APPLICATION OF MOLECULAR INTERACTIONS FOR BIO-SEPARATIONS AND DRUG DISCOVERY

A Dissertation

by

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ABSTRACT

Focus on microalgae continues to grow for the production of biomass and various value-added products including proteins, lipids, and pigments. The bioproducts from microalgae have found their applications not only in the field of petroleum industry but also in food and nutrition and pharmaceutical industries. However, due to several technical challenges associated with the inability to effectively dewater micro-algal biomass and extracting valuable compounds, microalgae-based bio-refinery is not yet economically feasible. To make the microalgae-based bio-refinery platform sustainable, less energy and resource intensive dewatering and harvesting techniques needs to be deployed. Part 1 of this dissertation focuses on a novel dewatering technique of microalgal biomass by using an amphiphilic polyelectrolyte, which upon adsorbing on the biomass, leads to formation of a net hydrophobic ensemble that consequently migrates into a hydrophobic organic solvent i.e., hexane. The technique also involves simultaneous separation of algal proteins by retaining them in the aqueous phase while migrating algal cellular debris to a hexane phase at the right system pH and polyelectrolyte concentration.

Separation and recovery of microalgae from the aqueous medium that they reside in is difficult as a result of the nature of the algal cells, i.e., small cell size, density close to water, low concentration, and ability to stay suspended in water due to surface potential. This study has been divided into four sections: 1) separation studies on model algal particles; 2) separation of model proteins (egg albumin); 3) Simultaneous separation of model algal particles and models proteins in hexane and aqueous phase; and 4) separating algal proteins and cellular debris in aqueous and hexane phase respectively. The technique involves the addition of a positively charged electrolyte, Mono/Poly-(diallyl dimethyl ammonium chloride, DADMAC) which interacts with negatively charged particles to form hydrophobic ensembles. The resulting hydrophobic ensembles, upon addition of a hydrophobic organic solvent, migrate from aqueous phase to the hydrophobic organic solvent phase.

From the studies conducted on *Chlorella sorokiniana*, the ability of polyDADMAC to dewater and extract cellular debris, lipids, and pigments to the hexane phase while retaining protein fraction in the aqueous phase was investigated. It was observed that different components could be migrated from one phase to the other by modulating the system pH. Close to the isoelectric point, proteins can be retained in the aqueous phase while selectively migrating algal debris to the hexane phase via targeted binding of the polyelectrolyte. Approximately 80% of total proteins were retained in the aqueous phase at pH 4, and 90% of cellular debris were migrated to the hexane phase at pH 4.5. Results indicate the possibility of separating multiple components from microalgae in an aqueous-organic solvent two-phase system using polyDADMAC.

Part 2 of this dissertation focuses on screening of highly specific RNA dependent RNA polymerase (RdRp) inhibitors for Tick-Borne encephalitis virus. Tick-Borne encephalitis virus (TBEV) in humans can be caused by direct tick bites or by consumption of non-pasteurized milk or milk products from TBEV- infected sheep, goats and cows. The TBEV genome encodes a single polyprotein, which is co/post-translationally cleaved into seven non-structural proteins. Of the non-structural proteins, NS5 contains the RdRp domain and a methyltransferase (MTase) domain that are responsible for the replication of the viral genome. The focuses of this section was on screening for potential antivirals using a hybrid receptor and ligand-based pharmacophore search. For identification of pharmacophores, a mixture of small probe molecules and nucleotide triphosphates (NTPs) were used. The ligand/receptor interaction screenings of structures using ZINCPharmer search engine in ZINC database resulted in five compounds that bound to the RdRp domain with high affinity. Compounds Zinc 9662, and Zinc 9041 had significantly lower binding energies than native NTPs at the RdRp binding site. Experimental studies indicated that Zinc 7151 substantially inhibited viral growth at 30 μM concentration while both Zinc 3677 and Zinc 7151 had antiviral activity at 100 μM.

DEDICATION

To my parents.

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NOMENCLATURE

*I*p	Isoelectric point
CC	carboxylated cellulose
ATPS	Aqueous Two Phase Separation
TPP	Three-phase partitioning
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
TCA	Trichloroacetic acid
EPS	Excreted organics
CTAB	N-cetyl-N,N,N-trimethylammonium bromide
SDS	sodium dodecyl sulfate
AOM	algogenic organic matter
PBRs	photo-bioreactors
PUFAs	polyunsaturated fatty acids
TAG	triacylglycerol
SDS-PAGE	sodium dodecyl sulfate-Polyacrylamide gel electrophoresis
FAO	Food and Agriculture Organization
PolyDADMAC	Poly-diallyldimethylammonium chloride
PEG	Polyethylene glycol
MV	Molecular vehicle

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1. LITERATURE REVIEW AND INTRODUCTION

Population growth that is projected to be >9 billion by 2050, compounded by climate change, competing demand for natural resources by industrialization along with urbanization [1], and resulting decrease in arable land has put significant strain on agricultural products and long-term food security. It is estimated that global food production should increase by 60 % by 2050 to meet the demand [2]. Various solutions have been proposed for increasing agricultural production without adversely increasing land expansion including the use of technology in farming smarter, breeding better seeds, crop rotation etc [1]. However, most of these techniques still fall short of giving a sustainable solution without creating severe distress on natural resources. Microalgae is a great alternative to traditional agriculture since it does not compete with arable land, could be grown year-round under all weather conditions, and produce a multitude of products using less resources.

Due to the aforementioned factors, microalgae have gained significant attention in the recent past to be a sustainable source of food, feed, bio-products, and biofuels. Microalgae is already looked-at as a source of lipids for biofuels, proteins for food, high-value pigments, bioremediation of water and other application/products. Biofuels from microalgae are considered as the most promising alternative to fossil fuels[3, 4]. Biodiesel generated through transesterification of lipids from microalgae has more advantages as compared to its terrestrial crop-based ones. Microalgae can be grown in year-round in any type (arable or non-arable) land and does not compete with agri- cultural land for space. It can be cultivated in water under atmospheric conditions with usage of a minimum amount of salts and other nutrients. If microalgae are grown under right cultivation con- ditions, it can accumulate up to 60-71 % proteins and 50-70% lipids/ dry weight [5, 6]. To support the global rising demand for lipids and proteins, microalgae can certainly be used as a source as they are its largest fractions [7]. In addition, microalgae can help as a CO_2 scavenger and assist in bringing the level of greenhouse emissions down.

Chlorella and *Spirulina*, in particular, have attracted significant scientific and commercial in-

terest because of their ability to generate a high amount of proteins and neutral lipids under appropriate growth conditions[8]. A high protein content (up to 70% of cell dry weight) along with being rich in minerals, vitamins, and carotenoids has made *Chlorella* an ideal nutritional substitute for human and animals[9]. To date, *Chlorella* is produced in over 70 companies with Taiwan Chlorella Manufacturing Co. (Taipei, Taiwan) being the largest producer with 400 t of dried biomass per year[10]. Chlorella also possesses the ability to synthesize large amounts (as high as 50% of dry weight) of storage neutral lipid mainly in a form of triacylglycerol (TAG)under stress conditions (e.g., high light or nitrogen deficiency), making it a promising candidate for lipid-based biofuels[9]. In addition, *Chlorella* is emerging as a cell factory to produce high-value chemicals, such as carotenoids, antioxidants, polyunsaturated fatty acids (PUFAs) and recombinant proteins[9, 11]. On the other hand, *Spirulina* can produce up to 64% of proteins of the dry weight of cells and is considered to be a rich source of high-quality proteins, vitamins, minerals and biologically active compounds[8]. *Spirulina* has also received a lot of attention for pigments of interest in food, pharmaceutical and cosmetic industry[12]. *Spirulina* is widely considered to be a viable photosynthetic organism for biomass production on an industrial scale[13]. Despite these benefits of *Chlorella* and *Spirulina*, the current technologies to harvest them are neither cost-effective nor energy-efficient, making associated algal biorefineries unsustainable.

To make algal biorefineries sustainable, it is necessary to identify the most suitable combination of methods for dewatering, harvesting and down-stream separation based on their cost effectiveness, energy efficiency and advantages/disadvantages. This chapter highlights the various techniques that are used for harvesting microalgae and advancements in protein harvesting to help make microalgae-based bio-refinery platform sustainable.

1.1 Microalgae cultivation overview

The composition of microalgae is significantly impacted by the growth conditions. Four major types of conditions for cultivation are known: photoautotrophic, heterotrophic, mixotrophic and photoheterotrophic[14]. Phototrophic cultivation of microalgae occurs when the inorganic carbon (CO_2) is used as a source of carbon under sunlight as an energy source. In the case of heterotrophic

cultivation, microalgae cannot survive under light conditions and need an organic source for energy under dark conditions. Mixotrophic cultivation involves the use of both organic and inorganic carbon source microalgal growth. As the name suggests, photoheterotrophic cultivation utilizes both light and organic carbon for energy. It involves the use of both carbon sources, organic and inorganic.

Phototrophic cultivation offers low cost of operation as compared to the other growth conditions and is widely accepted for cultivating algal biomass on a large scale. For the cultivation of phototrophic microalgae, two main alternatives have been reported: raceway ponds and photobioreactors (PBRs) [15]. Raceway pond is a closed loop oval-shaped channel. The pond is constructed in a way that the depth of the tank is shallow enough for the light to penetrate to the bottom. The entire system is open to air which provides algae with carbon dioxide for growth. A paddle is fixed at one end of the pond which prevents the algae from settling. PBRs, on the other hand, are closed transparent arrays of tubes under well-controlled conditions for better algae growth. They tend to be more costly as compared to raceway ponds in terms of maintenance and energy requirement.

1.2 Harvesting of microalgae

In order to harvest value-added products from microalgae, large quantities of water need to be removed first which may contribute to the total biomass production cost. There is no single-step separation technique available for dewatering microalgae. Generally, cell harvesting is done in two steps, namely: bulk harvesting and thickening. Total suspended solids reach 2-7% from 0.5% in bulk harvesting. It is further concentrated to 15-20% in the thickening step. The major techniques currently used in the harvesting of microalgae include centrifugation, microfiltration, tangential flow filtration, flotation, sedimentation, and electrophoresis [16]. Selection of a harvesting technique is dependent on the final use of the bio-product. Harvesting technique used for extraction of lipids may not be suitable for extracting edible proteins as it can involve the use of toxic chemicals. Figure 1.1 shows an overview of different stages in microalgae harvesting.

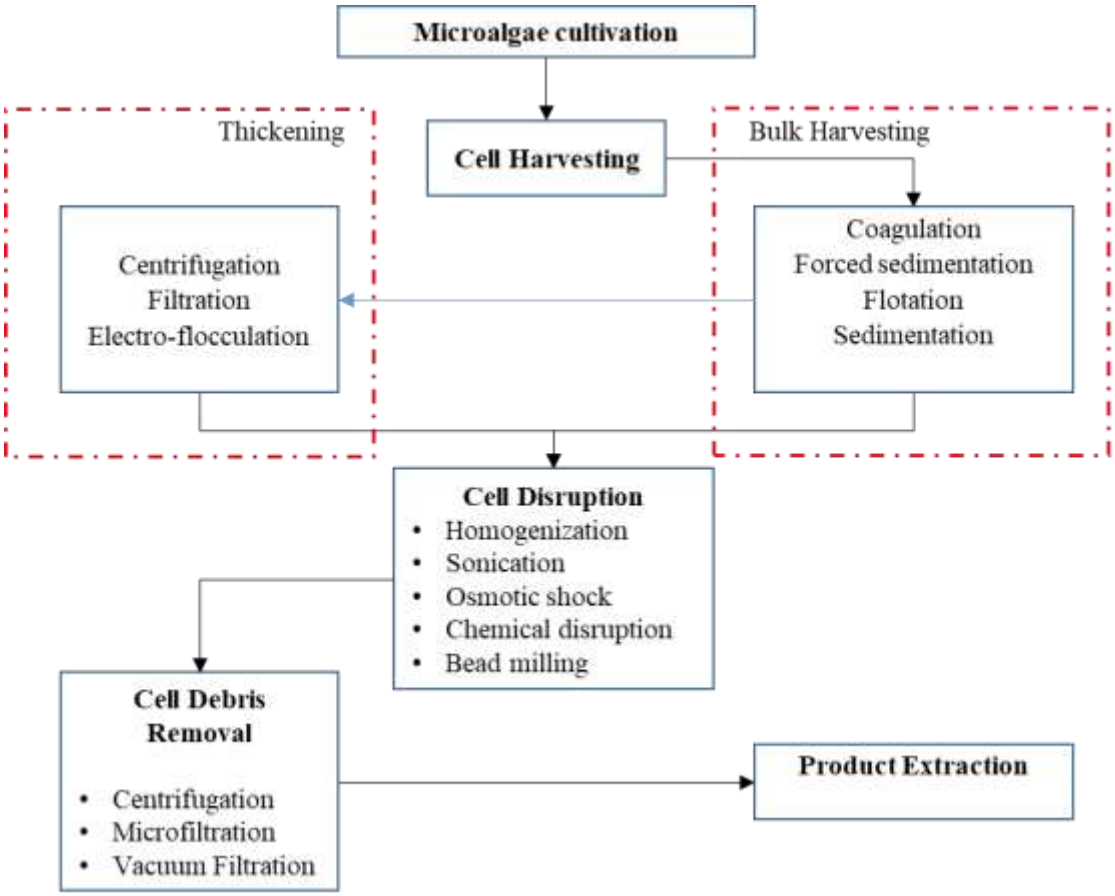

Figure 1.1: Overview of steps involved in microalgae bio-products harvesting.

1.2.1 Bulk harvesting

Due to the dilute nature of the algae growth culture (mass concentration less than 1g L^{-1}), bulk harvesting is done to reduce the initial volume of the growth media to be processed downstream. Generally, a less energy-intensive unit operation like flocculation, gravity/forced sedimentation, and flotation is chosen during this step.

1.2.1.1 Flocculation

Flocculation is a process in which the dispersed algal cells are aggregated together to form a clump for settling. It can be achieved either by auto-flocculation and/or chemical coagulation.

Auto-flocculation occurs as a result of precipitation of carbonate salt with algal cells in elevated pH, a consequence of photosynthetic CO_2 consumption with algae[17]. Hence, prolonged algal cultivation in sunlight under low carbon dioxide levels can trigger auto-flocculation.

Flocculation induced by addition of a chemical surfactant to neutralize the surface charge of microalgae cells is a common practice in the solid-liquid separation process. Microalgae cells carry negative charges owing to the presence of algogenic organic matter (AOM)[18] which keeps them in a well-dispersed state. By disrupting this stable state using an organic or inorganic surfactant, bulk harvesting of microalgae can be done. Inorganic flocculants like iron-based or aluminum-based coagulants neutralize the surface charge causing the cells to settle down[19]. Organic flocculants help in forming molecular bridges between cells thus increasing the floc size and improving the settling[20]. Biodegradable flocculants like chitosan that are naturally found and do not contaminate the culture have been successfully used to harvest microalgae[21]. The most effective polymers have been cationic as the charge on microalgae is negative under neutral pH condition. The efficiency of bio-flocculants is in the same range as that to chemical flocculants, around 60% [22].

1.2.1.2 Gravity sedimentation

Gravity sedimentation occurs when the force of repulsion between two microalgal cells is significantly small, consequently resulting in the amalgamation of biomass and settling down due to increased weight. Change in microalgal surface charge can occur at different stages of its growth. Gravity sedimentation is a time-consuming process and is often coupled with flocculation to enhance the rate of sedimentation. It is the most common method of harvesting algal cells in wastewater treatment because of the large volumes of water treated[23]. However, it is suitable only for large microalgae like *Spirulina* [24]. Maximum biomass recovery of 55% was achieved in a study conducted by Belovich and colleagues at the flow rate of 0.01 $cm^3\ cm^{-2}\ min^{-1}$ of *Chlorella* [25].

1.2.1.3 Flotation

Flotation works against the force of gravity. In the flotation separation process, air or gas bubbles are introduced in the cultivation medium. These air bubbles get attached to algal cells and then transport microalgae to the liquid surface. Flotation was reported to be more beneficial and effective than sedimentation with regards to removing microalgae[26]. Flotation is further divided into dissolved air flotation and dispersed air flotation. Dissolved air flotation entails the pressure reduction of a water stream that is pre-saturated with air at excess pressure to produce 10-100 μm bubbles[16]. Dispersed air flotation entails 700-1500 μm bubbles formed by a high-speed mechanical agitator with an air injection system[27]. Liu et al. found that around 20% cell removal was possible when flotation was done in presence of sodium dodecyl sulfate (SDS), 86% cell removal in presence of cationic N-cetyl-N,N,N-trimethylammonium bromide (CTAB) and over 90% removal in presence of chitosan[28].

1.2.2 Thickening

A high cell recovery efficiency can be obtained using centrifugation. Laboratory centrifugation tests show around 80-90% microalgae can be recovered at 500-1000 x gravity within 2-5 minutes [29]. Centrifugation is a preferred technique for harvesting microalgae because of producing extended shelf-life concentrates for aquaculture even though it is time-consuming and costly for large volumes of cultures [19]. Flocculation aided centrifugation has been studied to improve harvesting efficiency of microalgae. In a study conducted with *Chlorella sorokiniana*, a 20-50 fold reduction in volume was achieved which significantly reduced the energy input for centrifugation[30].

Table 1.1 shows harvesting deficiencies of microalgae using different techniques.

Table 1.1: Microalgae harvesting using various methods

Method	Technique	Harvesting Efficiency	Reference
Chemical	Cations from inorganic metal salts Metal hydroxide from inorganic metal salts Polyelectrolytes, biopolymers	90% 95% 90%	[16]
Auto-flocculation	Metal hydroxides, excreted organics	90%	[31]
Bio-flocculation	Excreted organics (EPS) from other algae, bacteria, fungi, GM organisms	60%	[32, 33]
Physical	Centrifugation Flotation	90% 90%	[15] [28]
Phase Separation	Use of cationic polyelectrolyte in water-organic solvent two-phase system	80%	[34]

1.3 Separation of proteins from microalgae

Separation of proteins and pigments from disrupted microalgal biomass is technically challenging due to the complex nature of the cultivation broth. Any technique(s) employed for the recovery of bio-products must be simple, less resource intensive, scalable and should not contaminate the product. Many techniques that are used for harvesting and separating proteins from bacteria are also used for microalgal systems and are discussed in more detail below.

1.3.1 Aqueous two-phase separation (APTS)

Aqueous two-phase separation is widely used for the separation of proteins from bacterial cells. In APTS, the solubility and hydrophilicity of proteins are influenced by the concentration of salts in the system. Salts like ammonium sulfate and potassium phosphate are added to reduce the solubility of the proteins in water consequently increasing their hydrophobicity. Polyethylene glycol (PEG) which is soluble in water in its native form, is also generally added to the mixture. However, due to high salt concentration, a second phase comprised of water and PEG is formed. As water molecules surrounding proteins migrate towards the salts, protein molecules migrate into the PEG phase.

Two-phase aqueous partitioning is considered mild for protein purification as denaturation of biological activity is generally not seen[35]. Based on the nature of target proteins, ATPS can be used for its separation from other junk proteins, nucleic acids, and cellular debris. Depending on the polarity of the molecules suspended, more polar particulate matter will settle down in the bottom phase containing salts and the non-polar proteins will eventually end up in the top phase, usually with PEG [36, 37, 38]. Selectively separating proteins by manipulation of their partition coeffi- cient can be easily achieved by changing the ionic strength of salts in the bottom phase, changing the molecular weight of the polymer, and by addition of salts like sodium chloride in presence of hydrophobic groups [39, 40, 41]. APTS has been used to separate human antibodies[42], for the recovery and partial purification of double layered Rotavirus-Like Particles [43] and purification of yeast like particles from yeast cells[44]. Approximately 84% proteins recovery from *Chlorella*

sorokiniana using aqueous two-phase separation was achieved in a 30% (w/w) K_3PO_4 and 20% (w/w) methanol system[45]. Aqueous two-phase separation process offers several advantages like scalability, ease of operation, use of non-toxic polymers, continuous operation, and better economics.

1.3.2 Three phase partitioning

Lovrien's group in 1987 first reported the three-phase partitioning system[46]. It is a relatively novel technique where an organic solvent and salt in water form two separate phases and the molecule(s) of interest interface between these two layers forming a three-phase partitioning system. TPP involves several sub-techniques including salting out, iso-ionic precipitation and co-solvent precipitation of proteins[47]. The suspension containing target molecules, i.e. proteins is saturated first using a salt, generally ammonium sulfate, and t-butanol is added to the mixture and vortexed. Proteins form an intermediate layer between the salt solution and t-butanol. TPP has been used to recover ficin from fig[47], proteins from *Chlorella pyrenoidosa* [48] and, isolation of trypsin inhibitor from legume seeds[49].

1.3.3 Chromatography

Chromatography is a technique that has been used for many years for separation and purification of proteins. It involves separation of different molecules from a mixture into fractions based on their molecular weight, charge, and adsorption. Commonly used techniques include size exclusion and ion exchange chromatography. Several others including expanded bed adsorption, reverse-phase, and counter-current chromatography have also been used for separation of proteins and pigments.

Gel-filtration, also known as the size exclusion chromatography, is one of the oldest and the most widely used methods for protein separation/ purification. Separation of proteins using gel-filtration is based on the molecular size of proteins. Proteins with large molecular size travel fast through the gel matrix than the molecules with smaller sized proteins. Molecules with larger size take a straight route during separation while the small sized proteins elute at a much slower rate as

they pass through the matrix and as a result, their speed is slowed down significantly[50]. The column in size exclusion chromatography can be made using different sieve sizes. The filtration gel is generally made up of cross-linked polyacrylamide and sugars like agarose and dextran or a combination of both[51]. Selection of the matrix primarily depends on the molecules to be separated and the molecular weight cutoff offered by the matrix to achieve separation. Use of a small size matrix to separate molecules with significantly large weight distribution reduces the elution rate consequently resulting in blocked column. Size exclusion chromatography has been used to purify phycocyanin from *Oscillatoria quadripunctulata* [52] and molecular weight estimation of phycocyanin from *Calothrix spp*. [53], and purification of proteins from *C. vulgaris*[54].

Ion exchange chromatography utilizes the native charges on the surface of the particles to promote separation. The stationary phase in the column consisting of resins carries a specific charge which is opposite to the charge of the particles to be separated. When analytes pass through the column, the compound of interest bind to the stationary column due to charge attraction. The molecules with same surface charge as the resin column are repelled and then continue to flow with the mobile phase out of the column. The molecule adsorbed on the packed column is released by using a salt solution with gradient ionic strength. The salts in the suspension have higher binding affinity towards the column and help dislodging the bound analytes. Molecules with low ionic strength desorb first followed by the molecules with high ionic strength. Quantification of amino acids [54], and purification of phycocyanin from *Phormidium ceylanicum* [55]has been conducted using ion exchange chromatography. Lipase separation from *Spirulina platensis* has also been studied using diethylaminoethanol bound sepharose gel[13].

1.3.4 Membrane separation

Membrane filtration has already been used to separate and purify high-value therapeutic proteins on a commercial scale[56]. Separation of proteins using membranes is dependent on the size of the protein to be separated and the permeability of the membrane, usually measured in Daltons. Membranes having a specific pore size are used to separate proteins of interest. A pictorial representation of a spiral wound membrane filtration unit is depicted in Fig 1.2.

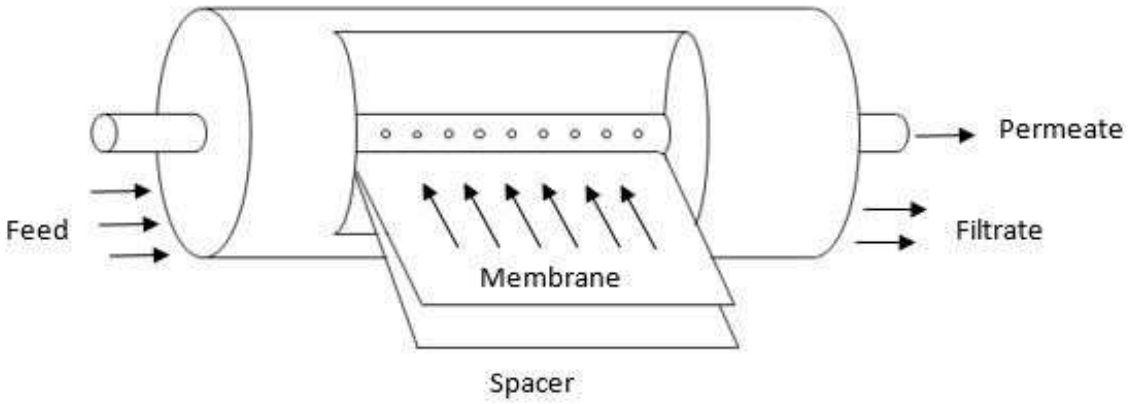

Figure 1.2: A spiral wound membrane module. The membrane is placed in a housing (not displayed) and the suspension is introduced from one end. Molecules that can pass through the membrane, flow towards the center of the module and flow into the centrally located pipe and exit the housing through permeate. The molecules that do not flow across the membrane flow towards the center exit the module as the filtrate.

Molecules with a size larger than the pores are rejected and continue to flow in the direction of the feed. Molecules with smaller size flow through the permeable membrane and enter a hollow pipe around which the membrane is wrapped and exits the module as permeate. Membrane filtration has been used in industries due to the ease of scalability, continuous operation and its ability to handle large volumes of suspension media. Three modules of membranes are widely used (figure 1.3): flat sheet, hollow fiber, and radial flow. Raja Ghosh reported that flat sheet membranes are widely accepted in protein separation as compared to the other two types [57].

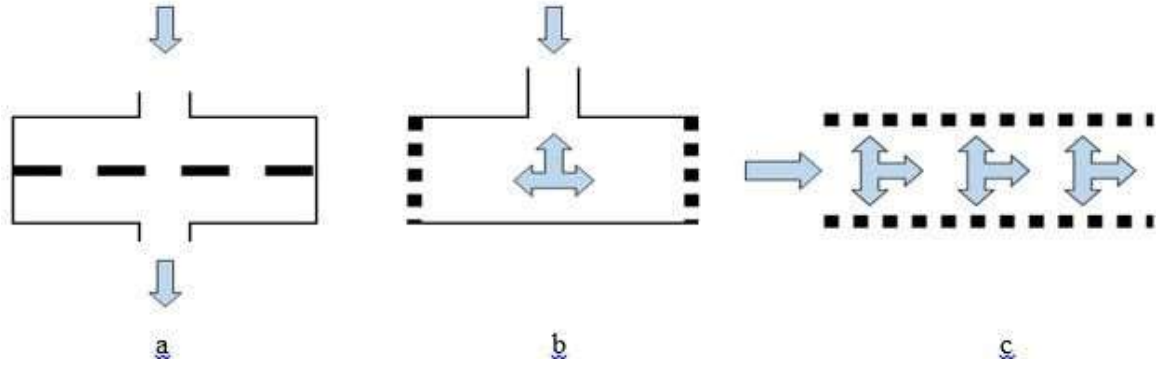

Figure 1.3: Flow pattern in different membrane modules. (a) Flat Sheet Membrane (b) Radial Flow Membrane (c) Hollow Fiber Membrane. The arrows show the direction of the flow of the aqueous suspension.

Recovery of proteins from *Nannochloropsis spp.* using a regenerated cellulose membrane was successfully conducted by Giorno et al [58]. In another study conducted on *Chlorella vulgaris*, proteins were concentrated using tangential ultrafiltration[54].

1.3.5 Electrophoresis

A protein molecule carrying a net surface charge can move in the presence of an electric field towards the electrode with an opposite charge. Depending on the charge density on the protein molecule, the rate of migration through the medium changes at any given electric field. The process is mostly done in gels which serves as a molecular sieve[59]. The rate of separation also depends on 1) frictional force between the gel and the molecule, 2) concentration of polymer that makes up the gel, 3) and the electric force driving the molecule. The separation of proteins is based on the size of the protein and their ability to flow through the porous gel. As the frictional drag is large for larger molecules, they travel slower in comparison to small molecules that move rapidly through the gel. Isolation of proteins from microalgae using electrophoresis is restricted to laboratory scale and is an unviable option on a commercial scale yet.

1.3.6 Magnetic separation

The use of magnetic separation for purification of proteins is fairly a new technique. Magnetic affinity separation, in general, is broadly divided into two categories: 1) direct method where the affinity ligand is attached to the magnetic beads first and then mixed in the solution containing the target molecules (Figure 1.4A) and 2) indirect method where the affinity molecules are first mixed in the solution containing the target molecules so that they attach to the molecule of interest and the resulting protein-ligand mixture is captured using appropriate magnetic particles (Figure 1.4B). In a system of particle mixture shown below (figure 1.4A), magnetic beads attached with ligands are introduced. The ligands get attached to the target molecule and form a ligand-protein complex. When an external magnetic force is applied to this system, the magnetic beads are attracted towards that system and the other debris material stays in a freely suspended form. These debris molecules are discarded and the molecules of interest are separated. The magnetic bead-ligand complex can be separated from the proteins of interest by further separation. Potential of harvesting microalgae using magnetic nanoparticles have already been investigated [60, 61]. Superparamagnetic iron oxide nanoparticles were used for separation of histidine-tagged Green Fluorescent Protein (His-GFP) from *E. coli* [62]. However, the use of magnetic nanoparticles for harvesting proteins from microalgae has not been investigated thus far.

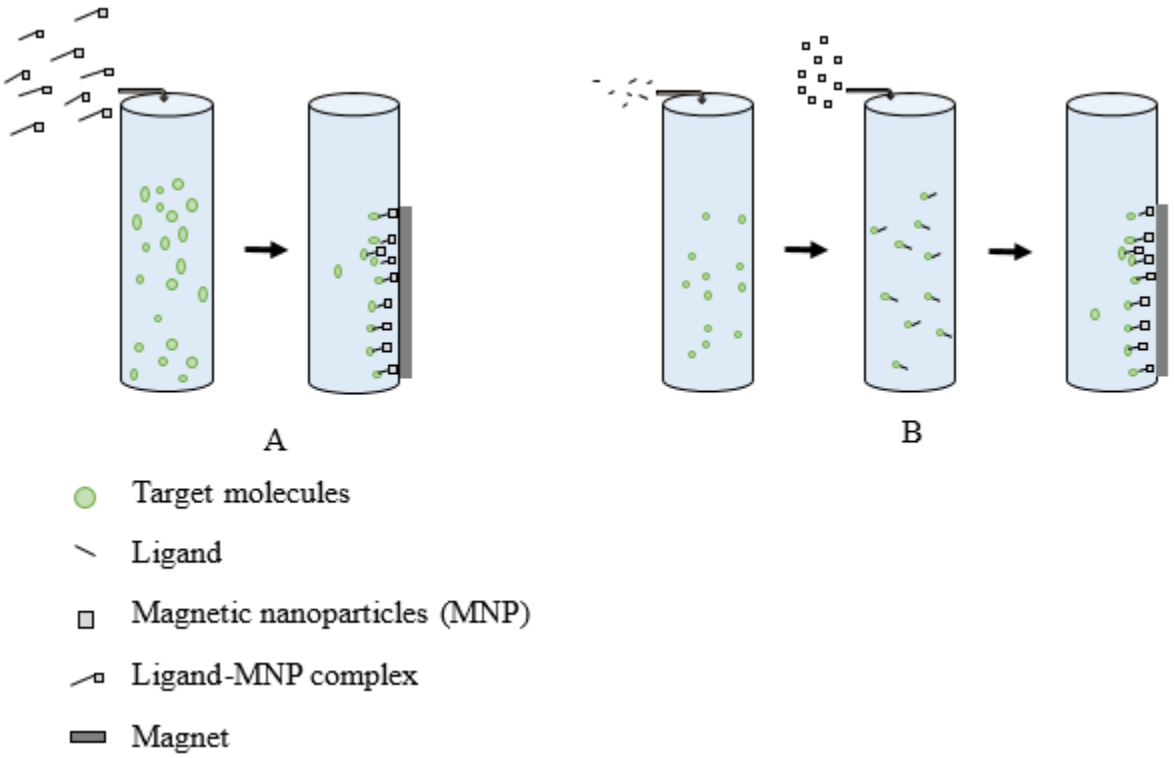

Figure 1.4: Magnetic separation using direct addition of ligand-MNP complex (A) and indirect MNP attachment to target molecule-ligand complex.

1.4 Separation of proteins using chemical techniques

Use of chemicals for separation of proteins from disrupted algal biomass generally involves the use of polymers as coagulants, salts in aqueous two-phase separation, and pH variation for precipitation. After separating disrupted biomass, the aqueous suspension contains total soluble proteins from microalgae. These proteins can stay suspended in water due to their small size and the intermolecular repulsive forces that exist between them.

