaffected while PAH becomes less positively charged. As a consequence, a pH decrease increases the total positive charge in the complex and is equivalent to lowering the F^- *via* compositional changes and vice versa. Additionally, lysozyme remains positively charged at pH < 10 (pI = 11.35), while succinylated lysozyme undergoes a net charge shift in the evaluated pH range (pI = 4.5) from negative to positive. [37] Lysozyme and succinylated lysozyme remain stable at room temperature for pH values as low as 3 and 3.5, respectively. [33,45] Earlier studies also suggest that proteins recovered from PECs remain functional. [46]

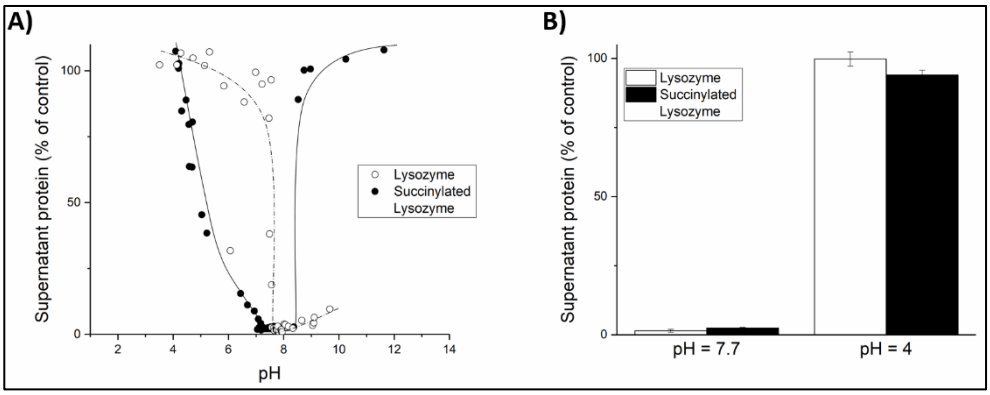


***Figure 3.2**. Effect of pH on the partitioning of lysozyme (○) and succinylated lysozyme (●) into PECs. (A) PECs are prepared at $F^- = 0.65$ or 0.55 for lysozyme or succinylated lysozyme, respectively, while the pH of the system is varied. Individual measurements are represented by dots. Lines are drawn to guide the eye. (B) Release of proteins from the PECs is done by lowering the pH from ~ 7.7 to 4. Data is shown as averages with error bars representing standard deviation, n = 3.*

To evaluate the effect of pH on the partitioning of the proteins, PAH/PAA PECs were prepared at $F^- = 0.65$ and 0.55 for lysozyme and succinylated lysozyme, respectively, at a pH between 4 and 12. In Figure 3.2A, we show that the shape of the partitioning curve of the proteins as a function of the pH is similar to the F^- dependence shown in Figure 3.1B: for both proteins, a region in which none to very little partitioning and a region of maximum partitioning into the PECs is observed. In the presence of lysozyme, at pH > 10, the presence of soluble complexes resulted in light scattering, which obscured the measurements and the protein concentration could therefore not be accurately determined.

The pH-dependent partitioning of proteins in PECs and the sharp transitions in partitioning as a function of pH and composition offer an interesting strategy to recover proteins from the PECs. This approach was previously shown to work for BSA in polypeptide complexes. [19] To investigate whether changes in pH also shift the equilibrium distribution and result in the release of lysozyme and succinylated lysozyme from PECs, the systems were first equilibrated at F_{opt}^-. Subsequently, the pH was lowered from ~ 7.7 to 4, where according to Figure 3.2A, the proteins are found in the dilute supernatant phase. Indeed, we show in Figure 3.2B that lowering of the pH recovers all lysozyme and nearly all succinylated lysozyme from the PAH/PAA PECs.

PECs are also known to be sensitive to ionic strength. An increase in salt concentration is known to disrupt polyelectrolyte complexes and recover partitioned protein. [47] Protein release using changes in ionic strength was, however, found to be less efficient than lowering the pH (Supplementary Figure S3.3). Additionally, the disruption of the complex *via* salt addition leads to soluble complexes, which interfered with the spectroscopic determination of the protein concentration.

Protein Separation Using PECs

The ability to selectively partition proteins based on F^- composition (Figure 3.1B) and release proteins by adjusting the pH (Figure 3.2B) opens up the possibility to separate lysozyme or succinylated lysozyme from a mixture of the two in PECs. Figure 3.1B shows that at the F^- for which maximal partitioning into PECs is observed for one protein, the other protein remains in the dilute supernatant phase. We therefore hypothesised that if we start with a mixture of lysozyme and succinylated lysozyme and add polyelectrolytes at F_{opt}^- for one of the proteins, it will selectively partition that protein, while the other protein remains in the supernatant.

Following this strategy, we separated a 1:1 mixture of lysozyme and succinylated lysozyme using PAH/PAA PECs *via* the procedure illustrated in Figure 3.3A. After each step, the total protein concentrations and compositions of the dilute phase were quantitatively and qualitatively investigated by UV-vis (Figure 3.3C) and gel electrophoreses (Figure 3.3B), respectively. The gel electrophoresis experiments (Figure 3.3B) verified that for each measurement, only one of the proteins was dominantly present in the supernatant, and thus, only one of the proteins was present in the PEC. Quantification by UV-vis spectroscopy (Figure 3.3C) shows that the total relative concentration of supernatant protein is either approximately half of the total protein concentration or nearly zero. Taken together, the results show that PAH/PAA PECs can be used to selectively separate either lysozyme or succinylated lysozyme from a mixture of the two proteins.

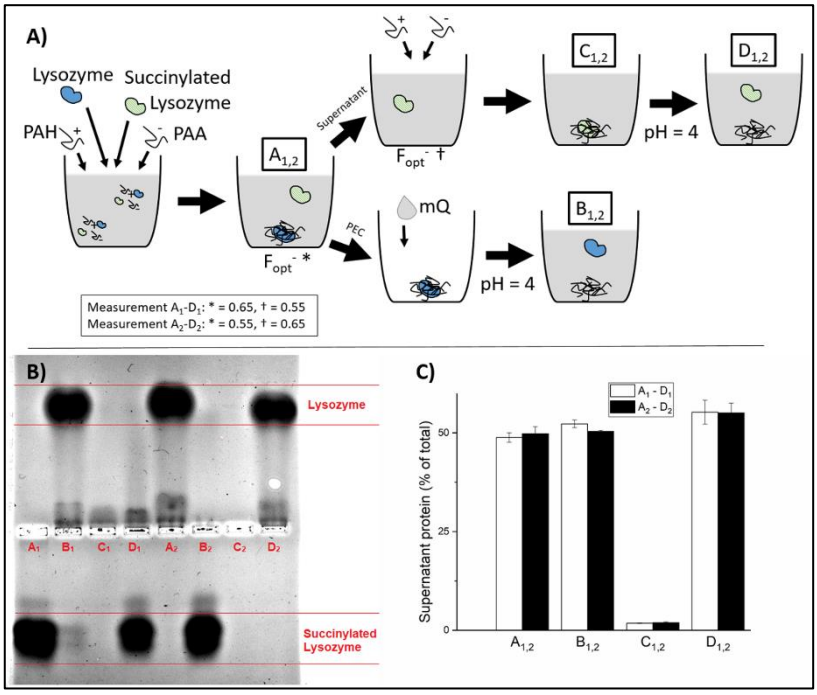


Figure 3.3*. Separation of lysozyme and succinylated lysozyme from a protein mixture containing 1 g/L of both lysozyme and succinylated lysozyme. (A) Schematic representation of the experimental procedure. The protein species were qualitatively and quantitatively measured at the points indicated as A1,2 - D1,2. (B) Qualitative analysis of the protein species present in the supernatant using SDS-PAGE. (C) Quantitative UV-vis analysis to determine total supernatant protein concentrations. Data is shown as averages with error bars representing standard deviation, n = 3.*

Discussion

Previously, single polyelectrolytes have been used to selectively form complexes with proteins from mixtures, resulting in the polyelectrolyte-protein complex forming a separate phase. [29-32] We have demonstrated that PECs consisting of oppositely charged polyelectrolytes PAH and PAA can also separate proteins based on charge. The protein partitioned by the PAH/PAA PEC was found to be dependent on the PEC composition F^-, which is a tunable factor. Depending on F^-, PAH/PAA PECs can act as selective solvents with high partitioning coefficients for either lysozyme or succinylated lysozyme. From Figure 3.1B and Figure 3.2A, we observe that PAH/PAA PECs have very steep transitions between no partitioning and full

partitioning of proteins with very high partition coefficients as a functions of PEC composition and solution pH. The exact region of the transitions depended on the charge of the protein, and we hypothesise that this region is also dependent on other physicochemical properties of the protein and the constituent polyelectrolytes. We suggest that membraneless organelles in biological systems may have similar steep transitions that can be manipulated by the cell *via* composition changes or variations in pH. Interestingly, we observed for lysozyme and succinylated lysozyme that maximum partitioning did not occur at $F^- = 0.5$.

Cells might be able to alter their MLO composition by manipulating the RNA or RNA-binding protein concentrations by production, recruitment from other cellular components, or degradation mechanisms. An early model suggests that cells could make such adjustments. [48] Protein modifications *via* phosphorylation, SUMOylating, and methylation are also known to influence phase separation, providing an additional mechanism for the cells to control MLO solvent properties. [44,48-50] In line with this, it has recently been shown that cells are able to regulate the dissolution and formation of specific MLOs during and after mitosis by regulating the presence of certain kinase enzymes. [51] Additionally, changes in the primary structure of RBPs may have drastic effects on complex coacervation and solvent properties as they affect the RBP's charge and isoelectric point. Minor protein modifications may thus result in a steep transition between maximum and no partitioning of proteins. One study where artificial membraneless compartments consisting of customised RNA and synthetic polycations were made showed that enzymes can indeed be partitioned and retain a level of activity in at least partially synthetic complexes. [52]

The cytosolic pH is generally very tightly regulated to a slightly alkaline (7 - 7.4) value. [53] However, Figure 3.2A shows that for PECs, only very slight variations in pH are required to make proteins switch from full to no partitioning in PAH/PAA PECs. Similar steep transitions might be found in MLOs, allowing changes in intracellular pH to influence protein partitioning behaviour. Variation in intracellular pH has been reported to vary depending on the cell's phase in the cell cycle and exact intracellular location. [53,54] Most notably, a consistent drop in cytosolic pH from physiological conditions to 5.5 has been observed for proliferating yeast. [55] Variations in both more alkaline and acidic directions occur at different phases during mitosis. [56,57] Interestingly, several MLOs have been observed to disappear during mitosis and reappear afterward, while the centrosome and spindle assemblies are MLOs that play key roles in cell division. [51,58] Additionally, pH gradients are present within migrating cells when different functionalities are required within the cell depending on the distance from the migrating leading edge. [59]

Beyond gaining insight into the discrimination of coacervate phases between proteins based on charge and into mechanisms by which MLOs can regulate protein

partitioning in the cell, we suggest possible applications. For these applications, it is important to realise that PECs behave as solvents. Understanding the factors that influence the partitioning behaviour of these tunable aqueous solvents may open new directions for the extraction and concentration of molecules from wastewater streams. Partitioning for various small molecules from solution has been reported. [60-62] The same principle is worth investigating for other compounds.

Another field where PECs might be promising is controlled drug delivery, [27,63,64] especially with the possibility of a triggered release system. [65] Early-stage experimentation has suggested that PECs can show reduced cytotoxicity compared to free drug [66] and can have a tunable drug release rate based on environmental pH. [67]

Conclusion

Membraneless organelles have the ability to partition intracellular proteins and act as an additional organizing mechanism for the regulation of intracellular processes. [3,11-14] The ability to selectively partition the desired protein(s) while excluding other cytosolic compounds is essential for MLO functioning. Polyelectrolyte complexes have been shown previously to be able to enrich a variety of proteins from solution into PECs, [17-21] but the ability to selectively partition proteins starting from a mixture using tunable PECs consisting of oppositely charged polyelectrolytes had not yet been shown. In this study, we showed that a high degree of selectivity is possible based on protein net charge, even when the proteins are otherwise structurally very similar.

Finally, beyond insight into MLOs, intracellular regulation, and potential new avenues to explore diseases, more direct applications of the ability of PECs to selectively and tunably partition proteins, biomolecules, or other organic compounds can be found in waste- or surface water treatment and in drug delivery systems.

Acknowledgments

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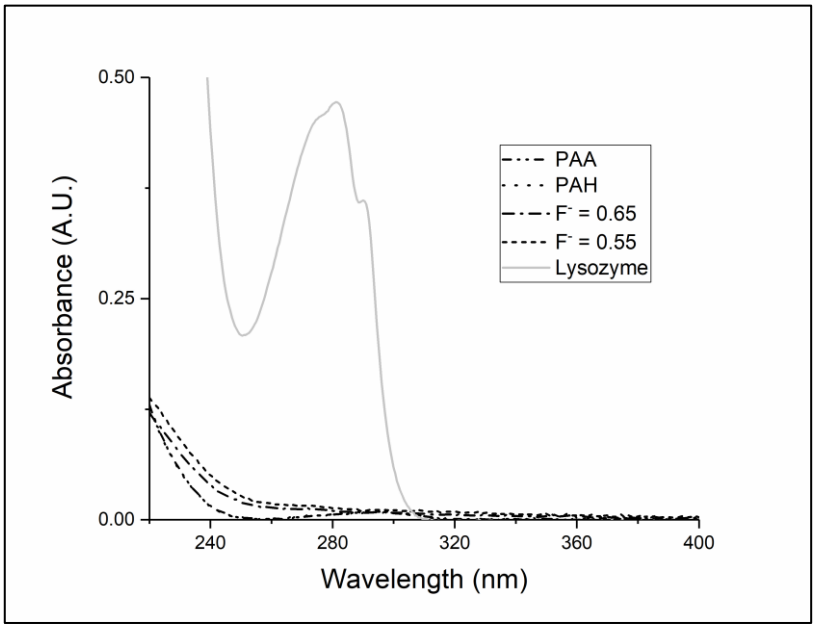
Supplementary Information

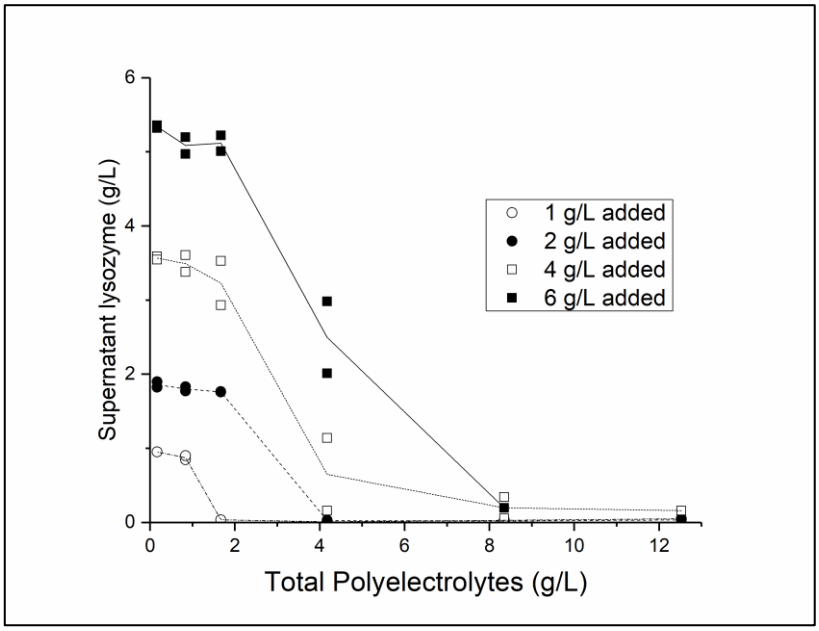
The Influence of PAA and PAH on absorbance spectra



Supplementary Figure S3.1. *The absorbance spectrum of PAA, PAH, and the supernatant of PAH/PAA PECs at specific F^- compared to the absorbance spectrum of 1 g/L lysozyme. All samples are treated identically prior to measurements. Lysozyme concentration is determined at 281.5 nm.*

Absorbance measurement at approximately 280 nm is an established, quick and simple method to determine protein concentration given a solution that contains only proteins with a known extinction coefficient. Our system consists of lysozyme (1 g/L), PAH and PAA. In Supplementary Figure S3.1 we show that both PAA and PAH (in the highest concentrations used) individually do not significantly influence the protein concentration measurement of lysozyme at 281.5 nm. Additionally, the presence of PAH/PAA PECs without lysozyme at the F_{opt}^- for (succinylated) lysozyme does not contribute in a meaningful way to the absorbance profile.

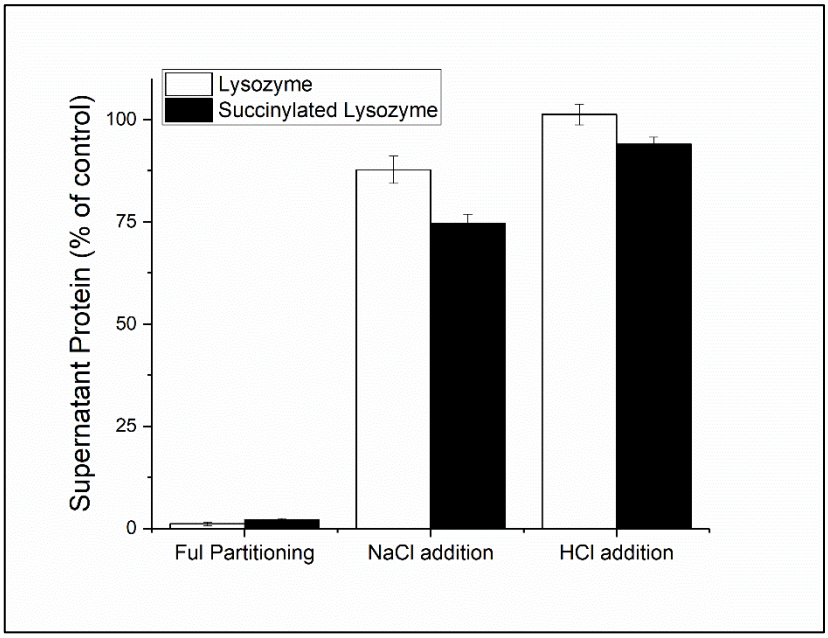
Effect of total polyelectrolyte- and protein concentration



***Supplementary Figure S3.2**. A comparison of the supernatant lysozyme as a function of total polyelectrolyte concentration and added lysozyme concentration. $F^- = 0.65$. Individual measurements are shown as dots, the lines connect averages.*

We varied the total amount of polyelectrolytes and lysozyme to identify suitable concentrations. The total amount of polyelectrolyte is based on a chosen concentration of PAA, where PAH is added to finalise into the F_{opt}^- for lysozyme ($F^- = 0.65$). The results are shown in Supplementary Figure S3.2. We observe that for all protein concentrations the PAH/PAA PECs have a capacity to strongly partition lysozyme into the PECs. However, if the total amount of polyelectrolytes is decreased below a certain number, protein concentration in the supernatant increases. The most suitable concentration was determined to be 1.67 g/L polyelectrolyte (corresponding with 1 g/L PAA for $F^- = 0.65$) and 1 g/L lysozyme. With higher concentrations of polyelectrolytes scattering was occasionally observed possibly due to slower kinetics of the system resulting in non-precipitated soluble complexes. When lower concentrations of polyelectrolytes were used, the PEC was saturated resulting in deviations from ideal solvent behaviour.

Release of Proteins from PECs *via* salt addition



Supplementary Figure S3.3. *A comparison of the effectiveness of both salt (0.5 M NaCl) and pH manipulation (using HCl to lower pH to 4) for releasing lysozyme (○) and succinylated lysozyme (●) from PAH/PAA PECs.*

In figure 2B we presented the ability to release (succinylated) lysozyme from PAH/PAA PECs by lowering the pH. In addition, we tested whether increasing the ionic strength of the solution *via* salt (NaCl) addition would disrupt the PECs and release the proteins (Supplementary Figure S3.3). Varying ionic strength *via* salt concentration is a commonly used technique for manipulating polyelectrolyte systems. Salt ions interact with the polyelectrolytes by screening polyelectrolyte charges and dissociating the PEC, resulting in the release of partitioned compounds back into the supernatant. [47]

We attempted to release (succinylated) lysozyme by replacing the dilute supernatant phase with 0.5 M NaCl. We observed that recovery of the proteins was possible, but only approximately 75 % compared to full recovery *via* addition of HCl. While it is possible to increase ionic strength further and eventually recover all protein by disrupting the complex completely, 0.5 M is already far in excess of physiological salt concentrations. For these reasons, we continued with the method of lowering pH instead of increasing salt concentration for further experiments.

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Chapter 4

Extraction of Lysozyme from Chicken Albumen using Polyelectrolyte Complexes

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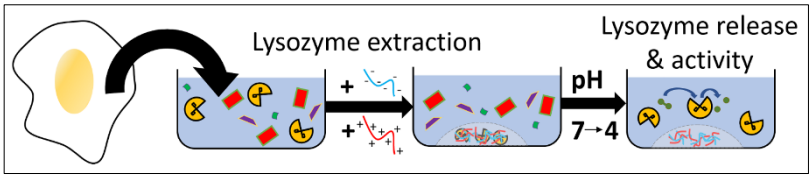
Abstract

Cells use droplet-like membraneless organelles to compartmentalise and selectively take-up molecules, such as proteins, from their internal environment. These membraneless organelles can be mimicked by polyelectrolyte complexes consisting of oppositely charged polyelectrolytes. Previous research has demonstrated that protein uptake strongly depends on the polyelectrolyte complex composition. This suggests that polyelectrolyte complexes can be used to selectively extract proteins from a multi-protein mixture. With this in mind, the partitioning of the protein lysozyme in four polyelectrolyte complex systems consisting of different weak and strong polyelectrolyte combinations is investigated. All systems show similar trends in lysozyme partitioning as a function of the complex composition. The release of lysozyme from complexes at their optimal lysozyme uptake composition is investigated by increasing the salt concentration to 500 mM NaCl or lowering the pH from 7 to 4. Complexes of poly(allylamine hydrochloride) and poly(acrylic acid) had the best uptake and release properties. These were used for selective extraction of lysozyme from a hen-egg white protein matrix. The (back)-extracted lysozyme retained its enzymatic activity, showing the capability of polyelectrolyte complexes to function as extraction media for proteins.



