PROTEIN ENCAPSULATION WITHIN OPPOSITELY CHARGED POLYELECTROLYTE COMPLEXES

by

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ABSTRACT

PROTEIN ENCAPSULATION WITHIN OPPOSITELY CHARGED POLYELECTROLYTE COMPLEXES

In this study, it is aimed to perform enzyme immobilization with the coacervation, which is used as a physicochemical encapsulation method. By using this encapsulation technology, active agents like drugs and enzymes can be protected from several non-desirable conditions of the environment such as high pH, organic solvents, and chaotic agents. In this research, one of the objectives is to form complex coacervate droplets with poly (diallyldimethylammonium chloride) (PDADMAC) which is a synthetic homopolymer, and pectin which is a carbohydrate found in the cell walls of plants. The biggest driving force in the formation of coacervation is the electrostatic interactions of the positive charges in PDADMAC with the negative charges in pectin. To learn more about protein encapsulation within complex coacervates, encapsulation of trypsin enzyme within complex coacervates comprised of a cationic polyelectrolyte, PDADMAC, and an anionic polyelectrolyte, pectin, was investigated as a function of mixing order of macromolecules and at different salt concentrations. Three different mixing orders was performed. Additionally, turbidimetric titrations of pectin – PDADMAC mixtures were done at six different salt concentrations. Formation of coacervate microdroplets was observed by light microscopy at these salt concentrations. Potentiometric titration experiments were performed for pectin to find the degree of ionization of pectin at the optimum pH of trypsin, which is 7.5. For further experiments, the most appropriate mixing order is selected, and the remaining experiments are performed with that addition sequence. Then trypsin and polyelectrolyte concentrations were changed and its effect on encapsulation was examined. After determining the most effective encapsulation method, enzyme activity was measured. Finally, it was investigated whether the secondary structure of trypsin changed with encapsulation by circular dichroism experiments.

ÖZET

KARŞI YÜKLÜ POLİELEKTROLİT KOMPLEKSLERİ İÇİNE PROTEİN ENKAPSÜLLEMESİ

Bu çalışmada fizikokimyasal bir enkapsülasyon metodu olarak kullanılan koaservasyon ile enzim immobilizasyonu yapılması hedeflenmiştir. Bu kapsülleme teknolojisini kullanarak, ilaçlar ve enzimler gibi aktif maddeler, yüksek pH, organik çözücüler ve kaotik maddeler gibi istenmeyen çevre koşullarından korunabilirler. Bu araştırmada, sentetik bir homopolimer olan poli (dialildimetilamonyum klorür) (PDADMAC) ve bitki hücre duvarlarında bulunan bir karbonhidrat olan pektin ile kompleks koaservatlarını oluşturmak amaçlandı. Koaservasyon oluşumundaki en büyük kuvvet, PDADMAC üzerinde bulunan pozitif yüklerin ve pektinin negatif yüklerin elektrostatik etkileşimleridir. Kompleks koaservat fazı içinde protein kapsüllemesi hakkında daha fazla bilgi edinmek için katyonik bir polielektrolit olan PDADMAC ve anyonik bir polielektrolit olan pektinden oluşan kompleks koaservatların içerisine tripsin enziminin kapsüllemesi, makromoleküllerin karıştırma sırası değiştirilerek farklı tuz konsantrasyonlarında araştırıldı. Üç farklı karıştırma sırası gerçekleştirilmiştir. Ayrıca pektin-PDADMAC karışımının pH'ı altı farklı tuz konsantrasyonunda bulanıklık titrasyonları yapılmıştır. Koaservat mikro damlacıklarının oluşumu, bu tuz konsantrasyonlarında ışık mikroskobu ile gözlenmiştir. Tripsinin optimum pH'ı olan 7.5'ta pektinin iyonlaşma derecesini bulmak için potansiyometrik titrasyon deneyleri yapılmıştır. İlerleyen deneyler için en uygun eklenme sırası seçildi ve geri kalan tüm deneyler bu seçilen ekleme sırasına göre yapıldı. Daha sonra seçilen bu yöntemle tripsin, polielektrolit konsantrasyonları değiştirilerek enkapsülasyona olan etkisi incelenmiştir. En etkili kapsülleme yöntemi bulunduktan sonra enzim aktivitesi deneylerine yapılmıştır. En son dairesel dikroism ile tripsinin bu enkapsülasyon ile ikincil yapısının değişip değişmediği araştırılmıştır.