1.4.1 Use of alcohol for protein precipitation

Proteins have a very low solubility in organic solvents. After the disintegration of cells, the cell debris stays freely suspended in the media along with the intracellular proteins and other components. The debris can be separated using either filtration or centrifugation. The protein-rich aqueous medium is treated with alcohol for solvent-based precipitation. Proteins and other nucleic

acids being less soluble as compared to cell debris in organic solvents can be easily separated. Studies using ethyl acetate, acetone, ethanol, methanol and a mix of ethanol and acetate have been conducted to separate protein-rich powders from *Chlorella protothecoides*[63].

1.4.2 Coagulation

After the disruption of cells, the most commonly used primary protein separation step is precipitation. This is done by reducing the repulsive forces that exist between two protein molecules. Addition of chemicals to induce flocculation in various solid-liquid separation process as a pretreatment is a common practice[64]. Flocculantion generally involves 1) pH modification and 2) addition of flocculants.

pH modifiers: After disruption of microalgal biomass, the pH is generally adjusted to 12 to solubilize all the proteins followed by filtering out cellular debris. From a study conducted on total proteins from *Chlorella vulgaris*, two groups of proteins with different isoelectric point ranges were identified[54]. Adjusting pH in these regions can help in initiating protein precipitation. Several studies have been conducted on protein precipitation via pH adjustment [65, 63].

Flocculants: Though the use of flocculants is more commonly done in the harvesting of microalgal biomass[66, 67, 68, 69], inorganic salts and natural polymer can also be used to separate proteins from the aqueous medium. A polymer carrying an opposite charge when introduced in the system binds to protein molecules resulting in the formation of net neutral complex. Flocculation of proteins is triggered by the formation of bridges via polymer networks and the choice of the polymer can vary depending on different proteins. Precipitation of a specific protein (lysozyme) using anionic surfactants without losing protein activity has already been studied [70, 71].

Table 1.2 gives a comparison of different protein separation techniques.

Table 1.2: Benefits and drawbacks of different protein separation techniques

Technique	**Advantages**	**Disadvantages**
Aqueous Two-Phase System (APTS)	Biocompatible Can be operated continuously Low toxicity Easy of scalability	Separation of two phases Potential back mixing between two phases
Chromatography	Can separate proteins based on molecular size and affinity Highly specific	Costly Cannot be scaled-up easily
Centrifugation	Continuous operation possible Can be scaled up	Expensive Can cause a loss in protein activity due to shear forces generated
Membrane Filtration	Continuous operation No mechanical moving parts Easily scalable Can be used for temperature sensitive proteins	The membrane can result in fouling Expensive setup
Electrophoresis	Separation based on a charge is possible	Can only be used effectively on lab scale
Magnetic Separation	Targeted separation of proteins possible Inexpensive Can be easily scaled up	Binding of proteins and ligands is highly dependent on pH, ionic strength and temperature
Precipitation	Can be easily scaled-up	Flocculants can be potential contaminants Recovery of flocculants is required

1.5 Extraction of pigments from microalgae

Different microalgae synthesize different photosynthetic pigments. *Cyanophyta* has chlorophyll a, β-cartonene, and mycoxanthin; *Prochlorophyta*, has chlorophyll a, chlorophyll b, and β -cartonene and zeaxanthin; *Rhodophyta* has chlorophyll a, chlorophyll d, α -, β -cartonene and lutein; and *Chlorophyta*, chlorophyll a, chlorophyll b, α-, β -, γ,-*cartonene*, lutein and zeaxanthin, *Euglena* has biotin and α-tocopherol, *Chlorella* has eicosapentaenoic acid(EPA), *Schizochytrium* has docosahexaenoin acid (DHA), and *Parietochloris incise* has arachidonic acid (AA)[72, 73]. Carotenoids find their application in nutraceuticals, cosmetics and as coloring agents, polyunsaturated fatty acids in food additives, nutraceuticals, pharmaceuticals, and baby food, polysaccharides in pharmaceuticals and cosmetics, and vitamins in nutrition and food supplements. Extraction and purification of these pigments have been done on a laboratory scale; however, for microalgae to be used as the primary source for pigments and oils, further work needs to be done.

Extraction of phycocyanin after harvesting microalgae is commonly done using ammonium sulfate precipitation. In a two-step purification process of phycocyanin, purification fold of 4.33 and a recovery of 33% was achieved [74]. Lutein which is a naturally occurring carotenoid in plants and flowers is also synthesized in microalgae. As compared to higher plants, synthesizing carotenoids in microalgae has an added advantage as it can be grown under controlled conditions in photo-bioreactors. Lutein extraction has been described in two schemes: 1. Suspending the harvested cells in acetone followed by multiple washes in diethyl ether and potassium hydroxide in methanol and finally precipitation using NaCl[75], 2. Suspension of lyophilized cells in potassium hydroxide containing ascorbic acid followed by extraction using dichloromethane till the cells become colorless. The purification step in the study was conducted using high-speed counter-current chromatography[76].

Polyunsaturated fatty acids (PUFAs) or essential oils that are primarily extracted from nuts, seeds, and fish have also been extracted from microalgae. As lipids are a major constituent of microalgae, PUFAs can produced on a large scale under controlled conditions. Typically used methods for extraction of lipids from microalgae include Soxhlet and Bligh and Dryer. However,

the use of ionic liquids or green solvents has been increasing to replace toxic organic solvents. The use of different ionic liquid-mediated lipid extraction from algal biomass has been described by Sang Hyun Lee et al.[77]. The use of supercritical CO2 for lipid extraction has also displayed substantial potential in a study conducted by Mendes and colleagues[78]. Along with lipids, supercritical CO_2 has been also used to extract chlorophyll and carotenoids from *Chlorella vulgaris* [79].

Table 1.3 provides a list of different components extracted from different microalgal species and their productivity.

Table 1.3: Current high-value product extracted from microalgae

Microalgae	**Compound**	**Compound Productivity**	**Reference**
Chlorella vulgaris	Lutein	1.98 mg/g	[76]
Chlorella sp. ESP-6	Lutein	2.1-2.3 mg/g biomass	[75]
Spirulina platensis	Phycocyanin	1.28 mg/ L	[80]
Chlorella vulgaris	Lipids	6.34 mg/ g biomass	[81]
Chlorella vulgaris	Lutein	3.16 mg/ g biomass	[82]
Chlorella vulgaris	Protein	0.5 g/g biomass	[54]
Spirulina platensis	Biopterin- α -glucoside	0.45 mg/ gm dried biomass	[83]
Chlorella vulgaris	Lipids and pigments	10 % lipids containing 1.61 mg chlorophyll and 1.72 mg carotenoids per g of biomass	[79]
Spirulina fusiformis	Chlorophyll	6 μg/mL	[84]
Chlorella vulgaris	Lipids	15 d incubation: 9.75 mg/ (L d) 20 d incubation time: 12.77 mg/ (L d)	[85]
Spirulina platensis	γ-linolenic acid	0.988 mg / L	[86]
Spirulina platensis	C-phycocyanin	0.17 g/l/d	[74]

1.6 Objectives

The overall objective of this dissertation work is to develop a unique approach to fractionate major classes of compounds selectively (proteins, lipids, and carbohydrates) from algal biomass while minimizing colloid formation.

1.6.1 Central hypothesis and specific objectives

The central hypothesis is that, a charged particulate substrate in an aqueous mixture could be migrated from the aqueous phase into an adjacent, immiscible (hydrophobic liquid) phase by using oppositely charged ionic polyelectrolytes (hereafter referred to as a molecular vehicles or MVs) that can transpose the hydrophilicity of the substrate's surface, making the MV-substrate ensemble hydrophobic. The approach is to use specific charges that the different classes of biological components carry (i.e., algal cell wall, proteins, and lipids) and utilize molecular vehicles to bind onto these components selectively via component-specific charges and mobilize them away from bulk medium to an adjacent solvent.

Specific objective 1: Develop a polyelectrolyte based technique for separation of carboxylate-functionalized cellulose (model algal biomass) from the aqueous phase to an organic solvent in an L/L (liquid/liquid) system. a) To identify parameters responsible for migrating carboxylate-functionalized cellulose model particles (resembling algal cell wall constituents) from aqueous phase to hexane phase in the presence of select Molecular Vehicles (MVs). b) To optimize parameters to increase the migration efficiency.

Specific objective 2: Develop a polyelectrolyte based technique for separation of protein from aqueous phase to an organic solvent in an L/L system. a) To identify parameters responsible for migration of proteins from the water phase to hexane phase in the presence of select MVs. b) To optimize parameters to increase the migration efficiency.

Specific objective 3: Develop a polyelectrolyte based technique to selectively fractionate protein and carboxylated cellulose mixture via a three-phase L/L/L system. a) To identify parameters and conditions conducive for selective fractionation of carboxyl functionalized cellulose beads and proteins via migration into a solvent using select MVs in a water/ hexane L/L two phase system.

b) To optimize parameters to increase fractionation efficiency in water/hexane L/L two phase system. c) To identify parameters and conditions conducive for selective fractionation of carboxyl functionalized cellulose beads and proteins via migration into a solvents using select MVs in water/ PEG/ hexane L/L/L three-phase system. d) To optimize parameters to increase the migration efficiency of select components in an L/L/L three phase system.

Specific objective 4: Test the multi-phase liquid fractionation system with algae. a) To identify parameters and conditions conducive for selective separation of intracellular proteins and lipids from *Chlorella sorokiniana* via multi-phase liquid fractionation system.

2. HARVESTING MICROALGAE USING IONIC POLYELECTROLYTES IN AN AQUEOUS-ORGANIC TWO-PHASE SYSTEM: SCREENING OF SEPARATION PARAMETERS USING MODEL ALGAL PARTICLES*

2.1 Introduction

The versatile nature of microalgae has made it an excellent choice as a source for numerous bio-products such as proteins, therapeutics, lipids, and other high-value polysaccharides [87]. But in the last few years, the focus has been primarily on lipids due to the high lipid yielding capacity of microalgae. Nonetheless, lifecycle assessment (LCA) and techno-economic analysis indicate that lipid recovery alone is economically unfeasible due to high energy demand for dewatering, drying and lipid extraction [88, 89]. Although some biomass and oil productivity improvements have been achieved, algae-for-biofuels-platform remains unsustainable unless a low-cost downstream processing technique is developed and other high-value metabolites are coproduced.

Harvesting microalgae is technically challenging because of a number of reasons including 1) the nature of the algal suspension[90], 2) the dilute nature of the microalgae suspension (mass concentration less than 1g L^{-1}) with densities close to that of water[91, 92, 19, 93], and 3) their stability in dispersed states due to the negative charges they carry owing to presence of algogenic organic matter [94, 18]. As the harvesting step could represent 20-30% of the biomass production costs, there is an absolute need to develop low-cost harvesting processes that could overcome all the physical, chemical and economical barriers [3, 92, 95, 4].

Aqueous two-phase separation techniques are widely used to separate biological entities like microbial cells, proteins, genetic material and organelles, but the separation of these biomolecules In the water-organic solvents is not practiced due to their low solubility in the organic phase. Though the water-organic system has been studied for separation of biomolecules by Lovrien's

* Part of this chapter is reprinted from Gejji, V., & Fernando, S. D. (2018). Harvesting microalgae using ionic polyelectrolytes in an aqueous-organic two-phase system: Screening of separation parameters using model algal particles. Process Biochemistry, 72, 188-197. Reprinted with permission from Elsevier.

group, the presence of the organic solvent resulted in the formation of a three-phase system[46]. The biomolecules resided in an intermediate layer between the aqueous and organic solvent phases which was proven to be quite challenging to separate. However, using the proposed technique, biomolecules can be wholly migrated into the organic solvent making the water-hexane phases easy to separate.

The solution proposed herein is to simplify the dewatering and separation of algal cells from aqueous media by adsorbing an oppositely charged amphiphilic polyelectrolyte on algal cells so that ensemble surfaces are hydrophobic and the algal-cells repel water molecules and migrate into hexane phase. The objective is to understand the migration behavior by capitalizing on particle's zeta potential and its ability to bind to the oppositely charged electrolyte and move the ensembles away from the water phase to a water-immiscible hexane phase. The charged particles and algal lipids can be easily recovered by evaporating hexane with relatively low energy input as compared to that required to evaporate water. In this work, the impact of various parameters like pH, type of electrolyte (monomer/ polymer), the concentration of the electrolyte, system temperature, and equilibration time on the migration percentage of functionalized beads from an aqueous medium to hydrophobic medium has been reported.

2.2 Material and methods

The chemicals used in this study were analytical grade. Sodium monochloroacetate was purchased from Sigma-Aldrich and sodium hydroxide and hexane from VWR chemicals. Asahi Kasei Chemicals Corporation provided cellulose beads (Celphere CP-102) to conduct this study. The average size of cellulose particles was 106 μm with a bulk density of 0.83 g/cm^3. DADMAC and low molecular weight PolyDADMAC (< 100,000) were ordered from Sigma Aldrich. Concentrated microalgae (15% w/w) were obtained from Texas A & M AgriLife Extension (Pecos, TX, USA). Algae were stored under dark conditions in the refrigerator at 4° C. During the experimentation, the microalgal suspension was diluted to 2% to mimic the natural conditions. Statistical analyses were done using JMP 13.

2.2.1 Carboxylation of cellulose particles

Using the process described by Magnus Bergh with a slight modification, carboxylation of cellulose particles was carried out [96, 87]. The modification being, cellulose beads mixed with sodium hydroxide solution before introducing it in sodium monochloroacetate solution. This was followed by addition of isopropanol. Carboxylation of cellulose particles was confirmed using FTIR analysis (figure 1) and the net surface charge was quantified in a flow cell cuvette arrangement using a Beckman Coulter' s Delsa nano C zeta-sizer.

The surface charge under certain condition determined the stability of the suspension. For this the zeta-potential had to be varied. The surface potential of the functionalized cellulose beads was varied by addition of acid or base.

2.2.2 Quantification of particles migrated

An initial weight of 0.2g of functionalized particles in 10ml water (or 2% w/w) were used for studies discussed herein. Cationic polyelectrolyte/ surface modifier (hereafter referred as SM) was added to the aqueous suspension of particles followed by addition of hexane, and the suspension was mixed thoroughly. The system was kept undisturbed for a set amount of time. The resulting two phases were separated carefully and used for gravimetric analysis (TGA, via TA Instruments - Q50). The weight pan of the TGA was loaded with 50 μl of a sample from the aqueous phase. The furnace temperature was maintained at 70° C for 15 minutes to evaporate hexane followed by 110° C for another 15 minutes to evaporate water. The constant weight was attributed to the amount of cellulose particles that did not migrate into the hexane phase. The difference between the amount of cellulose particles in the original suspension and the amount of particles in water phase after migration was equated to be the amount of particles that migrated into the hexane phase.

2.2.3 FAME Analysis

The analytical procedure was based on the transesterification of algal lipids to fatty acid methyl esters (FAME). Gas chromatography-mass spectrometry (GC/MS) was used to accurately quantify

and identify the FAMEs obtained from algae samples per gram of ash-free biomass (AFBM). The amount of each FAME in the algae samples was calculated based on the use of internal standards.

2.2.4 Experimental design

Preliminary screening was done to identify significant factors impacting the migration behavior. Equilibration time, temperature, electrolyte type (polymer/ monomer), pH, water to hexane ratio and electrolyte concentration were control variables and the amount of cellulose beads migrated was the primary response variable. A 2^{n-1} experimental design was used to identify significant parameters in the screening studies (Table 2.1). Three replicates for each experimental setup were done. The amount of particles migrated into hexane phase from the aqueous phase, and zeta potential (whenever applicable) were also used as response variables.

Table 2.1: Factor and levels used for screening studies

Factor	Level(s)
Type of Electrolyte Surface Modifier	PolyDADMAC and DADMAC
Electrolyte Surface Modifier (SM) concentration (w/w%)	2% and 5%
Temperature (°C)	4°C and 20°C
pH	4 and 11
Equilibration time (min)	60 and 180
Hexane : Water ratio (v/v)	1:1, 1:2 and 2:1

After identifying highly significant factors, more elaborate parametric studies were conducted to understand the migration behavior of the model particles. The impact of polyelectrolyte concentration (varied from 0.5 to 3 % w/w of the particles) and system pH (at 6, 7 and 8) on particle size, zeta-potential and amount of particle migration was studied based on the statistical significance resulting from the screening studies. Low polyelectrolyte concentrations and near neutral pH conditions were chosen looking at the high migration efficiency during the screening studies. The temperature was kept constant (at room temperature) considering the very low impact of it during the screening studies and the economics for large-scale algal separation. As the migration rate of the particles was observed to be rapid during the initial part of the migration process, to block the limiting factor, longer equilibration time was given for all the experiments.

2.3 Results and discussion

2.3.1 Carboxylation of cellulose particles

The presence of asymmetric stretch vibration of COO^- near 1560-1610cm^{-1} in the FTIR spectra (Figure 2.1) confirms successful carboxylation of cellulose particles[97]. The net negative surface charge on particles after functionalization reasoned the particles to be a suitable model as an algal emulate.

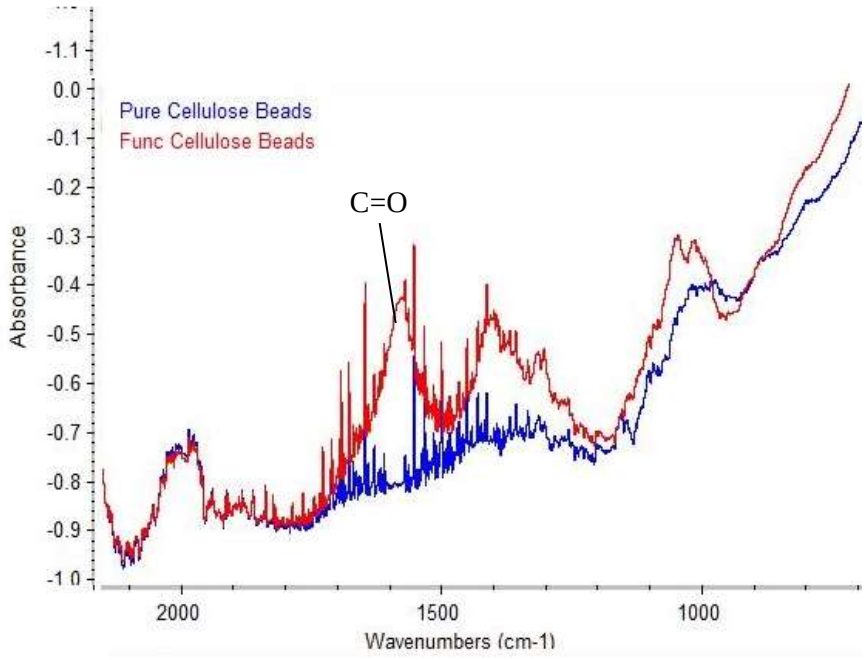

Figure 2.1: FTIR output of functionalized cellulose particles. An asymmetric stretch vibration of COO^- near 1560-1610cm^{-1}confirms the addition of (COO-) group on cellulose beads.

2.3.2 Screening studies

A set of screening studies were conducted to ascertain the impact of electrolyte addition and once added, the impact of the type (monomeric vs. polymeric) and the amount of electrolyte, time, pH and temperature. Samples volume used in TGA was 50µl; hence, the total amount of cellulose in the original suspension was 0.1 gm (figure 2.2).

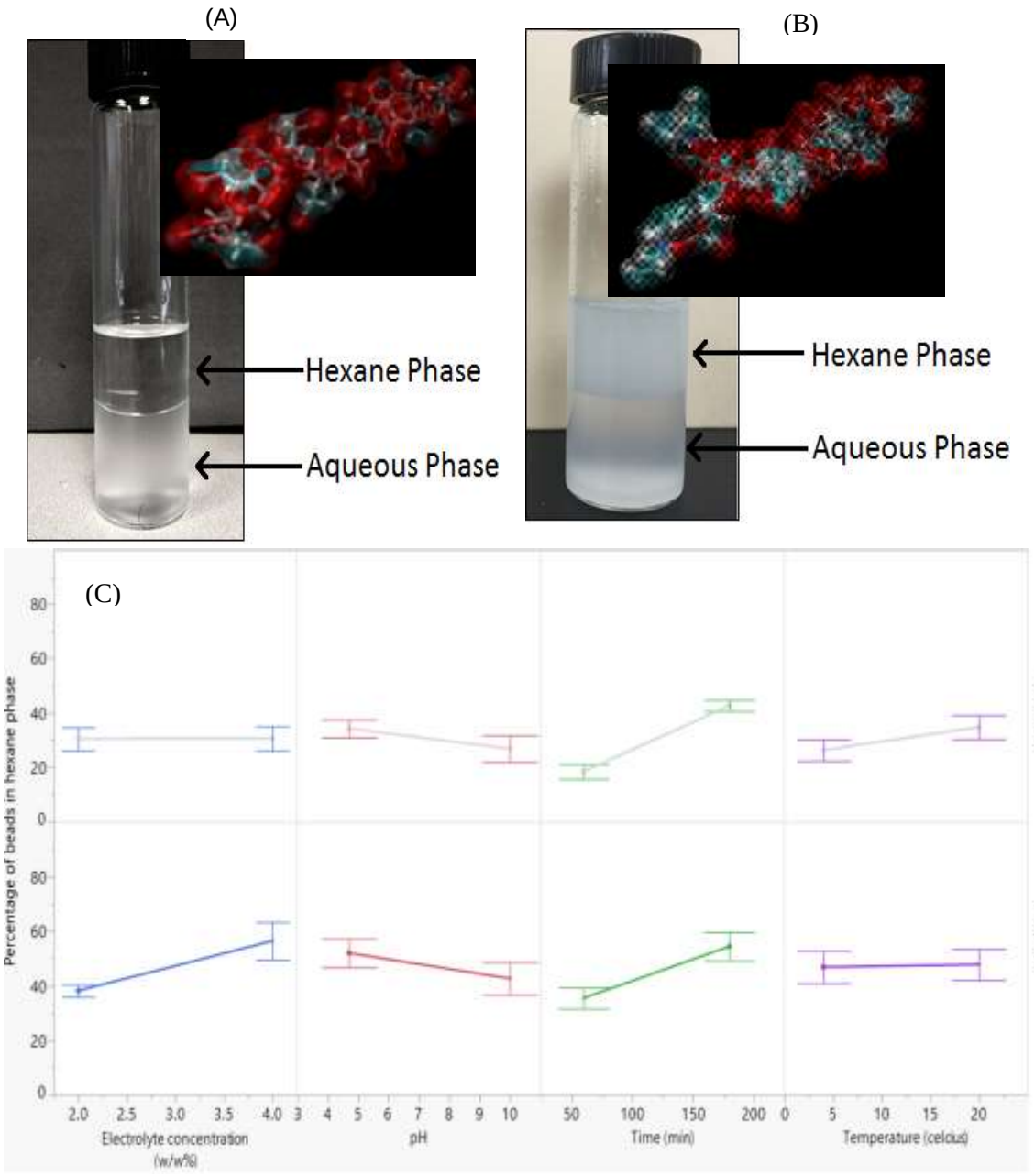

Figure 2.2: (A) Untreated functionalized cellulose particles stay suspended in the aqueous phase (hydrophilic surface is marked with red regions in the inset); (B) Polyelectrolyte treated cellulose particles (particle-electrolyte ensemble) becomes hydrophobic migrate toward the hydrophobic hexane phase (hydrophobic region is marked in cyan in the inset with only two DADMAC molecules interfaced with the cellulose crystal); (C) Impact of electrolyte type and concentration, pH, Time and Temperature on functionalized cellulose particle migration. Note: Each error bar is constructed using 1 standard error from the mean.

2.3.2.1 Effect of polymer addition

From the screening studies, it was clear that addition of the DADMAC electrolyte regardless of the type, i.e., monomeric or polymeric caused the particles to migrate into hexane phase from the aqueous phase (Figure 2.2 A, B, and C).

Functionalized Cellulose is hydrophilic (as can be seen by ionic surface charge distribution (red) in Figure 2.2A). Polar nature of poly-DADMAC makes it water soluble[98]. However, when poly-DADMAC is introduced into the solution containing negatively charged cellulose particles, positively-charged polyelectrolyte gets attached to the negatively-charged functionalized cellulose particles via charge neutralization. The polyDADMAC electrolyte has alkyl regions that are hydrophobic, and once these hydrophobic regions are preferentially exposed to the surrounding environment, the electrolyte-bound ensemble becomes hydrophobic (Figure 2B) making the cellulose- electrolyte ensemble water-insoluble. The charge distributions were mapped using Visual Molecular Dynamics (VMD)[99]. The hydrophobic nature of the organic solvent helps in increasing the solubility of the ensembles in it. Thus, forcing them to migrate away aqueous phase. Hexane being lighter than water, particles are seen to migrate into the top phase, i.e., into hexane, leaving the water at the bottom. In figure 2.2A, functionalized particles not treated with surface modifier are observed to remain in the aqueous phase as opposed to the DADMAC-treated particles readily migrate to the hexane phase (Figure 2.2B).

2.3.2.2 Effect of electrolyte type and concentration

Studies indicated that both electrolyte type (Prob > F = 0.0002) and amount (Prob > F = 0.0008) had a substantial impact on the migration system. As compared to the monomer, addition of polymer increased the migration efficiency. Higher concentrations of polymer had higher migration capacity, and this may be due to the formation of a net-like structure that entraps more solid particles Figure 2.2C.

2.3.2.3 Effect of pH

The impact pH was also statistically significant as per the screening studies (Prob > F < 0.0001). In-depth parametric studies conducted later would discuss the impact of pH in more detail.

2.3.2.4 Effect of equilibration time

Impact of equilibration times on the amount of bead migration to the hexane phase was significant (Prob > F < 0.0001). It was revealed as expected that longer equilibration times favored solids migration regardless of the level(s) of other variables.

2.3.2.5 Effect of temperature

The effect of temperature on the migration system was observed to be mildly significant (Prob > F = 0.0289). It was interesting that when the polymer was used, the impact was less prominent while with the monomer, higher temperatures slightly favored higher particle migration. This could be a result of a combination of the density change in the medium and variation of the molecular structure due to temperature rise. The structure of polymeric chains continually changes due to thermally mobility [100]. In case of polymeric-bound ensembles, as the temperature increases, the expansion of polymeric structure may have reduced the number of polymer molecules finding the charged surface due to steric hindrance and thus negatively impacting the overall hydrophobicity - reducing ensemble migration. In case of monomeric electrolyte, steric hindrance is less dominant, and as the temperature increased, the reduced density may have resulted in a lower drag assisting in migrating from aqueous phase to hexane.

2.3.2.6 Interactions between pH and electrolyte concentration

The degree of particle migration was significantly impacted by the two-factor interaction between electrolyte concentration and system pH (P< 0.0001). More details on this interaction are discussed under parametric studies.

2.3.2.7 Effect of water to hexane ratio

To test whether water to hexane ratio would be a limiting factor for the migration of particles, a study was conducted at three different levels of the ratio while keeping pH, time, temperature, polyelectrolyte concentration constant. The ratio of water to hexane was maintained at 2:1, 1:1 and 1:2. There was no significant impact of water to hexane ratio on the amount of particles migrated into the solvent phase. Obviously, for favorable economics and to have water to hexane ratio as a non-limiting factor during parametric studies, the solvent volume has to be kept to a minimum. Thus during subsequent parametric studies, the water to hexane ratio was kept at 1.

Above screening studies indicate that all the individual factors, i.e., electrolyte type and concentration, equilibration time, temperature, and pH significantly impacted the migration efficiency. Two-factor interactions between electrolyte concentration and system pH were found to have the most prominent impact on the migration behavior of the particles. Although temperature had a minimal impact on migration of particle when monomer was used, considering implications when translating this research to practice, temperature and equilibration time was decided to be kept constant (at room temperature) and 60 minutes respectively during parametric studies. The water to hexane ratio was kept constant at 1:1. Accordingly, for parametric studies, polyelectrolyte type and concentration and system pH were selected as primary variables.

2.3.3 Parametric studies

The parametric study performed to ascertain the impact of a broader range of select variables on particle migration showed that overall, the impact of pH (varied between 6, 7 and 8) to be significant, the polyelectrolyte concentration (varied from 0.5 to 3% w/w at 0.5% intervals) to be not significant, and the two-factor interaction between polyelectrolyte concentration vs pH interaction was marginally significant (Figure 2.3 A, B, C and D) on the amount of particle migration.

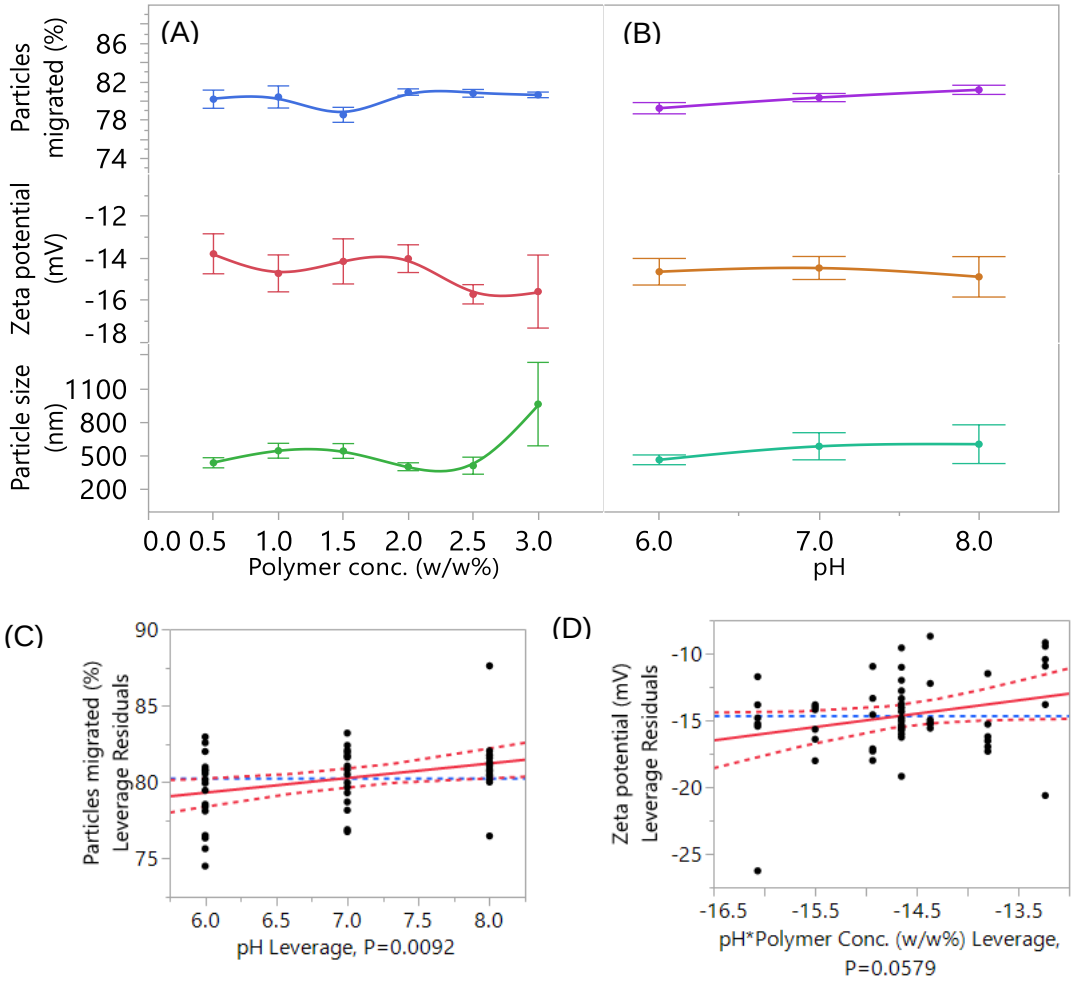

Figure 2.3: (A) Effect of electrolyte concentration and pH on particle size, zeta potential and particle migration. Particle migration remains almost constant (79%) irrespective of polymer concentration and system pH suggesting that the system is efficient at very low polymer concentration and over a wide pH range. Each error bar is constructed using 1 standard error from the mean.

The amount of particles that migrated was independent of the polymer concentrations that were tested, and this may be due to once charge-neutralization is attained, further increase in polyelectrolyte not impacting surface potential of the cellulose particles. Nevertheless, it was encouraging that the amount of particles migrated remained well above 75% regardless of the polymer concentration. This alludes that using polyelectrolytes as low as 0.5% is sufficient to

trigger migration.

The increase in the size of flocs formed as a result of polymer addition did not negatively impact migration. This alludes that the hydrophobic effect of cellulose-electrolyte ensembles alone was sufficient to mobilize the particles away from heavier aqueous layer to the lighter hexane layer against gravity.