Introduction

Cellular processes are extremely efficient and the most complex reactions occur in the blink of an eye. This efficiency requires extremely good spatio-temporal organisation of molecules and compartmentalisation of components is a way to achieve this. Traditionally, compartmentalisation has been assumed to occur in organelles surrounded by lipid membranes. However, recently membraneless organelles (MLOs) have been suggested as spatio-temporal organisers. MLOs are dynamic droplet-like condensates formed by aqueous liquid-liquid phase separation. Several kinds of MLOs have been reported, such as stress granules, nucleoli, cajal bodies, paraspeckles, and more. [1–8] The exact role and mechanism of many MLOs is still unknown, but it is expected that the separate chemical environment provided by these condensates allows for specific processes and reactions to occur. [2,3,6,9]

Cellular MLOs typically consist of charged intrinsically disordered proteins (IDPs) and oppositely charged polynucleotides. [2,6,7] The careful interplay of electrostatic interactions and other interactions such as cation-pi interactions, hydrogen bond formation, and hydrophobic interactions results in the formation of droplet-like condensates that are explicitly distinct from the surrounding fluid. A simple way to model these cellular condensates is by making use of oppositely charged polyelectrolytes. When aqueous solutions of oppositely charged polyelectrolytes are mixed, condensates will form that are distinctively different from the surrounding fluid. These polyelectrolyte condensates are called polyelectrolyte complexes (PECs). Factors that influence the phase behaviour of PECs include polycation to polyanion ratio, ionic strength, polyelectrolyte chemical structure, and pH. Unsurprisingly, the phase behaviour of PECs and their response to changes in the local environment is similar to the reported phase behaviour of MLOs. [10–14]

Once the PECs have formed, the partitioning of additional compounds, such as proteins, between the PEC and the dilute supernatant phase can be studied. The partitioning between PEC and supernatant is analogous to the partitioning between MLOs and the cytosol. For PECs, it has been found that proteins can partition into the polyelectrolyte-rich phase. [15–21] The partitioning behaviour of proteins also depends on factors that influence the phase behaviour of PECs. [17] This multi-parameter dependence on the partitioning of (macro)molecules makes understanding the exact molecular details challenging. [17] However, if we understand and can control the partitioning of molecules with a similar selectivity and efficiency as MLOs, PECs can be used as aqueous extraction media. [17] These extraction media are expected to show a high uptake of a specific molecule from an aqueous solution and a controllable release suitable for the recovery of bio-active compounds.

In this study we investigate the partitioning of the protein lysozyme in different model PEC systems. Lysozyme is a naturally occurring antibacterial enzyme of

industrial importance and is used in the food- and pharmaceutical industries as an additive to increase shelf life. [22–27] It is a relatively small protein with a molecular weight (MW) of 14.3 kDa and an isoelectric point of 11.35, making it cationic (net charge +7) at neutral pH. Lysozyme is also part of the innate immune system and present in human tears, mother's milk, and saliva. [26,28,29] A potent source of lysozyme is chicken albumen (i.e. egg white) Approximately 10 % of albumen is protein, and of these proteins approximately 3 - 4 % is lysozyme. [28,29]

Four model PEC systems were used in this study consisting of combinations of the polycations poly(allylamine hydrochloride) (PAH) and poly(diallyldimethylammonium chloride) (PDADMAC), and polyanions poly(acrylic acid) (PAA) and poly(4-styrene sulfonate) (PSS) as shown in Figure 4.1. PAH and PAA are weak polyelectrolytes with pH-dependent charge. PDADMAC and PSS are strong polyelectrolytes that bear their charge regardless of pH. All polyelectrolytes have a MW approximately an order of magnitude larger than lysozyme to facilitate separation of the polyelectrolytes from lysozyme, when desired.

First, we show the formation of PECs by mixing two oppositely charged polyelectrolytes. The PECs form a macroscopic polyelectrolyte-rich solid-like phase distinct from the polyelectrolyte-poor aqueous supernatant phase. The partitioning of lysozyme between the PEC phase and the supernatant phase is then measured as a function of the PEC composition to determine the optimal condition for lysozyme take-up. After lysozyme partitioning at the optimal composition, lysozyme is back-extracted from the PECs by increasing the salt concentration or lowering the pH. The PEC system with the best (back-)extraction properties is then used to extract lysozyme from a chicken albumen solution. Finally, we show that the (back-)extracted lysozyme retains its enzymatic function. The results from this study show the potential of PECs as selective extraction media for proteins.

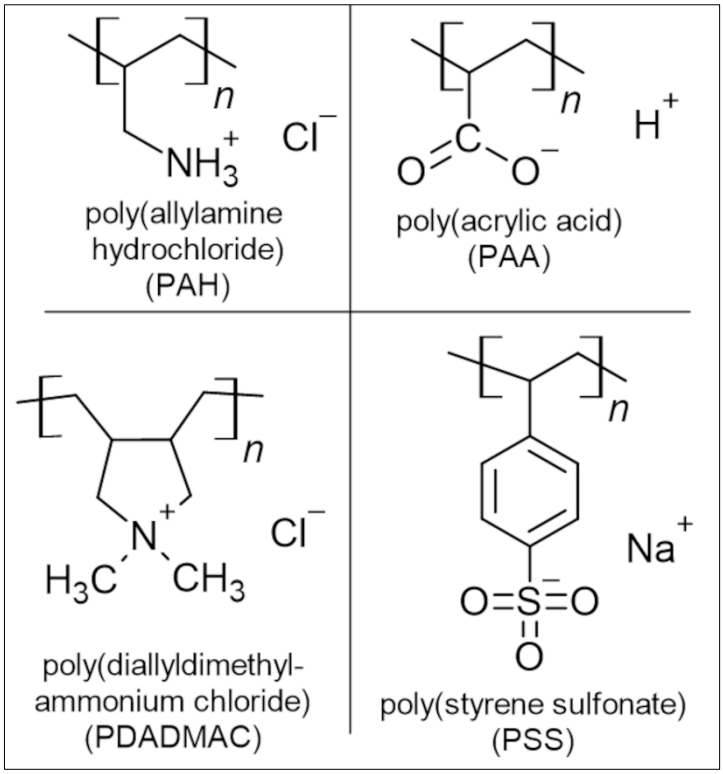


Figure 4.1. *Structure of the polyelectrolytes with their respective counterion used in this study. Top: weak polyelectrolytes where the charge depends on pH. Bottom: strong polyelectrolytes with charge independent of pH. Left: polycations. Right: polyanions.*

Materials and Methods

Materials

Poly(allylamine hydrochloride) (PAH) solution with a molecular weight (MW) of 150 kDa (monomer mass 94 Da) was purchased from Nittobo. Poly(acrylic acid) (PAA) acidic form solution (MW of 100 kDa, monomer mass 72 Da, product number 523925), Poly(diallyldimethylammonium chloride) (PDADMAC) solution (MW of 200-350 kDa, monomer mass 162 Da, product number 409022), poly(styrene sulfonate) (PSS) sodium salt solution (MW 200 kDa, monomer mass 206 Da, product number 561967), hen-egg lysozyme (MW of 14.3 kDa, isoelectric point of

11.35, activity of 40,000 U/mg, product number L6876), HCl solution, NaOH, NaCl, tris(hydroxymethyl)aminomethane (Tris), tetramethylethylenediamine (TEMED), sodium dodecyl sulfate (SDS), glycerol, glycine, methanol, acetic acid, isopropanol, and lyophilised *Micrococcus lysodeikticus* bacteria were purchased from Sigma-Aldrich/Merck. Ammonium persulfate (APS), Coomassie Brilliant Blue R-250, and bromophenol blue were purchased from Bio-Rad. PageRuler Plus prestained protein ladder was purchased from ThermoFischer Scientific. Lyophilised albumen powder was purchased from '*De Zuidmolen'* baking store. According to the manufacturer, albumen powder was made by freeze-drying the albumen directly after separation from the egg yolk. Unless otherwise specified, solutions were made with ultrapure water (mQ) filtered by an Advantage A10 water purification system (Millipore).

PEC Composition, Formation, and Lysozyme incorporation

Polyelectrolyte stock solutions were diluted from purchased solutions to working solutions (80 g/L). The pH of the working solutions was set to 7 (within the range of 6.8 to 7.2) with NaOH or HCl solutions. PECs are formed by mixing specific amounts of polycationic and polyanionic solutions. The composition, in term of the charge fraction, of the polyelectrolyte complex was quantified in F^-, which was defined as: [15–18]

$$F^- = \frac{[n^-]}{[n^-]+[n^+]} \qquad (1)$$

Where $[n^-]$ is the concentration of negative monomers and $[n^+]$ is the concentration of positive monomers upon mixing of polyelectrolyte solutions under the assumption that all monomers are fully charged. [15–17,33,59]

Unless otherwise specified, solutions were mixed so that the final total concentration of polyelectrolytes was 2 g/L and the concentration of lysozyme was 1 g/L. These concentrations were chosen based on earlier reports that for PAH/PAA PEC systems there would likely be a PEC composition F^- with complete lysozyme partitioning in the PEC. [16] Where possible, like-charged compounds were mixed prior to addition of an oppositely-charged compound and then thoroughly mixed. The total volume was set to 250 µl. Unless specified, no salt ions were added beyond those brought into the system as counterions to the polyelectrolytes and as a result of the setting of pH with NaOH and HCl.

Supernatant lysozyme concentration determination

After mixing, the samples were left to equilibrate for one day. Prior to measurement, samples were centrifuged at 12,400 g for 30 minutes. [15,16,20] The protein concentration of diluted supernatant was determined on a 2401PC spectrophotometer (Shimadzu) at 281.5 nm. The supernatant lysozyme sample concentration was expressed relative to a lysozyme control without polyelectrolytes. A lower amount of lysozyme in the supernatant corresponds with a larger amount of lysozyme in the PEC. Finally, the absorbance of a PEC control containing the same polyelectrolytes but not containing lysozyme was subtracted from the measured absorbance to compensate for absorbance caused by only the PECs or polyelectrolytes.

Lysozyme back-extraction from PECs

To determine the back-extraction of lysozyme from PECs as a result of added NaCl or HCl, PECs containing lysozyme were first formed at the F^- composition found to be optimal for lysozyme partitioning as previously described. The supernatant was then removed and replaced with mQ water containing either NaCl solution (0.5 M) or HCl solution (4 mM, corresponding to a pH of approximately 4 in the presence of the polyelectrolytes). After another day to equilibrate, the supernatant lysozyme concentration was determined as previously described.

Lysozyme extraction from lysozyme and back-extraction from PECs

Lyophilised chicken albumen powder was dissolved to prepare a stock solution (10/g), which was then diluted to 1 g/L for extraction. Extraction was done by adding PAH and PAA (in that order) at $F^- = 0.53$ at a total concentration of 4 g/L polyelectrolyte solution. After one day to equilibrate, PECs were centrifuged at 10,000 g for 30 minutes (Supplementary Figure S4.1). Supernatant samples were taken for evaluation of protein content via sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) after which the remaining supernatant was aspirated. New mQ (250 µl) was added as new supernatant with 4 mM HCl. After an additional day to equilibrate, supernatant samples were taken again for evaluation via SDS-PAGE and for evaluation of the enzymatic activity via an activity assay.

Protein analysis via SDS-PAGE

The protein composition of the albumen solution, supernatant during the extraction step, and supernatant after the back-extraction were determined via SDS-PAGE. Samples were stored at -20 ˚C before evaluation. Samples were thawed and mixed with an equal volume of loading buffer consisting of Tris (0.12 M), glycerol (20 %), SDS (4 %), and bromophenol blue (0.02 %) before electrophoresis on a polyacrylamide gel (15 %) in a Mini-PROTEAN vertical electrophoresis cell (Bio-Rad) for 30 minutes at 90 V, 30 minutes at 120 V, and 45 - 60 minutes at 150 V until the bromophenol blue indicator had left the gel. The gel was then transferred to and stained in a methanol (30 %), acetic acid (10 %), and Coomassie Brilliant Blue R-250 (0.05 %) solution for 1 hour. Destaining of the gel was done by submerging in flushes of methanol (30 %) and acetic acid (10 %) solution until background staining was removed. The gel was imaged with a Fluorchem M (ProteinSimple).

Lysozyme activity assay

Lysozyme has anti-bacterial properties by cleaving the cell walls of gram-positive bacteria. Enzymatic activity can be determined and quantified by adding lysozyme solution to a suspension of lyophilised *micrococcus lysodeikticus* bacteria (150 mg/L). The presence of bacteria results in a turbidity determined by optical absorbance at 450 nm, [60] which will decrease in the presence of active lysozyme or remain mostly constant in the absence of active lysozyme. The activity of lysozyme as determined *via* this protocol can be calculated by;

$$units\ ml^{-1} = \frac{A(T)-A(B)}{0.001*0.05} \qquad (2)$$

The amount of units/ml is derived from the decrease in absorbance at 450 nm (A(T)) relative to the decrease in absorbance at 450 nm of the bacteria suspension without the addition of active component (A(B)). The factor 0.001 is part of the unit definition, and the factor 0.05 is to translate the tested volume (50 µl) to units/ml. In this study, lyophilised bacteria suspension (2 ml) was used as the substrate, and solution containing active enzyme (back-extracted lysozyme solution) (50 µl) or purified commercially bought lysoyzme (7.5 mg/L, 50 µl) or additional substrate (50 µl) was added.

Results and Discussion

Previous studies have shown that lysozyme take-up by polyelectrolyte complexes can be very efficient and selective, that is; only lysozyme can be selectively extracted from an ideal mixture of two proteins by a PAH/PAA PEC system. [16] In this study we investigate the take-up and release of lysozyme by four different PEC systems enabling us to study the role of pH-dependent charge of the polyelectrolytes on the lysozyme partitioning. The goal is to identify the PEC system(s) that is/are most suitable for the extraction of lysozyme from a complex naturally occurring protein mixture. Here we use a weak and a strong polycation, PAH and PDADMAC, respectively, as well as a weak and a strong polyanion, PAA and PSS, respectively. These polyelectrolytes can be combined in four different ways allowing us to compare PECs consisting of weak/weak, strong/strong and two varieties of weak/strong polyelectrolytes. All polyelectrolytes have similar molecular weights (100 - 350 kDa) and their weights are an order of magnitude larger than the molecular weight of lysozyme.

We first investigated the partitioning of lysozyme as a function of the PEC composition quantified as the charge fraction (F^-). F^- is defined as the anionic fraction of polyelectrolyte charges (Equation 1) and it follows that at $F^- = 0$ only polycations are present, at $F^- = 1$ only polyanions are present, and $F^- = 0.5$ indicates charge stoichiometry. The PEC forms a distinct macroscopic solid-like phase in contrast to the aqueous supernatant phase (Supplementary Figure S4.1). The lysozyme concentration in the supernatant phase was measured using UV-vis spectrophotometry. The composition at which optimal partitioning occurs is the composition where the lysozyme concentration in the supernatant phase is the lowest.

The partitioning of lysozyme as a function of the composition for the four different systems can be found in Figure 4.2. For all PECs the general profile follows a similar shape. Starting at the cationic side ($F^- < F^-_{optimal}$) with a plateau region; at this composition range the lysozyme remains in the supernatant. At a certain composition a steep decrease is observed where a minimum amount of lysozyme is found in the supernatant. At this composition optimal lysozyme take-up occurs and we will refer to this composition at $F^-_{optimal}$. Finally, a more gradual increase in lysozyme concentration is observed at the anionic side ($F^- > F^-_{optimal}$) until lysozyme take-up no longer occurs. In all combinations there was an optimal PEC composition where nearly all lysozyme was partitioned in the PEC.

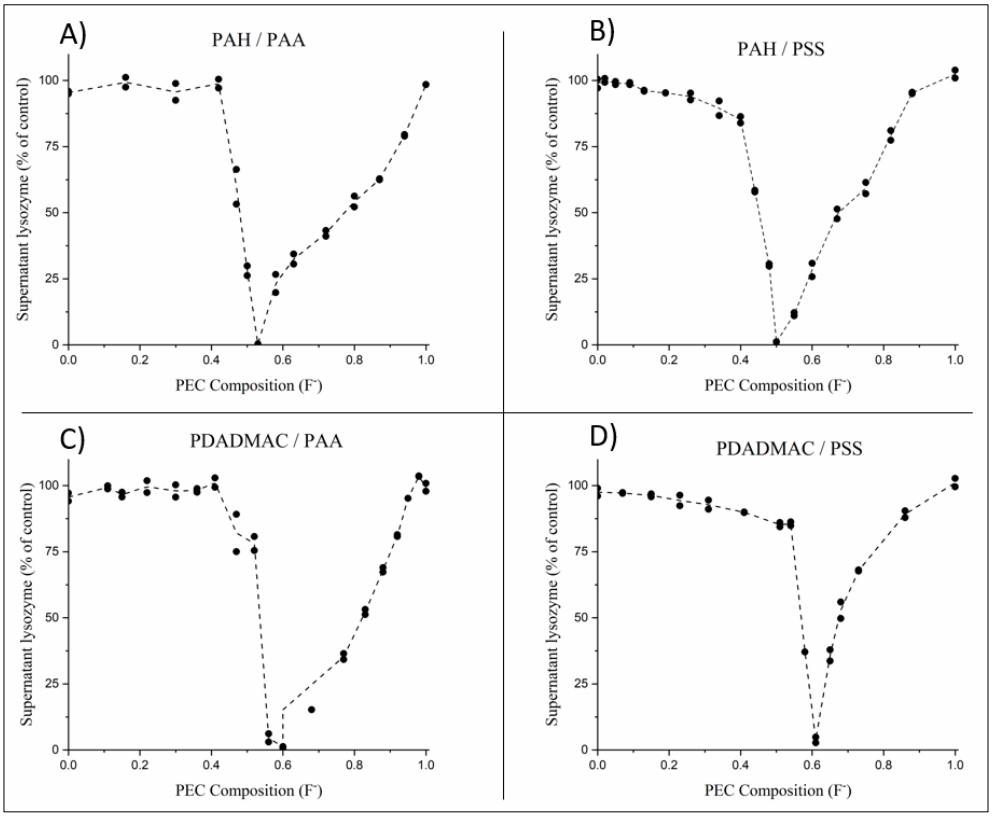


Figure 4.2. *The partitioning profiles of lysozyme in various PECs at pH 7. A) weak/weak PAH/PAA. B) weak/strong PAH/PSS. C) strong/weak PDADMAC/PAA. D) strong/strong PDADMAC/PSS. All show the supernatant lysozyme concentration as a function of different PEC compositions as expressed in F^-. A low supernatant lysozyme content corresponds to a high PEC lysozyme content and vice versa. Values represent individual measurements, lines connect averages of duplicate measurements.*

$F^-_{optimal}$ varied per system, from approximately charge stoichiometry for PAH/PAA ($F^- = 0.53$) and PAH/PSS ($F^- = 0.50$) to systems containing noticeably more negative charge for PDADMAC/PAA ($F^- = 0.60$) and PDADMAC/PSS ($F^- = 0.61$). Similar profiles have been reported before. [15,16] For all the evaluated PEC systems in Figure 4.2, the presence of both polycations and polyanions is necessary for partitioning of the lysozyme. At both $F^- = 0$ or 1, where the protein is only present with one polyelectrolyte, the solutions are optically clear indicating there are no soluble polyelectrolyte complexes present.

PEC systems containing PDADMAC consistently showed a $F^-_{optimal}$ at slightly anionic conditions ($F^- = 0.60$-0.61) compared to PAH systems at approximate charge

stoichiometry. This suggests that the partitioning is not solely a charge-driven process, in which case the expected $F^-_{optimal}$ would be found at charge stoichiometry for all PEC systems. [16] Including the charges of lysozyme does not significantly change the F^-. [16] The fact that no partitioning occurs at either $F^- = 0$ or 1 but instead is optimal at a very specific F^- suggests that specific interactions between the polyelectrolytes are an important step for the partitioning of lysozyme. The preferential interaction of proteins with polyelectrolytes at a more anionic charge ratio has previously been observed for some polyelectrolytes, although the exact reason remains unknown. [30,31] For the determination of F^-, the assumption has been that all polyelectrolyte monomers were charged. Recently, a study has demonstrated a method to measure the charge fraction of polyelectrolytes in PECs. [32] This technique may be applied in the future to further elucidate the exact role of charges on partitioning of proteins in PECs.

When the starting concentration of lysozyme is increased, the PEC becomes saturated. At high enough concentrations a fraction of the lysozyme will remain in the supernatant, even at the $F^-_{optimal}$. [16] This could be compensated for by increasing the total amount of PEC. [16] A previous study has suggested that within PECs the lysozyme concentration can be enriched up to 200 g/L. [15] This behaviour suggests a model of PECs as a phase that can be 'filled' with lysozyme and upon saturation the remaining lysozyme remains in the supernatant phase. In all the PEC systems of Figure 4.2, we see a clear F^- value where the supernatant lysozyme approaches zero. This suggests that for none of our systems we have reached the PEC saturation point.