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pH_{crit}	pH critical (pH value of formation of primary soluble complexes)
°C	Degree of Celsius
[S]	Substrate Concentration
Å	Angstrom
kDa	kilodalton
K_m	Michaelis Constant
M	Molarity
min	Minutes
mL	Millilitre
mM	Millimolar
M_w	Molecular Weight
N	Normality
nm	Nanometre
pI	Isoelectric Point
V_0	Initial Velocity
V_{max}	Maximum Rate
ΔV	Delta V
μm	Micrometre
pH_θ	pH phi (pH value of formation of coacervate droplets)
α	Degree of Ionization
MΩ	Megaohm

LIST OF ACRONYMS / ABBREVIATIONS

ADH	Horse Liver Alcohol Dehydrogenase
AIDS	Acquired Immune Deficiency Syndrome
ATP	Adenosine Triphosphate
BA	Nα-benzoyl-DL-arginine
BAPNA	Nα-benzoyl-DL-arginine-p-nitroaniline
BSA	Bovine Serum Albumin
CD	Circular Dichroism
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribose Nucleic Acid
E	Enzyme
EP	Enzyme-Product Complex
ES	Enzyme-Substrate Complex
HCl	Hydrochloric Acid
HIV	Human Immunodeficiency Virus
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
OD	Optical Density
P	Product
PAA	Poly (acrylic acid sodium salt
PAH	Poly (allylamine hydrochloride)
PDADMAC	Poly (diallyldimethylammonium) Chloride
PMETAC	(2-methacryloxy-ethyl trimethyl ammonium chloride)
pNa	p-nitroaniline
RNA	Ribo Nucleic Acid
S	Substrate
T1	Trial 1
T2	Trial 2
T3	Trial 3
UV-VIS	Ultraviolet and visible

1. INTRODUCTION

1.1. Encapsulation

The technique of entrapping or covering a substance or a mixture of materials (active agents) inside another substance or system (wall material) is known as encapsulation. This type of immobilization is done by enclosing the active materials like drugs or enzymes in a membrane bounded capsule. The encapsulating material is referred as the core, internal, fill, or payload phase, except for the active agent. Coating, membrane, shell, capsule, carrier material, or outer phase refers to the material that creates the coating [1]. Encapsulating technology is used in a wide range of disciplines and applications. For these reasons, those who want to use encapsulation technology have shown a great interest in polymer science. Advances in biopolymer and polyelectrolyte encapsulation-based technologies have driven development in a wide range of industries, including pharmaceuticals [2], foods [3], cosmetics and healthcare products [4], and agriculture [5].

The main purpose of encapsulation is to protect active materials by forming solid barriers from the foreign environment for a variety of reasons such as extreme pH, organic solvents, and high temperatures. The encapsulation process has many objectives, including protection of the encapsulated material against the external environment, controlled release of core ingredients, increasing, the activity of the substance being encapsulated, masking unpleasant odours or tastes, converting liquid droplets into solid particles, safe usage of toxic and harmful materials, and lowering flammability and evaporative loss of liquids [6].

The advantages of the encapsulation method, which has applications in many fields, are quite high. According to the literature, the advantages of encapsulation technique can be understood from the examples given especially in the fields of food industry and enzyme studies. The usage of encapsulation has increased significantly in the food industry. Encapsulated materials can be protected from moisture, heat, and other extreme conditions mentioned above, improving their stability and viability. Food encapsulation is also used to camouflage odours or tastes. The encapsulation of the contents helps to keep other

ingredients in the formulation from interacting and causing problems [7]. When the results of enzyme studies [8] are examined, it is shown that encapsulation has many advantages. The advantages of encapsulation method include the protection against harmful external environments, increase in the endurance of the enzyme, the shortening of the reaction time, and the fact that the enzyme stays in the reusable form. Even though there are many advantages of encapsulation method, there are some disadvantages, too. For instance, some active ingredients become unstable after encapsulation, some enzymes or drugs can get inactive or a decrease in effectiveness of these active materials can be seen after being processed and high cost for the isolation, purification, and recovery of active agents like proteins.