The increase of polyelectrolyte concentration tended to increase the magnitude of zeta-potential which is yet unclear. However, it is likely that pH may be playing a role (due to the statistically relevant interaction between pH and polyelectrolyte concentration, Figure 2.3D).

Figure 2.3C clearly shows that system pH had a significant impact on the amount of particles migrated. As the pH became basic, the amount of particles migrated increased. To further elucidate what may be occurring as the pH is increased from acidic to the basic range, an ancillary study was conducted on the impact of pH on zeta-potential of pure cellulose particles. The zeta potential was found to be -10.18mV, -14.44mV and -21.62 mV for pH 6, 7, and 8 respectively before electrolyte addition. It was noted that the magnitude of the potential increased as the pH increased (Figure 2.4A).

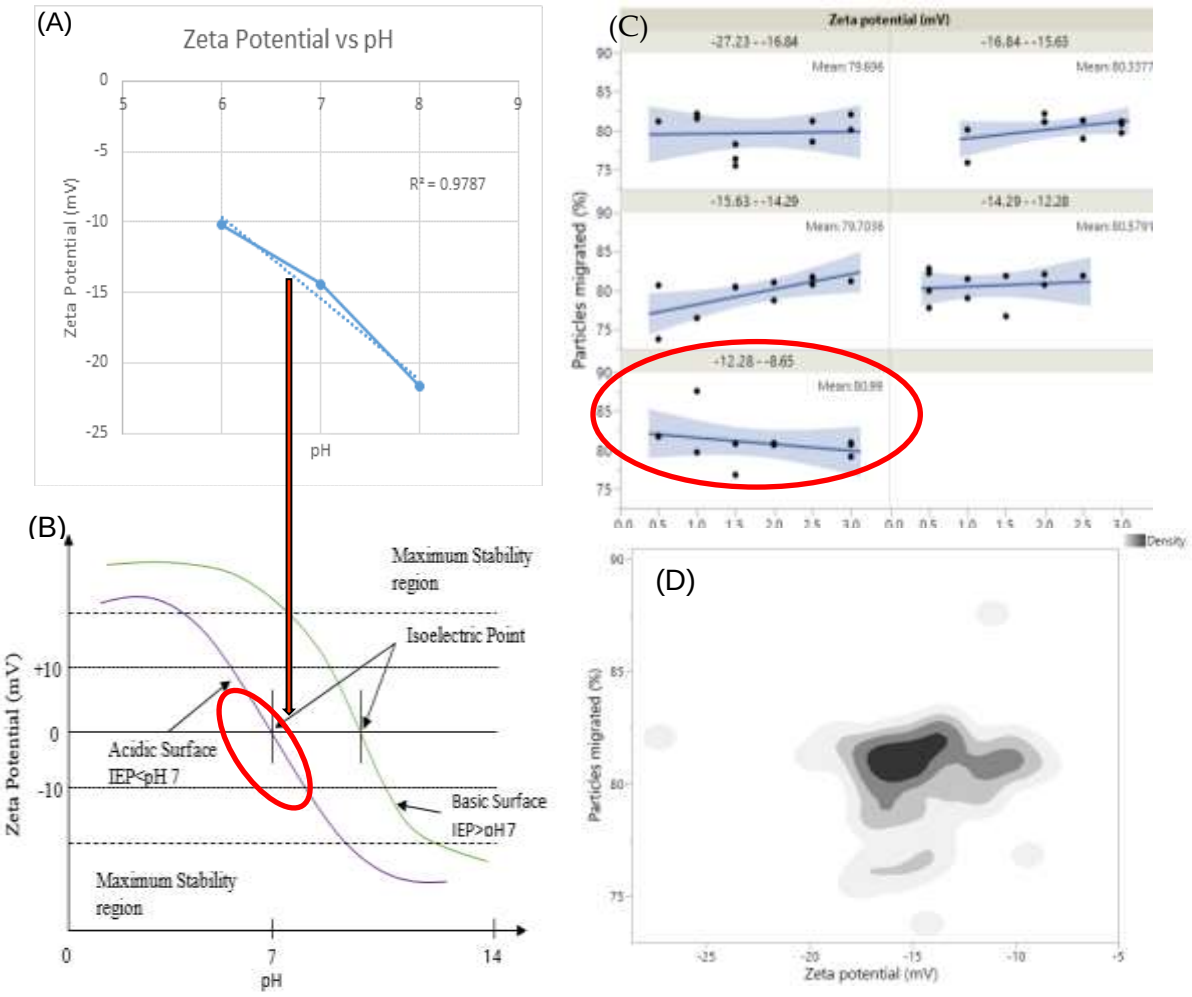

Figure 2.4: Effect of pH on zeta potential of carboxyl functionalized particles. (A) With the increase in pH, the net negative charge of the particles increased. (B) Particles with zeta potential > -10 mV are generally considered to be in the stable region i.e. stay suspended in aqueous solution. Purple and green lines indicates the change in zeta potential as a function of pH for acidic and basic surfaces respectively (C) Particles in the stable region migrated away from aqueous phase after treating them with polyelectrolyte, suggesting formation of hydrophobic ensemble. (D) Highest particle migration efficiency was observed when the zeta potential of particles was between -12 mV and -15 mV.

A further analysis (Figure 2.4B)[101] indicates that this system lies in the region marked in the generalized pH vs Zeta-potential curve alluding that the system is well dispersed, and a reduction of pH would force the system to arrive at its isoelectric point *I*p which also would lead to floc formation (indicating the possibility of better particle separation/migration from aqueous phase

given adequate hydrophobicity is attained). The purple and the green lines in figure 4B indicates the change in zeta potential as a function pf pH of acidic and basic surfaces respectively.

Once polymer was added, the zeta-potential reached around -15 mV (Figure 2.4D) indicating that the system was still in the stable region. Figure 2.4D represents the range of zeta potentials achieved by cellulose particles after migration under all the three pH conditions (i.e., 6, 7 and 8). It can be observed highest migration was achieved when particles arrived a zeta potential between -12 to -8 mV after polymer attachment. This indicates that the migration occurred while surface charges reached less negative (on its way to charge neutralization) which is a result of binding of polymer with hydrophobic ligands. Charge neutralization facilitates floc formation, but alone does not assist in the migration of the flocs. This means that usage of a cationic flocculation agent alone will not instigate the migration phenomena and the agent should essentially possess hydrophobic ligands. It was interesting to note that subsequent to polyelectrolyte addition; maximum migration occurred when the Zeta-potential was lowest (between -8.65 and -12.28mV, Figure 2.4C), i.e., the experimental units closest to charge neutralization. In fact, in experimental units that had higher zeta-potentials after polymer addition, the migration trends continued to improve with the increase in the polymer (see -12.28 - -27.27mV plots in Figure 2.4C). The results of zeta-potential before and after polymer addition confirms the charge neutralization effect since essentially the zeta-potential which was >-20mV before electrolyte addition at pH 8 (the condition at which migration was highest), reduced as a result of cationic polyelectrolyte addition. It is known that zeta potential is pH dependent and thus pH impacts particle migration. When applying this technique for fractionating real algal samples, it should be noted that the optimal surface charge could be arrived by changing the solution pH that algae reside.

Above observations indicate that an increase in pH results in a further increase in the magnitude of the surface potential which facilitates binding of the cationic polyelectrolyte. Essentially, the more polyelectrolyte bound, the more hydrophobic the surface would be which in turn would translate to more particle migration. Removal of H^+ from the system (via alkali addition), may also enhance the availability of a cationic portion of the DADMAC for charged particle surface due to reduced competition.

2.3.3.1 The impact of pH

Due to the statistically significant impact of pH during screening studies, a more in-depth look at how the system behaves under different pH conditions was done. Figure 2.5 depicts zeta potential and particle migration data sliced across individual pH condition(s).

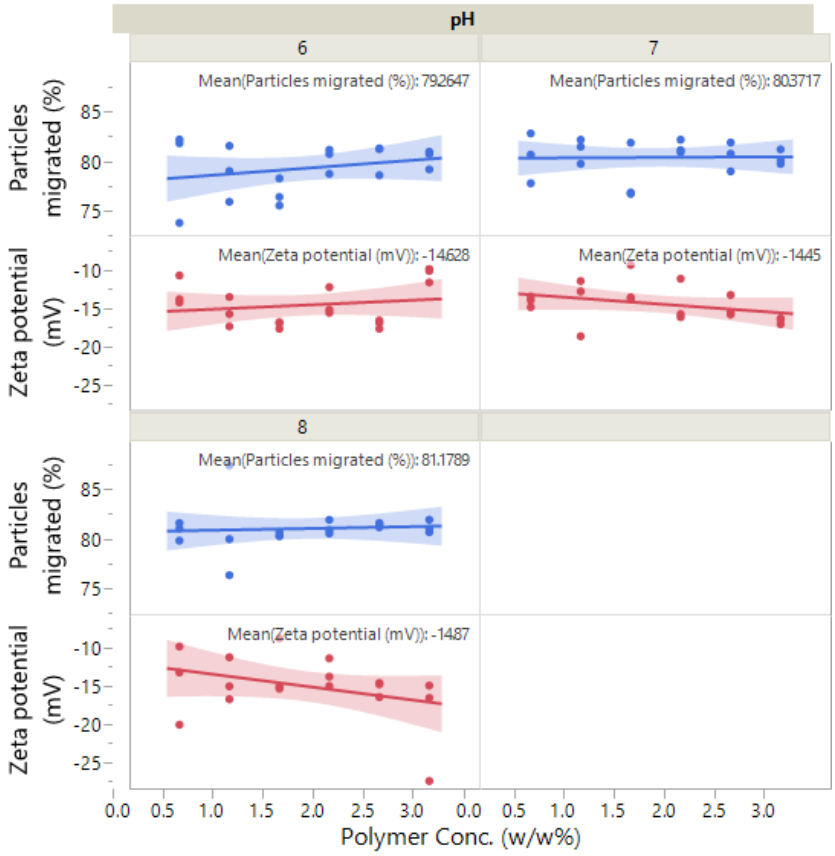

Figure 2.5: (A-C) Effect of electrolyte concentration and zeta potential on bead migration at different pH values.

Overall, regardless of pH, the system was able to migrate about 80% of the particles on average (Figure 2.5 A-C). The impact of different pH on Zeta-potential was quite evident; however, the impact on particle migration was not very significant. The system performed better in terms of migration at neutral and basic conditions as opposed to acidic conditions.

Acidic conditions worked well for this separation process with an overall mean of 79% of particles migrating from aqueous to solvent phase. It could be seen that as the polyelectrolyte concentration increased the zeta-potential decreased indicating that the charge-neutralization effect did materialize as expected; and the result was progressively increasing particle migration with increased polyelectrolyte concentrations. This was evident even during screening studies. It should be noted that even under acidic conditions the surface charge could be negative (see Figure 2.4) and this explains why the separation system behaved well at pH 6. However, the trend was that increasing pH favored particle migration.

At pH7, it is seen that as polyelectrolyte concentration increased, the magnitude of surfaces charges tended to increase while keeping the amount of particle migration essentially flat (80% migration). At low polymer concentrations, there is a higher availability for polyelectrolyte to bind to the negatively charged cellulose beads and to exert sufficient hydrophobicity (once the electrolytes latched onto the cellulose particles) and to facilitate ensemble migration; however, as the polyelectrolyte concentration increased the surface potential tended to increase slightly indicating less electrolyte binding (Figure 2.5B). This could be because higher polyelectrolyte concentrations inducing polymer aggregation via micellar formation that prevent the polymer interacting with the particle surface. This indicates that under neutral conditions, the lowest amount of electrolyte should be used for effective particle migration.

At pH8, it clear that the amount of particles migrated stayed relatively flat with increasing polyelectrolyte concentrations. The separation system performed best under basic conditions (with an average 81% of particle migration). Nevertheless, under basic conditions, the Zeta-potential seemed to increase with higher polyelectrolyte addition which is not advantageous (Figure 2.5C). This means that under these conditions, the addition of more polyelectrolyte does not help charge

neutralization which in turn should have translated to more particle migration. Accordingly, the system behaved well under basic conditions provided that the amount of polyelectrolyte not be in excess (i.e., <2%) i.e. to make the system imbalanced. Addition of excess cationic polyelectrolyte would introduce a net positive charge making the system unstable disrupting the migration process likely by impacting intermolecular interactions[102].

Above observations delineates that the addition of polyelectrolyte impacts surface charge (in addition to pH variation) and there seems to be an optimal level of polyelectrolyte along with the correct pH that promotes electrolyte-particle adhesion and in turn maximizes particle migration.

2.3.3.2 The impact of floc size

A key item that needs to be understood especially in a polymer-mediated system is how floc size impacts the system performance. It was noted that in general, the floc size remained 500nm (Figure 2.6A) and that of the flocs increased with increased polymer addition (Figure 2.6A). A closer look at how the floc size impacted particle migration reveals that smaller flocks favored more migration (Figure 2.6B); however, it was noted that acidic conditions favored smaller floc sizes (Figure 2.6C) likely since the system was still stable (i.e., with high zeta-potential) under low polymer concentrations (Figure 2.6A). This analysis reveals that conditions should be carefully modulated to improve further system performance such that the flocs are at the optimal size to facilitate migration.

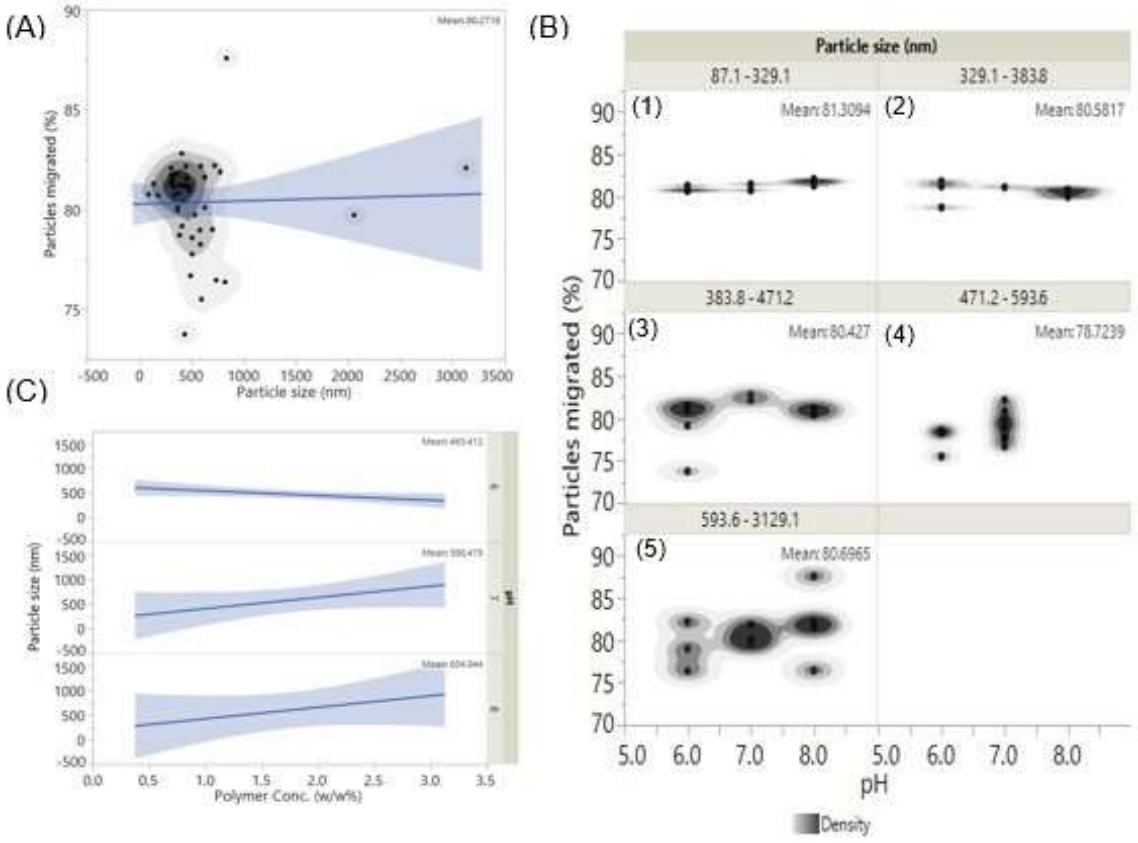

Figure 2.6: The impact of floc size on system performance. (A) Shows the particle migration efficiency remained 79% irrespective of the floc size. (B) Shows the system at different pH and particle flocs in different size ranges to have no significant change in the efficiency of migration system ($P>0.05$). (C) Change in polyelectrolyte concentration has no significant impact on particle size.

2.4 Validation studies with microalgae

The validity of the above premises of the ability of PolyDADMAC to attach onto algal cell surface and migrate the cells from aqueous phase to hexane was verified with Chlorella microalgae. As it could be seen from Figure 2.7, at 5% (w/w) level, more than 50% of the algal cells were migrated into hexane phase from aqueous phase when the algae were freshly harvested. The amount of cells migrated surpassed 80% with aged algae (four weeks in the refrigerator with no light) as compared to the fresh ones (within one week of harvesting). The likely reason for this is the increase of surface acidity when the enriched lipids (originally 15% w/w) hydrolyzed/oxidized

to acids[103]. In other words, the chemical changes that occur at the algal surface can influence the zeta potential and also increase the amount of extracellular organic matter[104]. Furthermore, the observed difference could also be the result of prolonged cold or dark exposure leading to exchange of fatty acids or to starvation. This observation is in-line with the findings with cellulose particles where increased initial surface potential tended to improve particle migration. It was noteworthy that the lower 1% (w/w) polyelectrolyte concentrations migrated lesser amounts of cells, possibly due to low surface coverage. These observations confirm the notion that there is an optimal amount of surface coverage required to exert an adequate hydrophobic effect to repel the polyelectrolyte-bound ensembles from aqueous phase; however, the polyelectrolyte concentration should not be too high so that the loosely bound and/or free polyelectrolytes can impart electrostatic forces overpowering hydrophobic effect.

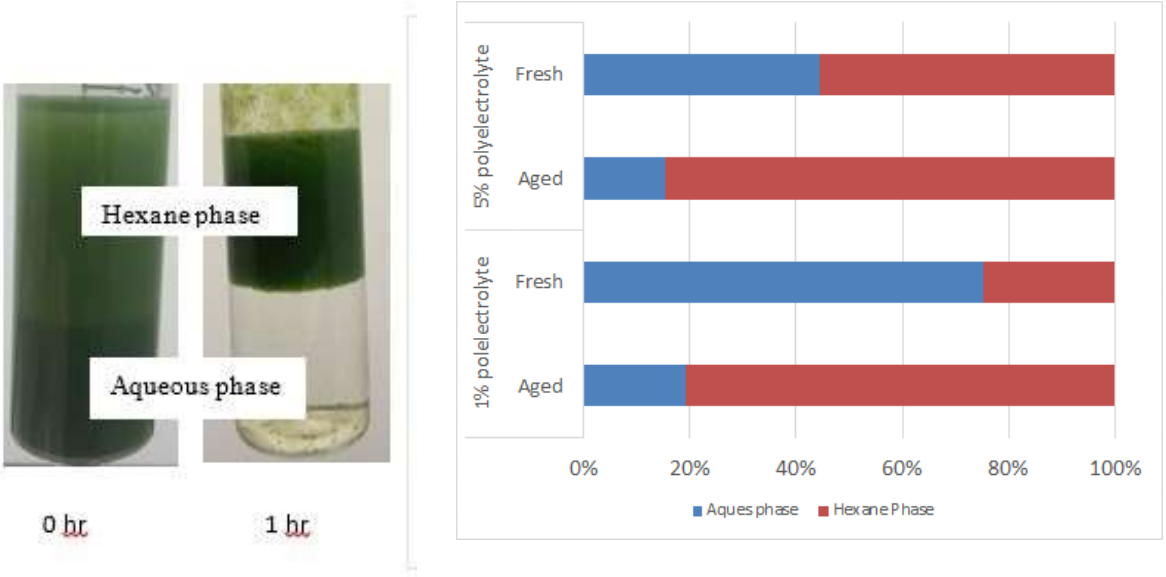

Figure 2.7: The amount of algal solids remaining in each phase after subjecting to PolyDADMAC functionalization. The image to the left depicts migration at the onset of separation and the one to the right depict near complete cell migration after 1hr.

Furthermore, the ability of the polyelectrolyte based system to remove lipids from algae with-

out subjecting them to any additional cell wall disruption was investigated. We anticipate an increased algal cell wall permeability as a result of interaction between the cell surface and poly-DADMAC. Similar observations were made, where cell membrane disruption was detected due to the addition of cationic polyelectrolytes in the system[105].

Form the lipid analysis conducted on the control sample (algae neither treated with the poly- mer not subjected to migration), algae treated with 1% and 5% (w/w) polyelectrolyte at 25°C, it was found that lipids can be successfully extracted into hexane phase without cell disruption. Table 2.2 represents the amount of fatty acid methyl esters (FAME) content per gram of hexane extracted (note: FAMEs extracted to hexane phase are given in per gram of hexane basis while to- tal FAMEs present in the original sample are given AFBM basis). The fraction of lipids extracted with 1% polymer and 5% polymer was 30% and 15% respectively based on the total lipid content. Nevertheless, considering only the algal fraction that migrated to the hexane phase, it was found that >90% of lipids were extracted with 1% polymer and 85-90% with 5% polymer. The amount of lipids extracted via polymer-based cell migration process is significantly higher than the previ- ous reported, i.e., 2.2%, from C. vulgaris without cell disruption and using hexane as extraction solvent[106]. The FAME yield using this technique is also significantly higher than the previous reported for Chlorella spp. using hexane as an extraction solvent in a Soxhlet setup (5.6%)[107]. The amount of lipids extracted with 1% polyelectrolyte was higher than 5%. From the algae treated with 1% and 5% polyelectrolyte, 53.77 mg FAME/ g AFBM and 28.47 mg FAME/ g AFBM was extracted respectively. The total milligram of FAME per gram of AFBM in the control sample was 178.22. The lower amount of lipids that were able to be extracted especially using 5% polymer could be attributed to the polymer disrupting the lipid leaching mechanism. These observations confirm that the electrolytes not only mobilize algae from water to hexane phase but also assists milk out lipids without disrupting the algal cells.

Table 2.2: Total FAME Content of Extracted Samples

	mg FAME/g Hexane		mg FAME/g AFBM
FAME	**1% Polymer**	**5% Polymer**	**Original Algae**
C14:0	0.004	0.003	2.46
C14:1 cis	0.002	0.002	0.00
C16:0	0.128	0.102	46.72
C16:1 cis	0.017	0.015	1.66
C16:1 trans	0.011	0.007	2.84
C16:2 cis	0.025	0.019	4.32
C16:3 cis	0.095	0.073	17.17
C18:0	0.015	0.011	3.56
C18:1 cis	0.093	0.065	24.58
C18:2 cis	0.166	0.133	29.01
C18:3 cis	0.223	0.182	38.64
C20:0	0.011	0.005	6.50
C24:0	0.000	0.000	0.77
TOTAL	0.791	0.617	178.22

2.5 Conclusions

Migration behavior of negatively charged particles resembling algal cells from the aqueous phase to hexane phase was studied using a net positively charged amphiphilic surfactant. Studies indicated that both monomeric and polymeric forms of DADMAC cationic electrolyte were able to successfully migrate carboxyl-functionalized cellulose particles. As compared to monomeric form, the polymeric form had a more positive impact on the migration behavior. Screening studies indicated that along with the form of the electrolyte (monomeric vs. polymeric), the amount, the equilibration time and pH affected the amount of particles that migrated into solvent phase. The temperature did not have a significant impact when the polymer was used in the migration system. For the volumetric ratios of the aqueous medium and the water-immiscible-solvent studied, it was observed that the ratios had no significant impact on the amount of particles that could be effectively migrated.

A more detailed parametric study using a broader range of polyDADMAC concentrations at acidic, neutral and basic conditions with 1:1 water to hexane ratio revealed

that the amount of particles migrated directly correlated with Zeta-potential, i.e., surface charges that the particles carry. It was revealed that the polyelectrolyte is functional over a broad pH range and can bind to the negatively charges cellulose particles resulting in particle migration. The magnitude of the surface charge of particles before polyelectrolyte addition correlated with system pH, i.e., high (basic)

pH resulted in higher surface potentials and low (acidic) pH resulted in lower surface potentials. After electrolyte addition, the system that initially was at a higher potential, i.e., more negative magnitude, migrated more achieving a lesser potential during the process. So, the amount of particles migrated was inversely proportional to the magnitude of surface charge after polyelectrolyte addition suggesting that particle migration correlated with bridge formation (by binding action of polyelectrolytes onto acid-functionalized cellulose).

Overall, the optimum combination of conditions was basic pH and low polymer concentration that facilitated particle migration from heavier water phase to the less dense hexane phase. To impart sufficient hydrophobicity to the particles with the purpose of repelling the ensembles away from the aqueous phase, pH of the system should be large enough to preserve negative zeta potential surface functionality and to provide the right amount of electrolyte to bind to it. The analysis also revealed that regardless of the system pH, the negatively charged particles could be mobilized to move away from water molecules to hydrophobic solvent phase by adding an adequate amount of cationic polyelectrolyte. The resulting floc size also had an impact on the system performance. The results leads to the possibility that particles with differing surface functionality (i.e., acidic, basic or zwitterionic) could be selectively migrated away from an aqueous mixture (containing acidic, basic, zwitterionic and neutral particles) to an immiscible solvent by modulating the system pH and allowing the particles interact with ionic electrolytes with hydrophobic residues. Thus ionic electrolytes could be used as potential molecular transport vehicles to adsorb onto particle surfaces with opposite charges, like those found in microalgae or microbial cells, to chemically dewater and separate them from a bulk aqueous medium where they originally resided to an organic solvent with this simple technique.

3. POLYELECTROLYTE BASED TECHNIQUE FOR SEQUESTRATION OF PROTEIN FROM AN AQUEOUS PHASE TO AN ORGANIC SOLVENT*

3.1 Introduction

Proteins play a vital role in human and animal metabolism [108]. With the sharp inclination towards a more health conscious life style, the demand for protein-rich food, especially from non-animal sources are on the rise. However, due to ever-increasing population and decreasing cultivable farmland, microalgae has gained increased attention as a protein source due to their ability to accumulate up to 60% proteins under the right growth conditions [109]. Despite these advantages, due to the dilute nature of the natural medium, microalgae harvesting and separation of proteins are still recourse intensive and expensive. Hence, there is a need for fractionation technique(s) that are economical, scalable and less energy intensive.

To separate biological materials (such as cells and cellular proteins), aqueous two-phase systems (APTSs) have been employed [41]. APTSs have been widely studied for protein separation because of its biocompatibility, ease of scalability, low toxicity of phase forming chemicals and continuous operation. APTSs involve the use of varying concentrations of a salt-polyelectrolyte combination or two polyelectrolytes and two salts in aqueous medium to form two distinctly separate aqueous phases that are immiscible in each other [110]. Due to the increasing salt concentration in the system, the polyelectrolyte losses surrounding water molecules and at an optimum salt concentration the polyelectrolyte (still surrounded by less number of water molecules) starts forming a distinct second phase. Increasing salt concentration in the medium also leads to precipitation of proteins forcing them to move away from the more hydrophilic phase to a less hydrophilic phase consequently results in an ATPS [35, 110]. However, an important issue pertinent to industrial scale ATPS is the phase separation rate [111]. The rate of phase separation can be

* Part of this chapter is reprinted from Gejji, V., & Fernando, S. (2018). Polyelectrolyte based technique for sequestration of protein from an aqueous phase to an organic solvent. Separation and Purification Technology, 207, 68-76. Reprinted with permission from Elsevier.

accelerated by centrifugation in disc stack centrifuges [112, 113], using an external microwave, and acoustic field and electrical polarization [114, 115]. However, this can substantially increase the downstream processing costs.

Recently, studies have also been conducted on the use of three-phase partitioning (TPP) systems where an organic solvent like t-butanol is used to separate proteins from aqueous medium [116, 117, 118, 119, 49]. In a TPP system, with the addition of salt, proteins precipitate out and form an intermediate layer between water and the organic solvent. Ammonium sulfate and organic solvent t-butanol reinforce each other's physicochemical effects, such as ionic strength effects, osmotrophy, osmotic stresses and exclusion crowding effect to partition the proteins as a mid-layer between aqueous and organic phases[117]. However, because of the low solubility of biomolecules (from cells to proteins) in the organic solvents, it is considered to be less suitable for commercially viable separation systems. Addressing the solubility issue of biomolecules like proteins in an organic solvent can help achieve a very efficient separation method. Instead of forming a three-phase partitioning system, increased solubility will result in the formation of just an aqueous-organic solvent two-phase system. Thus, during the separation of two phases, back mixing of the two phases will be avoided. It will not only reduce the strain on energy requirement for separating proteins from aqueous suspensions but also help reduce processing time and costs.

To address above issues with current separation systems targeting microalgae, we developed a technique that capitalizes on negatively charged surfaces that microalgae naturally possess. The processes separate algae from an aqueous suspension by migrating them away from the water phase into an adjacent immiscible (hydrophobic-liquid) phase by using an ionic polyelectrolyte that can transpose the hydrophilicity of the algal surface making it hydrophobic. The objective of this study is to evaluate if the aforementioned technique would be applicable for separating proteins. Such a technique can be easily scaled up and a superior replacement to energy-intensive alternatives like membrane filtration and centrifugation while also addressing the challenges of APTS and TPP.

3.2 Material and methods

Egg albumin extract and hexane were purchased from VWR and poly-(diallyl dimethylam-

monium chloride (average Mw 200,000-350,000 (medium molecular weight), 20 wt. % in H_2O) was purchased from Sigma Aldrich. Statistical analyses were done using JMP 13. The molecular simulation was performed using Autodock Vina, and interaction diagram was generated in Maestro.

3.2.1 Protein migration and quantification

An albumin protein solution (2% w/w) was prepared using deionized water. Low concentration of proteins was prepared to mimic comparable concentration of proteins from microbial sources, i.e., microalgae. An appropriate quantity of polyelectrolyte was added to each sample set and to ensure adsorption of the polyelectrolyte onto the proteins; the mixture was vortexed for 30 seconds. Hexane was added to the aqueous suspension, and the water-hexane system was again vortexed for 1 minute at maximum speed. The system was kept undisturbed for the phases to separate. Samples were drawn from both the hexane and aqueous phases and analyzed on the Thermo-Gravimetric Analyzer (TGA). The amount of protein in the original suspension control sample to calculate the amount proteins that migrated into the hexane phase. Figure 3.1 shows the schematic representation of the protocol used to migrate protein from aqueous phase to hexane phase.

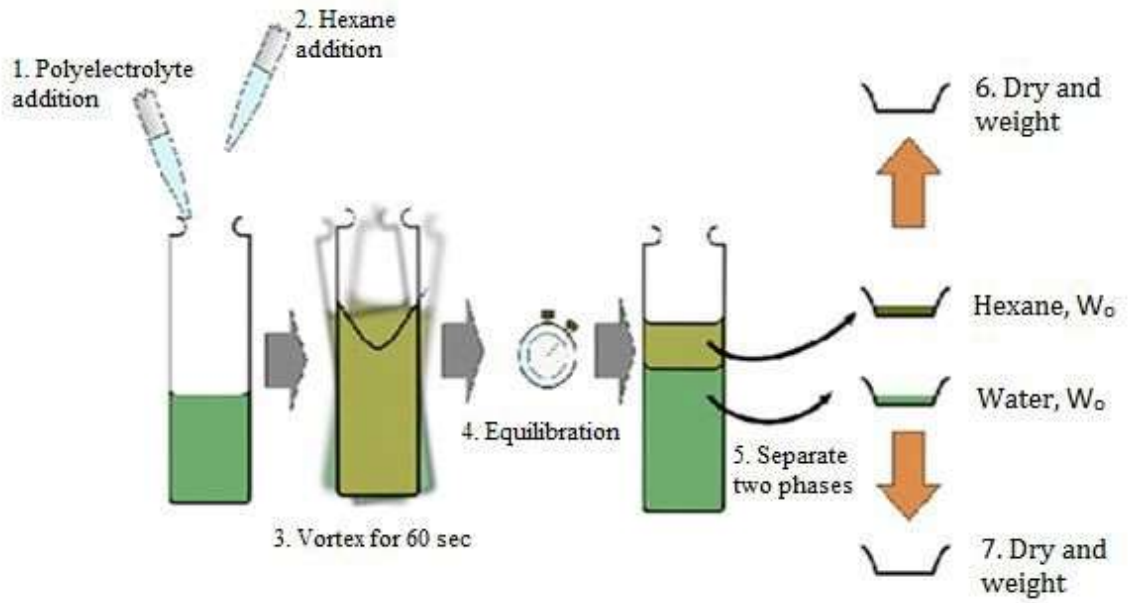

Figure 3.1: A schematic of the protein migration process and the analytical procedure (Note: W0, Water -Weight of particles in the water phase. W_0, Hexane- Weight of particles in Hexane phase).