All four evaluated PECs show effective lysozyme take-up at $F^-_{optimal}$. For extraction processes, recovery of lysozyme from the PEC is equally important. We therefore study the release of lysozyme from the PECs by addition of salt or lowering of the pH. First, we prepare PECs with partitioned lysozyme at their $F^-_{optimal}$ found in Figure 4.2. Then, we replaced the supernatant with either 500 mM NaCl or water with a pH of 4. We evaluated the concentration of lysozyme in the refreshed supernatant and express this value as the percentage of lysozyme released from the PEC. The process of releasing lysozyme in new supernatant is referred to as back-extraction. Salt ions can screen charges on the polyelectrolytes and proteins. By increasing the salt concentration, the interactions between the charged macromolecules can weaken and can result in the release of proteins. [33] Altering the pH will affect the degree of ionisation of weakly charged groups on the polyelectrolytes and proteins. If the interactions between the proteins and polyelectrolytes weaken as a result of a change in pH, proteins can be released from the polyelectrolyte complex into the supernatant phase. [16,20] Previously, we have reported that a decrease in pH from 7 to 4 gave the best result. [16]

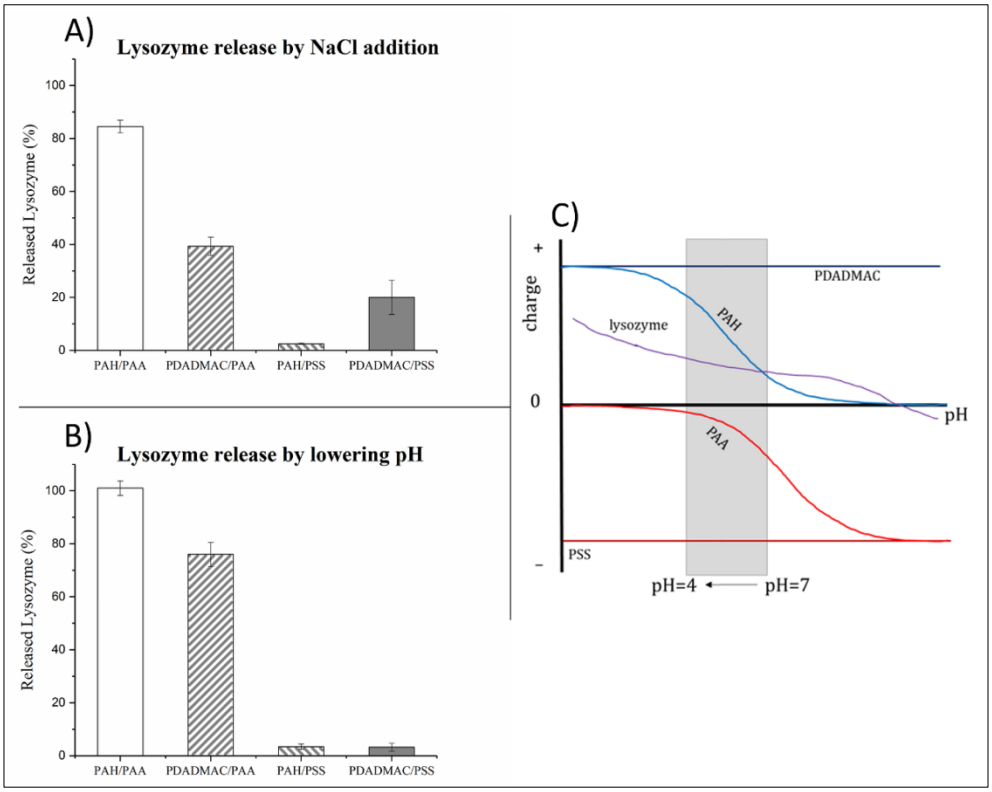


***Figure 4.3**. The back-extraction of lysozyme from the different PEC systems using A) 500 mM NaCl, B) 4 mM HCl (pH decrease from 7 to 4), C) schematic sketch that illustrates how the charge of the polyelectrolytes and lysozyme changes as a function of pH, [34] adapted with permission. [35] Columns represent the average of n = 4 with error bars indicating standard deviation.*

In Figure 4.3A and B, lysozyme back-extraction *via* the addition of salt or a decrease in pH are shown, respectively. For both methods the supernatant phase was removed and replaced by new aqueous supernatant with either a 500 mM NaCl concentration, or a lower pH (~4). The different PEC systems show widely different releases of lysozyme (Figure 4.3A) at 500 mM NaCl. The lowest release is found for PAH/PSS with 2.5 ± 0.3 % of lysozyme released. Second lowest is PDADMAC/PSS with 20.0 ± 6.4 %. Second-highest is PDADMAC/PAA with 39.3 ± 3.5 % released and the greatest back-extraction efficiency was found for PAH/PAA with 84.5 ± 2.4 % of lysozyme released.

An increase of ionic strength as a result of added salt ions is known to induce screening effects between oppositely charged polyelectrolytes, preventing the

polyelectrolytes from interacting (as strongly) with each other. Different PEC systems containing proteins respond differently to an increase in ionic strength. PEC micelle systems can either release the protein while remaining intact, or disintegrate completely and release the proteins based on the weak/strong nature of the polyelectrolytes. [33,36] There are several examples in literature where PECs consisting of weak polyelectrolytes *versus* PECs consisting of strong polyelectrolytes show a different resistance towards an increase in ionic strength. [37-40] In general, complexes consisting of only weak polyelectrolytes disintegrate at a lower ionic strength than complexes consisting of strong polyelectrolytes. [37] When weak and strong polyelectrolytes are combined the degree of ionisation and thus the pH of the system becomes important. In our case the lowest release was found with PAH/PSS. This is consistent with earlier reports that show that PAH/PSS PECs may even gain stability in the presence of up to 3 or 4 M NaCl. [41,42] PDADMAC/PSS PECs or multilayers have demonstrated the ability to form stable PECs in the presence of up to 2 M NaCl. [38,39] Lysozyme release from PDADMAC/PSS PECs is then more likely a result of reduced attraction between the PEC and lysozyme, which has a much lower charge density than the polyelectrolytes. PDADMAC/PAA PECs consisting of larger polyelectrolytes have been shown to be stable up to 1 M NaCl. [43] Interestingly, an increase in NaCl concentration does not result in a linear lysozyme release profile for PAH/PAA for the range of 0 to 1 M NaCl (Supplementary Figure S4.2).

A change in PEC size as a result of added salt or lowering pH was observed (Supplementary Figure S4.3) for both salt and lower pH in most PEC systems. The PECs either swelled or decreased in size. However, there was no clear correlation between change in PEC size and released lysozyme (Supplementary Table S4.1). For example, in the case of PAH/PAA PECs where all lysozyme was released by lowering the pH, the PEC size increased by approximately 10 %. Earlier reports on water content in PECs suggests that minor changes in PEC composition can affect their water content. This could explain the observed variations in PEC size as a result of pH change. [17] Size changes at increased salt concentration could be explained by the PEC network loosening due to screened polyelectrolyte charges and as a result more water is taken up by the PEC. An interesting observation was found for PDADMAC/PAA PECs, where lowering the pH to 4 resulted in a nearly 6-fold increase in PEC size. Visually the PDADMAC/PAA formed a gel-like structure specifically at the lower pH values, possibly due to hydrogen bonding. [44] For soluble PDADMAC/PSS PECs at stoichiometric compositions, an NaCl concentration of 0.5 M has been found to sharply increase the PEC hydrodynamic diameter. [45]

A decrease in the partitioning of proteins at higher NaCl concentrations has been reported earlier and it was hypothesised that the salt ions weaken the electrostatic and entropic interactions between the proteins and the polyelectrolytes. [18] That the

presence of salt ions influences the interaction between proteins and singular polyelectrolytes has been widely established and it stands to reason that salt ions can similarly influence the interactions between proteins and PECs. [46] It has been shown that protein charge is important in protein partitioning in PECs, and salt ion screening can influence the degree to which protein and PEC charges are able to see and interact with each other. [16,18]

Figure 4.3B presents the back-extraction of lysozyme when the pH of the supernatant is decreased from pH 7 to pH 4. This figure shows that a decrease in pH has a different effect on the four investigated systems. Both PAH/PSS and PDADMAC/PSS show the least amount of released lysozyme with 3.4 ± 1.0 % and 3.2 ± 1.5 %, respectively. PDADMAC/PAA released 76.0 ± 4.5 % and PAH/PAA released 101.0 ± 2.7 % of lysozyme.

For the strongly charged PDADMAC/PSS PECs it is expected that a change in pH will have little effect on the protein release as both polyelectrolytes bear a charge independent of pH. By changing the pH, the charge of protein will become more positive and the net charge of protein shifts from +7 to + 12. In Figure 4.3B it can be seen that the amount of lysozyme in the supernatant for this system at pH 4 is very low. The lysozyme that is released is likely the result of the change in lysozyme charge due to the decrease in pH. [34]

For the three other systems, the charge of one or two of the polyelectrolytes present will depend on the pH of the system. To illustrate, Figure 4.3C shows a sketch of the charge of the polyelectrolytes and lysozyme as a function of the pH. [34] Weakly charged polycations are fully charged at low pH and uncharged at high pH while the opposite will be found for weak polyanions. If we first consider the system consisting of two weakly charged polyelectrolytes (PAH/PAA), a pH decrease from 7 to 4 will result in a decrease of negative charge on the anionic PAA and an increase in positive charge on cationic PAH. For this system we find almost complete lysozyme release, as has been reported earlier for PECs consisting of shorter PAH and PAA. [16] The protein release can be explained by a change in complex composition that occurs when the net charge on the polyelectrolytes changes when the pH is decreased. Effectively the system obtains a different composition than $F^{-}_{optimal}$ and less protein can be taken up by the complex (Figure 4.2). [16] In principle an increase in pH will have the same, but opposite effect on the charge of the polyelectrolytes; now the polycation becomes less charged and the polyanion more charged. For lysozyme, with an isoelectric point of 11.65, a pH increase makes the protein less charged. Other interactions between the protein and the polyelectrolytes become stronger and make back-extraction at high pH difficult. [16]

The PAH/PSS and PDADMAC/PAA systems both consist of a weak and a strongly charged polyelectrolyte, but a clear difference in protein release is observed. The

relatively large amount of lysozyme released by PDADMAC/PAA PECs and relatively small amount of lysozyme released by PAH/PSS can be explained when considering the sketch presented in Figure 4.3C. At pH 4, PAA will become less charged and the interaction between the PEC and the protein become weaker and more lysozyme is released. For the PAH/PSS almost no lysozyme is released by a decrease in pH. In this system a decrease in pH will result in a stronger interaction between PAH and PSS, as PAH becomes more charged.

Protein release from PECs might be dependent on system parameters apart from an increase in ionic strength or a decrease in pH. For the lysozyme release as investigated in Figure 4.3, there was an abundance of supernatant phase compared to PEC phase of a factor of 30 – 60. In addition, lysozyme has high solubility in water. Together, this ensured that full back-extraction of lysozyme could be observed. However, care must be taken to design the back-extraction process for proteins with lower water solubility, or when working with different polyelectrolyte and protein concentrations.

The four PECs presented in this study display similar maximum lysozyme take-up (Figure 4.2). Contrary, the salt and pH-dependent release properties of these complexes are very different leading to strongly differing back-extraction efficiencies (Figure 4.3). Of the four polyelectrolyte combinations, PAH/PAA PECs demonstrated the greatest potential for lysozyme back-extraction by releasing all of its lysozyme with a decrease in pH. For this reason we continued with PAH/PAA PECs to evaluate whether they can be used to selectively extract lysozyme from a complex protein mixture. The protein mixture in this study is a lyophilised albumen powder, which is a commercially available protein mixture obtained by freeze-drying hen-egg white. It is commonly used for baking and cooking.

To extract lysozyme from albumen, PAH and PAA were added to a solution of albumen powder at the optimal lysozyme partitioning composition of $F^- = 0.53$ (Figure 4.2A). The supernatant phase was then refreshed and the proteins taken-up by the PEC were released by lowering the pH from 7 to 4, similar to Figure 4.2. The protein composition was then analysed via SDS-PAGE, which separates proteins based on mass (Figure 4.4). Columns on the gel represent different samples and bands within the columns represent proteins of similar mass.

In Figure 4.4, the protein composition of various steps in the lysozyme extraction process are shown. The three left columns contain reference samples of known protein sizes (R), pure lysozyme (A), and the albumen solution (B). Column (F) contains only PAH/PAA PECs. Columns (C-E) are triplicates of the supernatant phases after the addition of PAH and PAA at $F^- = 0.53$. Columns (G-I) are triplicates of the back-extraction.

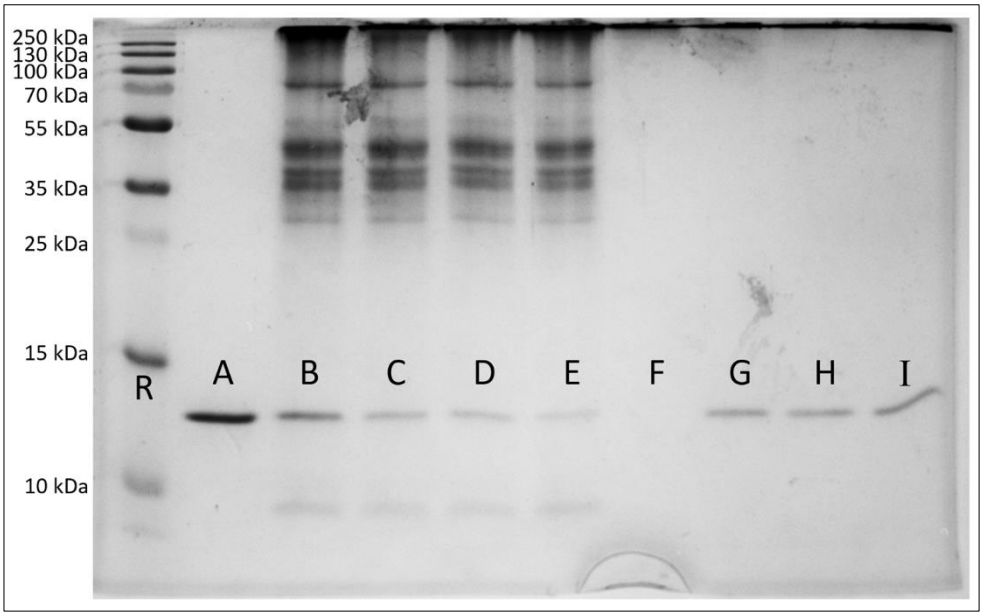


***Figure 4.4**. SDS-PAGE gels of the supernatant during extraction and release steps of 1 g/L albumen solution. R) Reference protein mixture, A) pure lysozyme, B) 1 g/L albumen solution, C-E) triplicate of supernatant after extraction, F) supernatant of PAH/PAA PECs without proteins, G-I) triplicate of the back-extraction supernatant after lowering the pH.*

In the albumen solution (B), a band indicating the presence of lysozyme, with a known molecular weight of 14.3 kDa, is visible between 10 and 15 kDa. Lysozyme is the only protein present in albumen with this molecular mass. [28] Other common albumen proteins are indicated in Supplementary Figure S4.4. When PAH and PAA are added (C-E) the protein band representing lysozyme becomes less intense, indicating a decrease in lysozyme. After the back-extraction (G-I), we find only lysozyme in the supernatant. This suggests that while not all of the lysozyme is extracted, everything that is extracted and back-extracted is lysozyme.

Columns (C-E) show that not all lysozyme of the albumen is taken up by the PEC. The presence of the (charged) albumen protein could alter $F^-_{optimal}$. The extraction with other PAH/PAA compositions around $F^-_{optimal}$ was investigated but no F^- was found where all lysozyme was clearly extracted (Supplementary Figure S4.4). A plausible explanation for the lack of full lysozyme extraction is the presence of the protein ovomucin; the most common albumen protein after lysozyme. [28] Ovomucin is known to bind to lysozyme via electrostatic interactions. [47,48] As we know that charge plays an important role in the partitioning of lysozyme in PECs, [16,18] the binding to ovomucin could prevent the lysozyme from interacting with the PECs. In

SDS-PAGE, the proteins are exposed to SDS which results in denaturation and separates ovomucin from lysozyme, resulting in lysozyme being visible as a band on the SDS-PAGE gel. This process is analogous to the known technique of separating ovomucin and lysozyme by increasing the salt concentration. [47,48] From earlier studies, we know that PECs can take-up high concentrations of lysozyme, therefore lysozyme saturation of the PEC is unlikely. [15,16]

Charge plays an important role in the uptake of proteins by PECs. From the release studies presented in Figure 4.3, screening of the charges on the polyelectrolytes as well as changing the amount of charge by a decrease in pH affects the partitioning of the proteins. In previous work we have shown that by changing the composition of the PECs, lysozyme and the oppositely charged succinylated lysozyme could be separated. [16] Albumen is a significantly more complex mixture than a mixture of lysozyme and succinylated lysozyme. Within the first ten most common proteins in albumen, lysozyme is the only protein with a positive charge at pH 7. [28] The results suggest that also in this study the charge of the protein species is a very important factor in selective extraction of a single protein from a mixture.

The concentration of the extracted lysozyme can be determined with UV-vis spectroscopy. Out of the initial concentration of 1 g/L albumen powder, the extracted concentration of lysozyme is 41.4 ± 2.8 mg/L (average ± standard deviation, n = 5). This is similar to the reported concentration of 34-35 mg/L. [28,29] It is possible that the lyophilised powder solution is more concentrated than native albumen or that the exact protein composition per egg depends on biological and external factors. Recently, a study has shown that the lysozyme content of hen eggs has notable variation depending on chicken breed as well as a large spread within breeds. [49]

Protein function is intrinsically linked to protein structure. Uptake and release of lysozyme in and from PECs could have a detrimental effect on the lysozyme structure either by the protein's temporary presence in a different environment or by structural disruption via direct interactions between either polyelectrolyte and the lysozyme. To confirm that extraction via PECs does not disrupt the structure (and thus function) of proteins, we evaluated the activity of lysozyme extracted and back-extracted from the albumen solution as shown in Figure 4.4. The assay we use is based on the turbidity of a bacteria suspension. A decrease of turbidity over time indicates the presence of active antibacterial enzymes like lysozyme.

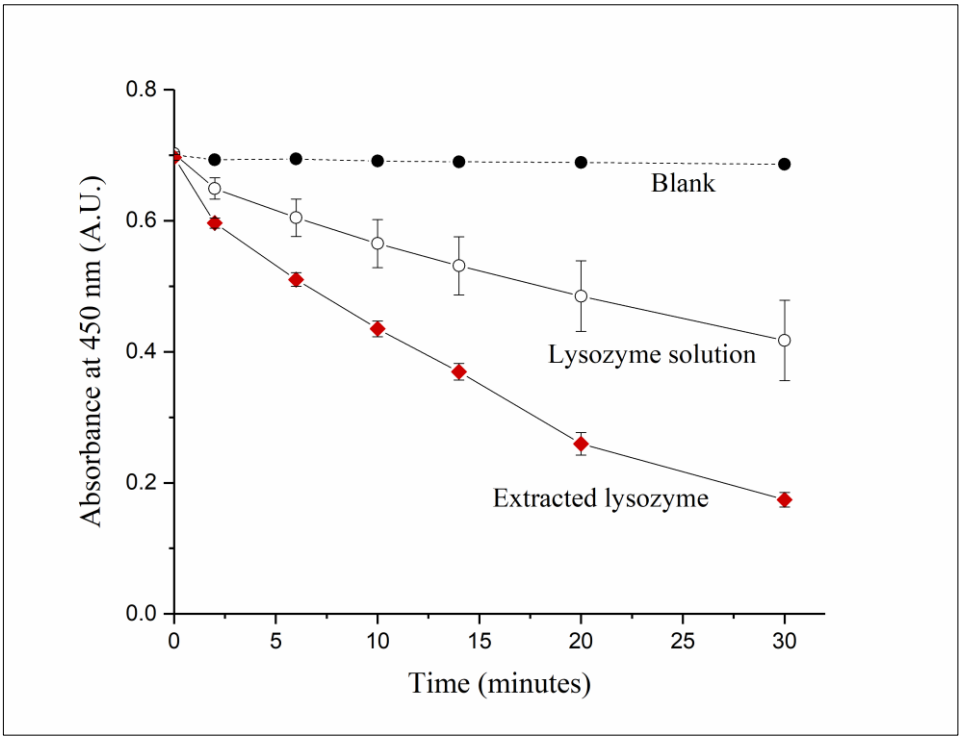


***Figure 4.5**. Enzymatic activity of the lysozyme extracted from albumen solution. A decrease in absorbance at 450 nm over time is associated with enzymatic activity. Filled circles (●) are substrate only. Empty circles (○) are 7.5 mg/L commercially bought purified lysozyme. Red diamonds (♦) are lysozyme extracted and back-extracted with PAH/PAA PECs. Symbols represent averages and the error bars represent standard deviations (n = 3). Lines connect the averages.*

In Figure 4.5 the activity of lysozyme extracted from albumen solution is compared to that of a negative control (substrate only) or a positive control (lysozyme solution). We observe a decrease of absorbance at 450 nm for both the positive control as well as the extracted lysozyme over time, indicating that both solutions contain active enzymes. The concentration of the lysozyme in the positive control was 7.5 mg/L and the measured concentration of the extracted lysozyme was 41.4 ± 2.8 mg/L.

The enzymatic activity in units/ml was determined from the decrease in absorbance using Equation 2. The activity of the extracted lysozyme was found to be 929 ± 85 units/ml. The activity of the commercial purified lysozyme was 457 ± 146 units/ml.

The activity of the extracted lysozyme seems approximately two times higher than commercial purified lysozyme. However, the concentration of the extracted

lysozyme was approximately 5.5 times higher than the commercial lysozyme. Effectively, this is a decrease in activity per amount of lysozyme of a factor 2.7 compared to commercial purified lysozyme. It is known that lysozyme activity varies between chicken populations as well as individual chickens. [50,51] The commercially available purified lysozyme is a composite of many albumen sources, whereas our extraction was done on one egg.

The activity of the complete albumen solution was 1490 ± 289 units/ml (Supplementary Figure S4.5). The (back-)extraction protocol in its entirety takes approximately 48 hours and occurs at room temperature. In this time the activity of the enzymes could have decreased due to naturally occurring enzyme denaturation. For the back-extraction a decrease in pH is used. This could also have an effect on the enzymatic activity. No significant effect of these factors on the activity of albumen solution was observed (Supplementary Figure S4.6). The difference in activity between the extracted lysozyme and the complete albumen solution can be explained by a combination of two factors. First, other albumen proteins such as ovotransferrin have been reported with similar antibacterial properties as lysozyme, [52,53] so the extracted lysozyme would be expected to show decreased antibacterial enzymatic activity even with a perfect back-extraction efficiency. Second, we know from Figure 4.4 that we do not extract all lysozyme.

It has previously been shown that the structure of BSA as determined by circular dichroism remained intact after a pH-induced release from liquid-like polypeptide PECs. [20] In this work we show that the selectively extracted and back-extracted lysozyme (Figure 4.4) also retains its enzymatic properties (Figure 4.5). Our results show the potential of PECs as extraction media for the recovery and isolation of proteins from complex protein mixtures.

The observed protein partitioning behaviour of the PECs used in our study has implications for the greater understanding of MLOs. In this study it is shown that PECs respond to changes in pH, salt concentration, and the PEC composition. These three factors can fluctuate in the intracellular environment: cells can produce more or fewer intrinsically disordered proteins (IDPs) or RNA and thereby alter the MLO composition, and the pH [54–56] as well as the concentration of salt ions [57] are known to vary within cells and as function of the cell cycle.

Two of the PEC systems in this study are expected to have a similar pH response as cellular MLOs. The weak/weak PAH/PAA PECs are analogous to MLOs consisting of two oppositely charged IDPs. [58] The PAH/PAA PECs will readily release its lysozyme due to a decrease in pH or due to increasing salt concentration, this behaviour could also apply to IDP/IDP MLOs. The weak/strong PAH/PSS PECs can be compared to IDP/RNA MLOs. PAH/PSS PECs are very resistant to releasing lysozyme despite a decrease in pH or an increase in salt concentrations. Therefore

IDP/RNA MLOs are expected to be relatively robust with respect to fluctuations in pH or salt concentration. Direct comparison between PECs and MLOs is difficult due to the complexity of the cellular environment and the unknown composition of MLOs. However, direct comparison between in vitro PECs and in vivo MLOs is difficult due to the complexity of the cellular environment and the unknown composition of MLOs.

Conclusion

Here we have shown that lysozyme can be (back-)extracted from a chicken albumen solution using polyelectrolyte complexes while retaining its enzymatic function. Although all the PECs studied here displayed similar partitioning profiles of lysozyme as a function of the complex composition, lysozyme release strongly depended on the type of polyelectrolytes and the release method used. For the lysozyme (back-)extraction, charge plays a dominant role, this will not necessarily be the case for all proteins. A systematic study of different types of proteins is required to make this method widely applicable for industrial applications.

Acknowledgments

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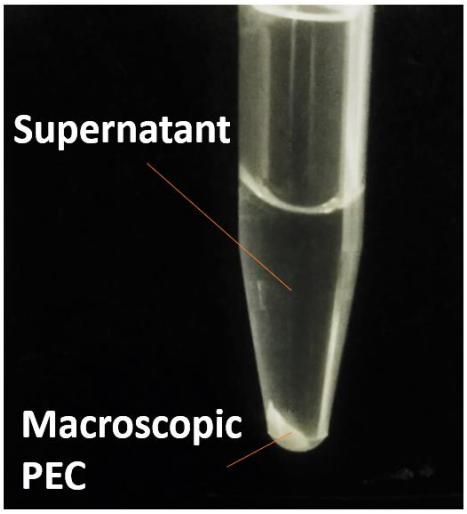
Supplementary Information

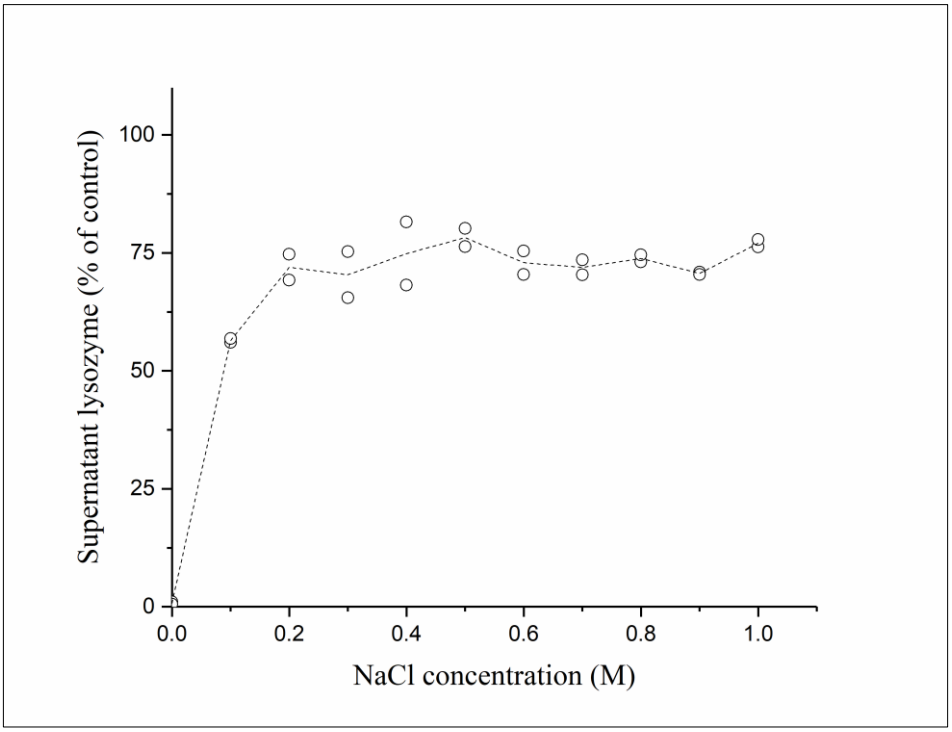
Preparation of PECs with albumen solution



Supplementary Figure S4.1. *Photograph of the macroscopic PAH/PAA PECs and the aqueous supernatant after centrifugation. Depicted is an PAH/PAA PEC with albumen, though this image is representative for all evaluated PEC systems with either albumen or purified lysozyme.*

Directly after addition of PAH and PAA to (colourless transparent) albumen solutions, there is a formation of macroscopic PECs as well as soluble PEC indicated by solution turbidity. In the course of the 24h incubation, the PEC largely settles at the bottom of the vessel. Finally, centrifugation merges and concentrates the soluble PECs down towards the macroscopic PECs. Supplementary Figure S4.1 shows the PEC-supernatant system for albumen solutions with PAH/PAA PECs, however the image is representative for all the evaluated PEC systems with either lysozyme- or albumen solutions.

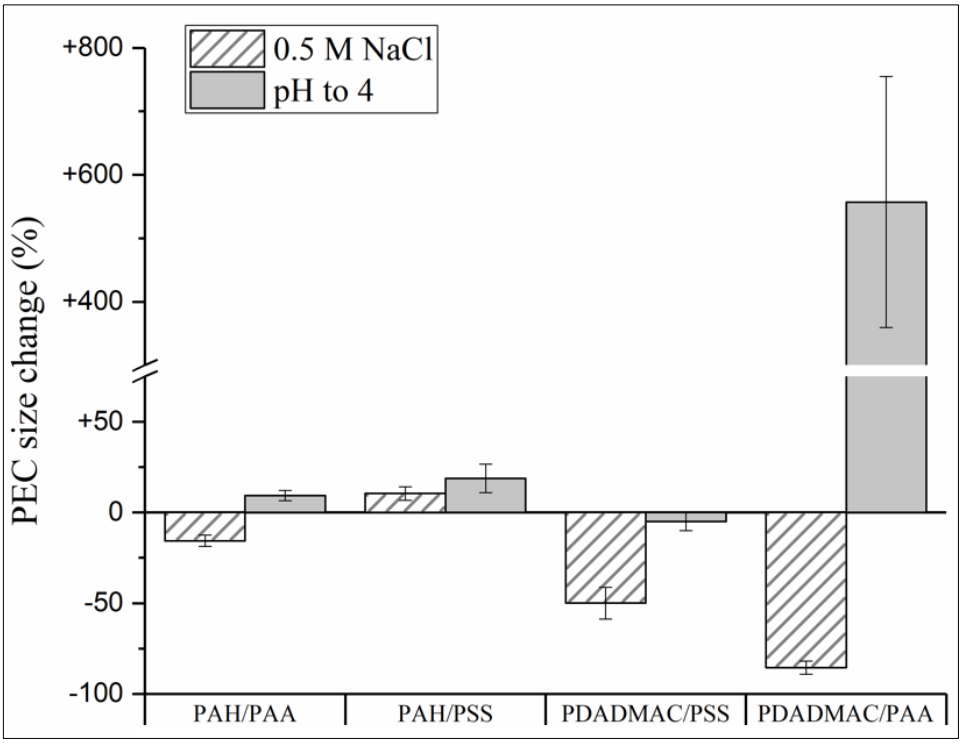
Release of lysozyme from PAH/PAA PECs as a function of NaCl concentration



Supplementary Figure S4.2. *The release of lysozyme from PAH/PAA PECs as a function of added NaCl concentration. Circles represent individual measurements of duplicates. The line connects the averages.*

In Figure 4.3, lysozyme was released from various PEC systems via the addition of 0.5 M NaCl in fresh supernatant. For PAH/PAA PECs, we also evaluated the effect of a range of NaCl concentrations on the lysozyme release (Supplementary Figure S4.2). Lysozyme release increases sharply and appears to reach a plateau at a NaCl concentration of 0.2 M. It is assumed that at a higher concentration, eventually all the lysozyme will be released as the PEC completely dissociates.

PEC size change as a result of adding 0.5 M NaCl or lowering pH to 4



***Supplementary Figure S4.3**. Size change of the four PEC systems as a result of refreshing the supernatant phase with either 0.5 M NaCl or with water of pH 4. Columns show averages and error bars show standard deviation of n = 3.*

Solution ionic strength influences the degree to which polyelectrolyte charges can interact with each other. Solution pH influences the ionisation degree of weak polyelectrolytes (i.e. PAH and PAA). In Supplementary Figure S4.3, we see that some PECs swell, while others decrease in size as a result of increased NaCl concentration or decreased pH. The PEC size change does not clearly correlate with the lysozyme release of Figure 4.3 for all PEC systems.

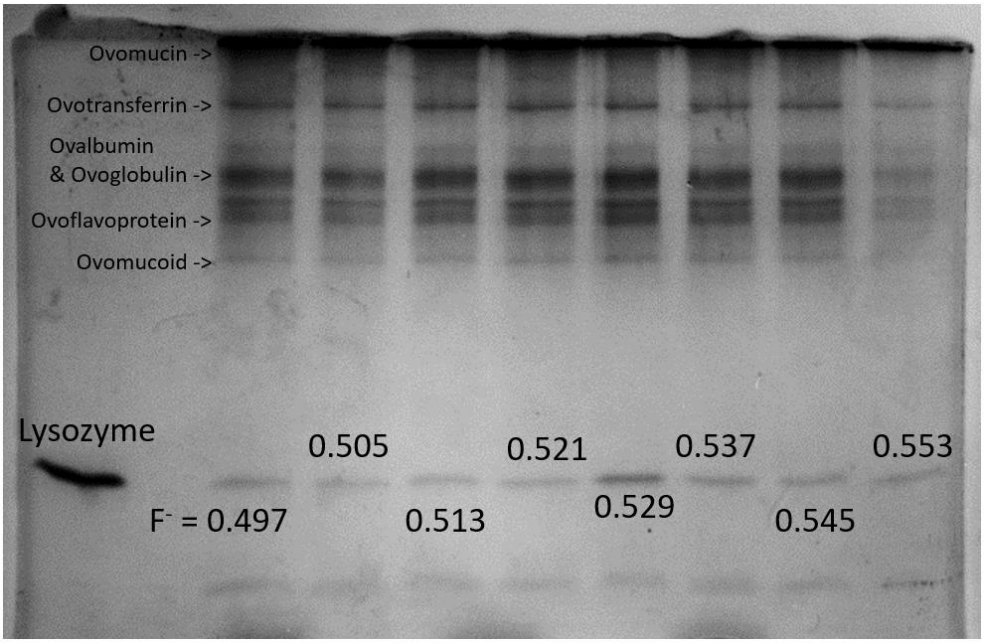
PEC size change and released lysozyme

Supplementary Table S4.1. *Numeric overview of the data from Figure 4.3 and Supplementary Figure S4.3, for comparison.*

PEC System	0.5 M NaCl size change	0.5 M NaCl lysozyme release	pH 4 size change	pH 4 lysozyme release
PAH/PAA	-15.6 ± 3.2 %	84.5 ± 2.4 %	18.9 ± 7.9 %	101.0 ± 2.7 %
PAH/PSS	+10.5 ± 3.8 %	2.5 ± 0.3 %	9.3 ± 2.8 %	3.4 ± 1.0 %
PDADMAC/PSS	-50.0 ± 8.8 %	20.0 ± 6.4 %	-5.0 ± 4.9 %	3.2 ± 1.5 %
PDADMAC/PAA	-85.5 ± 3.6 %	39.3 ± 3.5 %	557.2 ± 197.6 %	76.0 ± 4.5 %

In Supplementary Table S4.1, the numerical data from Figure 4.3 and Supplementary Figure S4.3 is consolidated. There is no consistent connection between size change and lysozyme release.

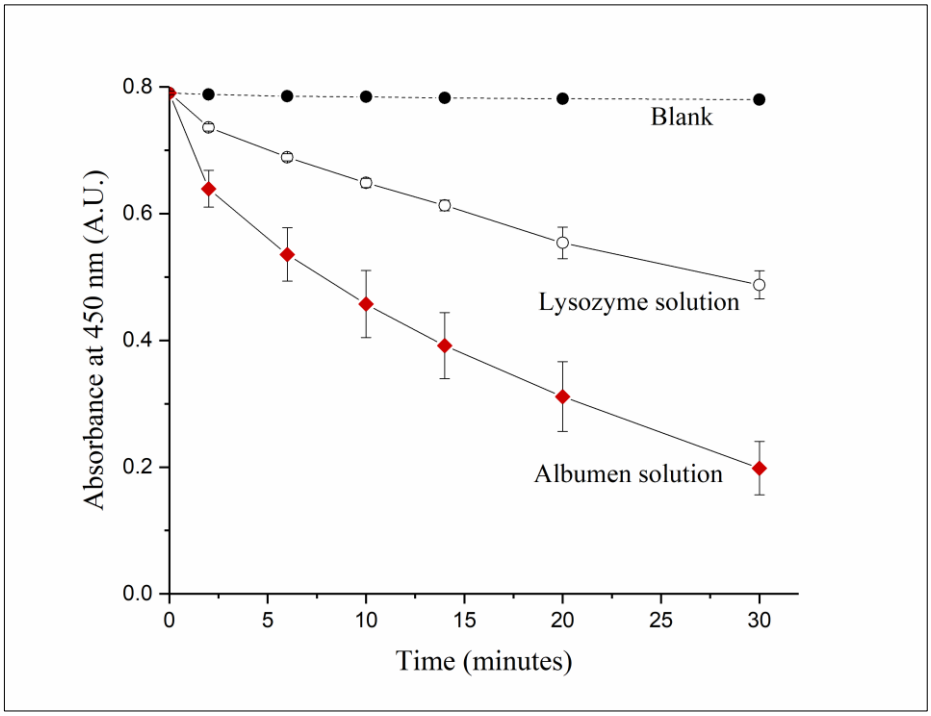
Partitioning of lysozyme from albumen solution at various F^-



***Supplementary Figure S4.4**. Protein profile of albumen solution supernatant in the presence of PAH/PAA PECs ranging in composition from F^- = 0.497 to 0.553. Additionally, several other proteins besides lysozyme that are present in the albumen are marked.*

As a result of Figure 4.4 and the non-complete partitioning of lysozyme from the albumen solution, we investigated whether the presence of many other proteins in the albumen solution could have changed the $F^-_{optimal}$ of lysozyme in PAH/PAA PECs to a different F^- value. In Supplementary Figure S4.4 the protein profile of the albumen solution in the presence of PAH/PAA PECs with a composition ranging from F- = 0.50 to 0.55 is shown. Within this range, there is no clear value of F^- where all lysozyme is clearly partitioned in the PEC. In addition, various other common albumen proteins have been indicated on the gel.

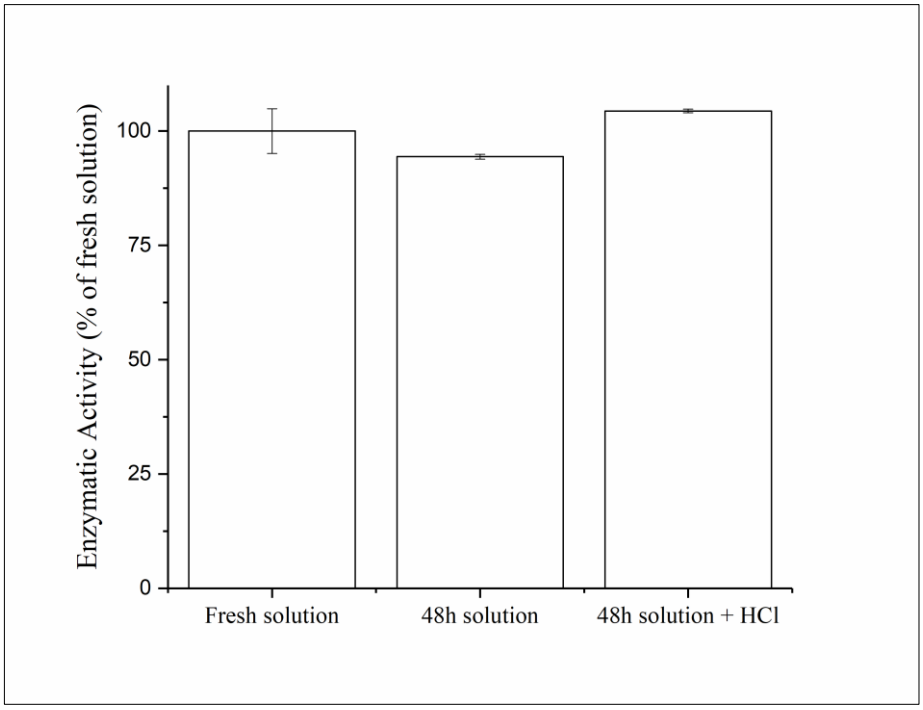
Activity of complete albumen solution



***Supplementary Figure S4.5**. Enzymatic activity of the complete albumen solution prior to lysozyme extraction. A decrease in absorbance at 450 nm over time is associated with enzymatic activity. Filled circles (●) are blanks consisting of the substrate only. Empty circles (○) are 7.5 mg/L lysozyme solutions. Red diamonds (♦) represent the 1 g/L albumen powder solution. Symbols represent averages and the error bars represent standard deviations (n = 3). Lines connect the averages.*

In Figure 4.5, the enzymatic activity of the lysozyme extracted from the albumen solution is shown. Supplementary Figure S4.5 shows the result of a similar assay but evaluates the enzymatic activity of the complete 1 g/L albumen solution. The activity is calculated via Equation 2 and corresponds to 1490 ± 289 units/ml.

Comparison of the activity of albumen solution before extraction, after room temperature equilibration, and as a result of the addition of HCl



***Supplementary Figure S4.6**. Variation in the activity of 2 g/L albumen powder solution as a result of either room temperature incubation for 48 h, or room temperature incubation for 48 h with the addition of 4 mM HCl after 24 h, compared to the activity immediately after preparation of the solutions (fresh solution). Data is shown as averages with error bars indicating standard deviation (n = 3).*

As a result of the extraction protocol the albumen solutions equilibrate at room temperature for a total of approximately 48 hours, and undergo a decrease in pH facilitated by the addition of HCl for 24 hours. We investigated the effect of both time and HCl addition on the activity of the albumen solution in Supplementary Figure S4.6. The activity of the albumen solution does not significantly change after 48 hours or with addition of HCl.

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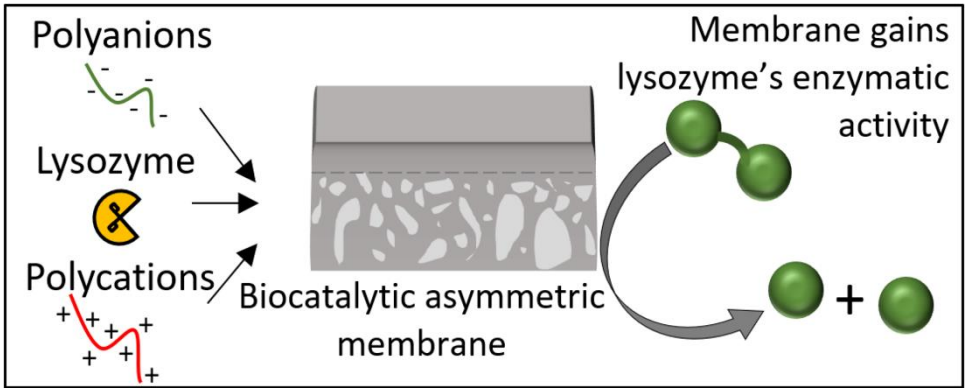
Chapter 5

Biocatalytic Membranes Through Aqueous Phase Separation

This chapter has been accepted for publication as:
J. J. van Lente, M. I. Baig, W. M. de Vos, S. Lindhoud. Biocatalytic Membranes Through Aqueous Phase Separation, *Journal of Colloid and Interface Science* ***2022***.

Abstract

Polymer membranes play a critical role in water treatment, chemical industry, and medicine. Unfortunately, the current standard for polymer membrane production requires unsustainable and harmful organic solvents. Aqueous phase separation has recently been proposed as a method to produce membranes in a more sustainable manner through induced polyelectrolyte complexation in aqueous solutions. We demonstrate that aqueous phase separation has another natural advantage that goes beyond sustainability: the easy incorporation of enzymes in the membrane structure. Biocatalytic membranes hold great promise in for example biorefinery, but the most common current post-production processes to immobilise enzymes on the membrane surface are complicated and expensive. In this study we demonstrated the first biocatalytic membrane produced via aqueous phase separation. We demonstrate an easy procedure to incorporate lysozyme in polyelectrolyte complex membranes made via aqueous phase separation. Our functionalised membranes have the same structure, water permeability (in the range of high nanofiltration, low ultrafiltration), and retention as membranes without lysozyme. Lysozyme is antibacterial by catalysing the hydrolysis of specific peptidoglycan bonds in bacteria walls. We demonstrate that the functionalised membranes are also capable of catalysing this reaction. The membranes remain enzymatically active for a period of at least one week. This opens new routes to produce polymer membranes with added biological function.