3.2.2 Measuring zeta potential of the egg albumin suspension

Zeta potential is the surface charge a particle carries. The zeta potential of the egg albumin was measured using Beckman Coulter (Delsa Nano C) Zeta Sizer via the flow cell assembly. The surface charge was measured for proteins at different system pH values before subjecting to the migration process. Water samples were collected and again analyzed for zeta potential after the system was subjected to the migration process to evaluate the effect of the polyelectrolyte addition on the zeta potential of the proteins remaining in the water system.

3.2.3 FTIR analysis

Proteins display two distinct FTIR peaks, amide I peak at wavenumber 1652 cm^{-1} and amide I peak at 1520 cm^{-1}. This analysis was done to reaffirm the polyelectrolyte assisted migration of proteins into the hexane phase and to understand the impact of the polyelectrolyte on the protein structure. FTIR was performed via a ThermoFisher Nicolet iS10 spectrometer. The results from

FTIR studies regarding protein conformation stability was reaffirmed by SDS-PAGE analysis.

3.2.4 SEM analysis

SEM analysis was conducted to observe the change in the protein surface before and after polyelectrolyte addition.

3.2.5 Molecular simulations

To ascertain interactions between protein and the polyelectrolyte, molecular simulations were performed using AutoDock Vina [120]. The structure of egg albumin was retrieved from protein data bank, and a model of polyDADMAC was built and optimized via ChemSketch. Monomeric, dimeric, and oligomeric forms (a maximum of 16 repeating units) of DADMAC molecule were used in the docking simulation. The results of molecular docking were visualized via VMD [99] and Maestro[121].

3.2.6 Experimental design

Table 3.1 depicts the independent variables and its corresponding levels chosen for detailed parametric studies.

All experiments were conducted in triplicate as a completely randomized design. JMP statistical tool was used to analyze data. The significance was set at p=0.05 level.

Table 3.1: Factor and levels used for screening studies

Factor	Level(s)
Type of Electrolyte (Surface Modifier)	PolyDADMAC
Electrolyte Surface Modifier (SM) concentration (w/w %)	0.5 -3 (at 0.5% intervals)
Temperature (^{o}C)	20^{o}C, 30^{o}C and 40^{o}C
pH	6, 7, and 8
Equilibration time (min)	20,30 and 40
Hexane : Water ratio (v/v)	1:1, 1:2 and 2:1

3.3 Results and discussion

3.3.1 Effect of time and temperature

Figure 3.2A shows the impact of different equilibration times on the amount of albumin migration from the aqueous phase to the hexane phase. It is evident that shorter equilibration times favored more proteins migration from the aqueous phase to the solvent phase. Nevertheless, the results suggested that the time was not statistically significant. However, to remove discrepancies associated, equilibration time was kept constant at 60 minutes for remaining experiments.

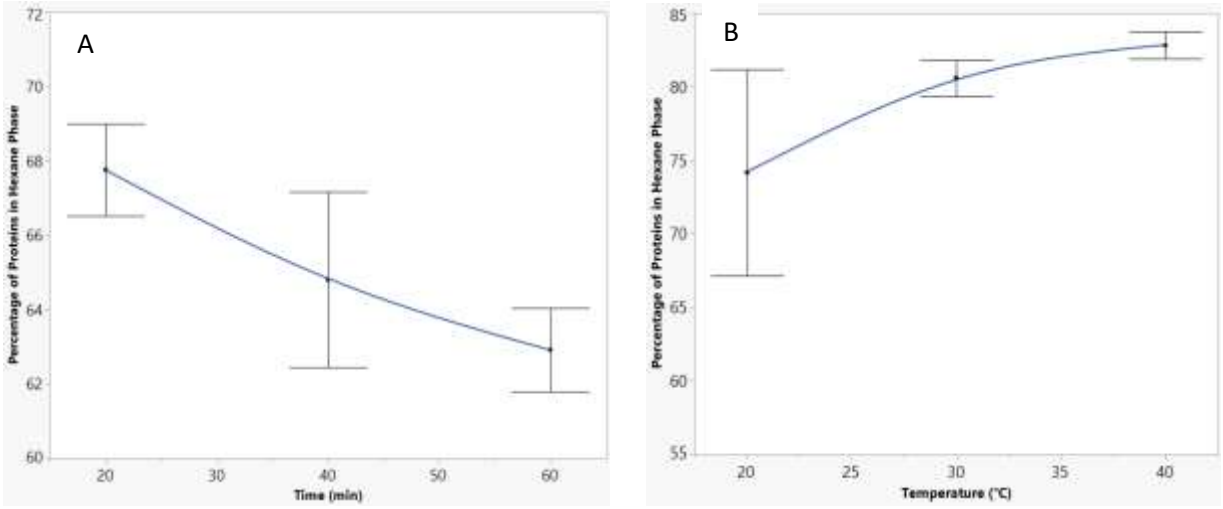

Figure 3.2: A) Effect of equilibration time on albumin migration from the aqueous phase to solvent phase (p value= 0.002); B) Effect of temperature on protein migration from the aqueous phase to solvent phase. As the equilibration time increased, the amount of proteins in the hexane phase seem to decrease slightly, and with the increase in the system temperature, there was a slight increase in the amount of proteins migration in the hexane phase (p value= 0.002).

Figure 3.2B shows the effect of temperature on particle migration from the aqueous phase to hexane phase with polyelectrolyte addition. It could be noted that the impact of temperature was not statistically significant. As a result, subsequent experiments were conducted at 20°C.

Maintaining the temperature at 20°C would also help in preserving the structural integrity of the protein.

3.3.2 Effect of hexane to water ratio

To test whether hexane to water ratio has an impact on the migration process, a study was conducted at three different hexane: water ratios, i.e., 2:1, 1:1 and 1:2, while keeping pH, time, temperature, and polyelectrolyte concentration constant. Figure 3.3 shows that maximum protein migration occurred at a hexane to water ratio for 1:2. Protein migration maximizing at high hexane to water ratio may be attributed to the time taken for the two liquids to form stable separated phases. The rate of phase separation is reported to be dependent on the relative size of the two phases where Salamanca, M., et al. observed that a larger top phase slowed phase separation [122]. When the equilibration time is constant, a low migration rate of proteins is observed in samples with higher top (hexane) phase as the time taken for the formation of stable hexane phase is extended for samples with lower hexane to water ratio. Nevertheless, during further studies, the hexane: water ratio was kept at 1:1 for the volume of hexane to be kept non-limiting. To eliminate the impact of time for separation, the amount of time left for phase separation was set at 1 h and non-limiting for the remainder of the experiments.

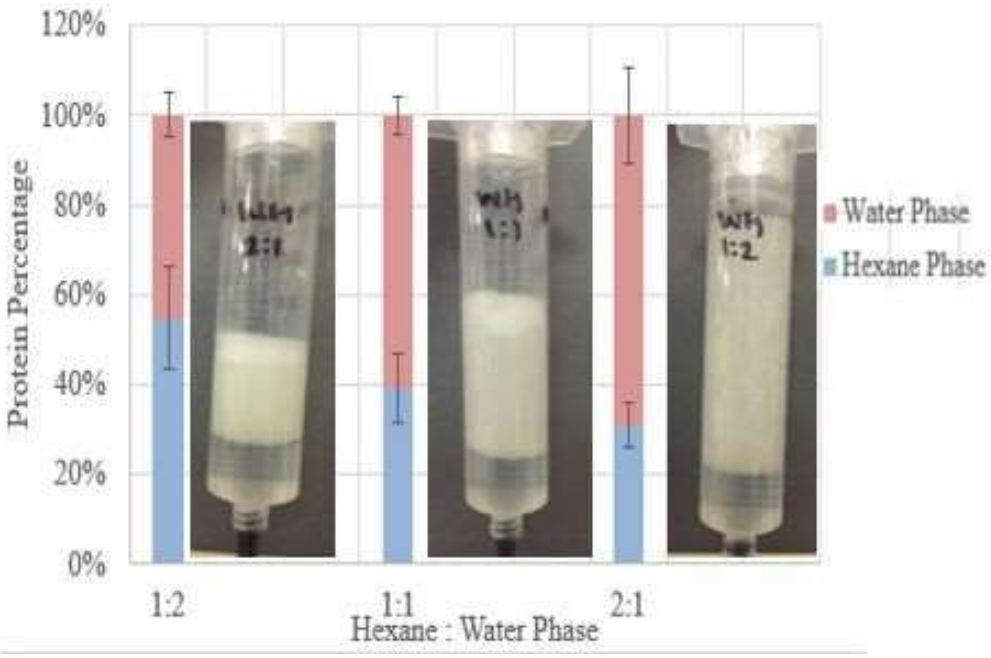

Figure 3.3: Solids migration as a function of hexane (H) to water (W) ratio. The amount of proteins (w/w) in the hexane phase was maximum for low hexane to water ratios. The stacked bars represent the amounts of proteins in respective phases. Note that the height of the white (protein) columns do not represent the mass; more hexane caused the protein to swell and occupy the entire volume fraction.

3.3.3 Effect of polyelectrolyte type on the protein migration

Figure 3.4 shows the impact of the two electrolyte types (i.e., monomeric and polyelectrolyte) and different molecular weights of the polyelectrolyte form (i.e., <100000 (low), 200000-350000 (medium) and 400000-500000 (high) on protein migration. Results indicate that the amount of the proteins migrated remained between 60-65% for the polyelectrolyte though there was no significant difference between migration efficiencies for the three forms with different molecular weights. The migration efficiency was lower for the monomeric form (52%). The higher migration efficiency for polyelectrolytes is attributed to the polyelectrolyte being able to better entrap protein molecules in the network strand and consequently leading to effective floc formation which in-

turn assists migration. For following experiments, low molecular weight polyelectrolyte was used which displayed to be the most effective for protein migration. It was also hypothesized that low molecular weight polyelectrolyte will form lighter electrolyte-bound-protein ensembles as compared to medium and high molecular weight ones and will face less resistance while migrating from bottom to top phase.

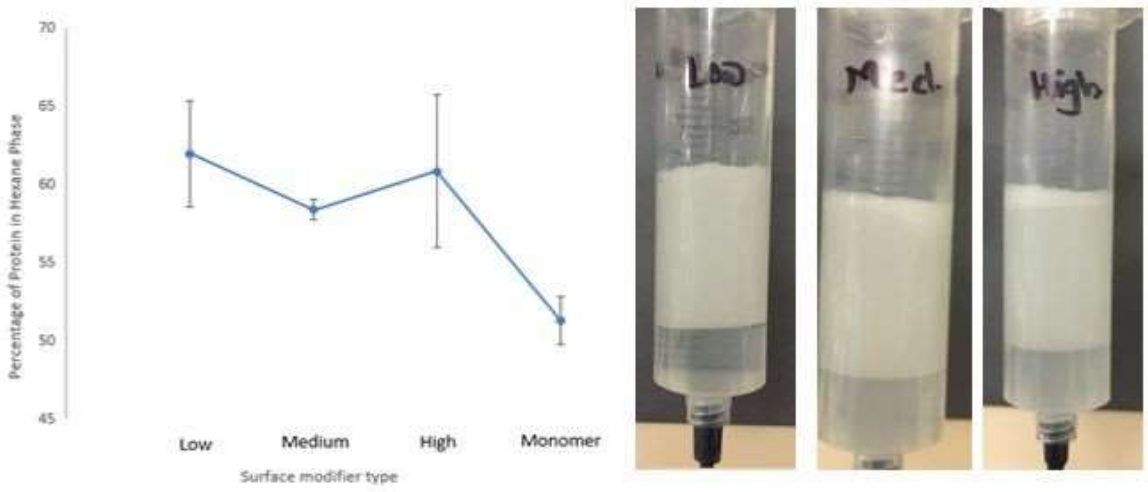

Figure 3.4: Effect of surface modifier type on the migration system. The polymeric forms were able to migrate more protein to the top hexane phase from bottom aqueous phase as compared to the monomeric form of the electrolyte. The performance did not change based on the molecular weight of the polyelectrolyte. The amount of protein migration was lower with monomer likely due to the inability of the monomer to trap proteins as effectively as the polymeric counterpart in the chain network.

3.3.4 Effect of polyelectrolyte concentration and pH on ensemble migration

The overall behavior of protein migration and zeta potential as a function of polyelectrolyte concentration is given in Figure 3.5A. Polymer concentration had a significant impact on the final zeta potential (i.e., surface potential after polymer interaction) and percentage of proteins that migrated to the hexane phase (p values = 0.028 and 0.002 respectively). However, pH had significant impact only on the percentage migration (p= 0.002) and not on zeta potential (p value= 0.57).

Overall, lower polyelectrolyte concentrations tended to favor protein migration. Zeta potentials subsequent to polyelectrolyte addition reached -5 to 0 mV range suggesting charge neutralization. The density map suggests most proteins migrated only once the system reached charge neutralization (Figure 3.5A).

Interestingly, the impact of polyelectrolyte concentration became evident once the data were clustered based on pH. Neutral and basic conditions favored protein migration (Figure 3.5B). Under neutral conditions, higher polyelectrolyte concentrations tended to favor protein migration whereas, under basic and acidic conditions, lower polyelectrolyte concentrations favored more protein migration (Figure 3.5C).

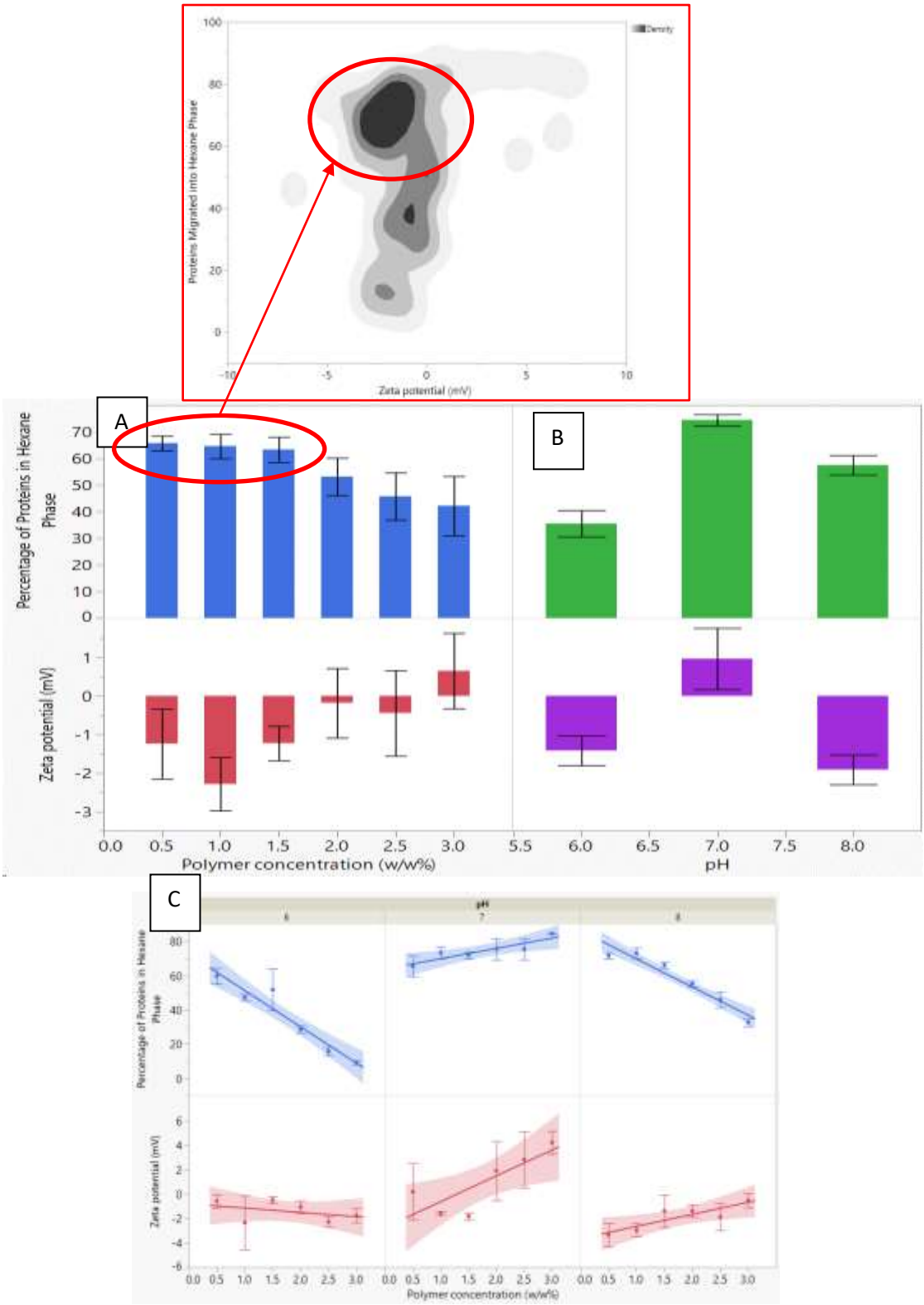

Figure 3.5: Effect of polyelectrolyte concentration and zeta potential on protein migration. Figure 3.5A shows the impact of polyelectrolyte concentration on protein migration and zeta potential. Figure 3.5B shows that under pH 7 the migration efficiency is the highest while the zeta potential reaches 0mV resulting in charge neutralization. Figure 3.5C shows impact of proteins migrated at different concentration under each pH condition.

To ascertain the reasons for higher polyelectrolyte concentrations favoring more protein migration under acidic and basic conditions as opposed to neutral conditions, the zeta potential of the system before polyelectrolyte addition was measured under varying pH. The zeta potentials before polyelectrolyte addition were found to be -3.72mV, -6.58mV and -25.1mV for pH 6, 7, and 8 respectively (Figure 3.6A). In order to ascertain where the present system lies in the pH vs. Zeta-potential continuum, the location of the current protein system within the generalized region of acidic (represented by a purple line) and basic (represented by a green line) particles under different pH conditions is presented (Figure 6 B) [101]. Between ±10 mV of zeta potential, the particles are observed to stabilize (and floc) over time; however, outside this zeta potential range, particles can stay well dispersed and suspended. The albumin system has an isoelectric point 5.3 pH while under neutral and basic conditions the zeta potential becomes more negative. This analysis indicated that this system lies in the region marked in the generalized pH vs. zeta-potential curve suggesting that the system is well dispersed before polyelectrolyte addition especially under neutral and basic conditions. A reduction of pH would force the system to arrive at its isoelectric point (at pH 5.3) which also would lead to floc formation (indicating the possibility of better particle separation/migration from the aqueous phase (given adequate hydrophobicity is attained)).

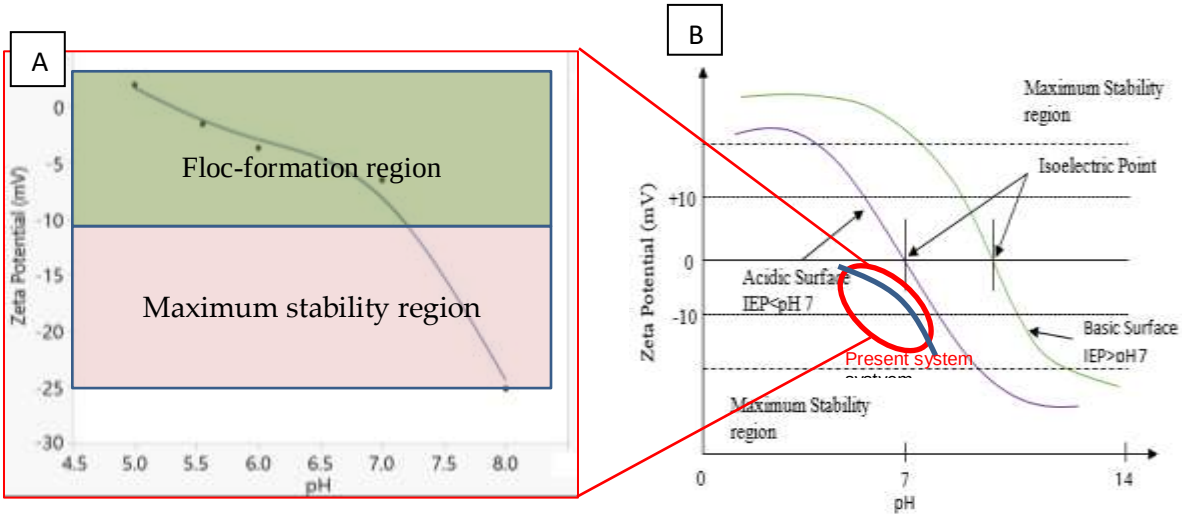

Figure 3.6: A) shows the change in zeta potential of egg albumin vs. a system pH. B) The location of the present protein system in the region that shows the generalized behavior of zeta potential for acidic (represented by a purple line) and basic (represented by a green line) particles under different pH conditions. Between ±10 mV of zeta potential, the particles are observed to stabilize (and floc) over time; however, outside this zeta potential range, particles can stay well dispersed and suspended. The albumin system has an isoelectric point 5.3 while under neutral and basic conditions the zeta potential becomes more negative. Note: IEP = Isoelectric Point.

Under low pH conditions, the system was closest to its isoelectric point and addition of only a small amount of cationic polyelectrolyte was able to bring the system to its isoelectric point forcing the system to charge-neutralize and in turn helping the system to migrate away from the aqueous phase due to the acquired hydrophobicity via hydrophobic ligands in the bound polyelectrolyte. However, excess polyelectrolyte sent the system further away from the isoelectric point (to the overall positive range) making the ensemble unstable again.

Under neutral pH, the system was more negative, and as more polyelectrolyte was added, the system reached charge neutralization and which in turn helped mobilize more protein to the solvent phase (Fig. 3.5C). However, under basic conditions, the addition of only a minimal amount of polyelectrolyte was able to maximize protein migration, and the addition of more polyelectrolyte, in fact, tended to negatively impact migration performance. The OH^- ions present in basic media likely influenced the performance of the system by excess cationic polyelectrolyte preferentially

interacting with OH^- as opposed to the relatively weak negatively charged particle surface.

Another factor that likely influenced protein migration to the top solvent phase from heavier aqueous phase would be the density of the polyelectrolyte-bound protein ensemble. The low net negative surface charge of the protein at pH 6 (-3.72 mV) and 7 (-6.58 mV) would adsorb a lower amount of polyelectrolyte than that of pH 8 (-25.1mV) due to decreased availability of oppositely charged groups on the protein in the acidic environment. This would consequently lead to the formation of less dense protein-polyelectrolyte ensembles helping them migrate against the gravity with minimum resistance. It should also be noted that in contrast, under acidic conditions, proteins low negative charge as compared to neutral pH attracts comparatively lower amounts of polyelectrolyte essentially making the ensemble even lighter, but less hydrophobic in turn reducing the amount of ensemble migration as compared to neutral solvent conditions. When the system pH is in basic, the highly negative surface charge adsorbs higher amounts of cationic polyelectrolyte making the protein-surfactant ensemble hydrophobic, but too heavy resulting in increased resistance for particles to move to the top phase. Accordingly, it is observed that there is an optimum pH and polyelectrolyte combination that promotes electrolyte binding, floc formation and increases the migration efficiency of proteins into the hexane phase. In this system, the most effective combination to obtain the desired output is 0.5% polyelectrolyte at pH 7.

3.3.5 Examination of the protein conformations before and after migration

To investigate the protein conformations before and after migration from aqueous to solvent phase, FTIR spectra of albumin were studied. Figure 3.7A shows the FTIR spectra of pure egg albumin extract and Figure 3.7B shows that of the proteins from hexane phase after the migration process was completed. As seen from the figure 3.7B, the amide I peak (1652 cm^{-1}) and amide II peak (1520 cm-1) of the protein were identified in the spectra. The two peaks were retained in the FTIR spectra of the extracted proteins; clearly indicating successful migration. The large peak in the extracted proteins spectra (3200-3550 cm^{-1}) corresponds to the O-H bond stretching from the polyelectrolyte. It also confirms the binding of the polyelectrolyte to the proteins.

From the FTIR analysis, it can be observed that the cationic polyelectrolyte helps in preserving

the structural integrity of the protein molecules. These findings are in line with the observation made in the earlier studies. It was observed that cationic surfactants are generally not as potent as anionic surfactants; in fact, in some cases, cationic surfactants manage to preserve protein activity for longer durations compared to native protein in the absence of surfactant additives[123]. The coexistence of protein and small amounts of ionic surfactant is believed to rely on specific interactions with the native state; thus surfactants assuming the role of a conventional ligand that stabilizes proteins and protects the helical structures[124].

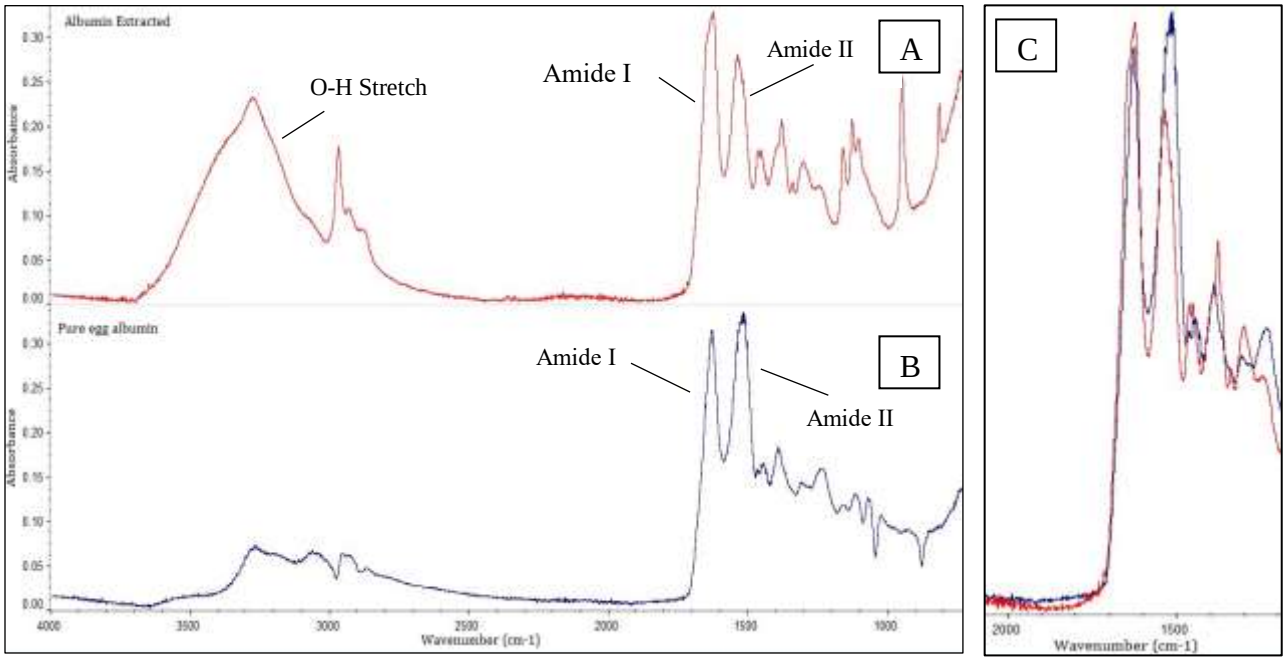

Figure 3.7: FTIR Spectra of (A) albumin after extraction and (B) pure egg albumin. The presence of both amide 1 and amide 2 peaks in the sample collected in hexane phase indicate successful migration of protein. (C) Stability of the amide peaks reaffirms the integrity of the proteins after migration.

3.3.6 SEM and autodock analysis

Scanning electron microscope (SEM) images of the protein and protein-polyelectrolyte complex are shown in figure 3.8. It is visible that the polyelectrolyte is well dispersed in water in its native state (Figure 3.8A) and once mixed with protein, forms clear flocs (Figure 3.8B) forming a blanket over protein aggregates helping migration.

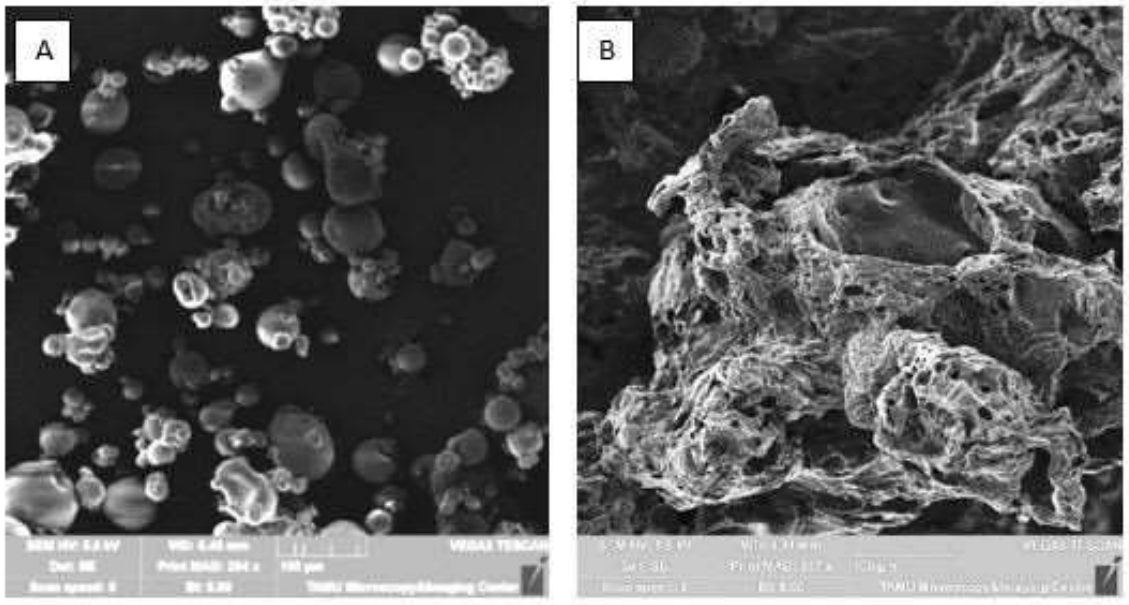

Figure 3.8: Scanning electron microscopy images of protein and protein-polyelectrolyte complex. (a) Image of proteins (294x) (b) Image of a single protein unit (944x) (c) image of proteins entrapped in the polyelectrolyte cover (337x) (d) close-up image of the protein-polyelectrolyte complex (2150x).

3.3.7 Molecular simulation

How the polyelectrolyte may interact with albumin surface was elucidated via docking simulations (figure 3.9 A, B and C). Docked conformations of DADMAC and polyDADMAC (comprising of 1, 2, and 16 monomeric units) shows that the positively charged electrolyte only binds to the oppositely charged (marked by green patches) areas of the protein's surface. It is observed that the 16-monomer PolyDADMAC wrapping a significant portion of the protein surface confirming the ability of a single chain polymer containing over 650 monomeric units (molecular weight <100,000) to entrap multiple albumin molecules. Inset in figure 3.9B shows the interaction diagram of the polyelectrolyte with the protein. Previous work suggests the ability of ionic head groups of polyelectrolytes primarily interacting with the anionic side chains of Asp and Glu amino acids[126]. The interaction diagram suggests similar phenomenon where the polar head of the polyelectrolyte forming close interactions with the negatively charged amino acid groups, Glu

and Asp. Furthermore, it is observed that the alkyl chains of these surfactants bind to the adjacent hydrophobic patches near the initial binding site of the protein as observed by others [126]. Hence it could be surmised that the neutralization of protein's charged groups via the interaction of polydadmac complex results in a net hydrophobic ensemble that in turn helps mobilize the ensemble from hydrophilic aqueous phase to a hydrophobic solvent.