Introduction

Polymer membranes are ubiquitous in water purification and industrial separation techniques. In the face of global water shortages as well as developing (bio)technology, the demand for more, better, and more versatile membranes continues to increase. [1–3] In addition, there are investigations into making membrane production processes more sustainable. [4–7] The common production method for polymer membranes is non-solvent induced phase separation (NIPS), also known as immersion precipitation. [8,9] In this process, polymers are typically first dissolved in an aprotic organic solvent and then cast on a substrate (e.g. a glass plate) as a thin liquid film. This film is subsequently submerged in a non-solvent bath, where the polymer then precipitates as the solvent migrates out of the polymer solution. The interface of the polymer solution and the non-solvent often forms a dense selective polymer layer while the polymer structure between the interface and the substrate will be more porous. This membrane asymmetry allows for higher water permeabilities due to the porous structure while maintaining desirable selective properties due to the dense selective layer. A disadvantage of NIPS is the requirement of repro-toxic organic solvents like *N*-methyl-2-pyrrolidone, which has recently been added to the restricted substances list by the European Commission. Therefore, alternative production methods have been proposed that use different, more sustainable solvents. [10–12] Still, it is difficult to find a more sustainable solvent than water.

In 2019 aqueous phase separation (APS) has first been demonstrated as a membrane production method in which both phases of the phase inversion are aqueous solutions. [13–20] Of specific interest is complexation-driven APS, where oppositely charged polyelectrolytes are first mixed in an aqueous solution under conditions that prevent polyelectrolyte complexation (e.g. high salinity or an extreme pH). Similar to NIPS, the viscous polyelectrolyte solution is then cast on a substrate and submerged in a different aqueous solution with conditions at which polyelectrolyte complexation occurs (e.g. low ionic strength or an opposite pH). Membranes produced via APS are also typically asymmetric. The membrane properties can be tuned by the manipulation of the precipitation conditions such as salt concentration or pH. [13–15,17,18] Since the complete membrane production process via APS occurs in aqueous environments, this method does not require the use of organic solvents.

In this study we suggest an additional advantage of APS: aqueous environments are more favourable to biomacromolecules such as enzymes, whereas organic solvents typically lead to enzyme denaturation and inactivation. For this reason, APS could allow easy incorporation of enzymes directly in the membrane production process instead of attaching enzymes as a post-production modification. In this way, existing APS techniques can be modified to create biocatalytic membranes. Currently there

are no reported APS systems that include enzymes as either part of their structure or as added functionality.

Biocatalytic membranes combine e.g. enzyme functionality with separation properties of polymer membranes. [21,22] Biocatalytic membranes potentially provide substantial benefits to the field of membrane technology due to the large variety of functionalities that biomacromolecules can give to membranes. [23,24] For example, biocatalytic membranes using hydrolase and oxidoreductase enzymes have been suggested for the removal of micropollutants and to reduce fouling. [23,25] Despite the potential of biocatalytic membranes, there are many limitations to their production and widespread use. Currently, enzymes are typically immobilised on the membrane surface after fabrication of the membrane. [21,22] The enzyme immobilisation can require various additional processing steps. In addition, enzymes can lead to a decrease in membrane permeability and selectivity, enzyme immobilisation or membrane production conditions can deactivate the enzyme, or enzymes can leach out of the membrane. [21,22]

Here, we demonstrate the incorporation of the enzyme lysozyme in polyelectrolyte complex (PEC) membranes produced via APS. For this reason we have adapted a previously reported APS system consisting of poly(allylamine hydrochloride) (PAH) and poly(sodium 4-styrenesulfonate) (PSS). [13] We propose a modification to existing APS protocols that allow for easy addition of enzymes directly to the casting solution, with the result that functional enzymes are incorporated in the membrane structure.

The enzyme chosen for this study is lysozyme. Lysozyme is an antibacterial enzyme that is part of the innate immune system and is found in saliva, tears, and other mucus secretions. [26,27] Its main function is destroying gram-positive bacteria by catalysing the hydrolysis of specific peptidoglycan bonds in bacterial cell walls. [28,29] Lysozyme is interesting for membrane technology due to its capacity to reduce membrane fouling by preventing or reducing the formation of a bacterial biofilm on the membrane. [30]

We demonstrate that the incorporation of lysozyme into PAH/PSS membranes produced via APS can be highly straightforward, especially when compared to the existing methods for functionalising membranes produced with traditional membrane fabrication processes. Moreover, the resulting membranes demonstrated enzymatic properties which persisted for at least one week. There was no detectable lysozyme found leaching out of the membrane during the membrane production process or during up to one week of storage. In addition, the membrane permeability, retention, and structure are not affected by the presence of lysozyme. The relative ease of production and biocatalytic activity opens a new route to produce sustainable membranes via APS with added biological functionality.

Materials and Methods

Materials

Poly(allyl hydrochloride) (PAH) 40 % solution with a molecular weight (MW) of 150 kDa was purchased from Nittobo. Poly(sodium 4-styrenesulfonate) (PSS) 30 % solution with a MW of 200 kDa (product number 561967), lyophilised hen-egg lysozyme (product number L876), lyophilised *micrococcus lysodeikticus* (product number M3770), 50 % glutaraldehyde solution (product number 340855), NaOH pellets, fuming HCl solution, and NaCl were purchased from Merck. Ultrapure Milli-Q water (mQ) is filtered by an Advantage A10 water purification system (Millipore), otherwise 'water' refers to demineralised water.

Membrane production

PSS stock solutions were diluted from 30 to 15 wt% with mQ or with 0.75 g/L lysozyme in mQ solution. PAH stock solutions were diluted from 40 to 15 wt% with mQ and 10 M NaOH to reach 7.5 wt% NaOH. These 15 wt% PAH and PSS solutions are then mixed 1:1.1 respectively to achieve a 2:1 positive:negative monomer ratio. The polyelectrolyte solutions are mixed and degassed to remove air bubbles, resulting in a viscous 15% mixed polymer solution.

The polymer solution is then cast on a glass substrate using a casting knife with a 600 µm gap before being submerged in 250 ml precipitation solution consisting of 2 % fuming HCl, 0.5 M NaCl, and 0.1% crosslinker glutaraldehyde in mQ. Precipitation took place for 15 minutes before the membranes were placed in water. In water, the membranes spontaneously detach from the glass plate within a few minutes. Membranes were stored in water until further analysis.

Membrane Characteristics Evaluation

The membrane morphology was determined by scanning electron microscopy (SEM). First, the membranes were submerged in a 20% glycerol solution for at least 4 hours. The samples were then air dried and submerged in liquid nitrogen for 10 seconds. For cross sections, frozen membranes were cracked. All samples were sputter-coated with 5 nm of Pt/Pd with a Q150T Plus (Quorum Technologies) before imaging with a JSM-6010LA electron microscope (JEOL).

The membrane pure water permeability (PWP) was determined by placing 3.0 cm^2 cut-outs of flat membranes (Figure 5.1B) in dead-end filtration cells (Amicon) where water was used as a feed. The feed vessel was pressurised by nitrogen gas and the

feed pressure was maintained at 1 bar. The permeate mass was measured as a function of time and calculated to provide the pure water permeability in $\frac{L}{m^2 * bar * h}$ with *bar* the pressure of the feed and *h* the time in hours.

The retention was measured in a similar set-up, except water was replaced with a 1 g/L bovine serum albumin (BSA) solution or a solution of 1 g/L polyethylene glycol (PEG) of 2, 6, 10, 20, and 35 kDa. PEG and BSA (66.5 kDa) concentrations in the feed and permeate were compared to determine the retention. The BSA concentrations were determined with a 2401PC UV-vis spectrophotometer (Shimadzu). The PEG concentrations were determined via gel permeation chromatography with a 1260 Infinity (Agilent) chromatographer using a 1000 Å, 10 µm 10 Polymer Standards Service Suprema 8x300 mm and a 30 Å, 10 µm column and a 50 mg/L NaN_3 eluent at 1 mL/minute.

Lysozyme concentration determination in solution

To determine the release of lysozyme from the membrane, 10 cm^2 of membrane with or without lysozyme was stored in 1 ml of mQ. At day 1, 2, 4, and 7 the supernatant was sampled and the mQ replaced. The supernatant samples were centrifuged for 30 minutes and evaluated for their absorbance at 281.5 nm, which is a characteristic absorbance peak for lysozyme. The absorbance of the supernatant for the membrane with lysozyme is compared to that of the membrane without lysozyme, as well as the theoretical absorbance that would be expected with lysozyme release based on the measured absorbance spectrum of lysozyme.

Lysozyme Enzymatic activity

Lysozyme activity of the membrane with and without lysozyme was determined *via* a lysozyme activity protocol. For this protocol, a 0.15 mg/ml suspension of lyophilised *micrococcus lysodeikticus* is prepared in a 50 mM potassium phosphate buffer at pH 6.2. Membrane cut-outs of 1 x 1 cm (Supplementary Figure S5.2) with and without lysozyme were incubated in the suspension and as dead-end measurements the suspension was evaluated for absorbance at 450 nm. Active lysozyme cleaves the *micrococcus lysodeikticus* in the suspension leading to a decrease in absorbance at 450 nm over time. In the absence of active lysozyme, the decrease in absorbance is much slower. The activity is expressed in units (U) calculated *via*:

$$Units\ cm^{-2} = \frac{A(T)-A(B)}{0.001*S} \quad (1)$$

Where A(T) and A(B) are the differences in absorbance at 450 nm per minute of the substrate suspension of the membrane with lysozyme and the membrane without lysozyme control respectively. The factor of 0.001 is part of the unit definition. The factor S represents membrane surface area, which is 1 cm^2 (1 cm x 1 cm membrane) unless otherwise specified. One piece of membrane was submerged in 2 ml of bacteria suspension.

Results and Discussion

Aqueous phase separation allows for membrane production to occur completely in an aqueous environment without requiring an organic solvent. This is not only an advantage because of sustainability, but also opens up a natural possibility to incorporate biomacromolecules in the membranes. These biomacromolecules would, with production methods like NIPS, be destroyed or denatured and rendered inactive. Incorporating biomacromolecules like enzymes can functionalise the polymer membranes.

We incorporate the antibacterial enzyme lysozyme by first mixing it with the anionic polyelectrolyte PSS before mixing with the highly alkaline PAH solution. At this high pH, PAH is uncharged. This viscous alkaline PAH/PSS(/lysozyme) solution is cast and coagulated in an acidic precipitation bath resulting in a polyelectrolyte complex membrane, as schematically illustrated in Figure 5.1A. We first evaluate whether the membrane properties such as morphology (via SEM), water permeability, and retention change by the addition of lysozyme. Then we investigate whether lysozyme leaches out of the membrane. Finally, we demonstrate that the membranes have biocatalytic properties.

Membrane characteristics

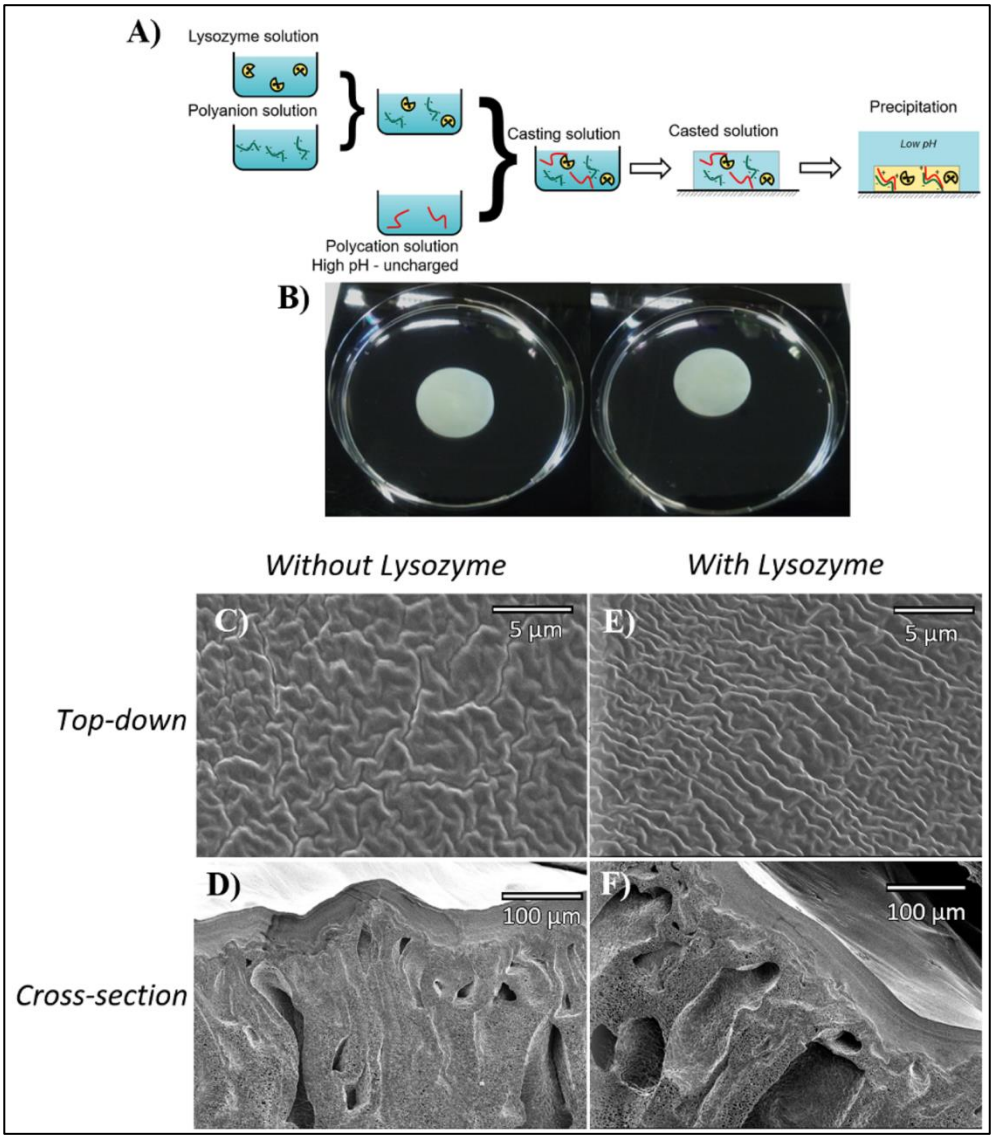


***Figure 5.1**. A) Schematic diagram of the production process. B) Photos of circular 3 cm^2 cut-outs of the membranes without (left) and with (right) lysozyme. C-F) SEM images of PAH/PSS membranes prepared via APS. C, D) Membranes without lysozyme. E, F) Membranes with lysozyme. C, E) Top view of dense selective layer. D, F) Cross-section.*

The PAH/PSS membranes with and without lysozyme appear as slightly elastic white sheets that can be cut in the desired shape (Figure 5.1B) regardless of the absence (left) or presence (right) of lysozyme. They can be comfortably handled with tweezers or hands without breaking. There was no noticeable difference in handling the two membranes. The morphology of the membrane structure was further evaluated with SEM. The surface of both membranes without (Figure 5.1C) and with (Figure 5.1E) lysozyme appear homogeneous without clear pores, where we stress that smaller pores (< 50 nm) would not be visible at these magnifications. Cross-sections of the membranes (Figure 5.1D and F) show a typical membrane structure for phase separation processes consisting of a dense selective layer supported by a porous structure. The thickness of the selective layer of membranes was 42 ± 10 µm (n = 5) without lysozyme or 39 ± 5 µm (n = 5) with lysozyme. Additional SEM images at different magnifications are available in Supplementary Figure S5.1.

Investigation with SEM found no observable difference in membrane structure. Both membranes showed structures consisting of a dense selective layer and an underlying porous structure (Figure 5.1D and F) consistent with previously reported PEC membranes produced via APS. [13–18] These structures are also consistent with what is otherwise expected from NIPS(-like) production processes. [31] There was no significant difference in the thickness of the selective layer or overall morphology of the membrane structure comparing membranes with and without lysozyme.

Enzyme loading has often been associated with a decrease in membrane function due to disruption of the membrane structure. [21,22,32] To study the effect of lysozyme incorporation in the membranes on the functioning of the membranes, the pure water permeability and retention properties of membranes with and without lysozyme were compared. PWP was tested by measuring water permeation over time under controlled pressure. Retention and molecular weight cut-off were determined by comparing PEG (2 to 35 kDa) or BSA (66 kDa) concentrations of the permeate and the feed.

In Figure 5.2, we show the PWP and BSA retention properties of PAH/PSS membranes with and without lysozyme. The PWP of membranes was $11 \pm 2 \frac{L}{m^2 * bar * h}$ without lysozyme and $12 \pm 2 \frac{L}{m^2 * bar * h}$ with lysozyme. The BSA retention of membranes without lysozyme was 95 ± 1 % while the BSA retention of membranes with lysozyme was 93 ± 1 %. The retention of the PEG particles up to 35 kDa was negligible (data not shown). The differences between the membranes for both PWP and BSA retention are not statistically significant.

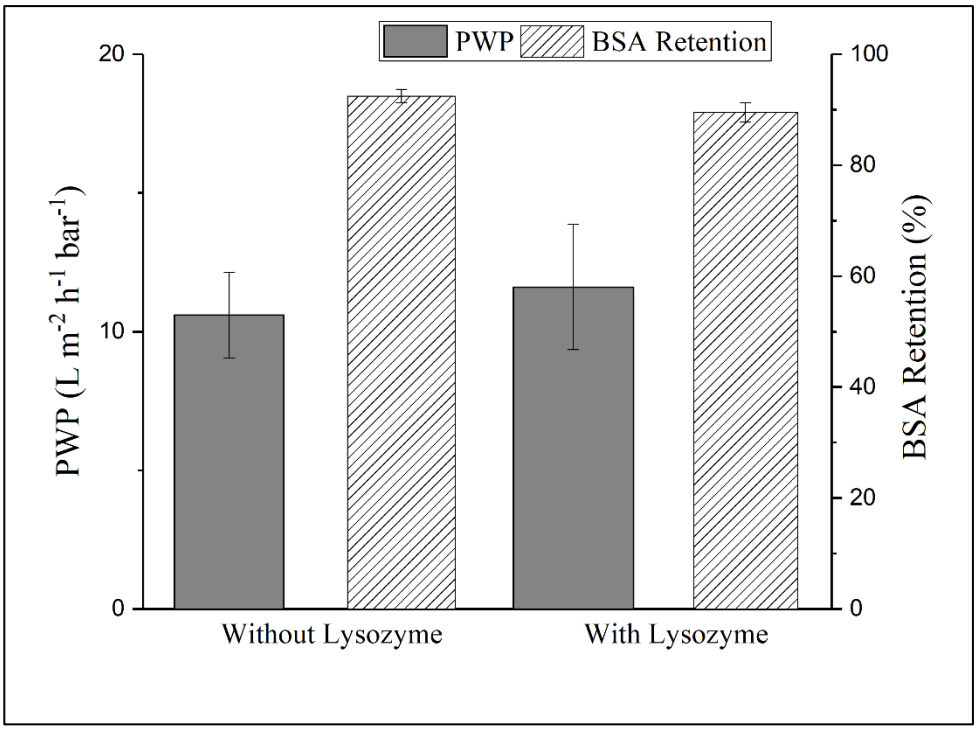


***Figure 5.2**. Membrane performance in terms of PWP and BSA retention. PWP is determined by measuring water permeability at 1 bar of pressure. BSA retention is determined by comparing feed and permeate of a 1 g/L BSA solution filtered through the membranes at 1 bar of pressure. Bars represent averages and error bars represent standard deviation. For PWP, n = 5. For BSA retention, n = 4.*

The PWP values fall within the expected range of high permeabilities for nanofiltration membranes to low permeabilities for ultrafiltration membranes, while the observed protein retention would fit with tight ultrafiltration membranes. Permeabilities of PAH/PSS PEC membranes have been reported to vary strongly depending on production parameters. By varying polyelectrolyte solution concentration, polyelectrolyte molecular weight, and salinity of the precipitation bath, the PWP of PAH/PSS membranes could be varied from ~3000 to ~2 $\frac{L}{m^2*bar*h}$. [13] Other reported APS systems use alternative polyelectrolyte pairs and/or phase inversions, making direct comparisons difficult. Noticeable differences are found in the casting solution total polyelectrolyte concentration, casting thickness, casting substrate, and precipitation bath composition (pH, ionic strength, ion species, crosslinker concentration). [13–19] It is likely that the exact production parameters have a large influence on the properties of the produced membranes, though the exact details are not yet fully understood. Interestingly, the thickness of

the selective layer of our PAH/PSS membranes is approximately an order of magnitude larger than that of a previous reported PAH/PSS membrane, though with a very similar PWP and BSA retention. [13]

Our APS-produced PAH/PSS membranes containing lysozyme are easy to produce and do not show a decrease in permeability or BSA retention when compared to membranes without lysozyme at the reported loading density (Figure 5.2). In contrast, other membranes functionalised with enzymes have previously reported a decrease in permeabilities up to 90 %. [32–36] The incorporation of lysozyme-containing nanotubes has been reported to lead to a doubling of the permeability but with decreased retentions. [37] A decrease in retention properties as a result of enzyme loading has been more commonly observed for other biocatalytic membranes. [21]

Lysozyme stability in the PAH/PSS membranes

Biocatalytic membranes rely on the continued presence of enzymes for their catalytic functionality. Enzymes that are physically or covalently bound to membranes may leach from the membrane over time resulting in a loss of biocatalytic activity or can become inactive. To test whether lysozyme leaches out of our PAH/PSS membranes, we stored membranes in ultrapure water and evaluated the absorbance of the storage water at 281.5 nm, a characteristic absorbance wavelength of lysozyme. We compare this absorbance with the storage water of lysozyme-free membranes after 1, 2, 4, and 7 days using UV-vis absorbance photospectrometry. From this comparison we determined whether lysozyme remained in the membrane.

For both membranes with and without lysozyme we observed increased absorbance at 281.5 nm in the aqueous storage medium (Figure 5.3). While the average absorbance of the storage water of the membrane containing lysozyme was consistently lower than the absorbance of the storage water without lysozyme, there is no statistically significant difference (t-test, $p < 0.05$) between the two conditions.

The observed presence of absorbance at 281.5 nm can be explained by the presence of soluble PAH/PSS complexes. Due to the processing of the PAH/PSS membranes in 1 cm^2 segments, trace amounts of PAH/PSS PEC fray from the edges and remained in solution. The absorbance detected is consistent with the absorbance spectrum for PAH/PSS PECs while the characteristic absorbance spectrum for lysozyme is not detected (Supplementary Figure S5.3). When lysozyme is incorporated in PAH/PSS PECs, the amount of lysozyme released in the aqueous phase was determined. Less than 1 % of lysozyme was released in the aqueous phase (Supplementary Figure S5.4). Only at salt concentrations above 0.5 M NaCl we observed a significant lysozyme release. PAH/PSS membranes produced via APS

have been reported to be stable in concentrations of 1 M NaCl or 1 M KBr for at least 5 days. [13]

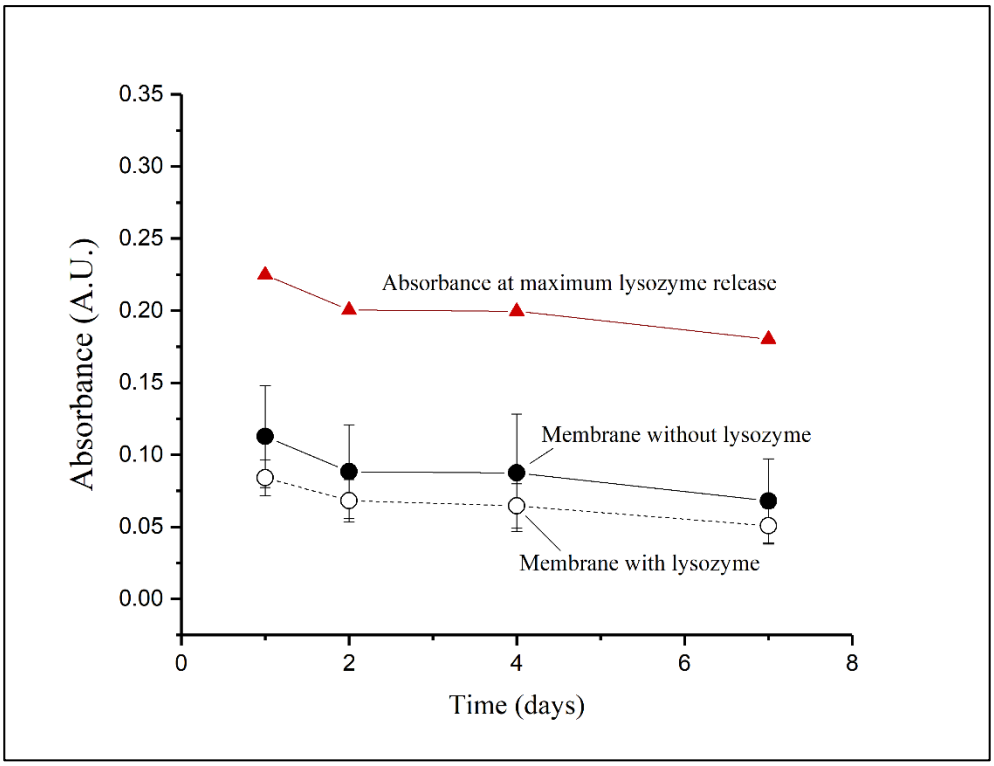


Figure 5.3. *Absorbance at 281.5 nm of supernatant storage water of PAH/PSS membranes with (○) lysozyme, without (●) lysozyme, and the hypothetical absorbance expected (▲) at complete lysozyme release. Values are represented as averages with standard deviation (n = 3).*

Lysozyme activity in PAH/PSS membranes

Lysozyme is an antibacterial enzyme that operates by degrading the cell walls of gram-positive bacteria. To study whether lysozyme remains active when incorporated into the PAH/PSS membranes, the enzymatic activity of lysozyme-containing membranes was investigated on a substrate of lyophilised gram-positive *micrococcus lysodeikticus* bacteria. We evaluated the enzymatic activity of the lysozyme-containing membranes on the day of membrane fabrication, as well as after one week of storage in water to determine the enzyme stability over time.

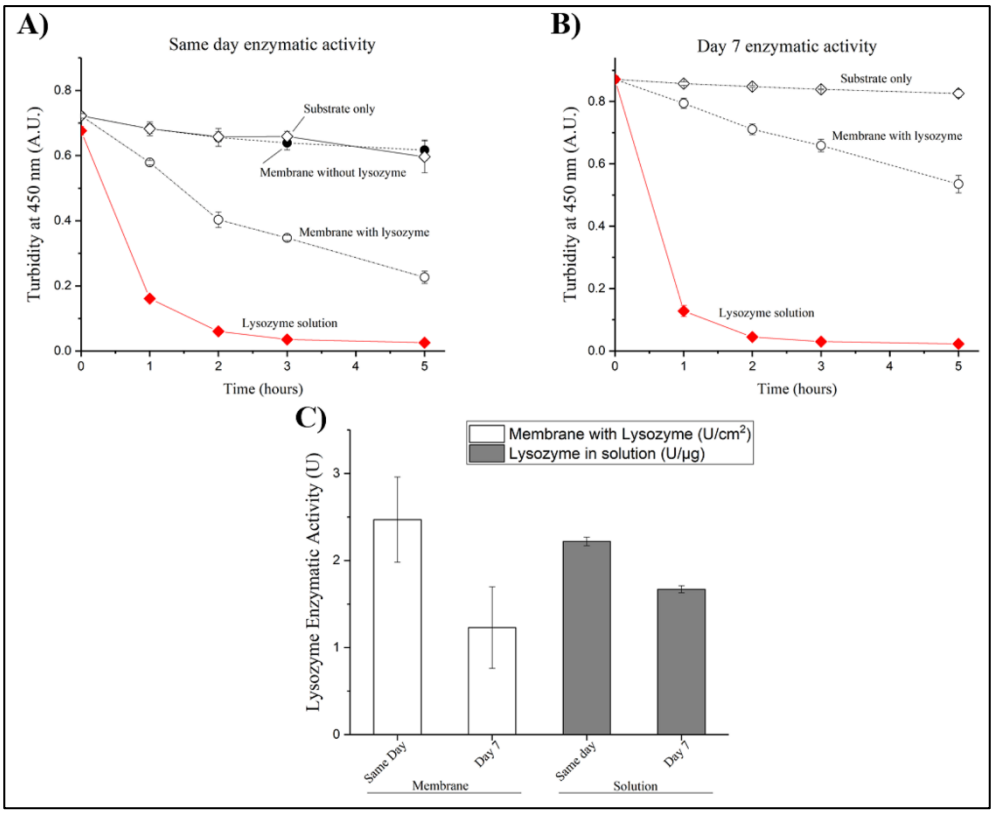


***Figure 5.4**. Enzymatic activity of PAH/PSS membranes containing lysozyme. The absorbance at 450 nm caused by the bacteria substrate in suspension decreases over time in the presence of active lysozyme. A) Lysozyme-containing membrane (○) made and evaluated on the same day compared to membranes without lysozyme (●), substrate only (♦), and 0.19 mg/L lysozyme in solution (♦) and B) lysozyme-containing membrane stored for one week in water, compared to 0.38 mg/L lysozyme in solution. In C) the enzymatic activity calculated from A) and B) are shown for both membranes with lysozyme as well as lysozyme in solution. All Values are shown as averages with error bars representing standard deviation (n = 3).*

The enzymatic activity of PAH/PSS membranes with and without lysozyme is shown in Figure 5.4. The activity of lysozyme is characterised by a decrease in solution turbidity at 450 nm caused by the degradation of the bacterial substrate by lysozyme (Figure 5.4A and B). Lysozyme-containing membranes had a lysozyme load of 4.49 ± 0.41 µg/cm^2 (n = 5). Using Equation 1, the activity of lysozyme-containing membranes was calculated (Figure 5.4C). For the membranes, the activity was 2.47 ± 0.49 U/cm^2 on the day of membrane preparation and 1.23 ± 0.47 U/cm^2 after one week in storage. PAH/PSS membranes without lysozyme did not display any enzymatic activity. The fresh lysozyme in solution had an activity of

2.22 ± 0.05 U/µg. After one week, the activity of the lysozyme in solution had decreased to 1.67 ± 0.04 U/µg.

The activity of membranes without lysozyme is similar to the activity of the substrate without added enzyme (Figure 5.4A), indicating that the PAH/PSS membranes do not have an inherent catalytic activity on the bacterial substrate that can be mistaken for lysozyme's enzymatic activity. Membranes containing lysozyme show a decrease in absorbance at 450 nm, indicating the presence of active lysozyme. The activity of lysozyme-containing membranes per mass of lysozyme (0.55 U/µg) is lower than that of lysozyme in solution (2.22 U/µg).

The decrease in enzymatic activity of lysozyme-loaded membranes compared to lysozyme in solution is likely the result of diffusion limitations of the bacterial substrate posed by the membrane matrix (Supplementary Figure S5). [38] Lysozyme molecules located deeper in the membrane structure might not react with the relatively large bacterial substrate that is unable to penetrate the membrane structure. The activity of lysozyme is approximately halved by the high and low pH conditions similar to those of the membrane production process when incubated at low pH, high pH, or both in sequence (Supplementary Figure S5.6).

For membranes with lysozyme immobilised on the surface, a large drop in activity has previously been reported. [39] Here, membranes were functionalised with 2.5 mg/cm^2 of lysozyme resulting in an activity of 1.5 to 4.8 U/cm^2. In contrast, our PAH/PSS PEC membranes were loaded with 4.49 µg/cm^2 of lysozyme resulting in an activity of 2.5 U/cm^2. [39] This comparison demonstrates that our method for the incorporation of lysozyme can be done with 500x less lysozyme, while still resulting in a comparable enzymatic activity.

The activity of lysozyme-containing membranes decreases by 50.2 % over the course of one week, compared to an activity decrease of 24.8 % for lysozyme in solution. A decrease in activity can be caused by inactivation of the enzyme over time as both the membrane and the lysozyme solution were stored in ultrapure water. Alternatively, the activity of lysozyme has been reported to decrease when complexed with PSS in solution. [40] We also observed a decrease in lysozyme activity when measured in the presence of PAH or PSS in solution (Supplementary Figure S5.7)

Decreased enzymatic activity has not been observed for for all enzyme-polyelectrolyte systems, suggesting that for future APS systems the polyelectrolyte pairs can be chosen specifically so that they conserve or even enhance the activity of the functional enzymes. [41]

Enzymes have also been incorporated in polyelectrolyte multilayer (PEM) membranes. With PEMs, oppositely charged polyelectrolytes are deposited on a support membrane to create alternating layers. Instead of exclusively using polyelectrolytes, biomolecules such as enzymes or nucleic acids can be assembled into the PEM structure. [42] In several studies, enzymes were incorporated in a PEM and enzymatic activity was still observed. [43,44] A study incorporating the protein-digesting enzyme trypsin in a PEM nanofiltration membrane reported that the relative activity of trypsin in solution was equal to that of trypsin embedded in two types of PEMs. [44] The incorporation of enzymes in PEMs can result in enhanced enzyme stability; while a decrease in enzymatic activity similar to that of our membranes was observed, this decrease took 10 - 12 days as opposed to our observed 7 days. [44] However, the increase in enzyme stability comes at the cost of enzymes leaching from the PEMs over time, which was not observed in our system. [45] Loading PEMs with biomolecules is often done in the context of drug delivery where loss of the loaded compound over time is a desired property of the system. [42] However, PEM fabrication is often time-consuming and additional chemical processing steps are often needed to guarantee successful enzyme loading.

The immobilisation of lysozyme (or other enzymes) on various films and membranes via traditional methods can also take many additional processing steps and require different chemical treatments and take up to multiple days. [21,38,39,46–51] In one study, where lysozyme was added directly to the membrane casting solution (more similar to membranes produced via APS), the lysozyme required (covalent) grafting to a protective agent via additional processing steps. [37] In contrast, incorporating lysozyme in PEC membranes produced via APS is as simple as diluting commercially available solutions of the anionic polyelectrolyte with a lysozyme solution (in ultrapure water) in place of diluting with ultrapure water. This process takes no additional time (excluding preparation of the lysozyme solution) and no additional chemical steps (Figure 5.1A). The ease of enzyme loading is a substantial benefit of APS over other biocatalytic membrane production techniques.

Conclusion & Outlook

Aqueous phase separation is a new membrane production method first reported in 2019. Since the first publications, various different polyelectrolyte pairings have been reported that can result in stable APS membranes. [13–20] In this study we demonstrated the first biocatalytic membrane produced via APS.

We found that the enzyme lysozyme can be incorporated into PAH/PSS membranes produced via APS by a single addition step during production and requires no additional post-production modification process. The APS-produced PAH/PSS

membranes functionalised with lysozyme show enzymatic activity consistent with that of lysozyme in solution. The enzymatic activity remains for at least one week. In addition, the membrane characteristics such as the morphology, pure water permeability, and BSA retention are unaffected by the addition of lysozyme at the tested lysozyme loading concentration. We envision that biocatalytic membranes produced via APS, based on the proof of concept reported here, can compete with biocatalytic membranes produced via traditional methods.

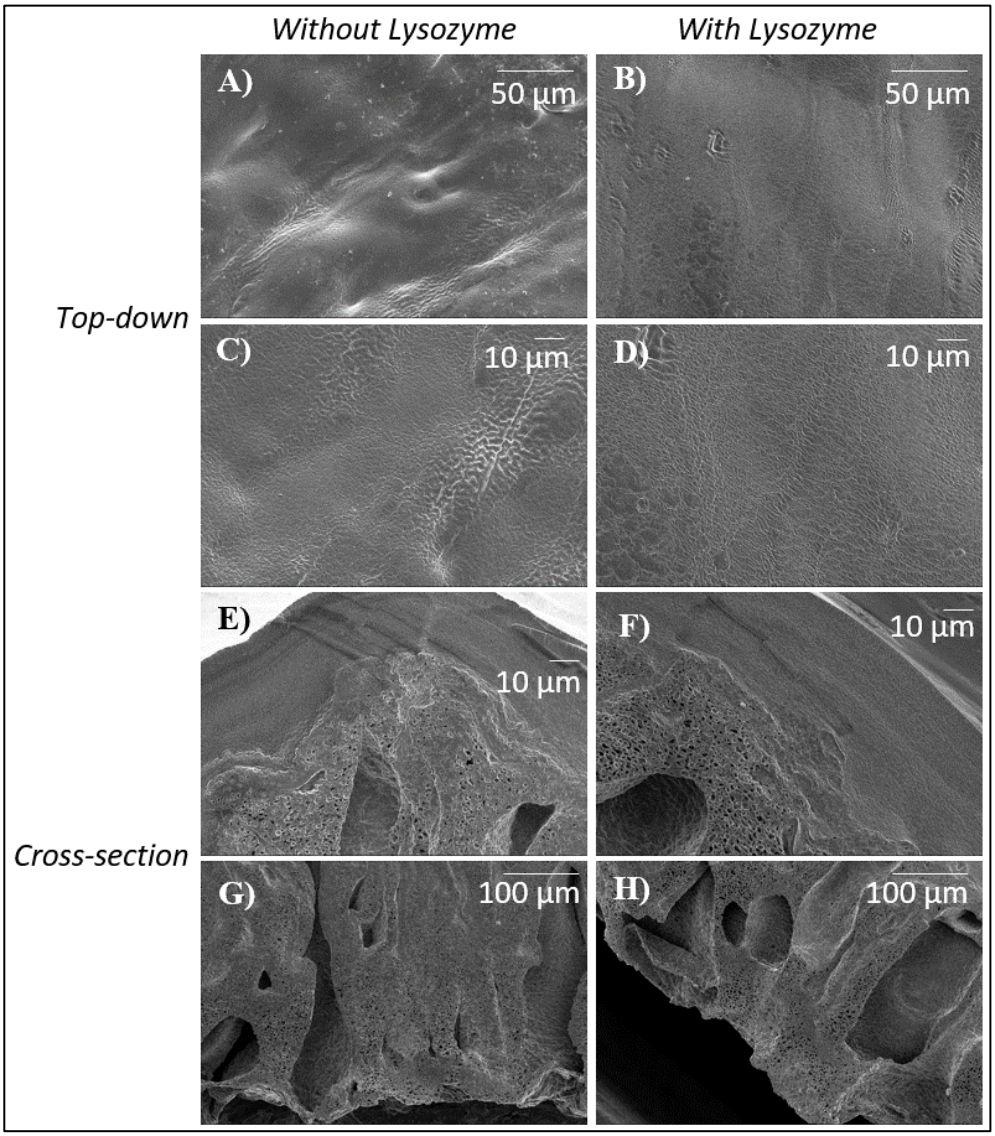
There are many enzymes which have been explored in membrane technology with the potential to provide interesting functionality. Biocatalytic membranes produced via traditional methods like NIPS have been modified with enzymes such as oxidoreductases, hydrolases, laccases, and horseradish peroxidases are currently immobilised on membrane surfaces in order to counteract membrane fouling or degrade various micropollutants. [23,52–55] Applications of biocatalytic membranes are also suggested in biomedical engineering. Hybrid transplanted tissue survival containing polymer membranes is often hampered by the slow growth of blood vessels which may be remediated by incorporating proteins such as vascular endothelial growth factor in the membrane structure, [56-58] In addition, biocatalytic membranes have also been suggested for use in dialysis. [59,60] The advent of laboratory-driven gain-of-function evolution may even open up the production of enzymes that do not occur naturally for customised biocatalytic activity. [61–63]

Acknowledgments

The authors acknowledge funding from the Netherlands Organization for Scientific Research (NWO) VIDI grant (# 723.015.003).

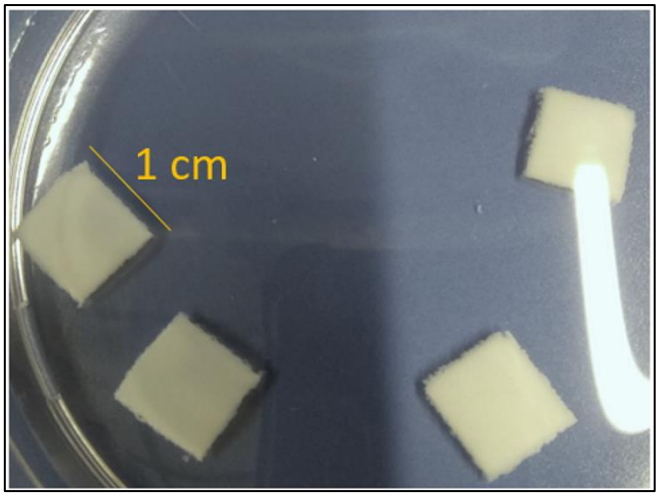
Supplementary Information

Additional SEM images



Supplementary Figure S5.1. *Additional SEM images of A-D) top-down selective layer view with A and C being membranes without lysozyme and B and D membranes with lysozyme. E-H) cross-section view with E and G being membranes without lysozyme and F and H membranes with lysozyme.*

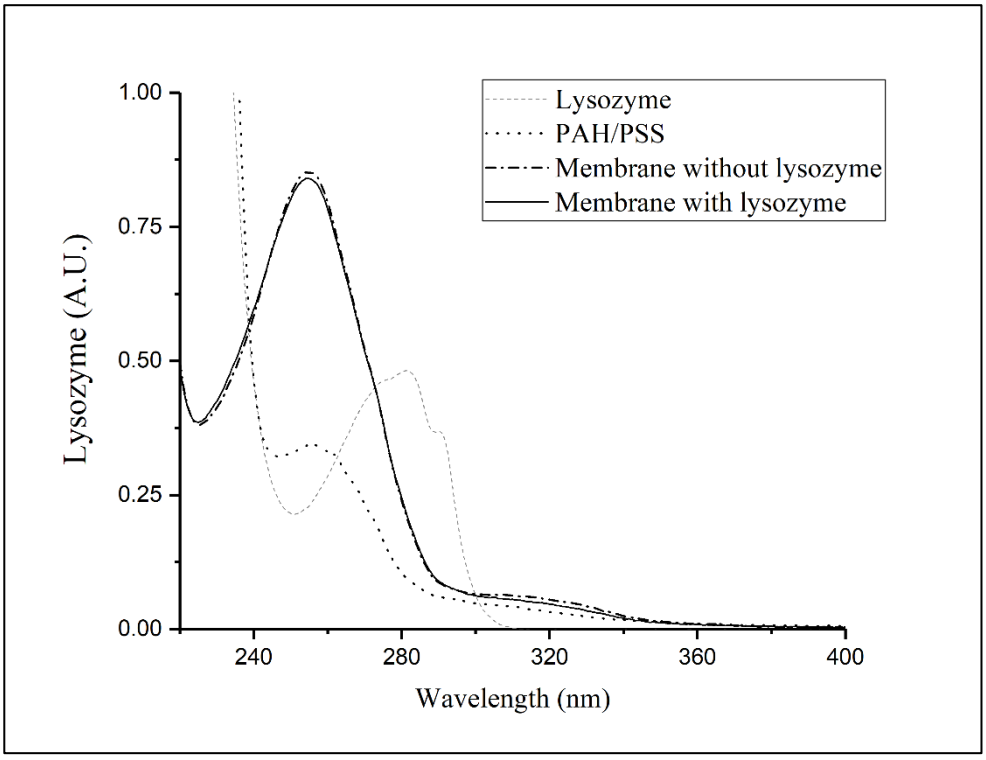
Post-production membrane processing



***Supplementary figure S5.2**. Post-production processing of PAH/PSS membranes resulting in square cut-outs of PAH/PSS membranes containing lysozyme for evaluation of enzymatic activity.*

PAH/PSS membranes produced via APS become white elastic sheets. These sheets can be manipulated by hands or tweezers without breaking. For further analysis of permeability, retention, and enzymatic activity, processing the membranes into specific shapes was desired. Besides the round shape of Figure 5.1B, the membranes could easily be cut into additional shapes such as 1 cm^2 squares (Supplementary Figure S5.2) and still be handled afterwards.