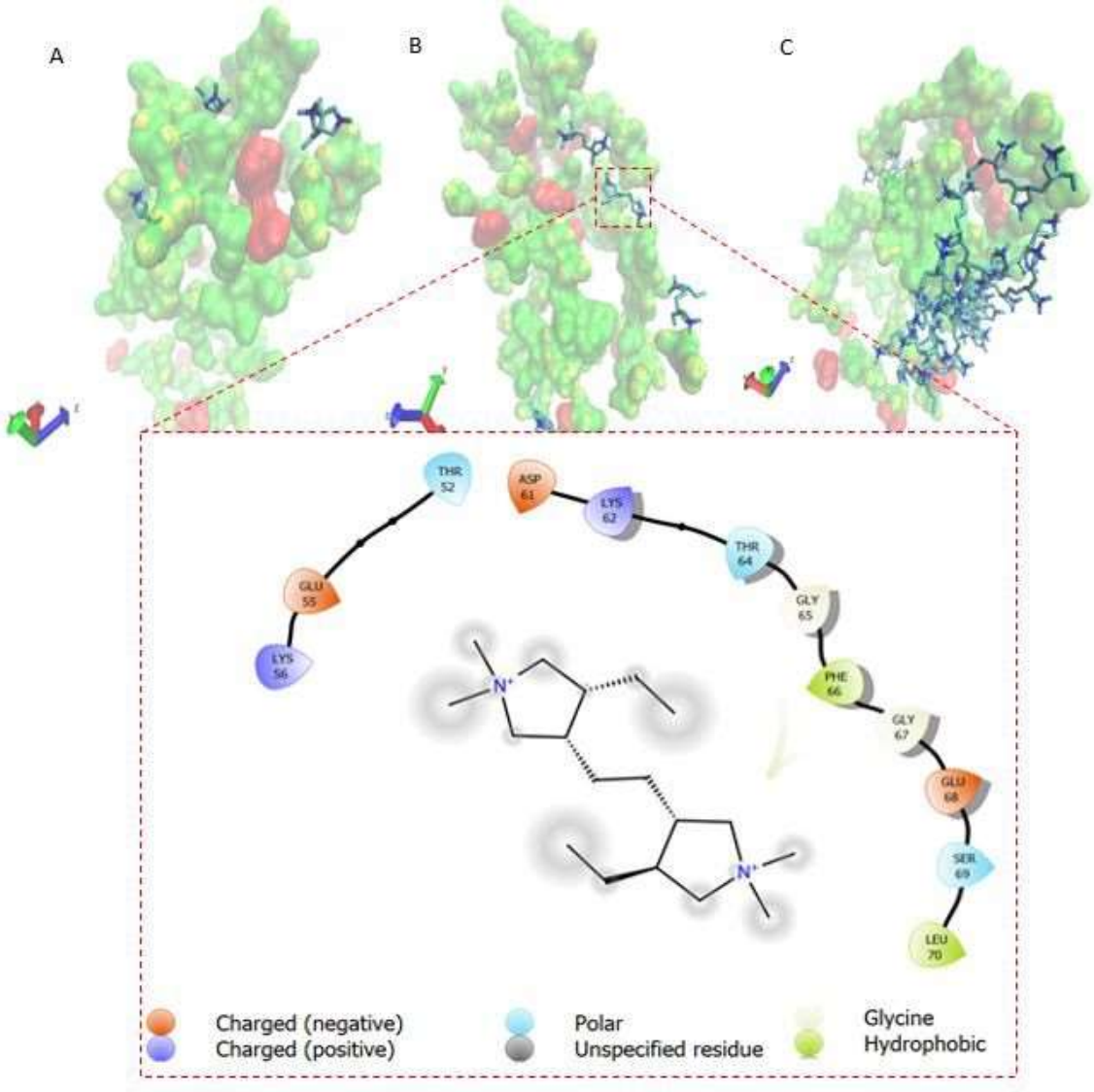

Figure 3.9: Interaction diagrams between (a) DADMAC and protein, (b) dimer of DADMAC and protein (inset shows the interaction diagram between an electrolyte molecule and the protein), (c) hexadecamer and protein.

3.4 Conclusions

The migration behavior of egg albumin from the aqueous phase to hexane solvent phase in the presence of polyelectrolyte PolyDADMAC was investigated. Studies indicated that the cationic polyelectrolyte was successfully able to migrate proteins from an aqueous phase to an adjacent hexane phase. Although higher times and higher temperatures (between 20°C and 40°C) tended to favor protein migration, this was not statistically significant. The impact of hexane to water ratios, i.e., 2:1, 1:1 and 1:2, on protein migration, indicated that maximum protein migration occurred at a hexane to water ratio of 1:2. Studies with low, medium and high molecular weight electrolytes indicated that there is no significant impact on the size of the polyelectrolyte on the efficiency of protein migration. However, the amount of proteins that migrated into the hexane phase were significantly low when the monomer was used as opposed to polymeric forms of the polyelectrolyte. Considering acidic, neutral and basic conditions, neutral pH appeared to be the best to promote protein migration. Neutral pH caused the zeta-potential of the final ensembles to reach near zero suggesting effective charge neutralization that in turn translated to better protein migration.

Although higher polyelectrolyte concentrations, in general, did not favor particle migration under acidic or basic conditions, higher polyelectrolyte concentrations did not negatively impact, in fact, favored protein migration under neutral pH conditions. FTIR studies indicated that the proteins were unfettered and preserved structural integrity after movement from the aqueous phase to solvent under the protection of the polyelectrolyte coating. The results suggest that ionic electrolytes with hydrophobic prosthetic groups could be used as molecular transport vehicles to protect and separate proteins from a bulk aqueous phase to an immiscible solvent phase.

4. SIMULTANEOUS SEPARATION OF CARBOXYL FUNCTIONALIZED CELLULOSE AND EGG ALBUMIN USING IONIC POLYELECTROLYTES IN AN AQUEOUS-ORGANIC MULTI-PHASE SYSTEM

4.1 Introduction

It is estimated that the demand for food and energy globally is set to increase by 50% in the next decade[127]. As a result of the rising global demand for both proteins and lipids, microalgae have been considered an excellent candidate to fulfill these needs. Though cultivation of microalgae is easier and faster as compared to conventional protein and oil sources, separation of algal biomass from the growth media for the extraction and fractionation of intracellular bio-products has been a major technical challenge due to the very dilute biomass concentration in growth media and the complexity of the matrix after disintegration. To make microalgae-biorefinery platform economically viable, a simpler and less resource intensive downstream unit operation for coextracting various bio-products together with lipids is a necessity.

Current unit operations involve the harvesting of microalgae in two steps, bulk harvesting, and thickening. Bulk harvesting is primarily done to increase the microalgae concentration from 0.5-1% to 2-7% using coagulation, sedimentation, and flotation followed by thickening process which involves the use of energy-intensive processes like filtration and centrifugation where the final concentration of the suspension is increased to 15-20%[15]. This is followed by disruption of cells to harvest the intracellular products of interest which could be furthermore challenging due to the complexity of the resulting matrix. It is widely accepted that a simple, low-energy, cost-effective and environmentally friendly downstream process is necessary, especially considering that the cost of harvesting can account for >30% of the total algal biomass production cost[19].

Recovery of proteins from microalgae faces significant technical challenges. High-value components in microalgae reside in the cytoplasm which is concealed by a tough cell wall which is known to restrict any extraction solvent's access [128, 129]. Accordingly, various techniques

have been employed for cell wall disruption such as bead milling [130, 131], ultra-sonication [132, 133, 134], microwave radiation [135], enzymatic treatment [136, 137], cell homogenization [138] and high-pressure cell disruption [139]. However, these techniques have been developed with extracting lipids as the primary focus disregarding what happens to the stability of other high-value bio-products in the process [129]. So, milder disruption techniques have to be developed taking into consideration the stability of all the intracellular bio-products.

Unfortunately, existing techniques are unable to dewater microalgae from its dilute suspension while simultaneously extracting lipids and other co-products in a cost-effective manner. Hence, there is a need to develop a technique which will not only effectively dewater microalgae but also separate the major components without compromising product quality.

The novel approach discussed herein can facilitate not only dewatering of microalgae from dilute growth culture but also extract various fractions of microalgae components simultaneously in the liquid-liquid multiphase system. The hypothesis here is to interface the charged algal cellular debris with oppositely charged amphiphilic polyelectrolytes which transpose the surface hydrophobicity of the debris. Upon attachment of the polyelectrolyte, the algal cell wall will result in the formation of a hydrophobic ensemble that will eventually be migrated into a hydrophobic solvent as reported earlier. Here we hypothesize that when several components are present in a mixture, a target component's surface charge could be altered by varying the system's pH. Studies presented herein are geared toward testing this hypothesis. The work conducted in this study is on model algal particles (consisting of carboxyl functionalized cellulose beads) and egg albumin (mimicking algal proteins) mixed together in an aqueous suspension.

4.2 Material and methods

Sodium monochloroacetate used for functionalizing cellulose beads was purchased from Sigma-Aldrich (Milwaukee, WI). Cellulose particles (Celphere CP-102) were kindly provided by Asahi Kasei Chemicals Corporation. Sodium hydroxide, hexane, egg albumin extract, PEG 8000, potassium phosphate, and ammonium sulfate and hexane were purchased from VWR (Radnor, PA). Polydialyldimethylammonium chloride (average Mw <100,000 (low molecular weight), 35 wt. %

in H_2O) was purchased from Sigma Aldrich. All the chemicals used were laboratory-grade. All the solutions were prepared in deionized water.

4.2.1 Functionalization of cellulose beads

Functionalization of cellulose beads with carboxyl groups was carried out as per the process described in the earlier work.

4.2.2 Two-phase and three phase separation of carboxyl cellulose particles from protein-cellulose mixture

For the separation of just the carboxyl-functionalized cellulose (henceforth referred to as CFC) from a mixture of CFC and protein molecules in a water-hexane two-phase system, their isoelectric point was studied separately. Protein and CFC particles in 1% (w/w) concentration each was suspended in DI water. pH of the suspension was adjusted using acetic acid. Adjusting the pH resulted in controlling the zeta potential of both proteins and cellulose. For selective migration of carboxyl cellulose beads from the aqueous phase to hexane, pH adjustment brought protein molecule close to their isoelectric point while keeping the cellulose beads negatively charged. This led to targeted binding of the polyelectrolyte to the cellulose beads. After adjusting the system pH, the calculated amount of polymer was mixed with the protein-CFC suspension. To ensure maximum adsorption of the cationic polymer onto the particles carrying the opposite charge, the mixture was vortexed at maximum speed for 30 seconds. Hexane was added to the aqueous suspension, and the water-hexane system was again vortexed for roughly one minute at maximum speed to ensure contact between the particle-polymer ensemble and hexane. The system was kept undisturbed for the phases to separate. Samples were collected from both the hexane and aqueous medium for further analysis.

For the three-phase system, additional steps were performed for the selective migration of cellulose away from proteins. A salt solution containing potassium phosphate (20% w/w) and ammonium sulfate (5%w/w) was prepared. A 30% (w/w) solution of PEG 8000 was prepared separately using DI water. The protein-cellulose suspension was prepared as mentioned in the two-phase

separation above. After the pH adjustment and polymer addition, salt solution followed by PEG solution was added to the suspension. The entire system was mixed using a vortex machine. Hexane was added to this mixture and vortexed again for a minute. The system was kept undisturbed for an hour for the formation of three separate phases. Samples from the lower (salt) phase and middle (PEG) phase were collected for further analysis.

4.2.3 Quantification of proteins and cellulose

Quantification of proteins and cellulose in aqueous phase (in two phase and three phase system), PEG phase and hexane phase was done using Thermofischer' s GenesysTM 10S UV-vis spectrophotometer. The amount of proteins and cellulose beads in the original suspension were measured at 280 nm and 205nm respectively. After the completion of the migration process, samples from different phases were collected and used to measure the absorbance at 280 and 205nm using a quartz cuvette. The absorbance values were used to quantify the amount of proteins and cellulose in the respective phases.

4.2.4 Measuring zeta potential

The zeta potential of the particles suspended was measured at different pH conditions using Beckman Coulter's zeta analyzer. The analysis was done using a flow cell module. Zeta potential of the mixture before and after the treatment with polyDADMAC was done to see the impact of the polymer on the surface charge.

4.2.5 Characterization of particle separation

To understand the cause behind low migration efficiency in three phase system, confocal laser microscopy was used. Images were taken using an Olympus FV1000 confocal microscope for the system in which control parameters displayed lowest separation efficiency. Proteins were stained using Rhodamine B dye to distinguish them from CFC.

4.3 Results and discussion

4.3.1 Effect of pH

Zeta potential of the particles was directly proportional to the pH of the system. Under low pH (acidic conditions), the presence of H^+ ions resulted in the lowering of the potential of the particles due to shielding of negatively charged carboxyl groups on cellulose particles and proteins. Figure 4.1 shows the isoelectric curve of albumin and functionalized cellulose beads.

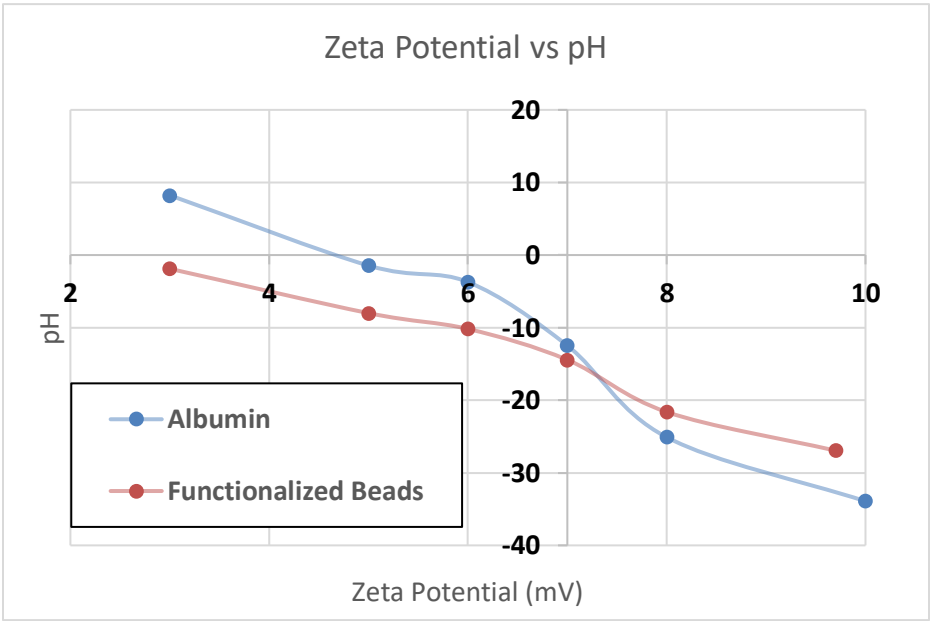

Figure 4.1: Isoelectric curve of egg albumin and carboxyl functionalized cellulose beads under different pH conditions. The isoelectric point for proteins was 4.7, and 2.9 for CFC particles (n=1).

The cellulose suspension was stable when the zeta potential remained above -10 mV. However, after the zeta potential went below -10mV, the particles settled over time. A similar phenomenon was observed in the case of proteins. Isoelectric point (*I*p) for CFC was calculated to be pH 2.87, and pH 4.69 for egg albumin. The differences between the *I*p of cellulose and proteins assisted in the selective binding of the polymer to cellulose beads alone.

4.3.2 Water- hexane two-phase system

4.3.2.1 Effect of polyelectrolyte addition on zeta potential

Figure 4.2 shows the changes in the zeta potential of the suspension after the addition of polyelectrolyte and subjecting the particles to migration. As it is evident from figure 4.2, as the polymer concentration in the suspension increases, the zeta potential decreases. As the concentration of the polymer increased, the negatively charged particles under pH 4, 4.5 and 5 reached charge neutralization. Under pH 3 and 3.5, proteins were positively charged, and cellulose particles were close of their isoelectric point; and, the addition of polymer under these conditions increased the overall positive charge of the suspension.

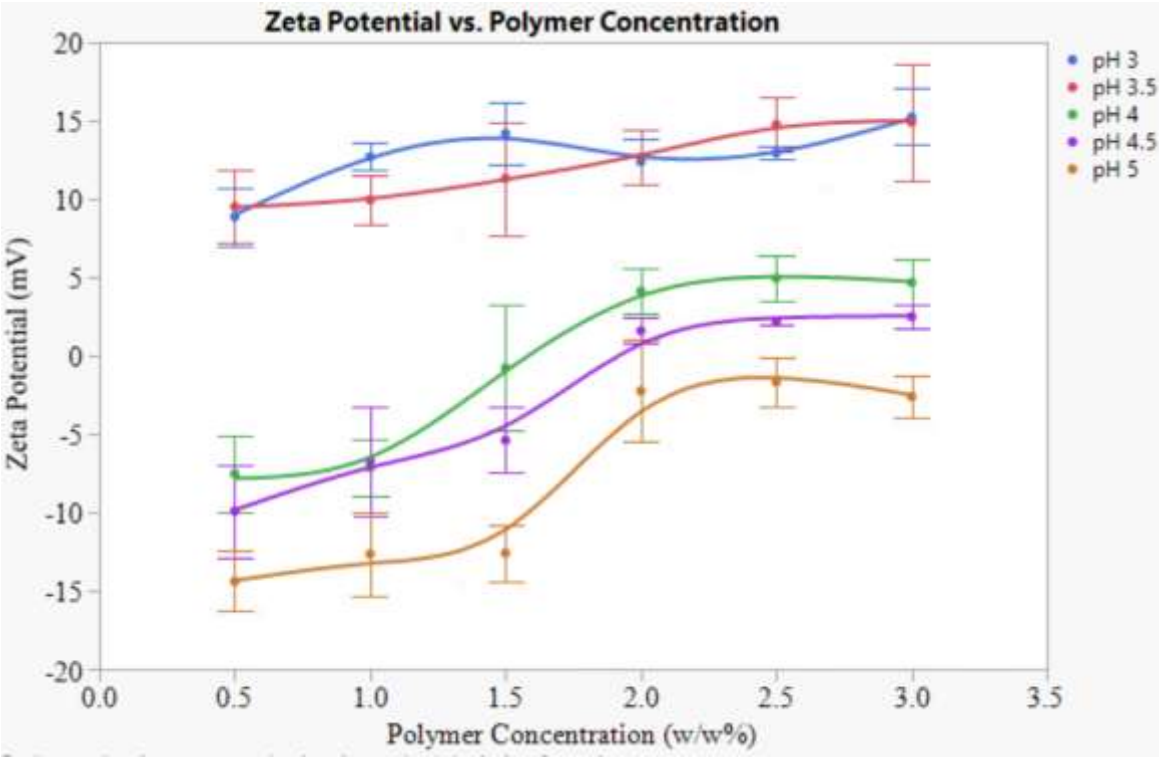

Figure 4.2: Zeta potential of the suspension under different pH and polyelectrolyte concentration conditions.

The zeta potential of the control suspension was varied from pH 3 to 5. It was observed that

the net charge on the particles varied as the pH of the system changed, and the *I*p of the particles influenced the partitioning of the proteins in the aqueous phase and cellulose in hexane phase after polyelectrolyte attachment. With the increase in the acidity of the suspension, the net positive charge of the particles increased as a result of consumption of H^+ ions by the particles (without polyelectrolyte addition). With the addition of polymer to this suspension, the cationic polyelectrolyte was bound to the negatively charged particle surfaces, either cellulose or proteins or both resulting in destabilization of the system. Particles were seen to settle down under gravity over time. A similar phenomenon has been observed previously, and it is believed to be due to the dominance of van der Waal forces over repulsive electrostatic forces[140].

4.3.2.2 Impact of polymer on the particle size

Figure 4.3 shows the impact of polyelectrolyte concentration on particle size. The particle size of the suspension without polyelectrolyte adsorption was found to vary between 196 nm to 211 nm range (represented by the red in figure 4.3).

A significant increase in the particle size was observed (400-570 nm) with the addition of polyelectrolyte which could be a result of entrapment of particles in the polymeric net. Though the overall particles grew with polymer addition, the increase in the polymer concentration further did not increase ensemble size. In fact, a slight decrease in the particle size was observed with the increased polymer concentration likely due to the dominance of repulsive forces breaking large clusters forming multiple smaller polymer-particle ensembles.

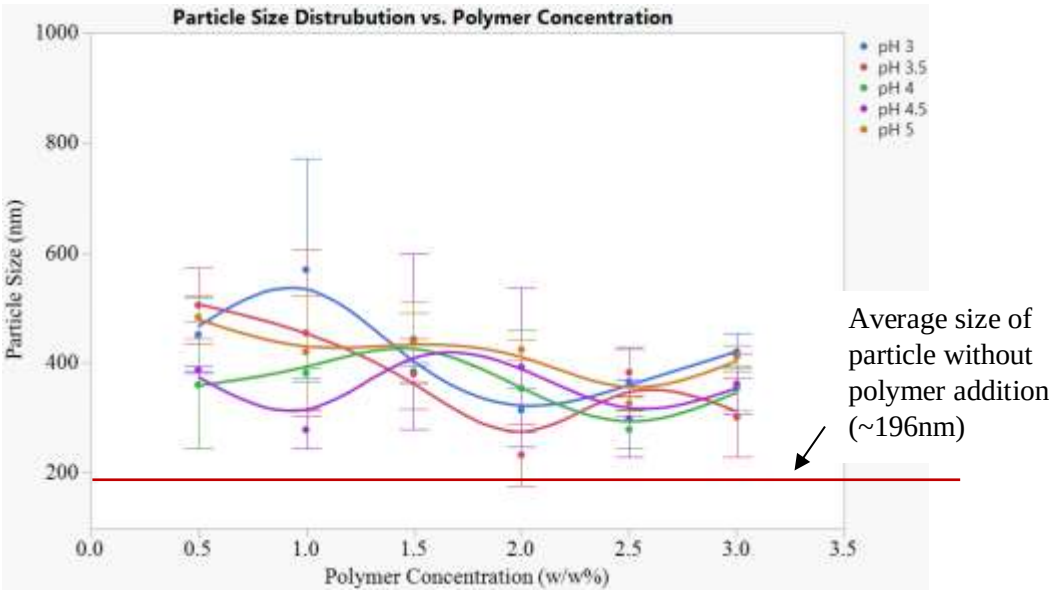

Figure 4.3: Impact of polymer concentration on the particle size distribution. It is evident that the particle size distribution is independent of the polymer concentration at different system pH. There is a slight reduction in the particle size as the polymer concentration increases, but the change is non-significant.

4.3.2.3 Effect of polymer concentration and pH on cellulose migration

Figure 4.4 shows the migration efficiency of cellulose in the hexane phase under different polyelectrolyte and pH conditions. The movement of cellulose from the aqueous phase to hexane did not improve with the increase in the polymer concentration; lower polymer concentrations had comparable migration efficiencies to the highest ones for a given pH condition. From figure 4.4C, it can be observed that the percentage of cellulose migrated into the hexane phase is lower under acidic conditions (40-50 %) but is relatively high under pH 4, 4.5, and 5 conditions (85-95%). As previously mentioned, the CFC beads have higher zeta potential under these pH conditions. A high negative charge allows increased interaction between the cationic polyelectrolyte and the cellulose molecules leading to increased migration efficiency to the hexane phase. On the other hand, the migration efficiency did not change with the amount of polyelectrolyte added under a given pH (fig 4.4B). The migration efficiency might be dependent on the coverage of cellulose by the polymer and simply having sufficient polymer to cover the surface would achieve desired migration.

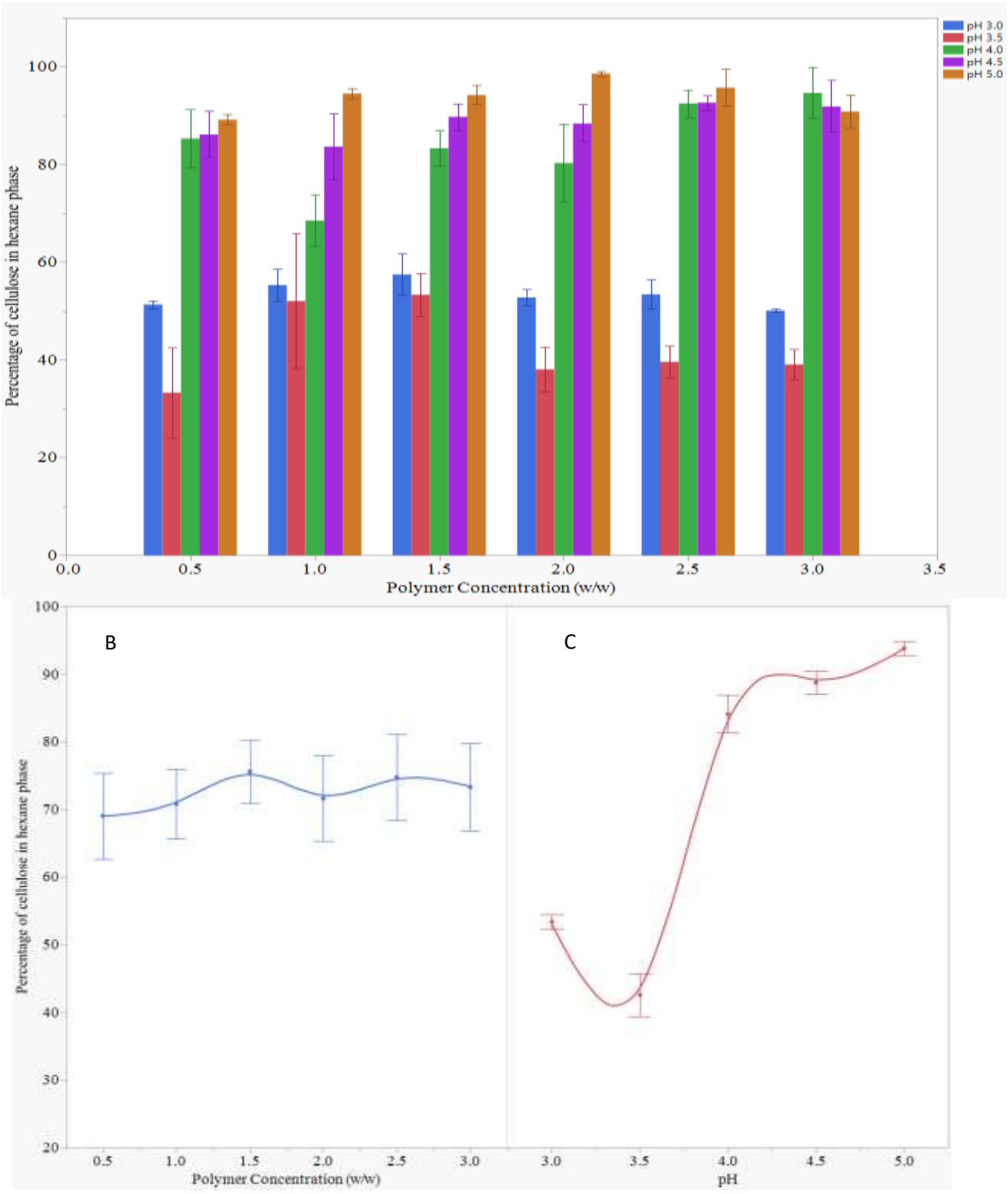

Figure 4.4: (A) Cellulose migration as a function of polymer concentration under different pH conditions, (B) % cellulose migration under different polymer concentrations, and (C) % cellulose migration under pH conditions.

The formation of the ensembles followed by migration can be explained as follows. The net positively charged polyDADMAC, upon binding onto the negatively charged cellulose beads, forms a hydrophobic cellulose-surfactant complex (figure 4.5). After addition of hexane into the system, water molecules surrounding the polyelectrolyte-cellulose complex are replaced by the organic solvent resulting in the migration of the ensemble to the organic solvent phase.

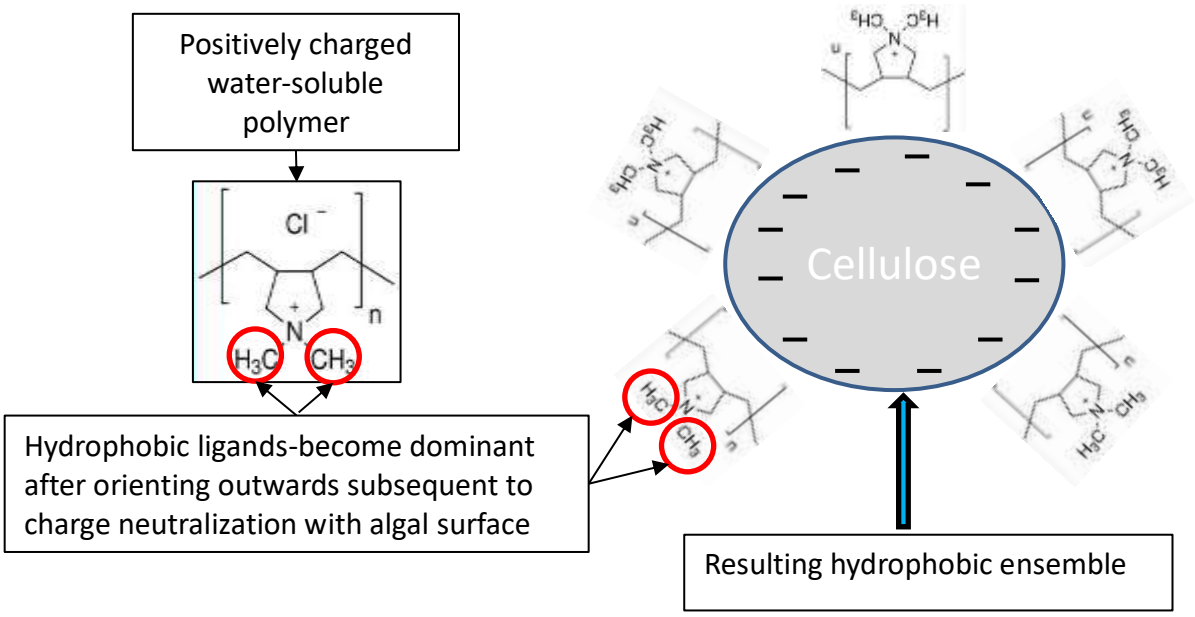

Figure 4.5: Negatively charged cellulose surface is functionalized to be hydrophobic via attachment of net positive amphiphilic polymer which consequently results in the formation of a hydrophobic ensemble.(A) Cellulose migration as a function of polymer concentration under different pH conditions, (B) % cellulose migration under different polymer concentrations, and (C) % cellulose migration under pH conditions.

The amount of polyelectrolyte bound to cellulose was dictated by the zeta potential of the cellulose and zeta potential is dependent on the system pH. To evaluate the migration efficiency as a function of pH, the acidity of the system was increased by adding acetic acid. It was found that, as the pH reaches closer to the isoelectric point (pH 2.89) of cellulose, their migration efficiency dropped down to 40% from 95% at pH 5. This was due to the shielding of carboxyl groups on cellulose by H+ ions from the acid leading to the reduced electrostatic interaction between

the positively charged polymer and negatively charged cellulose. Cellulose is approximately net neutral under these conditions cannot bind to cationic polyelectrolyte. At pH 3, the migration pattern of cellulose reversed. This could be as a result of the charge reversal of the ensembles. After charge neutralization under pH 3, the non-polar tail of polyDADMAC interfaces to the near neutral particles through hydrophobic interaction. These chains carry N^+, causing the ensemble to become positively charged and thus re-stabilizing the system. Similar observations were made by A. Ariffin and co-workers while working on flocculation of paper mill wastewater using polyDADMAC[141]. PolyDADMAC is also known to interact with particles under a wide range of pH which explains its ability to interact with particles under near charge neutral conditions [142].

4.3.2.4 Effect of polymer concentration and pH on protein migration

Figure 4.6A shows the impact of polymer concentration on protein retention in water phase under different pH conditions. It can be observed that, as the polymer concentration increased, the amount of proteins retained in aqueous phase increased slightly from 50% to 60%; however, the change was not statistically significant change (figure 4.6B). On the other hand, as the pH reduced, the amount of proteins retained in the aqueous phase reduced significantly (figure 4.6C). The maximum protein retention in the aqueous phase was observed at pH 4.5, i.e., close to the isoelectric point of the proteins under investigation. The surface of proteins became positive as the acidity of the system was further increased. Near the isoelectric point, a maximum amount of proteins (75%) were retained in the water phase as a result of the low binding capacity of proteins to the polymer. As the acidity of the system was further increased, the proteins became positively charged. We believe that the positively charged proteins not only repel the cationic polymer but also compete with proteins to bind to the negatively charged cellulose beads to form ensembles.

A small amount of proteins is seen to migrate to the hexane phase under each pH. This is possibly due to the entrapment of proteins in the polyelectrolyte matrix. As the protein becomes positively charged below their isoelectric point, they could potentially compete with the cationic polymer to bind with negatively charged cellulose beads resulting in the formation of protein-cellulose complexes. These complexes can get entrapped in the polymeric matrix along with a few positively charged polymer and negatively charged cellulose. Cellulose is approximately net neutral under these conditions cannot bind to cationic polyelectrolyte. At pH 3, the migration pattern of cellulose reversed. This could be as a result of the charge reversal of the ensembles. After charge neutralization under pH 3, the non-polar tail of polyDADMAC interfaces to the near neutral particles through hydrophobic interaction. These chains carry N^+, causing the ensemble to become positively charged and thus re-stabilizing the system. Similar observations were made by A. Ariffin and co-workers while working on flocculation of paper mill wastewater using polyDADMAC[141]. PolyDADMAC is also known to interact with particles under a wide range of pH which explains its ability to interact with particles under near charge neutral conditions [142].