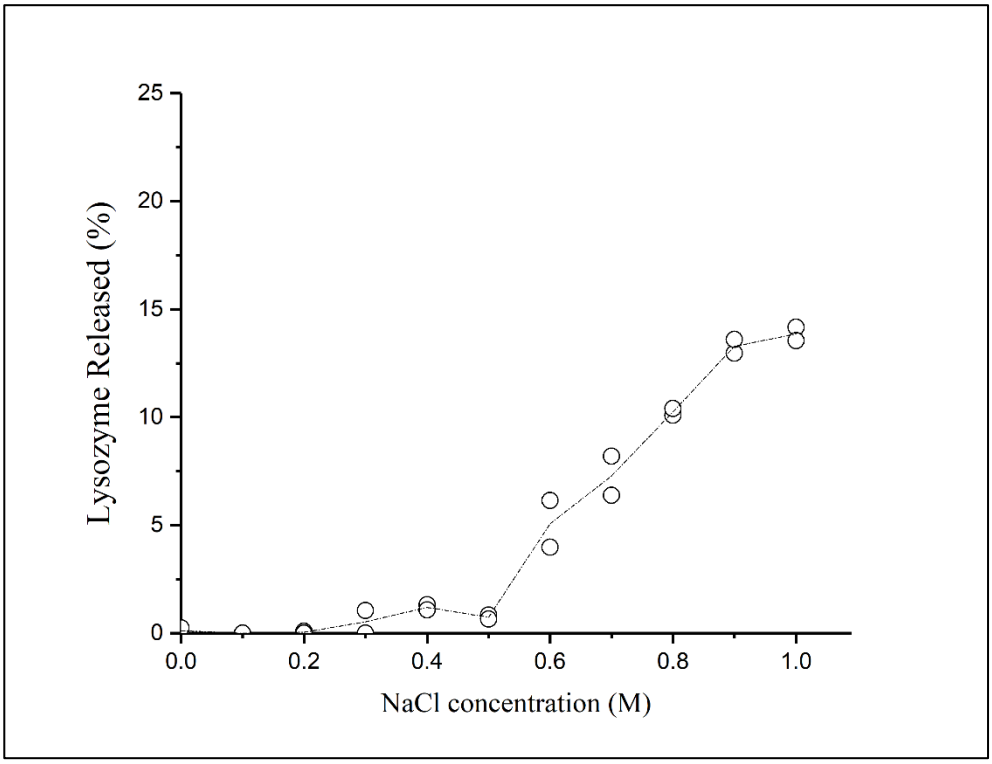
Absorbance spectra of lysozyme, soluble PAH/PSS PECs, and storage water



***Supplementary figure S5.3**. Absorption spectra for 0.2 g/L lysozyme (dashed line), soluble PAH/PSS complexes taken from the supernatant of a 2 g/L PAH/PSS complex (dotted line), the storage water of 10 cm^2 membranes without lysozyme at day 1 (solid line), and storage water of 10 cm^2 membranes with lysozyme at day 1 (dash-dot line). The spectrums are based on single representative measurements.*

In Figure 5.3, the amount of leaking lysozyme from the PAH/PSS membrane containing lysozyme is determined via UV-vis measurement of the water in which the membrane is stored. In Supplementary Figure S5.3, several absorbance spectra of the relevant components are shown. The lysozyme concentration is determined relative to its peak at 281.5 nm. Soluble PAH/PSS complexes show a peak at 255 nm and an elevation without a peak at 281.5 nm. The storage water of both membranes with and without lysozyme similarly show a peak at 255 nm and an elevation without a peak at 281.5 nm. We conclude that the absorbance seen in Figure 5.3 is then likely the result of the absorbance caused by dissolved PAH/PSS complexes and not by lysozyme.

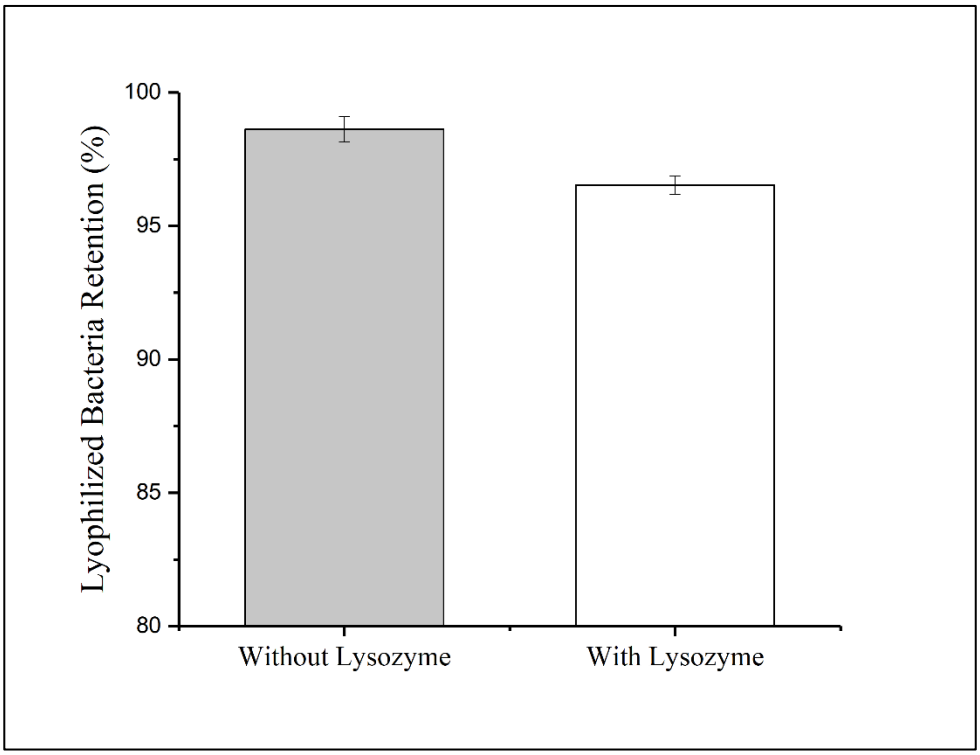
Lysozyme stability in PAH/PSS PECs



Supplementary figure S5.4*. The release of lysozyme from PAH/PSS PECs as a function of increased NaCl concentration. Empty circles (○) represent individual measurements from duplicates. The line connects the averages of duplicates.*

Lysozyme, once incorporated in PECs, is resistant to expulsion from the complex. Increasing ionic strength by addition of NaCl is a common method to disrupt the PECs. First, 1 g/L lysozyme was incorporated in 2 g/L PAH/PSS PECs at charge stoichiometry. Following, the PECs are submerged in NaCl solutions of 0 to 1 M. After one day the released lysozyme is measured with UV-vis spectrophotometry after which this is expressed as a percentage of total incorporated lysozyme (Supplementary Figure S5.4). Without significant concentrations of NaCl, lysozyme remains within PAH/PSS PECs. Without NaCl addition (0 M), only 0.12 % of the lysozyme content was released after one day.

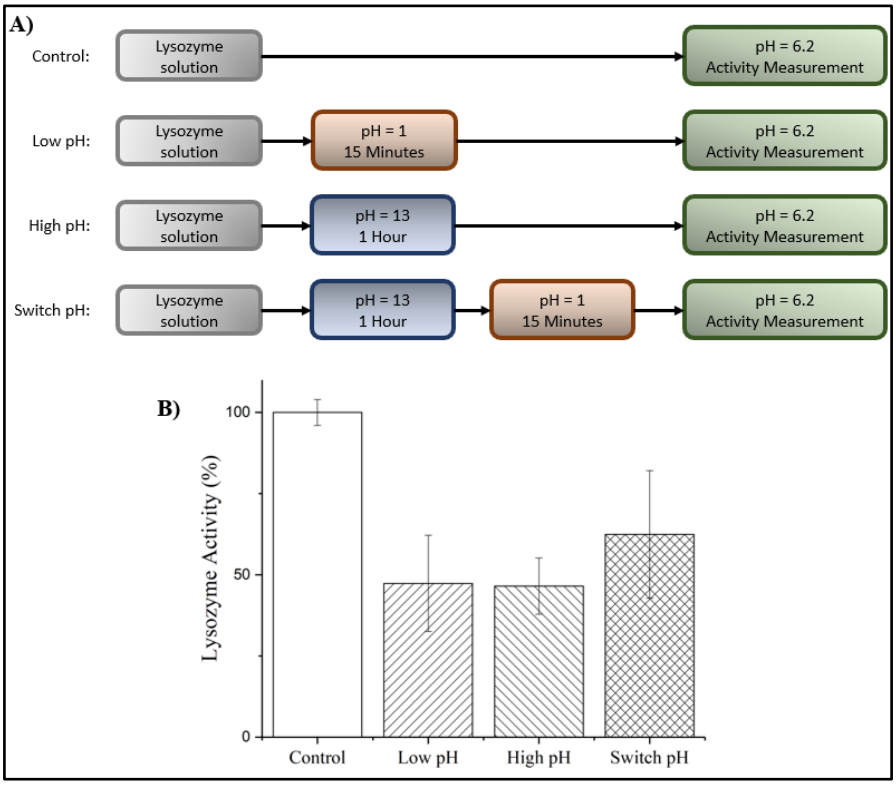
PAH/PSS PEC membranes are impermeable for a bacterial suspension of lyophilised *micrococcus lysodeikticus*



***Supplementary figure S5.5**. The retention of lyophilised micrococcus lysodeikticus bacteria suspended in ultrapure water by PAH/PSS PEC membranes without (left) and with (right) lysozyme. Values represent averages and error bars represent standard deviation with n = 4.*

The PAH/PSS PEC membranes produced via aqueous phase separation as described retain BSA dissolved in water (Figure 5.2B). A similar retention test is done for Supplementary Figure S5.5 with 150 mg/L suspension of lyophilised *micrococcus lysodeikticus* in ultrapure water in place of a BSA solution. The retention is determined by comparing the concentration of the bacteria suspension (determined by turbidity at 450 nm) in the permeate with that in the feed.

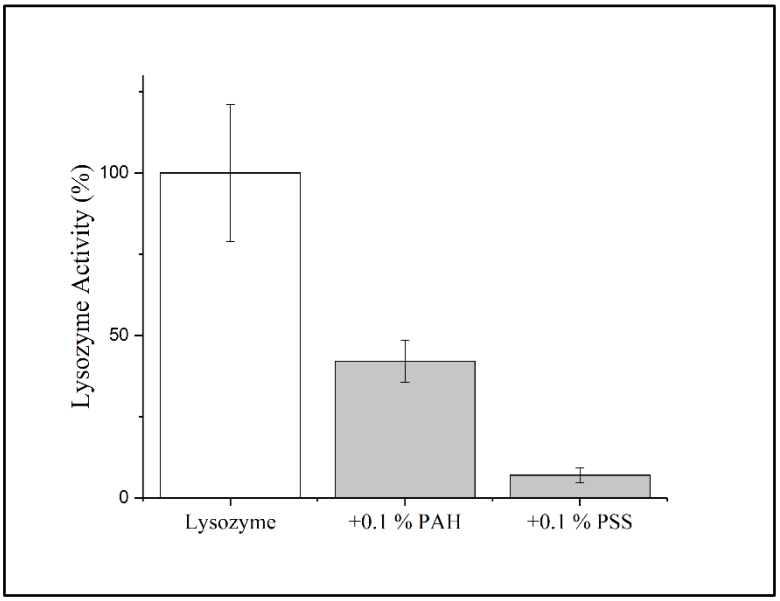
The activity of lysozyme after incubation at low and high pH



Supplementary Figure S5.6. *The activity of 7.5 mg/L lysozyme solution after it has been incubated at low (1) and high (13) pH analogous to the membrane production method. Activity was determined by adding 50 µl of the final lysozyme solution to 2 ml of bacteria suspension. A) Schematic representation of the lysozyme incubation conditions and B) activity of the various conditions. Values are averages and error bars represent standard deviations with n = 3.*

Enzymes can be sensitive to environmental conditions. In the membrane production process lysozyme is exposed to high and low pH conditions for approximately an hour and approximately 15 minutes respectively. To investigate whether the activity of lysozyme was (negatively) affected by this process, lysozyme was incubated in these conditions, and then evaluated for activity (Supplementary Figure S6A). The activity of lysozyme approximately halves after pH treatment regardless of whether it is incubated at a high or low pH (Supplementary Figure S6B). While activity decreases as a result of the pH inversions used in the production process, it does not disappear entirely.

The activity of lysozyme in the presence of PAH or PSS



Supplementary Figure S5.7*. The activity of lysozyme solution (7.5 mg/L) compared to the activity of lysozyme in the presence of 0.1 % PAH or 0.1 % PSS. Activity was determined by adding 50 µl of the final lysozyme solution to 2 ml of bacteria suspension. Values represent averages and error bars represent standard deviation with n = 3.*

The activity of a lysozyme solution is compared to the activity in the presence of 0.1 % PAH or PSS in Supplementary Figure S7. In the presence of PAH, the activity of lysozyme drops to 42.0 ± 6.4 %. In the presence of PSS, the activity of lysozyme drops to 7.0 ± 2.3 %. There are several possible mechanisms for the decrease in activity. The polyelectrolytes can interfere with the proper functioning of lysozyme's active site, preventing the cleaving reaction. Likewise, the polyelectrolytes can interfere with the bacterial membranes, preventing access to lysozyme's active site. It is also possible that the polyelectrolytes simply act as a spacer material, decreasing the likelihood that lysozyme's active site successfully finds a suitable cleavable site on the bacterial membrane.

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Chapter 6

Outlook

This dissertation is about the use of polyelectrolyte complexes (PECs) for two types of separation processes. In the first type of separation process, PECs are used as extraction media for several molecules. This topic is covered in chapters 2 to 4. The second type of separation process is the use of biocatalytic membranes consisting of PECs produced *via* aqueous phase separation (APS) and supplemented with enzymes. This topic is covered in chapter 5. Consistent themes for both separation processes is the focus on proteins and PECs. For the extraction this is primarily the ability to (selectively) partition lipases, lysozyme, and succinylated lysozyme into the PECs. For the membrane this is the ability to incorporate lysozyme in its structure resulting in a bioactive membrane.

But no (scientific) work is ever complete or without limitations. The work presented here is no exception. In this chapter, the research of the previous chapters is reflected upon and future research paths in the field of separation processes using PECs and biocatalytic membranes produced *via* APS are suggested. This chapter is divided into thematic sections. Each section covers avenues of research towards a better understanding of the phenomena which we observe and/or suggests other applications.

Polyelectrolyte Choice

When polyelectrolytes were introduced in chapter 1, it was briefly addressed that the structure of (synthetic) polyelectrolytes is only really limited to imagination and practical synthesis feasibility. Yet the choice of polyelectrolytes used throughout this manuscript has been limited to only a few. Poly(acrylic acid) (PAA) is used for chapters 2 to 4. Poly(allylamine hydrochloride) (PAH) is used for chapters 3 to 5. Poly(styrene sulfonate) (PSS) is used for chapters 4 and 5. If we indeed have a near-infinite choice of polymer structures, there must have been reasons for these choices. A straightforward reason is availability. If PECs are ever to become a relevant extraction media, it helps if the polyelectrolytes used are readily available and inexpensive. All of the polyelectrolytes used in this dissertation are already in use for different applications and both science and industry are generally familiar with their behaviour. Another reason is that PAA and PAH are some of the most structurally simple synthetic weak polyelectrolytes available. Similarly, PSS and poly(diallyldimethylammonium chloride) (PDADMAC) are two of the most commonly used synthetic strong polyelectrolytes. In Figure 4.2 the partitioning behaviour of lysozyme in four different PEC systems is indeed similar (but not identical) which suggests that partitioning is a generic effect for a specific protein depending on the composition of the complex (expressed in polyanion fraction F^-) and that it is mostly (but not completely) independent of the polyelectrolytes used.

If the partitioning potential of lysozyme is indeed not polyelectrolyte-specific, it makes little sense to use expensive and complicated polyelectrolytes.

Natural polyelectrolytes

A future perspective on the choice of polyelectrolytes is the use of natural polyelectrolytes. While PAA, PAH, PSS, and PDADMAC may be relatively inexpensive and widely available, they are still derived from petroleum chemistry. With an increase in attention for green chemical processes and natural alternatives it will be beneficial to look at natural polyelectrolytes. Polycarbohydrates, most commonly carboxymethyl cellulose (CMC), are already safely used as additives to foodstuff to enhance viscosity and stabilise emulsions. [1,2] Future extractions may benefit from the use of hybrid synthetic/natural PECs (like PAH/CMC) or fully natural PECs (like chitosan/CMC). We have performed some initial experiments with chitosan/CMC PECs and studied the partitioning of lysozyme. The partitioning profile of lysozyme into these PECs is broadly similar to the partitioning into PAH/PAA PECs, though more experiments are needed to be able to definitively conclude if using natural PECs as extraction media is feasible.

A similar approach is possible for PEC membranes produced *via* APS, the technique used in chapter 5. One or both of the polyelectrolytes used in APS could be substituted by natural polyelectrolyte alternatives. Considerable research is already underway regarding the use of natural polymers for non-APS membrane production and it makes sense to extend this to APS. [3–6] A major challenge with using natural polyelectrolytes is decreased mechanical stability as a result of weaker intermolecular interactions and high hydrophilicities compared to synthetic polymers and polyelectrolytes. [7,8] Some suggestions on how to overcome these disadvantages are by using a blend of natural polymers and synthetic materials, or by crosslinking the natural polymers more thoroughly. [7,8] Some inspiration for how natural PECs can be made with good mechanical properties can be found in how certain animals use PEC-based adhesives. [9–11]

Understanding Partitioning Behaviour

Throughout chapters 2 to 4 there has been an elephant in the room; the lack of an encompassing mechanism to explain and predict the partitioning behaviour of compounds within PECs. From chapter 3, it is clear that the (net) charge of proteins plays a very important role in the partitioning behaviour. Modelling has shown that protein charge heterogeneities and net charges affect the structure of the protein-

polyelectrolyte aggregates, with a higher degree of charge heterogeneities leading to the formation of more tightly-packed aggregates. [12] This charge anisotropy (charge 'patchiness') also influences the behaviour of protein-polyelectrolyte mixtures. [13-14] Charge anisotropy has also been suggested as an explanation for the lysozyme partitioning behaviour in chapter 3 where the ideal lysozyme partitioning composition of the PEC is not found at PEC charge stoichiometry. [15,16] The suggested explanation is that the electrostatic interaction between proteins and PECs primarily takes place at charge patches and as a result the patch location and structure are more important than the overall net charge of the whole system.

The ionic strength of the solution, typically controlled by varying the NaCl concentration also plays a role in partitioning behaviour, as shown in chapter 2. A slightly higher ionic strength (in the range of 0 – 100 mM NaCl) generally improves partitioning, except when it doesn't (such as for the CALA lipase in Figure 2.3D, where low ionic strength first results in a decrease in partitioning). At the relatively low ionic strengths we use, charge regulation plays an important role and may influence the total charge of the complex even if the polyelectrolytes themselves were mixed at charge stoichiometry. Ionic strength affects the degree to which protein charge is affected by pH. [17] It is similarly possible that the ionic strength influences the degree to which charge regulation effects between proteins and the PEC operate. Ionic strength could influence the electrostatic interaction between PEC and protein resulting in different portioning behaviour at different ionic strengths.

As a final major factor, chapters 2 to 4 noted that the composition of the PECs (quantified as the fraction of negative monomer charge F^-) was an extremely important factor in determining partitioning behaviour. For lysozyme, maximum partitioning was found close to charge stoichiometry (*i.e.*, $F^- = 0.50$) or in the slightly anionic region (F^- around 0.60 - 0.70). For lipases it was found consistently in the cationic region (F^- around 0.25 to 0.40). While it is tempting to attribute this difference to the expected charge of the enzymes (lysozyme is cationic at pH 7 while most lipases are anionic), this does not seem to explain all observations. The maximal partitioning PEC composition for lysozyme in chapter 4 was found to have slight variation based on the polyelectrolyte chemistry, with PECs containing PDADMAC showing a small shift to the anionic region ($F^- = \sim 0.60$) compared to PECs containing PAH ($F^- = \sim 0.50$). A method has recently been published that suggests a method to measure the actual charge of a PEC using H-NMR. [18] For the future, comparing the calculated PEC charge and comparing it to the measured charge might provide insights on how complexation influence the PEC charges and potentially how the protein and PEC influence each other.

There is quite some literature available on the extraction of proteins from a solution using single polyelectrolytes where the mechanisms are more straightforward and

more clearly understood. [19] There are comparatively few other studies that explore the partitioning of proteins or even other compounds in PECs. [16,20-24] Translating all these findings into concrete design rules for PECs specialised in extracting a single compound is tricky; all these studies use slightly different system parameters and as we have shown, small differences in system parameters can be extremely important for the partitioning of specific molecules into the PECs.

Partitioning of α-lactalbumin, eGFP, and BSA in polyelectrolyte complexes

We have investigated additional protein-PEC systems not discussed in any of the previous chapters. A selection is shown in Figure 6.1. The protein α-lactalbumin is from the same protein superfamily as lysozyme and the two are structurally similar. [25] However, the surface charge distribution between the two is very different (Figure 6.1E). [26] Perhaps as a result its partitioning behaviour (Figure 6.1A) in PAH/PAA PECs is different from that of lysozyme. Where lysozyme has a distinct optimal partitioning composition around F^- = 0.5, 0.6 (chapter 4), or 0.65 (chapter 3) where (nearly) all lysozyme has partitioned into the PEC, α-lactalbumin shows no such clear and narrow optimum and instead the maximal partitioning into the PEC hovers around 20 – 40 %.