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Near the isoelectric point, a maximum amount of proteins (75%) were retained in the water phase as a result of the low binding capacity of proteins to the polymer. As the acidity of the system was further increased, the proteins became positively charged. We believe that the positively charged proteins not only repel the cationic polymer but also compete with proteins to bind to the negatively charged cellulose beads to form ensembles.

A small amount of proteins is seen to migrate to the hexane phase under each pH. This is possibly due to the entrapment of proteins in the polyelectrolyte matrix. As the protein becomes positively charged below their isoelectric point, they could potentially compete with the cationic polymer to bind with negatively charged cellulose beads resulting in the formation of protein-cellulose complexes. These complexes can get entrapped in the polymeric matrix along with a few cellulose beads and migrate into the hexane phase. Figure 4.7 shows a schematic representation of possible reasons for the proteins to migrate into the hexane phase even when they are not directly bound to the polymer below their isoelectric point.

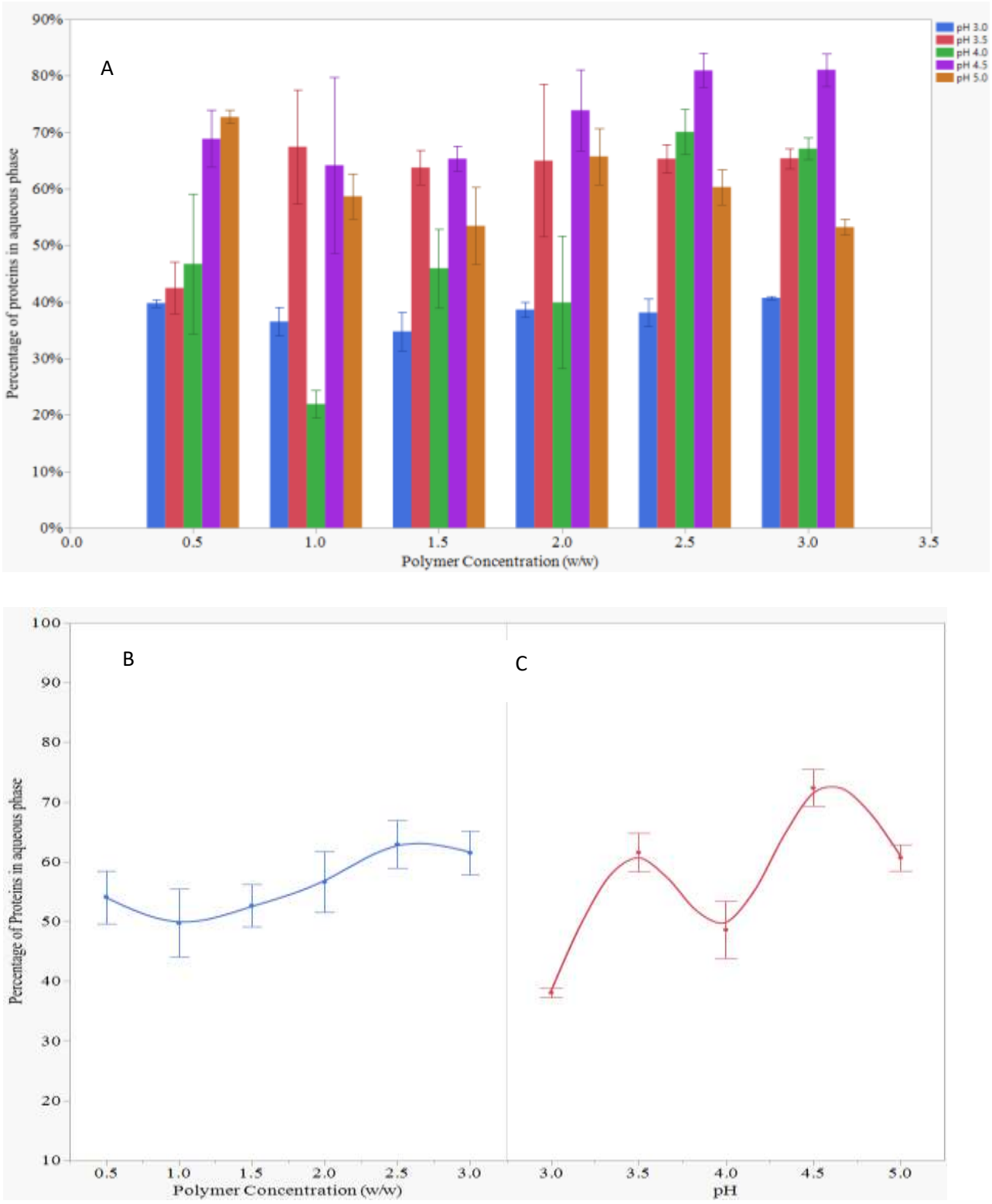

Figure 4.6: (A) Protein retention in the aqueous phase as a function of polymer concentration under different pH conditions. (B) % proteins retained in aqueous phase under different polymer concentrations, and (C) % proteins retained in aqueous phase under pH conditions.

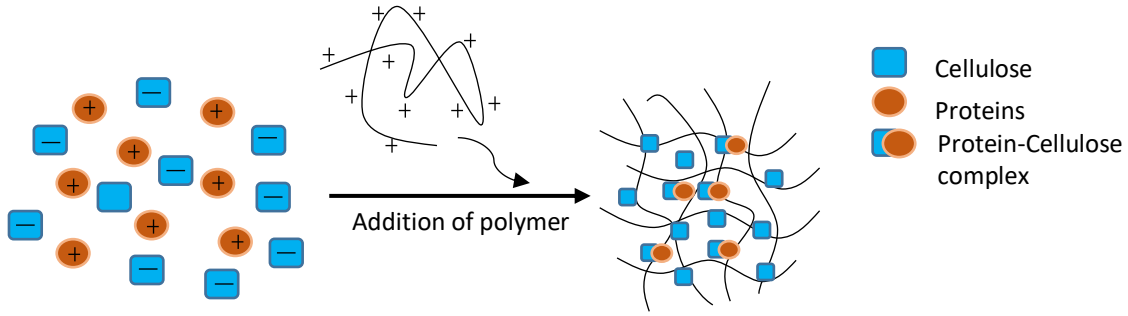

Figure 4.7: Schematic representation shows the occurrence of events when proteins carry a net positive charge (below isoelectric point), and cellulose is net negatively charged. The negatively charged cellulose attach to the polymeric chains forming a net-like structure. Proteins could also bind to cellulose as they both carry opposite charges and form a stable entity. The protein-cellulose complexes could get entrapped in the polymeric net resulting in an overall reduction of protein retention in water.

4.3.3 Water-PEG-hexane three-phase system

To study the migration behavior of cellulose into the hexane and of proteins in the PEG phase, the pH of the system and the amount of polymer added was varied.

Figure 4.8 and figure 4.9 shows the migration behavior of proteins and cellulose under different pH conditions and polymer concentration in a salt-PEG-hexane system. From the studies conducted, it was clear that approximately 70-80% of proteins and cellulose independently migrated from the aqueous phase to the hexane phase under a broad range of pH and polymer concentrations. The separation of proteins from cellulose remained quite low in the three-phase system as compared to the water-hexane two-phase system. This may be as a result of the complex nature due to the presence of a high ion concentration of salts (ammonium sulfate (5w/w%) and ammonium phosphate (20w/w%)) in the three-phase system. As the ionic strength is increased, the electrostatic interaction weakens leading to aggregate formation of soluble complexes through hydrophobic interaction. Similar observations were made by Karayianni, M., et al. [143]. In this study, the high ionic strength of the solution could lead to aggregate formation between polyelectrolyte, proteins, and cellulose where the polyelectrolyte is wrapped around proteins and cellulose

leading to their migration into the hexane phase.

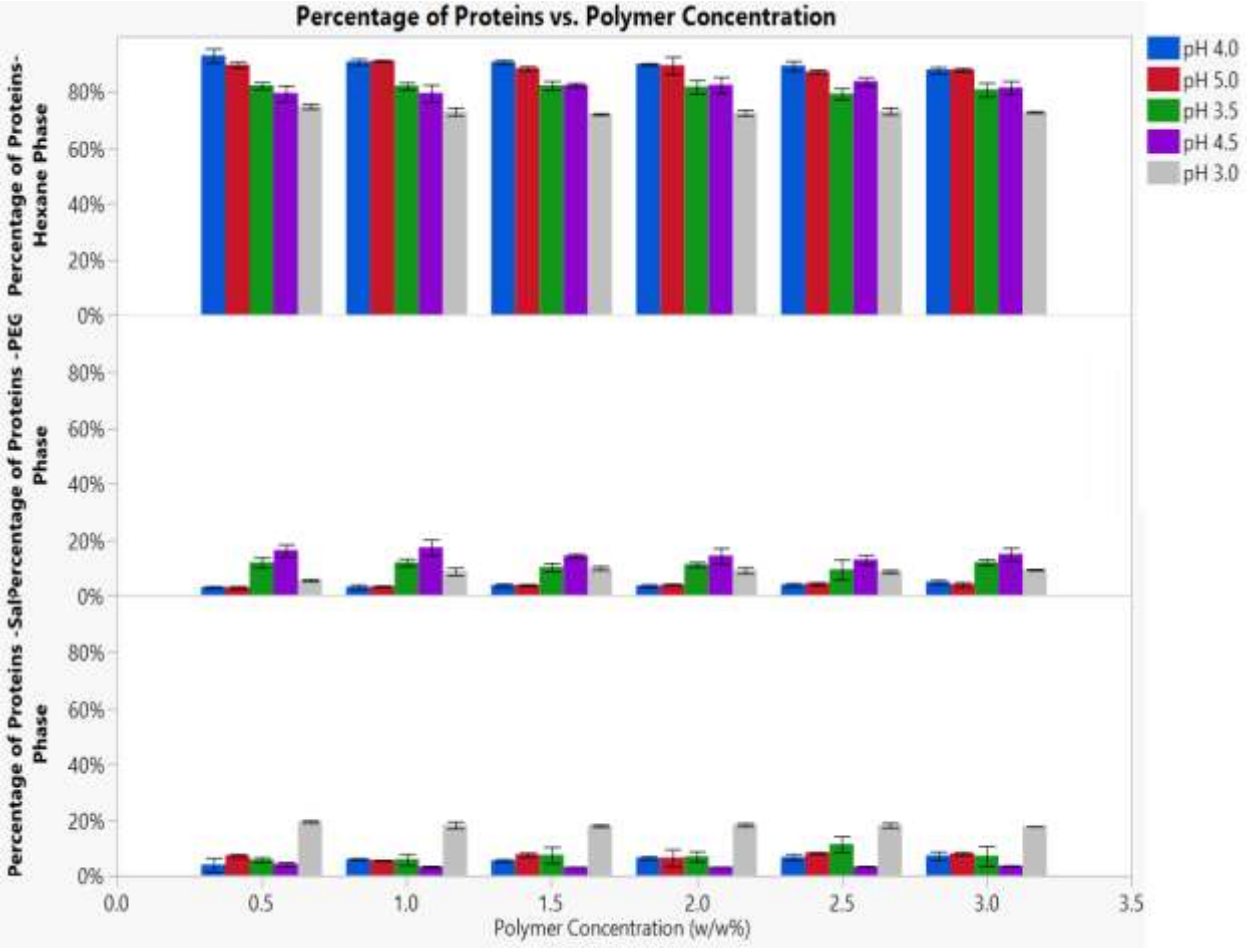

Figure 4.8: Proteins % retained in each phase as a function of polymer concentration and system pH in the salt phase (top) and PEG phase (bottom).

Figure 4.9 shows the migration behavior of cellulose under different pH conditions and different polymer concentrations in the salt-PEG-hexane system.

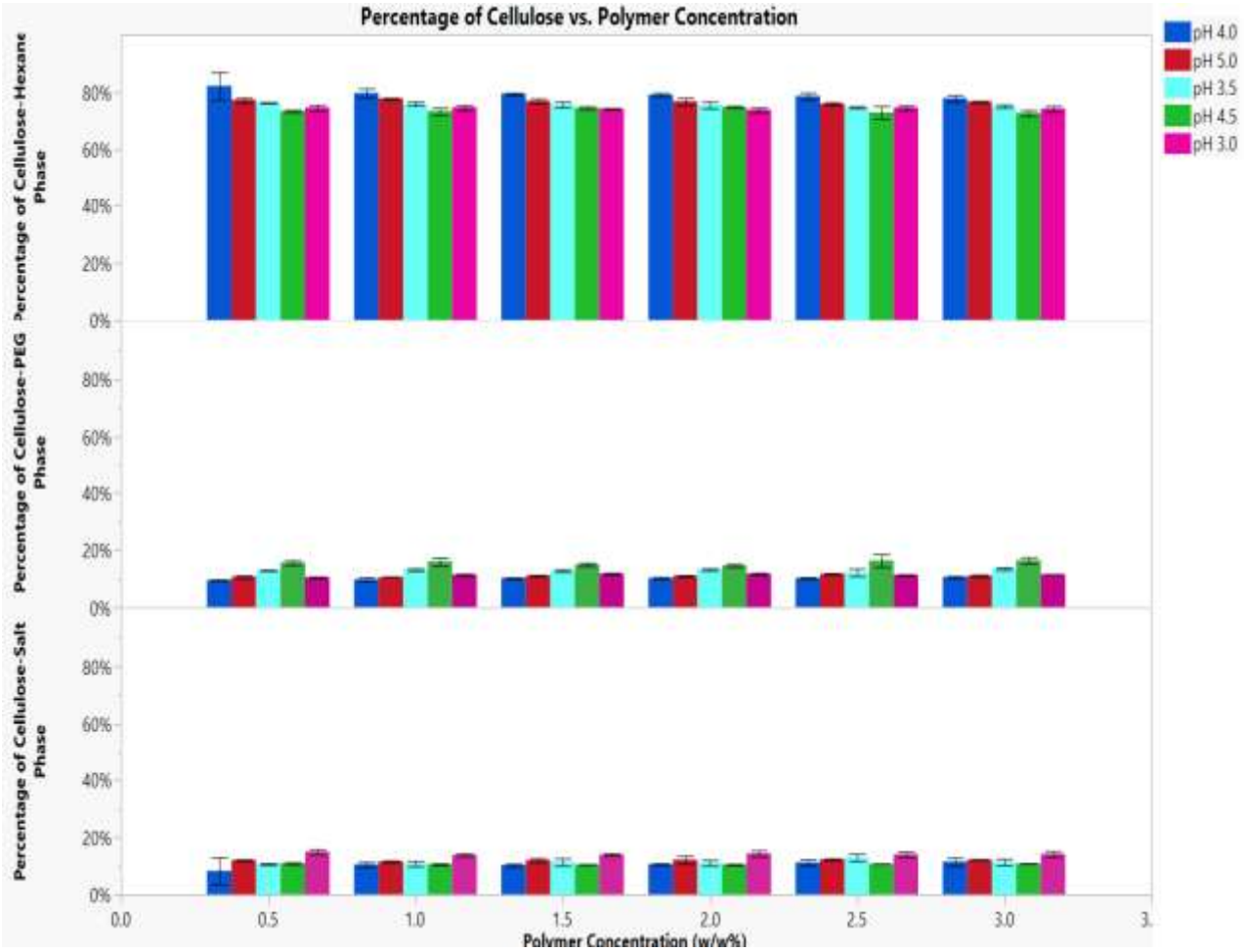

Figure 4.9: Cellulose % as a function of polymer concentration and system pH in the salt phase (top) and PEG phase (bottom).

Under a broad range of pH, the surface charge of proteins and cellulose varied potentially initiating an interaction between the two molecules. Due to the high concentration of salts, the particles can lose water molecules surrounding proteins and/or cellulose due to increasing hydrophobicity. Under such high salt concentrations, the protein molecules and the CFC can form clumps as a result of increased hydrophobic interactions between them.

The formation of protein-cellulose ensembles was verified using confocal laser microscopy (Figure 4.10). Here, proteins are depicted in red and cellulose in green in all the three phases. The clumps can get entrapped in the polymeric matrix stabilizing the system. Also, under high salt concentrations, the size of the reverse micelles decreases due to the onset of destabilization of the system[144] leading to the formation of many small-sized reverse micelles. These small reverse micelles can entrap the hydrophobic ensembles leading to reduced separation efficiency.

Formation of protein-cellulose ensembles is evident in all three phases. As a result, migration of ensembles to all the three phases is evident. And thus, the two-phase system performs better as compared to the three-phase system for individual component separation.

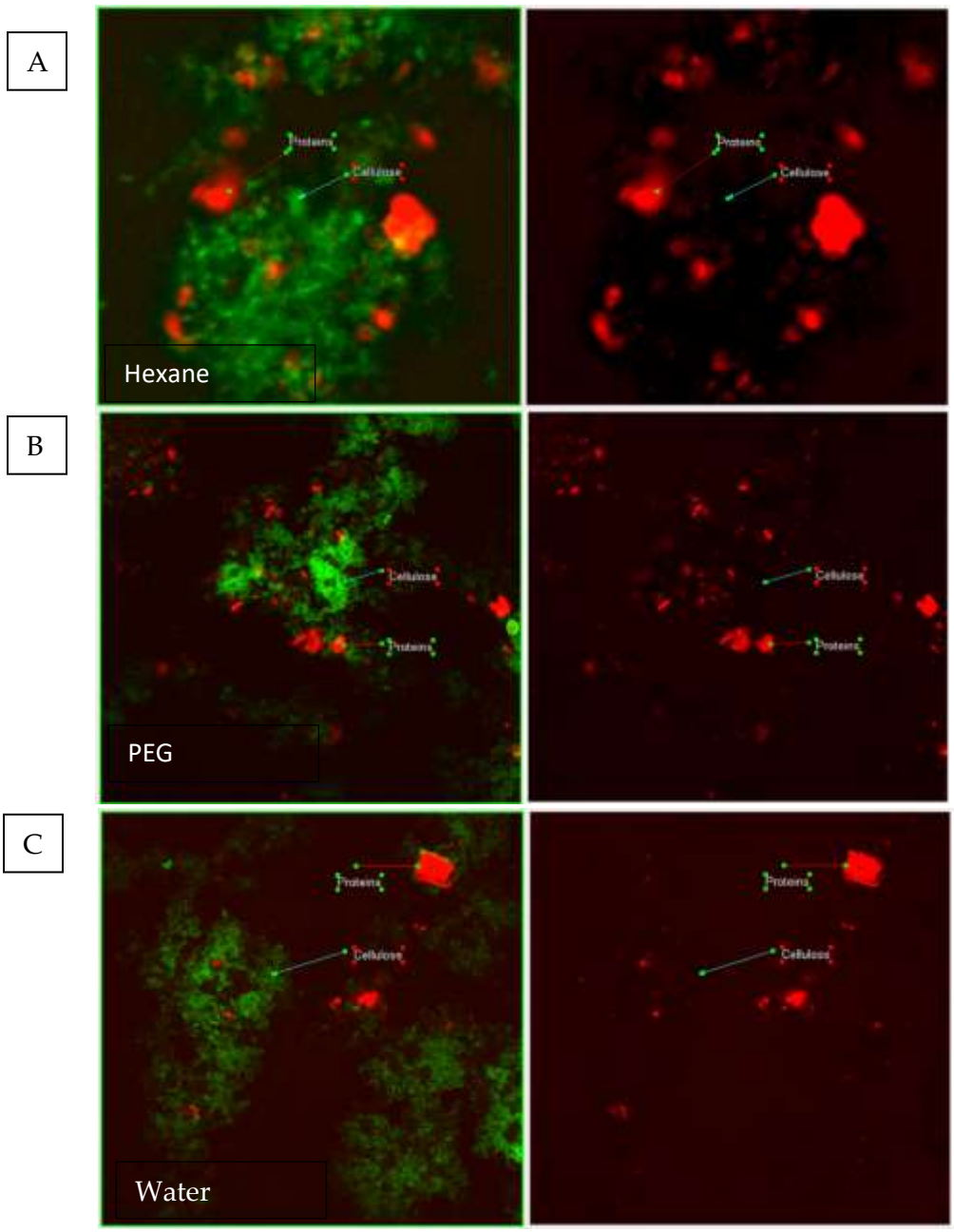

Figure 4.10: Images of proteins and cellulose by fluorescence microscopy in A) Hexane (top Phase), B) PEG (middle phase) and, C) salt (bottom) phase. The images on the right show the proteins molecules in bright red. The images on the left are superimposed images of proteins and cellulose. Cellulose particles are marked in green. Rhodamine B dye was used to stain the proteins molecules red.

4.4 Conclusions

This study demonstrated the capability of an amphiphilic polymer, polyDADMAC, to selectively separate carboxyl functionalized cellulose (model algal cells) from proteins under different pH conditions in a water-organic solvent two-phase system and a PEG-water-hexane three-phase system. Results indicate that water-hexane two-phase system performed better for individual com- ponent separation as compared to the PEG-water-hexane three-phase system. Presence of high amount of salts in the three-phase system seemed to interfere with the interfacation of polyelec- trolyte with the cellulose beads. In water/ hexane two-phase system, under pH 4.5 (close to the isoelectric point of protein) 75% of the proteins were retained in the water phase while simulta- neously migrating around 85% of cellulose beads into the hexane phase. The use of the cationic polyDADMAC appears to be a promising technique for selective separation of proteins from a mixture containing negatively charged particles paving the way for separating high-value proteins from cellular debris via a water-organic two-phase system.

5. THE USE OF POLYELECTROLYTES FOR SIMULTANEOUS DEWATERING AND FRACTIONATION OF MICROALGAL PROTEINS, LIPIDS, AND PIGMENTS

5.1 Introduction

With a projected population growth from 7 to 10 billion by 2050 and consequent increase in the demand for better nutritional sources, microalgae have gained a lot of attention in the recent past. Microalgae has proven to be a beneficial source of proteins, lipids, and pigments that have applications in food, biofuel, nutraceutical, and pharmaceutical industries [145, 11]. For several years, microalgae cultivation and harvesting have been done with lipid synthesis for biofuel production in mind; however, several life-cycle and techno-economic analyses have indicated the importance of co-extraction of value-added products to make microalgae sustainable source for biofuels [146, 147]. Nevertheless, co-extraction of several products from microalgae is challenging due to their small size, density close of that of water, and colloidal stability [19, 61, 148]. Therefore, it important to develop a simple technique which allows simultaneous separation of several components in a minimum number of unit operations.

To extract and separate intracellular products, microalgae needs to be first dewatered and disrupted. Currently, practiced algal biomass harvesting techniques include coagulation, sedimentation, flotation, passage through an electrical field, filtration and centrifugation[91]. In general, the harvesting technique should be non-toxic and environmentally friendly irrespective of the final use of the biomass. An ideal process should be effective and provide high biomass throughput with minimal costs, energy and other resources[149].

Chemical coagulation/flocculation is commonly practiced for algal biomass separation because of economic viability [91]. Flocculation involves adjustment of system pH to reduce the intermolecular repulsion forces and to coalesce the small well-suspended algal cells. Coagulation involves the use of chemicals/polymer to bind algal cells and form agglomerates through charge neutralization. Although these processes have been widely investigated for algal biomass harvest-

ing, none of the above techniques address the issue of handling large amounts of water and effective separation of bioproducts. So, there is a need for a technique that can dewater and simultaneously extract as many products as possible in a single unit operation.

Accordingly, the overall goal of this study is to develop a simpler technique that can harvest algal cells while extracting several valuable intracellular components like lipids, proteins, and carotenoids by manipulating their innate surface charge and their solubility in organic solvents. The basic principle of flocculation/coagulation (manipulating and neutralization of surface charge) was used with a major modification; here, an oppositely charged amphiphilic polyelectrolyte was used to bind onto the algal cells such that the resulting ensemble via charge neutralization becomes hydrophobic. The addition of a hydrophobic organic solvent immiscible with the aqueous phase helped in migrating the algal cells and any hydrophobic components from the aqueous phase into the solvent and making it easier to recover the cells/debris using very low energy input. Specifically, the impact of polyelectrolyte concentration and pH on the extractability of proteins, lipids, and pigments using a hexane/water two-phase system was investigated.

Chlorella was used for the study due to a multitude of reasons. This organism is known to have a capacity to accumulate several valuable components like proteins (51-58%), lipids (14-22%), carbohydrates (12-17%) vitamins, minerals, and pigments [150, 151]. Also, proteins from *Chlorella* is reported to be of high quality comparable with eggs, soy protein, and meat as recommended by World health organization(WHO) [152] and Food and Agriculture Organization (FAO) [153].

5.2 Materials and methods

Chlorella sorokiniana with 75%(w/w) solids content was provided by Texas A & M AgriLife Extension at Pecos, Texas in frozen form. Biomass was transferred to the refrigerator and store at -4°C. For use, the biomass was thawed at room temperature and diluted to 2% using deionized water to mimic actual growth conditions. Sodium hydroxide, sulphuric acid, hexane, vanillin extract, Bradford' s reagent, and 50 ml syringes were purchased from VWR. PolyDADMAC (average Mw <100,000 (low molecular weight), 35 wt. % in H2O) and protease inhibitor cocktail was purchased from Sigma Aldrich. All the chemicals used were laboratory-grade. All the

solutions were prepared in deionized water. Canola oil, used for developing a generalized standard curve for total lipids quantification, was purchased from a local store.

5.2.1 Cell disruption method for protein extraction

Biomass stored at 4°C was thawed at room temperature before use. Ultra-sonication (UP440St, Hielscher sonicator with a 3mm diameter probe, 24kHz) was used to disintegrate cells. Sonication was performed at 30 % amplitude and 0.5 cycles for one hour. The cell suspension was kept on an ice bath to prevent denaturation of intracellular material due to heat. After the sonication process was complete, the suspension was diluted to 4%w/w. A volume of 10 ml of the diluted suspension was centrifuged at 7000 rpm for 15 minutes, and the supernatant was analyzed for total soluble proteins using Bradford assay. The amount of soluble proteins extracted from the algal biomass was treated as 100% in the later experimental section.

5.2.2 Effects of pH

Seven different pH conditions including 3, 3.5, 4, 4.5, 5, 6, and 7 were adopted for the study. The main focus was to evaluate the impact of pH on protein retention in the water phase and migration of algal cellular debris to the hexane phase and to determine the optimum pH value for the separation process. All the pH values were adjusted to the nearest ±0.05 units at 25°C.

5.2.3 Effect of polymer concentration

Based on favorable results from a previous study[154], low molecular weight polyDADMAC (average Mw <100,000 instead of high or medium) was used in this study at six different concentrations ranging for 0.5% to 3% (w/w) (in 0.5% increments) of microalgae. The experiment was conducted as a completely randomized design with the aforementioned six levels of polymer concentrations and seven levels of pH.

5.2.4 Preparation of two-phase separation system

After preparing the diluted suspension of sonicated cells, the pH of the suspension was adjusted as per the required test conditions. A volume of 5 ml of DI water was added to the syringes, and the

appropriate amount of polyDADMAC was added to each syringe. The suspension was vortexed at maximum speed for 10 sec to ensure a homogenous suspension. A volume of 5 ml of microalgae suspension was added to the polymer suspension and vortexed for 1 minute at maximum speed to ensure polymer microalgae interaction. The final concentration of microalgae was 2%. A volume of 10 ml of hexane was added and vortexed again for 2 minutes at maximum speed and then allowed to sit undisturbed for one hour. Separation of water and hexane molecules was observed instantaneously. Algal cells along with hexane molecules formed the top phase. The migration system was kept undisturbed or one hour to attain equilibrium. The two phases were then separated for further analysis.

5.2.5 Quantification of proteins and algal cells in the two phases

Quantification of proteins and algal cells in the aqueous phase was done using Thermofischer's Genesys™ 10S UV-vis spectrophotometer. Quantification of proteins was done using Bradford's standard assay (Absorbance at 595nm), and for algal cells, it was done by measuring turbidity at 680 nm. The amount of particles migrated from the aqueous phase into the hexane phase was calculated using the following equation:

$$\text{Proteins in the aqueous phase (\%)} = \frac{(A595\ before\ migration - A595\ after\ migration\ in\ water)}{A595\ before\ migration} \times 100 \quad (1)$$

$$\text{Cells in the aqueous phase (\%)} = \frac{(A680\ before\ migration - A680\ after\ migration\ in\ water)}{A680\ before\ migration} \times 100 \quad (2)$$

5.2.6 2D SDS-PAGE

Isoelectric points of the proteins extracted from microalgae were determined using sodium-dodecyl sulfate at the Protein Chemistry Lab at Texas A & M University. The 2D SDS-PAGE was performed on a non-linear 3-10 pH range.

5.2.7 Lipid analysis

5.2.7.1 Preparation of lipid standard

Quantification of lipids was done using the method previously described by Butovich et al. [155] with modification. Sulfo-Phospho-Vanillin reagent (SPV) was prepared by dissolving 0.6 g of vanillin in 10 ml of absolute ethanol and 90 ml DI water followed by addition of 400 ml concentrated phosphoric acid. The reagent was stored in the dark.

Canola oil was used to develop a standard curve. An increasing amount of canola oil (2-10 mg) was taken in fresh glass vials, and 100 µl of sulphuric acid was added to each vial. The reaction mixture was incubated at 95°C for 25 minutes. It was then cooled for 5 minutes by placing it on an ice bath. A volume of 5 ml SPV reagent was added and allowed to react for 20 minutes at 37°C with constant shaking in the dark. It was then diluted with the addition of 15 ml of water and then used for recording absorbance at 535 nm.

5.2.7.2 Sulfo-phospho-vanillin assay

After disruption of cells, the suspension was centrifuged at 5000 rpm for 20 minutes. The supernatant was siphoned off in a fresh glass vial. Water was evaporated in a hot air oven at 105°C for 24 hours. Following the drying step, samples were used for lipid quantification using the aforementioned SPV mechanism. The amount of lipids in original algal suspension pre-separation was determined and treated at 100% extract. For quantifying the lipid content in the harvested cells, hexane phase was transferred into fresh glass vials and kept in a hot air oven at 70° C for 3 hours to evaporate all the solvent and later used in SPV assay.

5.2.7.3 Fatty acid analysis

For lipid analysis, the algal lipids were derivatized to fatty acid methyl esters (FAME) via transesterification. Gas chromatography-mass spectrometry (GC/MS) was used to quantify the fatty acid composition. An internal standard was used for FAME Quantification process.

5.2.7.4 Effect of temperature on lipid extraction

After separation of the two phases, water and hexane, the hexane phase was transfer into a fresh glass vial. It was capped and kept in a water bath at different temperatures, i.e. 20°C, 30°C, 40°C, 50°C. Quantification was done with SPV assay.

5.2.8 Extraction of carotenoids

After separation of the two phases, hexane was transferred to a fresh glass vial, and the solvent was evaporated in a hot air oven at 70°C for 4 hours. The samples were cooled to room temperature, and 10 ml of ethanol (95% v/v) was added to each vial. Determination of chlorophyll a (C_a), chlorophyll b (C_b) and total carotenoids (C_{a+b}) was done using the following equations [156]:

$$\text{Chlorophyll a (µg/ ml)} = \frac{13.36 \times A664}{5.19 \times A649} \quad (3)$$

$$\text{Chlorophyll b (µg/ ml)} = \frac{24.43 \times A649}{8.12 \times A664} \quad (4)$$

$$\text{Total Carotenoids (µg/ ml)} = \frac{1000 \times A470 - 2.13 \times Chlorophyll\ a - 97.64 \times Chlorophyll\ b}{209} \quad (5)$$

5.2.9 Statistical analysis

All the experiments were conducted in triplicate and as completely randomized designs. Statistical analyses were conducted using JMP Pro 14.1 (SAS).

5.3 Results and discussion

5.3.1 Separation of *chlorella*

With the objective to selectively migrate algal cellular debris from aqueous phase to the hexane phase, the impact of pH on the debris separation efficiency was evaluated. Here, after disintegrating the algal suspension, the pH of the system was adjusted according to the experimental design. At every pH condition, the volume of cationic polymer added was varied between 0.5-3% (w/w) of algal cells. The rationale here is that pH adjustment can assist in modifying the surface charge of the cells thus affecting the repulsion behavior between particles[34]. Figure 5.1a shows the impact

of polymer concentration and pH as two-factor interaction on algal cells migration into the hexane phase. Figure 5.1b shows the impact of polymer concentration and figure 5.1c shows the impact of pH on the migration of algal cells into hexane phase at different polymer concentrations.