Unlike lysozyme, both enhanced Green Fluorescent Protein (eGFP) (Figure 6.1B) and bovine serum albumin (BSA) (Figure 6.1D) lack a narrow optimum F^- where a maximal amount of protein into PAH/PSS PECs is observed. We instead see a gradual partitioning of BSA or eGFP as a function of F^-. The result is a broad distribution around an optimum PEC composition in contrast to the sharp peak seen with lysozyme. To further complicate this matter, BSA partitioning in PDADMAC/PSS PECs (Figure 6.1C) does show a sharper peak in protein partitioning as a function of PEC composition more reminiscent of lysozyme partitioning. Currently we have no verified explanation for these differences in protein partitioning as a function of PEC composition, though we can speculate. For instance, lysozyme is a more structurally compact globular protein whereas BSA is larger and more structurally flexible. [27] This flexibility might help BSA structurally reform to engage in a more electrostatically or entropically favourable interactions in the PEC. This flexibility translates to a larger range of PEC compositions where the proteins can successfully engage in electrostatic interactions with the PECs. In addition, BSA is simply larger than lysozyme and has more charge patches on its outer surface that can interact with the PECs. [16]

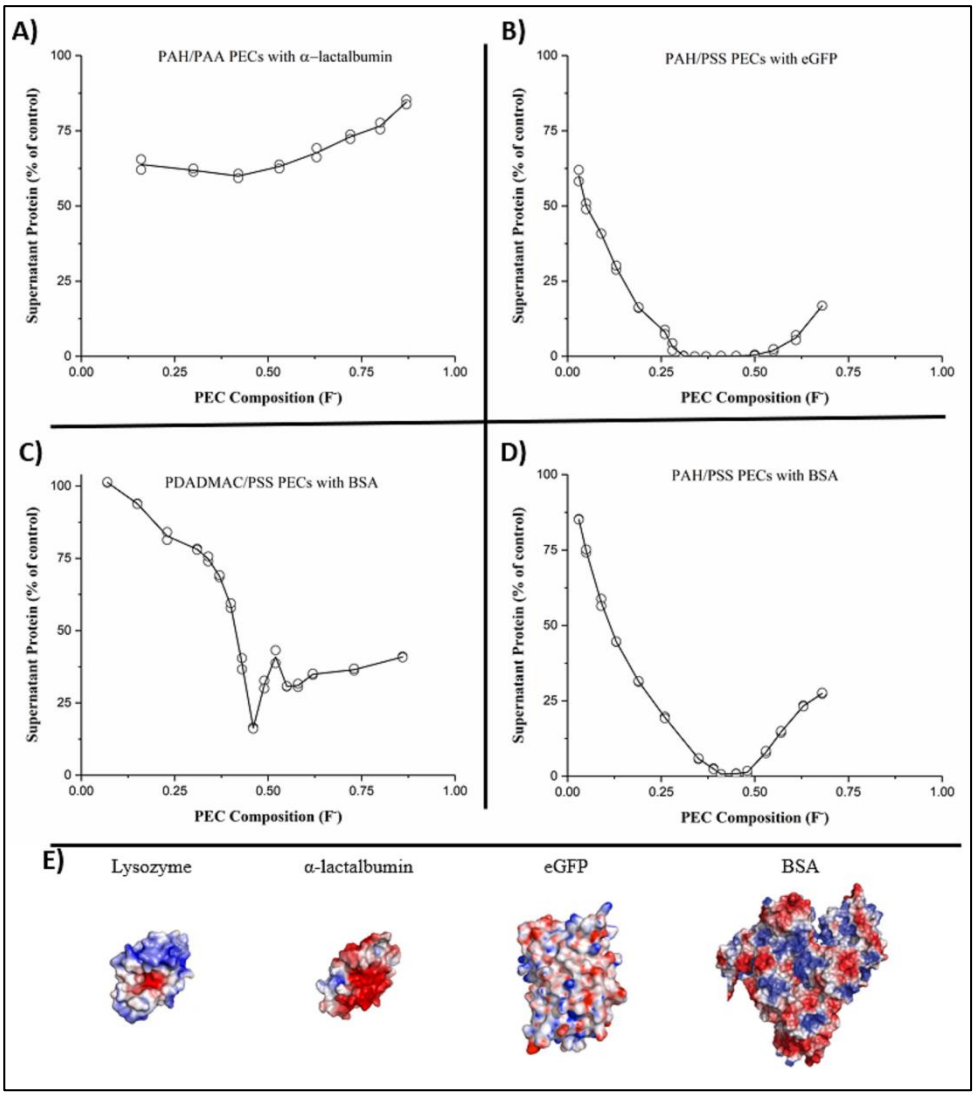

Figure 6.1. *Partitioning behaviour of A) α-lactalbumin in PAH/PAA PECs at various compositions, B) eGFP in PAH/PSS PECs at various compositions, C) BSA in PDADMAC/PSS PECs at various compositions, D) BSA in PAH/PSS at various compositions, E) surface charge distribution plots of the relevant proteins, sourced with permission from* [26,28,29]. *Red indicates negative charge and blue indicates positive charge. Data points show individual duplicates, and the line connects the average of two duplicates. Total polyelectrolyte concentration was 2 g/L, α-lactalbumin and BSA concentrations were 1 g/L, eGFP concentration was 0.28 g/L. The concentration of α-lactalbumin and BSA was determined via tryptophan emission fluorescence (excitation 280 nm, emission 350 nm), the concentration of eGFP was determined via emission fluorescence (excitation 488 nm, emission 507 nm). Quantity is expressed relative to a control without polyelectrolytes.*

Research paths to understand partitioning

While speculation on the nature of the partitioning behaviour is all well and good, the fact remains that for now the exact mechanism remains unclear. There are two structural pathways to gain a better understanding of the mechanism; directed high-throughput empirical analysis and *in silico* simulations validated by empirical analysis. High-throughput analysis of the various system parameters (such as ionic strength, temperature, PEC composition, compound concentrations) can help map out which parameters are important for which polyelectrolyte pair and partitioned compound. We already know from chapter 3 that succinylation of lysozyme drastically alters the partitioning behaviour. By looking at the (changes in) partitioning behaviour as a result of other post-translational modifications such as methylation or phosphorylation or pre-translational modifications such as point mutations in the primary structure (i.e. changing the amino acid sequence) we can further investigate what drives the partitioning. We do have a lead. Charge 'patchiness' is a broad concept, and there are various parameters that can be evaluated, such as the patch size, the number of patches, the distance between patches, *etc*. One study has found differences in phase separation behaviour between proteins which are isotropically charged or proteins *versus* proteins engineered to have charge patches. [30]

However, it is near-impossible to investigate the partitioning behaviour fully empirically for all polyelectrolyte pairs and compounds. By comparing the result from models *in silico* to empirical results it is hopefully possible to develop functional and predictive theories that describe (a part of) the partitioning behaviour. This would hopefully drastically reduce the parameters necessary to investigate *via* high-throughput analysis.

Understanding complexation

In addition to partitioning, the exact behaviour of PEC formation can be frustratingly complex. We have validated that, for PAH/PAA PECs as used in chapter 4 at approximately $F^- = 0.50$, all polyelectrolytes (within the margin of error of the detection equipment) were present in the PEC with no detectable amount in the supernatant. However, this was not the case for chitosan/CMC PECs, where only about 75 % of the polyelectrolytes formed the PEC. This raises the question of how other polyelectrolyte pairs behave. Especially at different F^- values, different ionic strengths, different pH, and different concentrations.

It was also noted visually that complexes behaved slightly different in the presence of lysozyme. At the concentrations typically used in chapters 3 and 4, the total mass of the polyelectrolytes is only two to three times higher than the total mass of

lysozyme. It is unlikely that at these amounts the lysozyme is merely an inert presence without any influence on the PEC behaviour. This influence can vary with different proteins. All of these complexities reiterate the need for greater theoretical knowledge of the interaction between proteins and polyelectrolytes.

Alternative Extractions

In chapter 4, we presented a PEC system capable of selectively extracting lysozyme from egg albumen. Albumen is a natural protein mixture with relatively few non-protein components. Selective extraction is possible due to the affinity of lysozyme to partition into PECs as a function of the PEC composition. It stands to reason then, that different compositions could result in the selective extraction of different proteins. It can be interesting to try if other proteins besides lysozyme can be selectively extracted from the albumen using the same fundamental method of changing the PEC composition.

Similarly, expanding the PEC extraction method to other (natural) mixtures is an important step if this method wants to be elevated to an applied technology. First, lysozyme can (attempt to) be extracted from other mixtures. While chicken albumen is the standard source for commercial lysozyme, it is also naturally present in saliva, tears, blood serum, and human milk amongst many other sources. [31,32] Some of these sources are easily accessible for motivated researchers. The second pathway is to extract different proteins from different mixtures. Casein is a common milk protein and its extraction from cow milk has been on our radar as a candidate. It is used in various nutritional applications. [33] Preliminary experiments suggest that there is an F^- composition (around $F^- = 0.30$) at which casein proteins were removed from store-bought whole cow milk using PAH/PAA PECs.

We are not restricted to proteins. In chapter 2 we extract and back-extract butanol from an aqueous butanol solution to a new aqueous solution. Similar to how we expanded lysozyme extraction from pure lab conditions in chapter 3 to a realistic mixture in chapter 4, expanding a butanol (or lactic acid) extraction to a process where it is selectively extracted from a fermentation broth could be a logical step. These fermentation broths are currently some of the most sustainable processes to produce either butanol or lactic acid. [34,35] However, the downside is that purification of the desired product from the broths is an energy-intensive process.

The realistic feasibility of these initially suggested extractions is partially dependent on our understanding of the partitioning of these compounds into the PECs as discussed in the previous section.

With the eye on sustainability PECs may also find roles in creating a more circular economy. For example, wastewater streams can contain high protein concentrations that are currently unused. [36] PECs could potentially be used to extract a relevant fraction of these proteins from the wastewater stream for alternative use. Even better if this will be possible with natural polyelectrolytes.

Biocatalytic Membranes

Biocatalytic membranes with lysozyme

In chapter 5, we prepared a biocatalytic PAH/PSS membrane *via* APS that contained functional lysozyme enzymes. Cut-outs of the membranes showed enzymatic activity on a substrate of lyophilised gram-positive bacteria. However, the enzymatic activity was restricted to cleavage of the substrate by the lysozymes located on the membrane surface exposed to the substrate solution. This limited interaction was a result of the substrate being too large to penetrate into the membrane structure completely. When trying to evaluate the membrane's enzymatic activity when the bacterial substrate was present in the feed, we found that the membranes retained most of the substrate. Unfortunately, this meant we could not confirm that the enzymatic activity would also be present when the membrane is used in a filtration set-up.

We attempted to circumvent this limitation by trying an alternative lysozyme substrate; the small molecule 4-methylumbelliferyl-beta-D-N,N',N''-triacetylchitotrioside (4MBT). 4MBT has a molecular weight of 786 Da and is substantially smaller than bacterial fragments. Its small size is also far below the molecular weight cut-off of the membrane. Lysozyme cleaves the 4MBT structure resulting in the fluorescent 4-methylumbelliferone product. Unfortunately, while the 4MBT substrate passes through the membrane, both membrane with and without lysozyme show the presence of the fluorescent product in the permeate, with no spectral difference, making this also an unsuitable substrate to test enzymatic activity during residence time in the membrane.

However, the failure to verify that the proteins in the membrane are still enzymatically active using these two substrates need not be the end of experimentation with the PAH/PSS membrane containing lysozyme. There are more known substrates that are catalysed by lysozyme of various sizes and origins. [37,38] As an alternative, lysozyme-containing membranes could be evaluated by counting (the lack of) bacterial growth on or near their membrane surface compared to membranes without lysozyme. [39]

While we demonstrated in Figure 5.4 that lysozyme does not leach out of the membranes under the tested conditions (room temperature storage in demineralised water), we did not investigate under what conditions lysozyme would be lost. From chapter 4 we know that PAH/PSS PECs are relatively resistant to releasing their lysozyme load compared to other PEC systems. However, these results do not necessarily translate completely to the PAH/PSS membranes, which include the use of (a small amount of) glutaraldehyde as a crosslinker. Additional experiments that test under which conditions (e.g. salt concentration, pH, temperature) lysozyme is released from our biocatalytic membranes would give important data about their applicability for industrial situations.

Biocatalytic membranes with GFP

Besides lysozyme, we also attempted to incorporate eGFP into the PAH/PSS membrane structure. The eGFP was produced in-house *via* a plasmid vector (pEGFP-N1, Clontech) expressed in bacterial culture. One of the advantages of using eGFP (and related proteins) is the ease of readout and relation to protein structure: if eGFP is fluorescent, it is structurally intact. [40] We incorporated eGFP in the PAH/PAA membranes in the same way as described in chapter 5 for lysozyme.

Initial results for the incorporation of eGFP were promising. We see that a membrane not containing eGFP (Figure 6.2A) showed no fluorescence, while a membrane containing eGFP (Figure 6.2B) had become fluorescent. In similar behaviour to free eGFP in solution, exposure to a fluorescent lamp for 24 resulted in photobleaching of the eGFP and a loss of fluorescence of the membranes (Figure 6.2C).

Sadly, the incorporation of eGFP was hampered by reproducibility issues. While a series of membranes produced in early 2020 showed results like those in Figure 6.2, subsequent membranes produced later in that year and onwards did not show fluorescence distinct from PAH/PSS membranes without eGFP. The eGFP stock solution had not decreased in fluorescence activity (both in terms of fluorescence emission intensity as well as emission spectrum), and the same protocols and PAH batch of PAH was used as with the successful initial experiments. This demonstrates the need for further investigation into the mechanisms of protein-PEC interactions in the context of APS; clearly something had changed, though which factor or combination of factors responsible for the difference in eGFP performance when incorporated into PAH/PSS membranes remains elusive.

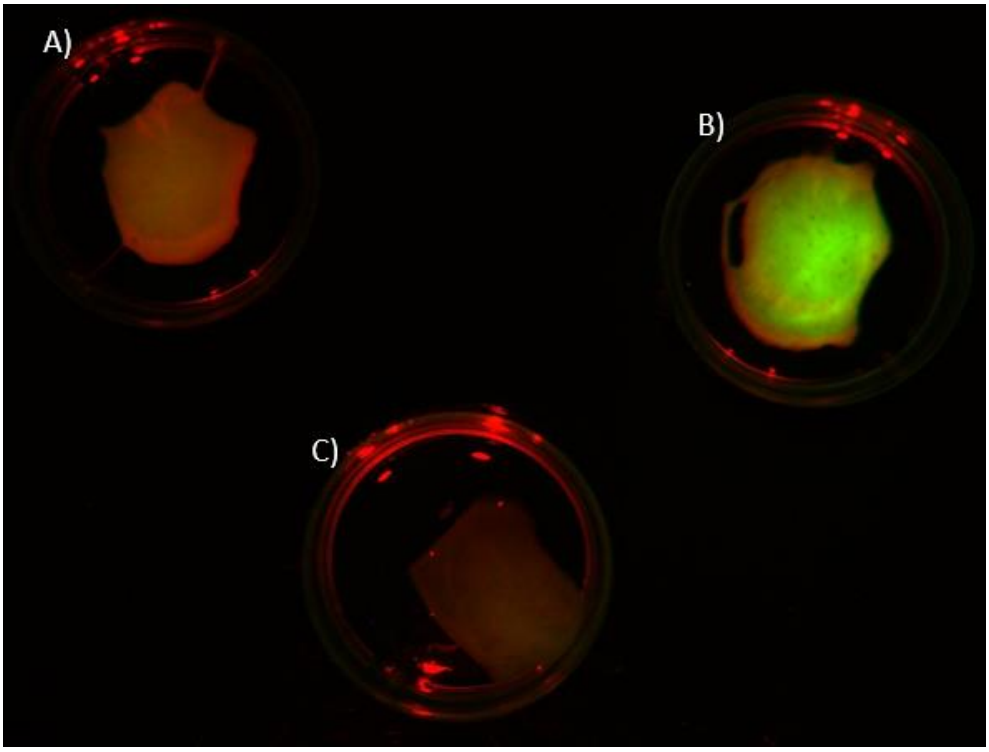

***Figure 6.2**. PAH/PAA membranes produced via APS containing either A) no eGFP, B) eGFP directly after membrane fabrication, or C) eGFP after 24 hour of photobleaching. Image is a composite of brightfield imaging (red channel) and fluorescence with excitation at 475 nm and emission at 537 nm (green channel) using a FluorChem M (Proteinsimple).*

Besides the ease of fluorescent readouts, eGFP would allow us to study the localisation and possible diffusion behaviour of proteins in our membranes. We know from Figure 5.3 that at least lysozyme remains within the membrane. It is unclear if lysozyme is mobile within the membrane structure or remains bound in place. Fluorescence recovery after photobleaching (FRAP) might shine light on this question. With FRAP, a section of membrane containing eGFP would be selectively photobleached and the possible diffusion of fluorescent molecules into the photobleached area observed over time. Such an analysis can provide important information on the interaction of incorporated proteins with the PEC membrane as well as quantitative data on protein mobility in PECs. [41] FRAP is not strictly limited to eGFP, as any protein incorporated in the structure can theoretically be labelled with a fluorescent marker.

Biocatalytic membranes with other proteins

In order to further develop the concept of APS-produced biocatalytic membrane, more possible of these membranes consisting of different polyelectrolyte pairs and biomolecules will have to be investigated. Alternative polyelectrolyte pairs are currently investigated, as well as different phase inversions to produce the membranes are currently researched and demonstrate that many possible polyelectrolyte combinations exist that produce useful and stable membranes. [42–48] In a relatively small amount of time, various functional systems have been identified. This is good news as a larger possibility space of constituent polyelectrolytes offers up more possibilities for functional biomolecules. A specific protein might lose functionality in a PEC membrane of one set of polyelectrolytes but retain functionality in another set.

To bring APS into the spotlight in the field of biocatalytic membranes, it might be worthwhile to attempt to create biocatalytic membranes this way that incorporate some of the currently most commonly used enzymes for biocatalytic membranes. Proteases, glucose oxidases, lipases, laccases, and peroxidases are all widespread names within the world of biocatalytic membranes. [39,49] Some of applications for these biocatalytic membranes are the creation of biosensors, the removal of organic micropollutants, antifouling, and the facilitation of specific reactions. [49–54]

Biocatalytic membranes with other biomolecules

The research presented in dissertation is mostly limited to proteins as a biomolecule, but there are more categories of biomolecules that could add functionality to membranes.

Poly(dopamine) coatings, inspired by mussels, have become an interesting modification that improve mechanical stability of membranes. Much like enzymes, treatment with (poly)dopamine is currently typically a post-production process. Using the APS process for incorporating biomolecules suggested in this dissertation, poly(dopamine) functionalisation of membranes may be greatly simplified. [49,55] In addition, poly(dopamine)-functionalised membranes may themselves be suitable platforms for more classic biocatalytic membranes produced by enzyme adsorption. [56] Tannic acid and gallic acid, both common polyphenols found in plants have been suggested for a similar purpose as poly(dopamine). [57–59]

Shielding Proteins for incorporation

While APS *via* the pH phase shift presented in chapter 5 is much more gentle to proteins compared to the organic solvents commonly used in non-solvent induced phase separation, it is still a limiting factor in producing biocatalytic membranes. The biomolecule that is to be incorporated needs to survive both the high (14) and low (1) pH of the casting solution and the precipitation bath respectively in addition to surviving interaction with the polyelectrolytes. These restraints limit the protein choice significantly. Fortunately, investigation is making progress into easing these restraints. First, a milder pH shift (12 to 4) has been demonstrated to be feasible for membranes consisting of branched polyethyleneimine and PSS. [47] Second, different phase inversions are investigated, such as moving from high to low salt concentrations. [42–46] These new developments will hopefully open up the choice of proteins for incorporation into these membranes.

Another strategy to maintain enzymatic functionality could be to protect the proteins during the incorporation process. Stabilizing and protecting proteins (and even activity enhancement) by association with polyelectrolytes is a known phenomenon. [60–63] While we still observed a decrease of lysozyme activity in the PAH/PSS membrane over time in chapter 5, it is possible that there are polyelectrolyte pairs that are suitable for APS that provide specific protection to lysozyme or other proteins. In addition, proteins may be pre-complexed with a (third) polyelectrolyte for stability or protection that still allows incorporation in the greater membrane structure. [20] The concept of a protein protected during a phase inversion process to facilitate membrane production has been demonstrated for lysozyme immobilised on halloysite nanotubes. [64] These nanotubes protected the lysozyme during the phase inversion process using organic solvents to create poly(ethersulfone) membranes. A complex of lysozyme with an additional polyelectrolyte could essentially fulfil the same function.

Protein conformational changes in the presence of polyelectrolytes

As most of the chapters discuss some form of interaction between proteins and polyelectrolytes, further investigating into the possible structural modifications will be of interest. For some enzymes, it is already known that complexation with a polyelectrolyte does not have to negatively affect the enzyme's structure or function. [65] Förster resonance energy transfer (FRET) could be used to investigate differences in protein conformation when confined in PEC(s) (membranes). FRET has been used to study the kinetics of protein exchange between complex coacervate core micelles. [66]

A different study has investigated the conformational changes of lysozyme when it complexes with PSS using small-angle neutron scattering. They found that the lysozyme conformation when complexed with PSS is dependent on (amongst others) the PSS chain length and the relative quantities of lysozyme and PSS. [67] Longer PSS chains and a greater relative abundance of positive charge (brought in by the protein) were associated with lysozyme retaining its structure. Learning more about which factors influence protein structure when proteins are associated with polyelectrolytes will help in the future when selecting suitable polyelectrolyte pairs for the preparation of biocatalytic membranes *via* APS.

Conclusion

The use of PECs as extraction media as well as the use of biocatalytic PEC membranes produced *via* APS are both in early stages of development. While we show with proof-of-concept studies that both ideas have merit, there is plenty of room for research into pushing towards more sustainable and green polyelectrolyte choices, better theoretical insights of the underlying mechanisms, and more practical applications.

We currently mostly use synthetic polyelectrolytes for our complexes, research in the future focussing on the use of (modified) natural polyelectrolytes may contribute to more sustainable separation processes. This has been challenging especially for the development of membranes, as the use of natural polyelectrolytes often leads to strongly reduced mechanical properties.

The exact mechanism of how and why proteins and other compounds partition into PECs remains elusive. Suggestions such as charge patchiness on the proteins have been offered and the research in this dissertation presents some empirical trends, but there lacks a predictive theoretical model. The development of a model that explains partitioning behaviour as a function of the various system parameters either through high-throughput testing, *in silico* modelling, or both, will be of importance.

Demonstrating the feasibility of PEC extractions by partitioning different molecules from different mixtures will be an important step to bring them to the attention of the scientific and commercial communities. Similarly, demonstrating APS is a robust technique to produce a variety of membrane consisting of different polyelectrolyte pairs and biomolecules will surely benefit the growing APS field.

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