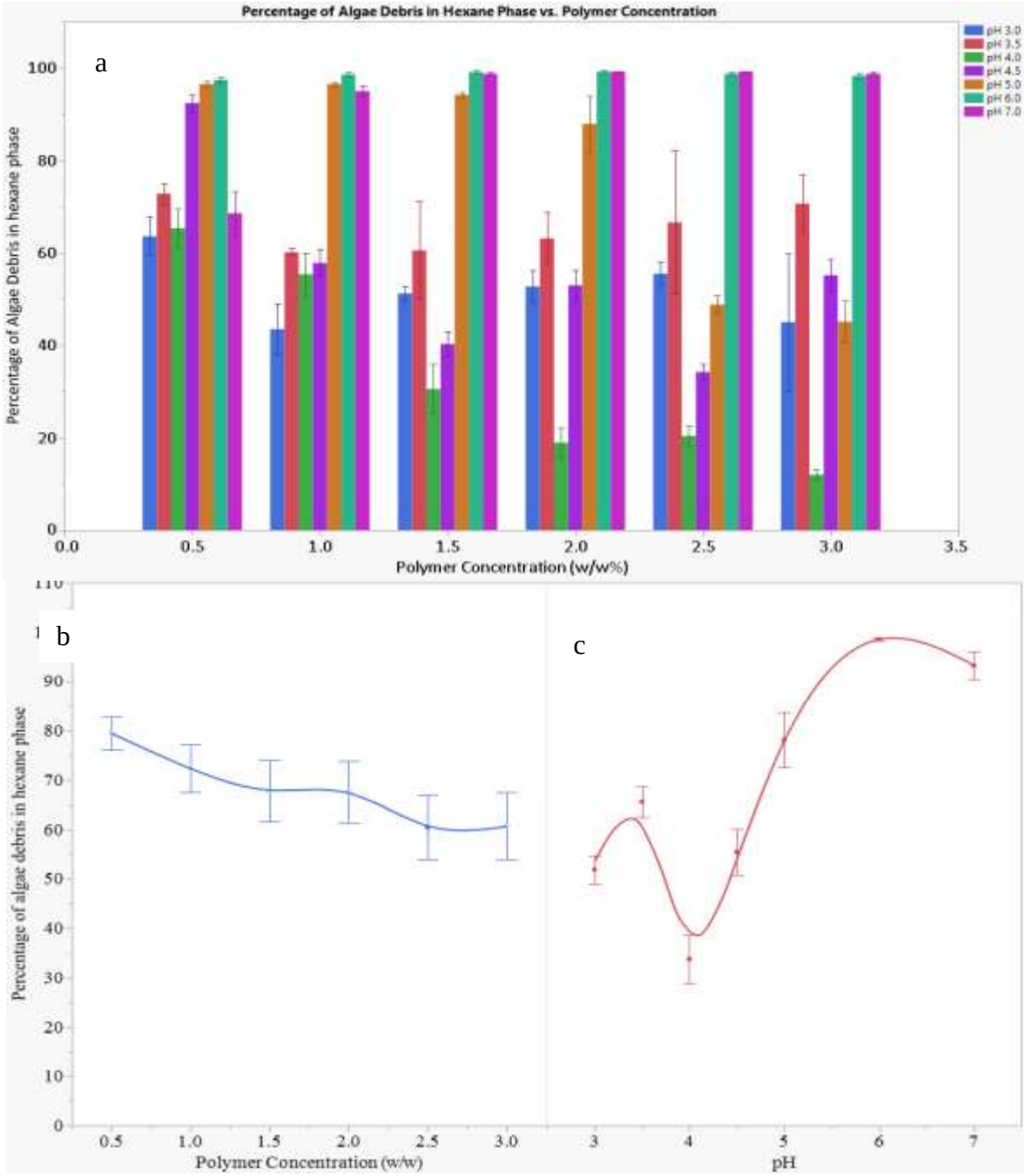

Figure 5.1: The amount of microalgal debris migrated into the hexane phase as a function of (a) polymer concentration and pH (two-factor interaction), (b) different polymer concentrations, and (c) different system pH. Values are shown as mean +/- standard error, n=3.

It can be seen that as the pH increases, the percentage of algae debris that migrated to the hexane phase increased ($P<0.0001$). The electrostatic interaction between two particles is governed by the degree of protonation or dissociation of carboxyl groups in the suspension, which is directly controlled by the system pH. Similar observations were made earlier where adsorption of surface modified chitosan was done proteins[157, 158]. It was also evident that high pH conditions (5-7) heavily favored algal cell debris migration as compared to low pH conditions (3-4). Under acidic conditions, due to the presence of excess H^+ ions in the system, the negative charge on the particles reduces via protonation of the groups carrying negative charges, resulting in reduced interaction between the negatively charged algal cells and the cationic polymer. Similar observations were made during the study of adsorption of chitosan, and bovine serum albumin (BSA) were decreasing pH increased the positive charge on chitosan and BSA through amino group protonation, eventually leading to reduced adsorption of chitosan and BSA[159]. The amount of cellular debris is significantly lower at and below pH 4 as compared to pH 5, 6, and 7. From figure 5.1b, it can be seen that as the polymer concentration increases, the amount of algal debris migrating to the hexane phase decreases ($P=0.0004$). Algal debris migration to the hexane phase was highest at a polymer concentration of 0.5% (w/w).

With the shift in pH towards alkalinity, the negative charge on the particles increased thus increasing the interaction between the cationic polyelectrolyte and the algal cells. It was also observed that there is a significant influence of interaction between pH and polymer concentration on the migration system efficiency ($P=0.039$). The reduced migration efficiency at higher polymer concentration could be due to re-stabilization of the suspension due to the presence of excess polymer. Once a certain number of polymeric strands bind onto the negatively charged particles, the unbound/ free polymeric strands might interact with the once that are already adsorbed on the algal cells rather than the free algal cells via hydrophobic-hydrophobic interaction, consequently resulting in reduced migration efficiency. Steady flocculation rate via charge neutralization followed by abrupt stabilization is a common characteristic of the presence of excess polymer in the system[160].

5.3.2 Retention of proteins in the aqueous phase

Figure 5.2a shows the impact of two-factor interaction of pH variation and polymer concentration in the system on the retention of total proteins from microalgae. Figure 5.2b shows the impact of polymer concentration while figure 5.2c shows the impact of pH on the retention of proteins in the aqueous phase. The system pH played a significant role in regulating the amount of proteins retained in the aqueous phase ($p<0.001$). It is evident that the amount of proteins that stayed back in the aqueous phase were highest at a pH range from 3.5-4. At pH 3, 25% of total proteins stayed back in the aqueous phase indicating that most of the proteins migrated into the hexane phase. The amount of proteins in the aqueous phase stayed relatively low between pH 5 to 7.

The migration behavior of proteins into the hexane phase was also governed by the amount of polyelectrolyte added into the system (figure 5.2c). As the polymer concentration increased, the retention of proteins in the aqueous phase also increased ($p<0.0001$). This again is likely due to re-stabilization of the system as a result of the excess polymer as described earlier. The increase in the amount of proteins retained in the aqueous phase as a result of polymer concentration was not as steep as it was due to the increased pH.

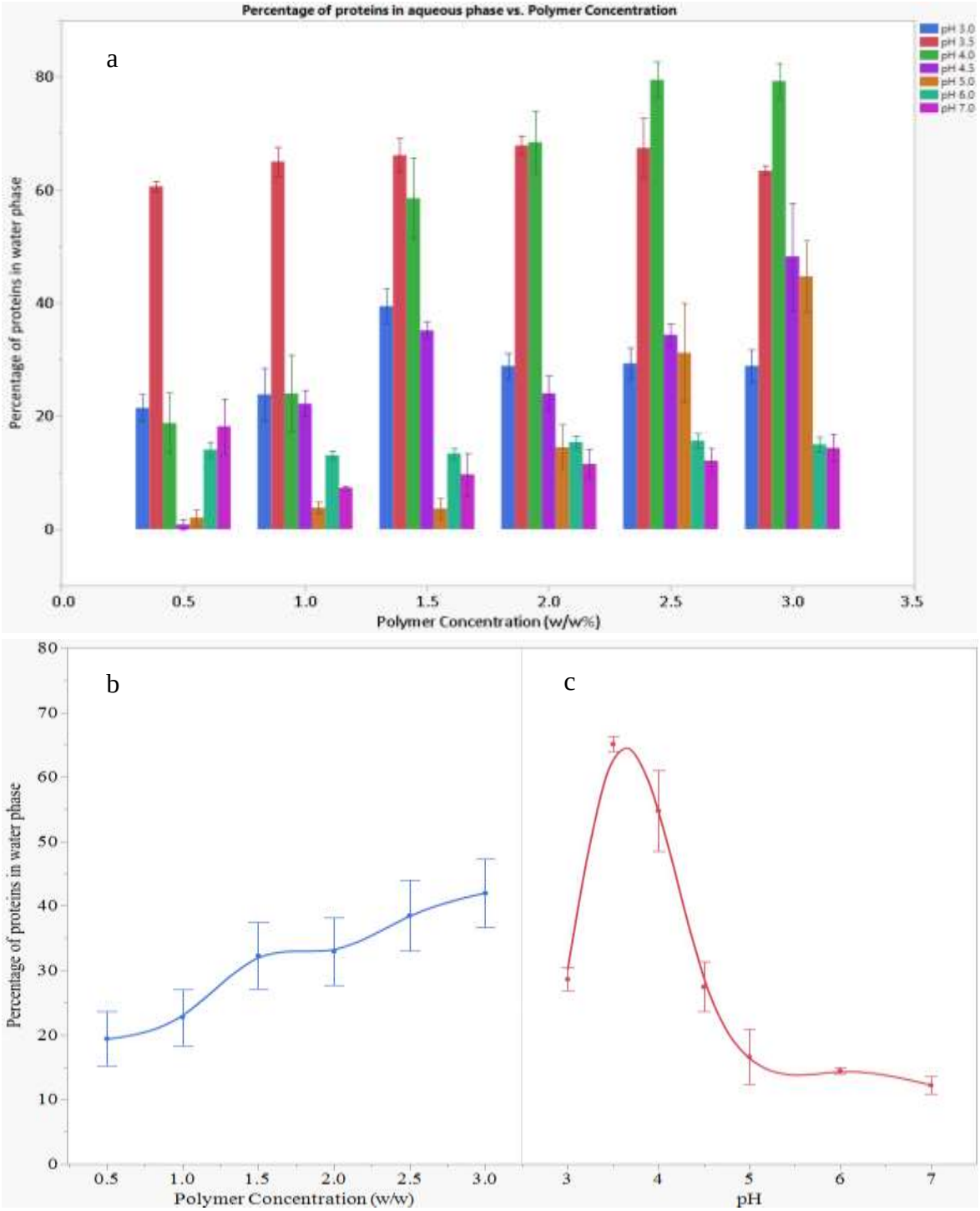

Figure 5.2: The amount of microalgal proteins retained in the aqueous phase as a function of (a) polymer concentration and pH (two-factor interaction) (b) different polymer concentrations, and (C) different system pH. Values are shown as mean +/- standard error, n=3.

To understand the impact of pH on protein retention in the aqueous phase, a 2D SDS-PAGE

analysis was conducted to evaluate the isoelectric point distribution of total proteins from microalgae (figure 5.3). Similar results were observed for the isoelectric point focusing done for proteins extracted from *Chlorella vulgaris* where a group of proteins was identified to have isoelectric point range of 4-5.5 pH [54]. It is evident that the major group of proteins that were retained in the water phase are those with an isoelectric point close to pH 4.

The highest retention of protein in the aqueous phase can be attributed to minimum interaction between the net charge neutral proteins at pH 4 and the cationic polyelectrolyte. There will be no formation of the hydrophobic ensemble if there is no electrostatic interaction between the two entities. However, as the pH tends towards alkalinity, the negative charge on the proteins increases due to the presence of OH^- ions in the suspension. Except for a small fraction of proteins with an isoelectric point between pH 6 and 7, all the remaining proteins can interact with the cationic polymer resulting in an ensemble; thus, lowering retention of proteins in aqueous phase above pH 5.

A small group of proteins with isoelectric points close to pH 3 was identified in the 2D SDS-PAGE analysis (figure 5.3). The remaining proteins will carry a small positive charge below their respective isoelectric points. This can potentially result in an interaction between the proteins and the polyelectrolyte via hydrophobic-hydrophobic interaction; therefore, resulting in the migration of ensembles into the hexane phase. These observations indicate the possibility of targeted retention of a certain group of proteins in the aqueous phase by adjusting the system pH to their respective isoelectric point. In such a situation, any of the proteins at a pH above its isoelectric points will carry a net negative charge and could be mobilized to the hexane phase by using a cationic polyelectrolyte. Analogously, any protein(s) at a pH below the isoelectric pH should be able to be mobilized to the hexane phase by using an anionic polyelectrolyte.

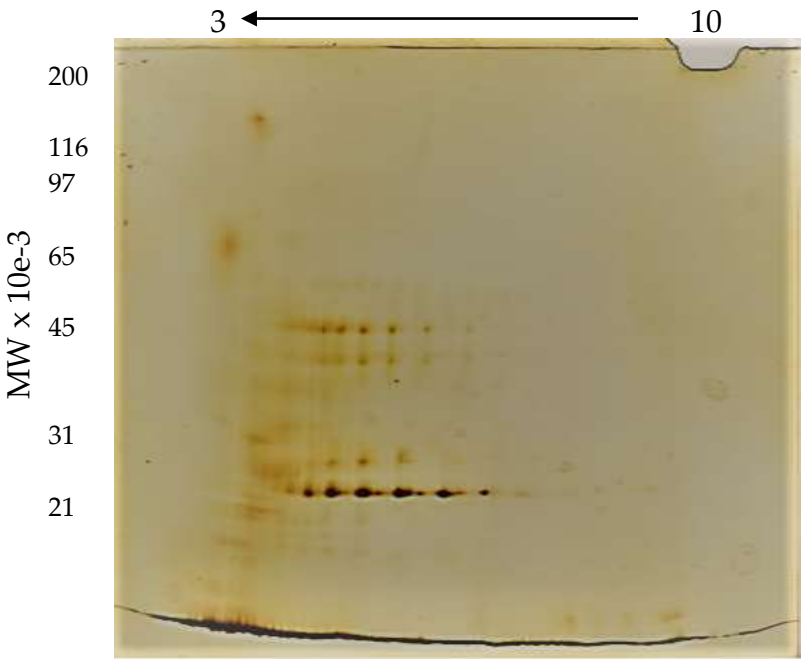

Figure 5.3: 2D SDS-PAGE for total proteins extracted from the microalgae.

5.3.3 Lipid extraction from microalgae

The primary reason to investigate lipids extracted from microalgae during the migration process was to test the ability of the migration system to co-extract lipids while selectively retaining proteins in the aqueous phase and migrating micro-algal debris to the hexane phase. The co-extracted lipids were quantified using the SPV assay. Figure 5.4 shows the amount of lipids coextracted in hexane phase during algal cellular debris migration at different polymer concentration and pH (figure 5.4a, two-factor interaction), different polymer concentrations (figure 5.4b), and system pH (figure 5.4c). It was noticeable that low pH conditions (pH 3) tended to favor lipid release; however, no statistically significant correlation (p=0.9547) was observed.

Nevertheless, the lipids extracted as a function of varying polymer concentration was statistically relevant ($p=0.0528$). This may be due to the ability of the polyelectrolyte to milk-out lipids from microalgae via forming perforations on the cell wall. Similar observations were made by Hong, Seungpyo et al., where interaction between polycationic polymers and live cell membranes induced the formation of holes or expansion of preexisting defects[105].

The amount of lipids extracted in the hexane phase under different pH and polymer concentrations did not follow a specific trend, and this may be attributed to differences in the level of cell disruption via sonication. Studies have shown that ultra-sonication has relatively low cell disruption efficiency[161]. The amount of lipids that can be extracted in the hexane phase will primarily rely on the degree of cell disruption.

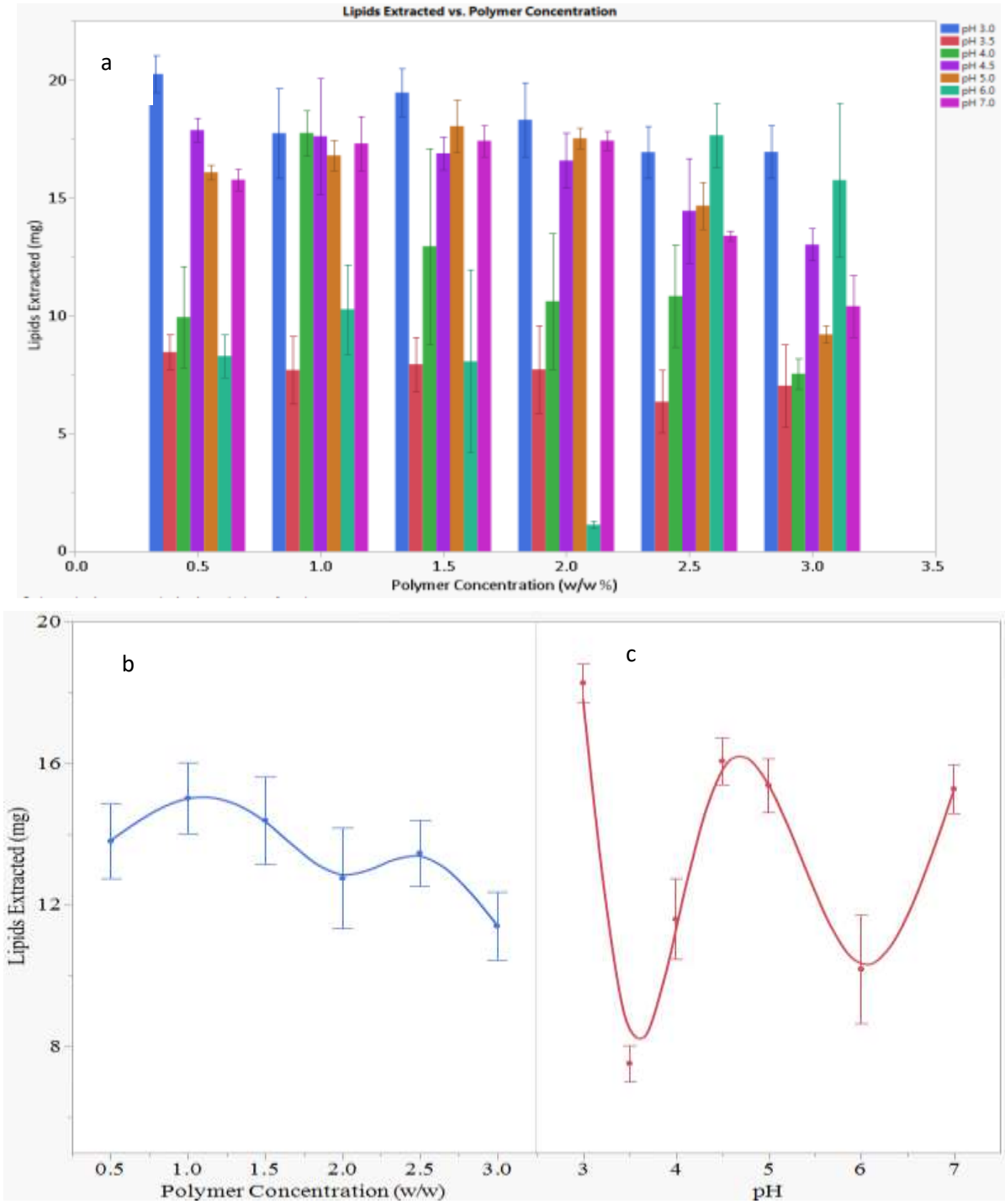

Figure 5.4: Quantity of algal lipids extracted into the hexane phase as a function of (a) polymer concentration and pH (two-factor interaction) (b) different system pH, and (c) polymer concentration conditions. Values are shown as mean +/- standard error, n=3.

To elucidate the lipids milking-out phenomenon via cationic polyelectrolyte, separate studies were

conducted. Lipid analysis was performed on 1) control sample (microalgal suspension in water), 2)microalgae separated with 0.5% and 3% polymer, 3) whole and disrupted (using sonication) microalgae, 4) and microalgae from hexane phase subjected to heat (70°C) and no heat conditions (room temperature, 25°C). Table 5.1 represents the amount of fatty acid methyl esters (FAME) content per gram of hexane-extracted (note: FAMEs extracted are represented in per gram of hexane basis while total FAMEs from original biomass are represented on AFBM basis). Percent lipids extracted for each condition was calculated based on the total lipids present in microalgae. There was no particular trend observed in the amount of lipids extracted at different polyelectrolyte concentrations, 0.5% or 3%. From the data (table 5.1), it can be seen that under no sonication, at room temperature, high polymer concentrations resulted in 60.49% lipids extracted vs. only 48.34% at low polymer concentrations. This indicates that lipids can be milked-out from whole micro-algal cells even without any cell disruption via sonication likely as a result of the ability of the polymer to make perforations in the cell wall. However, lipid extraction efficiency is better when cells were subjected to ultrasonication. Furthermore, it was also observed that the temperature of the system (hexane phase) after migration had no impact on lipid extraction efficiency. In fact, better extraction efficiencies were observed at room temperature; 89% of total lipids were extracted in hexane phase under room temperature with 0.5% polymer treatment.

Table 5.2 shows the fatty acid profile under different lipid extraction conditions. No significant difference in the fatty acid profile was observed for the microalgae treated with 0.5 or 3 % polyelectrolyte, sonicated and non-sonicated cells or the cells that were subjected to 70° C as against 25° C.

5.3.3.1 Extraction of lipids at different temperatures

After migration of algal biomass into hexane phase, the two phases were separated. Hexane phase was kept at different temperatures to extract lipids (Figure 5.5). No significant change was observed in terms of lipids extracted in hexane. These results as in line with the observations made earlier in GS-MS studies.

Table 5.1: Total FAME extracted for different conditions. Quantification of fatty acid methyl esters was done using internal standards (n=1).

Sample Treatment	Temp (° C)	Polyelectrolyte % (w/w of microalgae)	Lipid Content mg FAME/ g Hexane	hexane:AFBM ratio (mL of hexane / g of AFBM)	g of hexane/g AFBM	mg FAME/g AFBM
No Sonication	25	0.5%	0.19	381.56	238.93	45.64
Sonication	25	0.5%	0.29	374.76	234.67	67.82
No Sonication	70	0.5%	0.22	381.56	238.93	51.85
Sonication	70	0.5%	0.22	374.76	234.67	50.92
No Sonication	25	3.0%	0.24	381.56	238.93	57.11
Sonication	25	3.0%	0.21	374.76	234.67	48.11
Sonication	70	3.0%	0.24	374.76	234.67	56.32
No Sonication	70	3.0%	0.19	381.56	238.93	46.35

Table 5.2: Fatty acid profile of Chlorella from different harvesting conditions. Quantification of fatty acid methyl esters was done using internal standards (n=1)

	No Sonication		Sonication		No Sonication		Sonication	
Polyelectrolyte Concentration	0.5%	0.5%	0.5%	3.0%	3.0%	3.0%	3.0%	
Fatty Acid Methyl Ester	No Heat	Heat	No Heat	Heat	No Heat	Heat	No Heat	Heat
C16:0	0.062	0.069	0.086	0.066	0.085	0.067	0.066	0.076
C16:1 cis	0.002	0.004	0.006	0.005	0.005	0.004	0.004	0.004
C16:1 trans	0.009	0.014	0.019	0.015	0.012	0.011	0.012	0.016
C16:2 cis	0.013	0.016	0.024	0.015	0.014	0.011	0.014	0.015
C16:3 cis	0.047	0.051	0.073	0.049	0.049	0.037	0.050	0.054
C18:0	0.035	0.035	0.041	0.036 0	.050	0.039	0.036	0.039
C18:1 cis	0.012	0.014	0.018	0.017	0.012	0.013	0.010	0.019
C18:2 cis	0.004	0.007	0.009	0.008	0.005	0.007	0.007	0.009
C18:3 cis	0.006	0.007	0.010	0.007	0.006	0.005	0.007	0.009
	mg FAME /g Hexane							

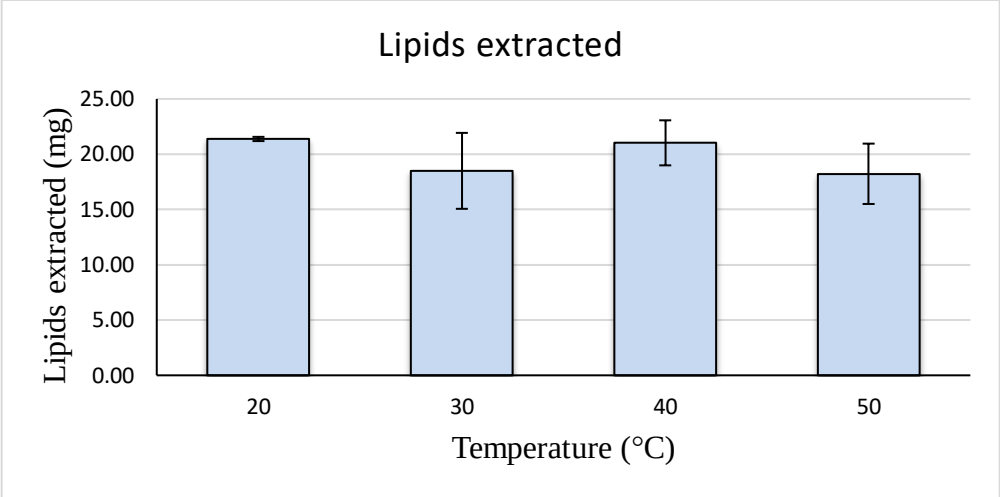

Figure 5.5: Lipids extracted at different temperatures. Values are average of three replicates and +/- standard error.

5.3.4 Carotenoids extracted from microalgae

After the migration of algal cells, carotenoids extracted in the hexane phase were investigated. Carotenoids are an important byproduct of the algal extraction process. Figure 5.6 shows the amount of chlorophyll a, chlorophyll b, and total carotenoids extracted under different pH conditions. It is interesting to note that as the amount of algal cellular debris increased in the hexane phase with the pH tending towards basic conditions, the amount of chlorophyll and carotenoids extracted in hexane also increased. Although only a small quantity of carotenoids and chlorophyll was extracted during the process as compared to other processes that are dedicated to extracting these molecules, this technique can potentially be used to co-extract such high-value products. It should be noted that for maximum yield, chlorophyll and carotenoids have to be extracted via a multi-step solvent washes as described by Xu Zhang et. al. [162].

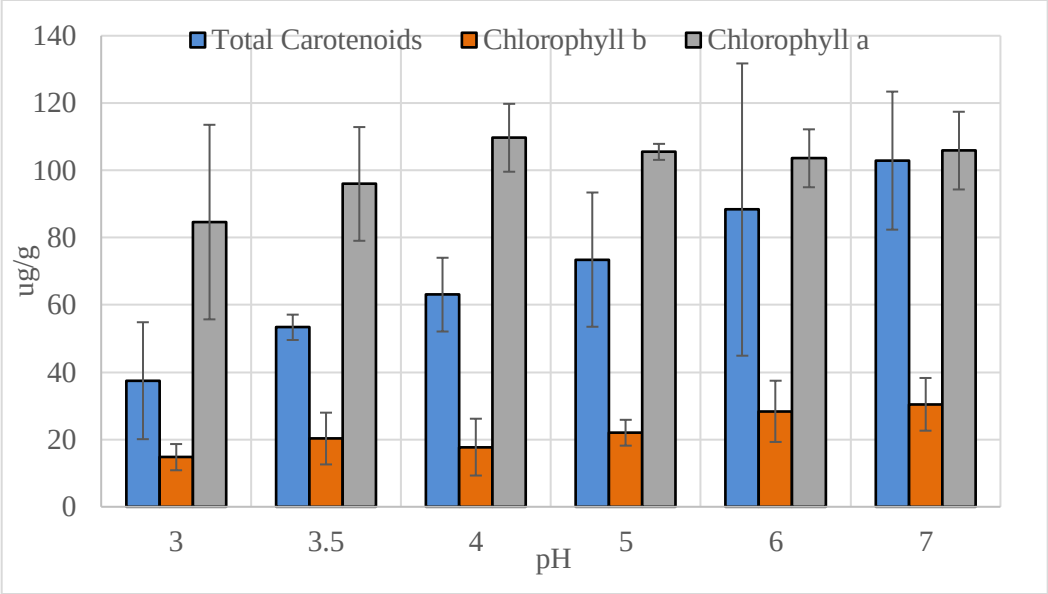

Figure 5.6: Chlorophyll and carotenoid extraction results. Values are average of three replicates and +/- standard error.

5.4 Conclusions

PolyDADMAC was successfully used for targeted separation of cellular debris along with lipids and other pigments to the hexane phase while retaining proteins in the aqueous phase of *Chlorella* microalgae. Isoelectric points of suspended particles are critical to determine the separation efficiency of each component. Approximately 80% of total proteins were retained in the aqueous phase at pH 4, and 95% of algal cellular debris were migrated into hexane phase at pH 5, 6 and 7. Lipids, chlorophyll, and carotenoids were also extracted in hexane during the process.

6. HIGHLY SPECIFIC RNA DEPENDENT RNA POLYMERASE INHIBITORS FOR TICK-BORNE ENCEPHALITIS VIRUS

6.1 Introduction

Tick-borne encephalitis virus (TBEV) causes tick-borne encephalitis (TBE), a severe neuro-infection in humans[163, 164]. Common symptoms of TBE are fever, fatigue, joint pain, rashes and body ache. TBEV circulates between ticks and hosts in geographically strictly limited natural foci, which can range in size from large to very small [165]. TBE in humans can be caused by direct tick bites or by consumption of non-pasteurized milk or milk products from TBEV-infected sheep, goats and cows. Oral consumption of non-pasteurized milk has contributed to recent outbreaks in several European countries including Slovakia, Czechia, Hungary, Austria and Estonia [166, 167, 168].

Vaccination is the most specific preventive tool against TBE [163]. Registered vaccines contain inactivated virion antigens as the active compound [169, 170]. Vaccines available today are known to induce a high degree of protection against the virus. However, numerous cases involving previously vaccinated persons against TBE have been positively diagnosed [171, 172]. Vaccine failures have been reported and may be overlooked due to different, and sometimes confusing, antibody kinetics in vaccines with TBEV infection[173]. Recent vaccine failures against TBEV [173, 174, 175] directs researchers to revisit paths for preventing and/or treating TBE.

TBEV genome encodes three structural proteins (C, M, and E) and seven non-structural proteins ((NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) and these proteins play a vital role in viral replication [55]. The largest TBEV non-structural protein NS5 (100kDa) is involved in viral RNA replication [176]. NS5 has a positive charge and is conserved in flaviviruses[177]. NS5 protein also contains methyltransferase (MTase) and RNA-dependent RNA polymerase (RdRp) domains [178]. Many inhibitors of flavivirus MTases and RdRps have been identified through various techniques like cell-based assays, virtual screening, fragment-based and structure-based

design [179, 180, 181, 182, 183, 184, 185]. However, the majority of these compounds have not shown antiviral efficacy [186], whereas few of them have displayed relatively low potency, high cytotoxicity, and/or low therapeutic index[180, 187, 188].

This work looked at potential antagonists that have a high potential to hinder viral replication via NS5 silencing while maintaining a low and/ or no toxicity to humans by parsing interactions between dynamically changing receptor and impinging ligands by doing the initial screening in silico via molecular dynamic (MD) simulations.

6.2 NTP interactions with RNA-dependent RNA polymerase domain of TBEV NS5

TBEV replication is complemented by rearrangement of cellular membranes that facilitate viral genome replication and protect viral components from host cell responses[189]. Replication of positive-strand flaviviruses is facilitated by the viral RdRp. The RdRp region in positive-strand viruses is one of the most conserved motifs [190]. Additionally, NS5 from these viruses contain conserved motifs found in NA 5′ -methyltransferase[178]. RdRp is an essential protein in RNA-containing viruses[191, 192] and catalyzes the replication RNA from an RNA template. Replication of viral RNA proceeds in two steps. Firstly, RdRp binds to the RNA template at the 3′ promoter region initiating the replication process followed by addition of nucleotide triphosphates to the 3′ end leading to elongation phase. The crystal structure of TBEV NS5 has not yet been resolved. So, the RdRp domain of TBEV was identified by mapping the conserved RdRp domain (that lies between residues 530-682) of Japanese Encephalitis Virus (JEV) crystal structure to TBEV NS5 (Fig. 6.1).

Present studies were conducted on an NS5 bound model [193] (Fig. 6.1).

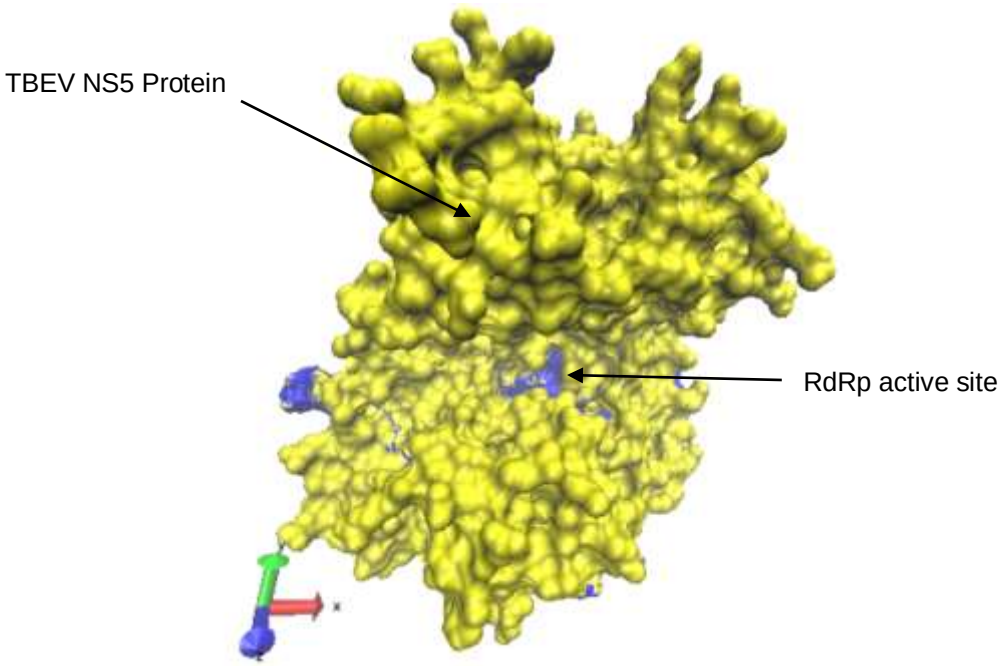

Figure 6.1: NTP binding RdRp domain of TBEV NS5. The active site is modeled on a bound form of NS5 homology model

6.3 SAMe and NTP interactions on TBEV Protein

Mtase and RdRp conserved regions on TBE viral protein were analyzed using AutoDock Vina [120]. S- Adenosylmethionine (SAMe), a standard substrate involved in methyl group transfers and adenosine triphosphate (ATP), cytidine triphosphate (CTP), guanosine triphosphate (GTP) and uridine triphosphate (UTP) were used as ligands. The docking results generated multiple conformations of NTPs binding onto RdRp domain (Fig. 6.2A) while the SAMe binding onto Mtase domain (Fig. 6.2B).

The simulations revealed that top conformations of NTPs bound to the same pocket that coincided with the previously identified RdRp domain of NS5. The interaction diagrams re- vealed that the NTP binding pocket resided among residues VAL 132, ASN 135, ALA137, GLY 139, ALA 140, TRP 141, SER 142, ARG 152, and SER198; ARG201; SER 331, THR336, TYR 337, ASB 340, and SER 390; and GLY 520, ARG 521, SER 525, ILE 526, and HIS 527. It was observed that adenosine triphosphate (ATP), cytidine triphosphate (CTP), guanosine triphosphate (GTP) and uridine triphosphate (UTP) bound to the RdRp active site with average affinities of 8.2, 8.6, 7.6 and 8.2 kcal/mol respectively. It was interesting to note that SAMe bound in the vicinity of the RdRp site with a reasonable affinity of 7.7 kcal/mol (Fig 6.2, Table 6.1).

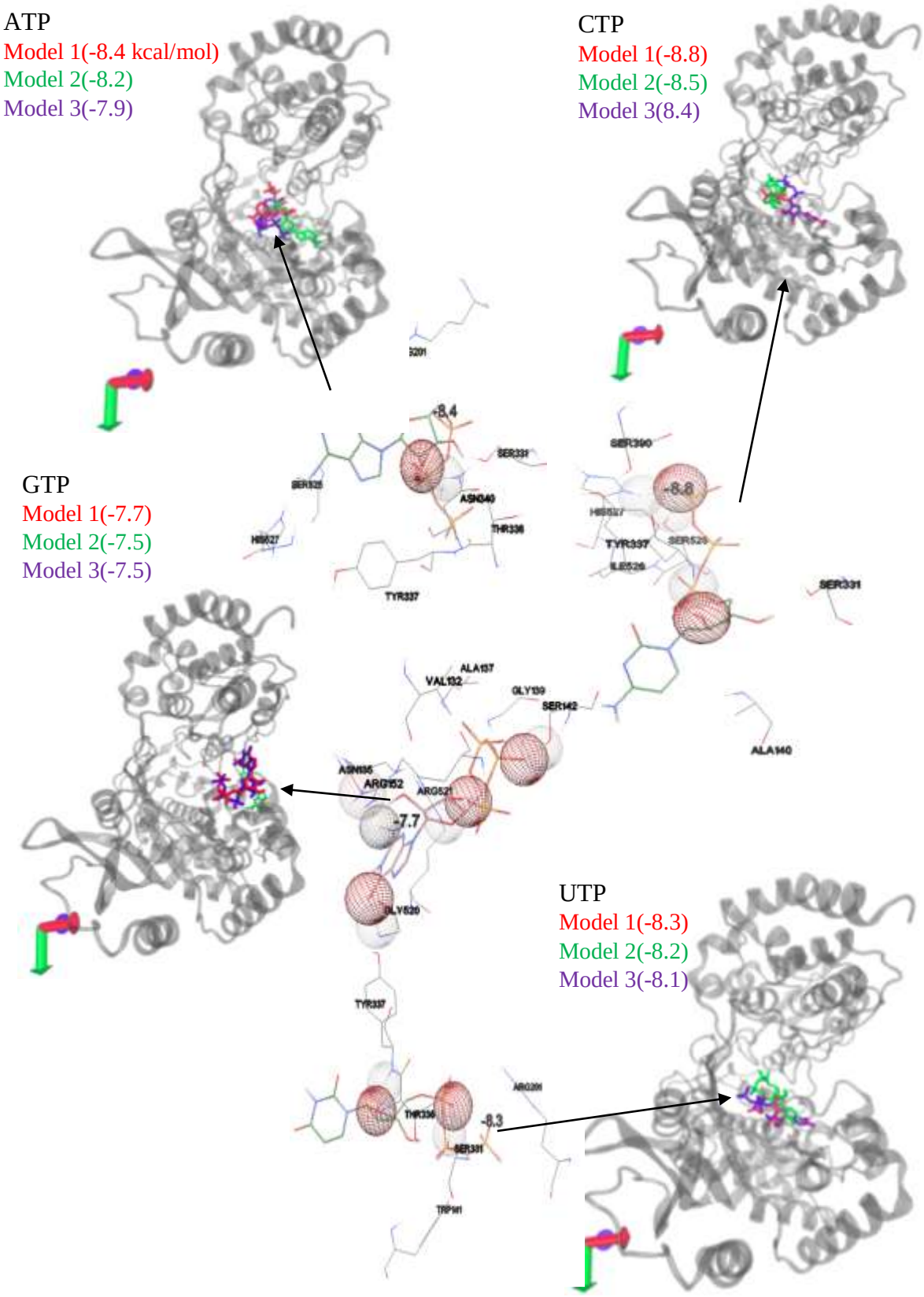

Figure 6.2: Nucleotide triphosphate interaction with TBE viral protein's RdRp domain

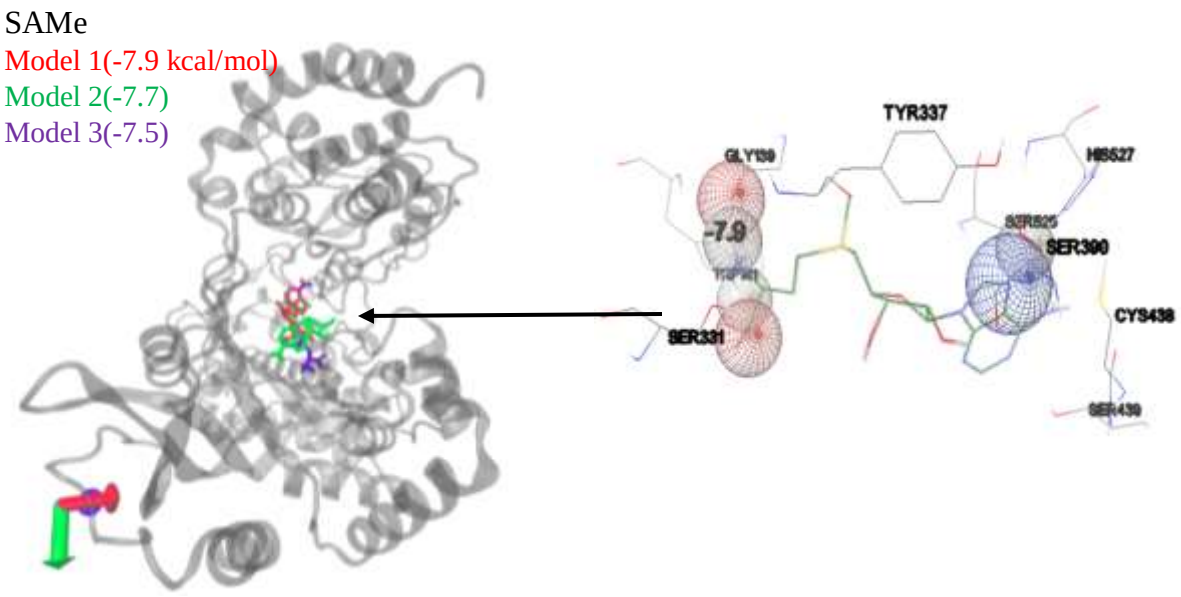

Figure 6.3: S-Adenosyl methionine (SAMe) interaction at the methyl transferase domain

Table 6.1: Hydrogen bonds formed by NTPs and SAMe with various amino acids on NS5

Ligand Name	**Receptor amino acid number**	**Maximum binding energy** (kcal/mol)
ATP	TYR 337	-8.4
GTP	ASN 135, ALA 137, GLY 139, SER 142, GLY 520 and ARG 521	-7.7
CTP	HIS 527, ILE 526, SER 525	-8.8
UTP	SER 331	-8.3
SAMe	GLN 333, TYR 337, ASP 392, SER 390, HIS 527, CYS 438	-7.9 -7.9

The output from docking simulations predicts nucleotides and SAMe binding to the respective

sites with high affinity. Interaction diagrams also suggest that the RdRp and methyltransferase domains reside close proximity to each other suggesting that compounds that would bind to the same region with a higher affinity than native substrates could be potential candidates as antivirals.

6.3.1. Druggability assessment and identification of pharmacophores

Once the interaction sites of NTPs on NS5 were revealed, a druggability analysis[194] was done to determine how potential ligands would interact with NS5 via NAMDsimulations[195]. The molecular dynamic (MD) simulations were done in the presence of small organic molecular probes. The probes, which are the most commonly used for druggability analyses consisted of 60% isopropanol and equal 10% concentrations of isobutene, acetamide, acetate, and isopropylamine (Fig. 6.3A). The druggability assessment was performed with the intention of unraveling any "hot spots" or "clusters" of hotspots that indicate the existence of druggable receptors. The system equilibrated to contain 10200 water and 510 probe molecules (i.e., 306 isopropanol, 51 isobutene, 51 acetamide, 51 acetate and 51 isopropylamine).

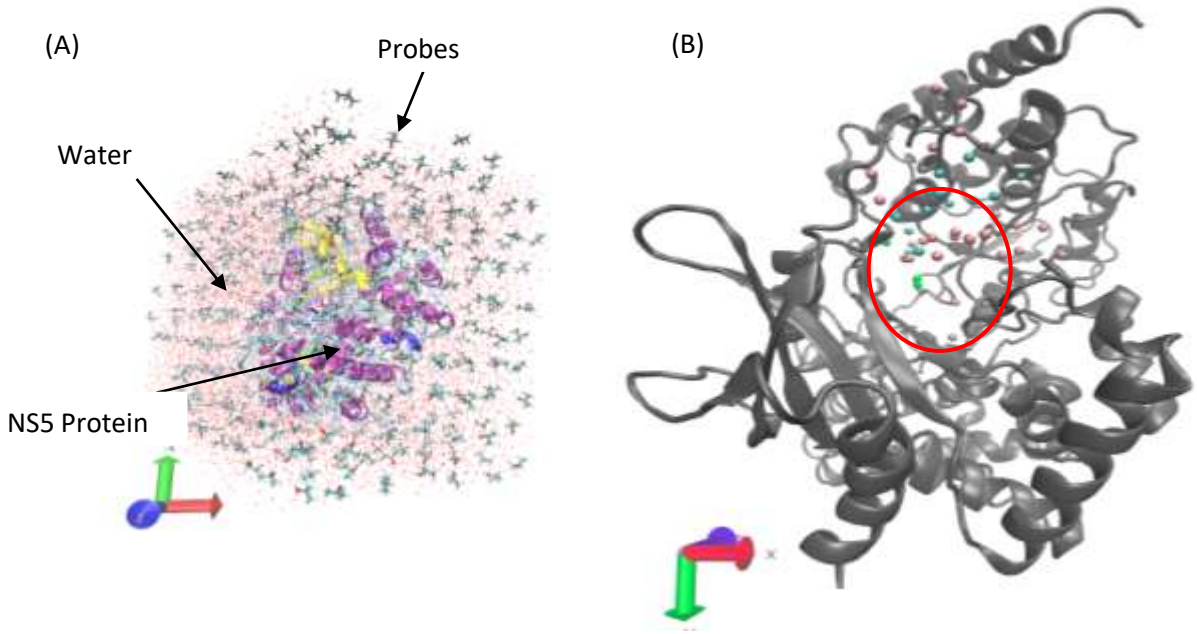

Figure 6.4: Probe interaction with TBE viral protein.

The druggability analysis revealed 254 small-molecule binding hotspots ranging from a minimum ΔG of -2.41 kcal/mol and maximum of -1.00 kcal/mol. The protein surface was enriched with 137 binding hotspots of isopropanol with the lowest binding free energy of -2.11 kcal/mol. Nevertheless, isobutene (21 hotspots, -2.10 kcal/mol), isopropylamine (26 hotspots; -2.25 kcal/mol), acetamide (9 hotspots, -2.41 kcal/mol), and acetate (61 hotspots, -2.38 kcal/mol) enrichment were more isolated. The analysis predicted the presence of one druggable domain clustered and overlapped with the RdRp domain (Figure 6.3B) with an achievable binding affinity of -12.70 kcal/mol and highest drug-like affinity 0.556nM occupying 458.86 $Å^3$. The pharmacophore distribution of the NS5 RdRp druggable receptor is shown in figure 6.4. From the probes tested, it is clear that isopropanol, and isobutene followed by acetamide have the maximum affinity at the RdRp domain. The pharmacophores were resolved by matching the location with the activity of the probes that were clustered together at the site.

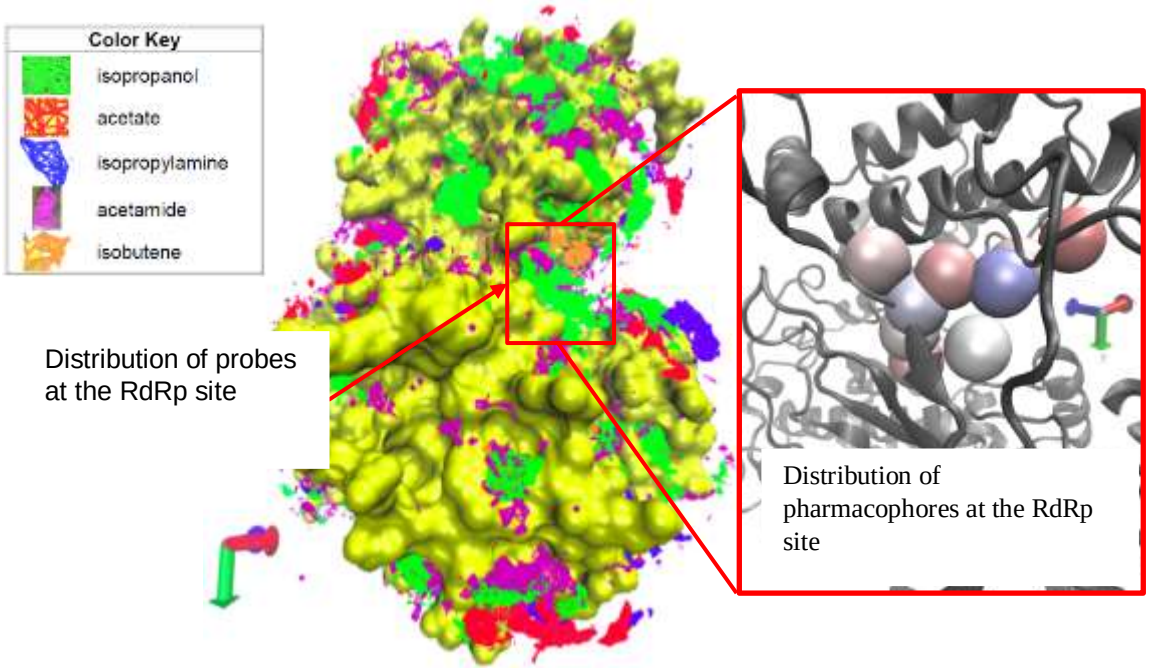

Figure 6.5: Pharmacophore point distribution of TBEV protein.

The hotspots isolated from druggability analysis along with active sites of NTPs were used in

the Enhanced Ligand Exploration and Interaction Recognition Algorithm (ELIXIR-A) to set up the system for ligand screening.

6.3.2 Ligand screening and verification

With the pharmacophore point information gathered from the ligand/receptor interaction analysis, a ligand-screening was performed via Zincpharmar facility[196]. For ligand screening, common pharmacophore coordinates identified by Druggui analysis that coincided with the RdRp site and active sites of the NTP ligand that had the highest binding affinity, i.e., cytidine triphosphate (CTP) were used. Initial screening pooled 20 pharmacophores which were narrowed down based on most dominant in- teractions as identified by interaction diagrams. Initial screening resulted in dozens of potential targets which were further narrowed based on above criteria and availability of compounds for experimental verification.

Final analysis resulted in five potential ligands, Zinc 7065 (Citicoline also known as cytidine diphosphate-choline (CDP-Choline) or cytidine 5′-diphosphocholine (ZINC id: ZINC6507065)); Zinc256079662; ZINC id: ZINC00010733677; Zinc 9041 (Rhodamine-12-dUTP triethylammonium (ZINC id: ZINC000328579041)); and Zinc 7151 (Benzoxaborole). Properties of all the potential inhibitors are shown in Table 6.1.

CDP-Choline (Zinc 7065) is an intermediate in the generation of phosphatidylcholine which is a common biochemical process in the neural cell membrane [197]. Administered orally or by injection, citicoline is non-toxic and very well tolerated compound that is known to be catabolized quickly[198]. Zinc 9662 is a uridine di-phosphate-α-D-galactose disodium salt and thus a nucleoside analog. Zinc 9041 is recommended for direct enzymatic labeling of DNA/cDNA. Zinc 7151 has gained significant attention due to its therapeutic properties in recent years. It has low bio-toxicity combined with high target specificity[199]. Zinc 3677 has not been reported in any trials so far.

Table 6.2: Information on potential inhibitors

Id	ZINC7065	ZINC9662	ZINC3677	ZINC9041	ZINC7151
Octanol-water partition coefficient logP	-1.598	-4.86	2.617	-1.42	1.04
molecular weight	489.335	566.302	442.493	992.718	133.943
number of hydrogen bond acceptors	14	16	6	-	1
number of hydrogen bond donors	3	9	1	-	0
"Rule of five" violations	2	3	0	3	0
Number of Rotatable Bonds	10	9	7	20	0
Enzyme inhibitor score	1.63	1.24	-0.23	-3.2	1.92
Average docking affinity kcal/mol	-7.63	-9.3	-6.67	-10.5	-5.93

The highest affinity (i.e., lowest binding energy) of the most stable conformation (at the RdRp binding site, RMSD=0), the average affinity of all conformations at the RdRp site (RMSD < 4), and the average affinity of sites other than the RdRp site (RMSD > 15) are depicted in Fig. 6.5A. The most stable conformations of the molecules are depicted in Fig. 6.5B. It is noted that Zinc 7065, Zinc3677 and Zinc 7151 had a lower binding affinity than native NTPs; however, Zinc 7151 and Zinc 3677 displayed zero violations for Lipinski's rule of 5 while also displaying a high bioactivity score indicating a better inhibition as compared to other compounds. Zinc 9662, and Zinc 9041 had significantly lower binding energies (average affinity -9.3, and -10.5 kcal/mol respectively) than native NTPs (i.e., ATP -8.6, CTP -8.5, GTP -8.4 and UTP -7.8 kcal/mol) at the RdRp domain. It was interesting to note that Zinc 9041 did not have any conformations that bound away from the RdRp site portraying their high specificity to the site. It is clear that these

three molecules would preferentially bind onto the RdRp site and thus have the most likelihood of acting as potential inhibitors.

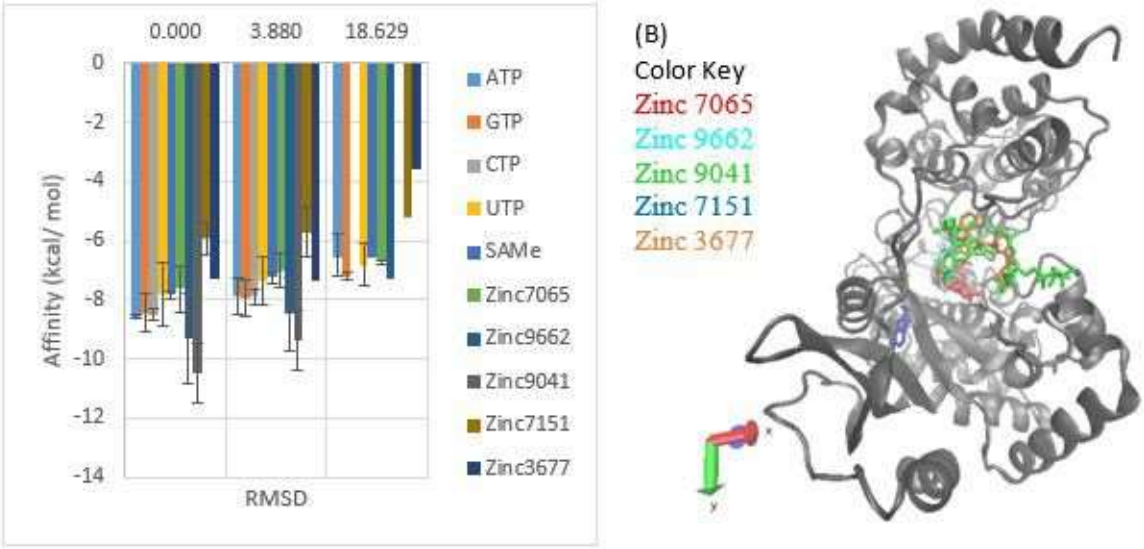

Figure 6.6: (A) Binding affinity of all the NTP molecules, SAMe and all the potential drugs, (B) Drug binding sites.

6.3.2.1 Testing of the anti-TBEV activity in-vitro

Antiviral testing was performed as described previously [200], with slight modifications. Low-passage TBEV strain Hypr (a typical representative of the European TBEV subtype) was used for the antiviral testing. Porcine kidney stable (PS) cells, a cell line widely used for TBEV growth (Kozuch et al.[201]), were cultured in Leibovitz (L-15) medium, supplemented with 3% newborn calf serum and a 1% mixture of antibiotics and antimycotics and 1% glutamine (Sigma-Aldrich, Prague, Czech Republic). The compound cytotoxicity was determined in terms of cell viability using the Cell Counting Kit-8 (Dojindo Molecular Technologies, Munich, Germany) according to the manufacturer's instructions. A viral titer reduction assay was performed to determine the anti-TBEV activity of the tested compounds in cell culture. PS cells were seeded in 96-well plates (approximately 2 x 10^4 cells per well) and incubated for 24 h at 37 °C to form a

confluent monolayer. The medium was then aspirated from the wells and replaced with 200 μL of fresh medium containing the tested compound at concentration 0, 10, 30, and 100 μM. The cells were incubated for 24 h; the medium was then removed and replaced with a fresh medium containing the drug and virus at a multiplicity of infection of 0.1. After 2 h incubation, the medium was replaced with fresh medium containing the drug and incubated for 24 h at 37 ℃. Then, the supernatant medium was collected and viral titers were determined by plaque assays as described previously. Immunofluorescence staining assay was performed to measure the expression of TBEV surface antigen (E protein), as previously described [195]. Cytotoxicity testing revealed that compounds Zinc 7095, Zinc 9662 and Zinc 9041 are cytotoxic and these compounds were excluded from further testing. In-vitro antiviral effect, demonstrated by both immunofluorescence staining and viral titer reduction assay, was demonstrated in TBEV cultures treated with Zinc 3677 and Zinc 7151 (figure 6.7). In case of Zinc 7151, TBEV growth was substantially inhibited at concentrations 30 and 100 μM. Zinc 3677 exhibited lower antiviral activity with TBEV titer reduced only if the cells were treated with the compound at 100 μM concentration. Both compounds at concentration 100 μM inhibited substantially expression of viral antigen in TBEV-infected culture.

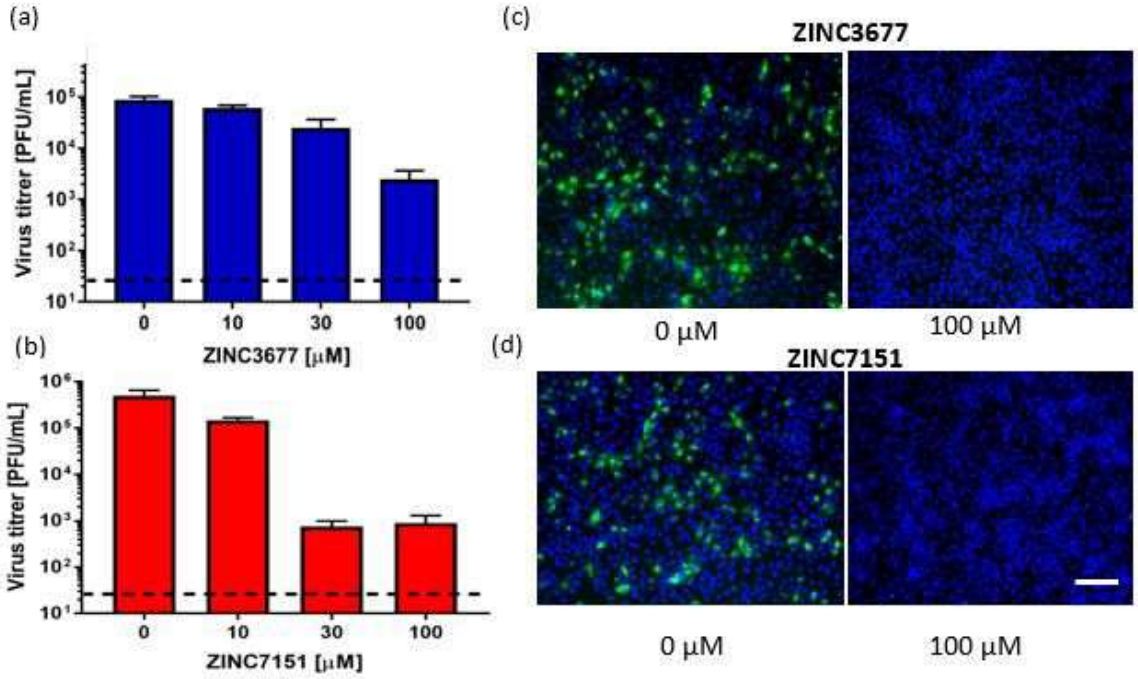

Figure 6.7: (a-b) displays potency of Zinc 3677 and Zinc 7151 for inhibiting of TBEV replication in PS cell culture at the indicated concentrations. (c-d) Immunofluorescence staining determined that Zinc 3677 and Zinc 7151 at concentrations of 100 μM inhibited TBEV surface antigen (protein E) expression in-vitro.

6.4 Conclusions

By using NTPs and select probes as model ligands, pharmacophores at the RdRp domain were isolated using a combination of techniques that included NAMD molecular dynamic simulation based Druggability analysis, docking. After in-vitro testing, three of five compounds, Zinc 7095, Zinc 9662 and Zinc 9041 were found to be cytotoxic and were excluded from further testing. Zinc 3677 and Zinc 7151 exhibited antiviral activity with no cytotoxicity. The efficacy of Zinc 7151 was higher with only 30 μM inhibiting viral growth while Zinc 3677 showing antiviral activity only at 100 μM.

7. SUMMARY AND CONCLUSIONS

Microalgae, due to several advantages including the possession of high protein content, PUFAs, pigments, and carbohydrates, are a potential alternative for food and nutrition. It can be used in a wide array of industries including food, nutrition, and pharmaceuticals.

Dewatering of microalgae remains the most significant bottleneck for microalgae-based biorefinery to be sustainable. The cost of the bioproducts from microalgae is governed by the cost of dewatering and harvesting of algal biomass. In this work, a novel technique to dewater microalgal biomass using polyDADMAC was developed. The technique involves use of an amphiphilic polyelectrolyte which selectively binds onto the negatively charged biomass surface, thus transposing the hydrophilicity of the ensemble and subsequently migrating it in the hexane phase. Simultaneous co-extraction of proteins, lipids, and carotenoids have also been discussed.

Results from this work have revealed that an amphiphilic polyelectrolyte can be successfully used to dewater microalgal biomass from the aqueous phase into an inorganic solvent phase, i.e. hexane. The positively charged head upon binding onto the negatively charged microalgae forms a net hydrophobic ensemble. After addition of hexane to the aqueous phase, the ensemble migrates into the upper hexane phase. The results also revealed that several value-added bioproducts could also be simultaneously extracted and separated using the same amphiphilic polyelectrolyte. When the surface charge on different components from the biomass is controlled by adjusting the pH of the system, the attachment of the polyelectrolyte can be controlled. As the pH of the system is brought close to the isoelectric point of the target molecules, i.e. proteins, the low surface charge density of the proteins does not allow the interaction between the proteins and the cationic polymer while the negatively charged cellular debris interacts with the polymer at the same pH and forms a hydrophobic ensemble. This allows the selective migration of the algal biomass-polyDADMAC ensemble to migrate into the hexane phase. The free proteins are retained in the aqueous phase. Lipids and carotenoids were simultaneously extracted in the hexane phase.

The two dimensional SDS-PAGE revealed that the total algal proteins were divided into two distinct regions based on their surface charge. This difference in the surface charge can be utilized to selectively separate target proteins of interest. In the case of *Chlorella sorokiniana*, a majority of proteins had their isoelectric point close to pH 4-4.5. Approximately 80% of total proteins were retained in the aqueous phase at that pH range. The amount of algal biomass dewatered using this technique was approximately 95% from pH 5-7. The neutral lipids that are soluble in hexane can easily be extracted in the hexane phase without performing any additional unit operations. Chlorophyll a, chlorophyll b and carotenoids are also extracted in the hexane phase. After separating the two phases, the temperature adjustment did not show any significant increase in the extractability of lipids and other pigments from the biomass, multiple washes can certainly improve the extraction deficiencies of these molecules.

Lastly, the efficiency of the system is seen to be dependent on the surface charge density of the target molecules. The concentration of polymer needed for good separation percentage is dependent on the coverage of the molecules to be migrated. A low concentration (0.5% w/w) of the polymer can be used to achieve high migration efficiency. Increase in the polymer concentration beyond optimum concentration can re-stabilize via hydrophobic-hydrophobic interaction between the ensemble and the free polymer and reduce the overall migration efficiency.

In the studies conducted on TBEV by using NTPs and select probes as model ligands, pharmacophores at the RdRp domain were isolated using a combination of techniques that included NAMD molecular dynamic simulation based Druggability analysis, docking studies and ELIXIR-A, an enhanced ligand exploration platform. Using Zincpharmer pharmacophore screening engine, five molecules that had a high affinity of which three would preferentially bind onto the RdRp site were identified. After in-vitro testing, Zinc 7095, Zinc 9662 and Zinc 9041 were found to be cytotoxic and were excluded from further testing. Zinc 3677 and Zinc 7151 exhibited antiviral activity with no cytotoxicity. The efficacy of Zinc 7151 was higher with only 30 μM inhibiting viral growth while Zinc 3677 showing antiviral activity only at 100 μM. These compounds represent excellent targets for structure-activity optimizations leading potentially

to a discovery of effective TBEV antiviral drugs.

7.1 Further studies

1. Study impact of cationic polymer on the integrity of the algal biomass. Observations indicated the ability of the polyDADMAC to disintegrate the cellular structure resulting in milking out of lipids without subjecting the biomass to any disruption method.

2. Conduct a techno-economic analysis based on process parameters to determine the scalability and commercial feasibility.

3. Test the feasibility of using polyDADMAC for harvesting bacterial biomass in aqueous-organic two-phase system.

4. Evaluate the feasibility of using green switchable solvents instead of hexane.

5. To test natural compounds as potential inhibitors for the proliferation of TBEV.

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