The encapsulation method depends on the type and physical properties of the core and capsule materials. Economic assessments, sensitivity and durability of materials, desired capsule size, and chemical and physical features of both the wall and core ingredients influence the process used. Capsules should not aggregate, precipitate or adhere, and the chosen encapsulation process should have a high encapsulation performance and active material-loading capacity [9]. The sensitivity or durability (e.g., thermal stability) and nature (e.g., solubility in polar or non-polar solvents) of active molecules determine the conditions for encapsulation. Meanwhile, release of active agents like cancer drugs can be controlled by environmental pH fluctuations, mechanical processes, enzymatic activity, or other external stimuli [6].

Many encapsulation methods have been reported in the literature. In general, there are three types of encapsulation techniques: chemical, physico-chemical, and physico-mechanical procedures [10]. The table below summarizes some key methods, which are classified into main groups.

Table 1.1. Encapsulation methods.

Chemical Processes	**Physico-chemical Processes**	**Physico-mechanical Processes**
Interfacial polycondensation	Coacervation and phase separation	Spray drying

Table1.1. Encapsulation methods. (cont.)

Chemical Processes	Physico-chemical Processes	Physico-mechanical Processes
Suspension, dispersion, and emulsion polymerization	Layer-by-layer assembly	Centrifugal techniques
	Sol-gel encapsulation	Fluid bed coating
	Supercritical CO_2 assisted microencapsulation	Pan coating

There are different encapsulation types according to the materials and methods used. Interfacial polycondensation [11], suspension [12], dispersion [13] and emulsion [14] polymerization are encapsulation types made by chemical methods. Coacervation or phase separation [6], layer-by-layer assembly [15], sol-gel encapsulation [16] and supercritical fluid microencapsulation [17] can be examples of widely used physico-chemical methods. Physico-chemical methods which can be utilized in encapsulation studies are spray drying, centrifugal techniques, fluid bed coating and pan coating. Looking a little deeper into the scientific studies, there may be more different and interesting methods that can be found regarding the developing technology and scientific advances. Here, more frequently used, and widespread techniques are mentioned, and examples are given.

1.2. Proteins

Proteins are one of the four groups of carbon-based biomacromolecules. These macromolecules can consist of one or more polypeptide chains formed by 20 different standard amino acids which are monomers of proteins. According to the properties of these amino acids, proteins can be classified as polar, non-polar, acidic, or basic. The linear chains formed by amino acids are called polypeptides. Proteins must have at least one long polypeptide chain in their structure. This structure, by which the order and types of amino acids can be determined, is called the primary structure of proteins. The alpha-helix and beta-sheet structures obtained by hydrogen bonds formed between the amino and carboxylic acid groups that make up the amino acids are called secondary structures of proteins. This

structuring is observed in all proteins. However, the densities of alpha-helix, beta-sheet and random coil structures differ in each protein type. Thanks to the bonds established between the R-groups, which allows there to be 20 different types of amino acids, proteins can fold and acquire their tertiary structure. Among the R-groups, bonds such as hydrogen bonds, ionic bonds, hydrophobic interactions, and disulphide linkages can be observed. These bonds or interactions will be established on polypeptide chain depending on the chemical property of the amino acid and the code from the deoxyribonucleic acid. For a protein to function properly, it must have a tertiary structure. Finally, there are proteins that have a quaternary structure because of interaction between two or more polypeptide chains. Not every protein in living things must have this structure. The best example of this type of protein is haemoglobin. These four levels determine the shape and function of proteins and are distinguished from one another by the degree of complexity in a polypeptide chain. The primary level is the most fundamental and basic, whereas the quaternary level describes advanced bonding.

Proteins perform a wide range of functions within organisms, including catalysis of metabolic reactions, DNA replication, protein synthesis, responding to stimuli, providing structure to cells and organisms, and transportation of molecules. Proteins differ primarily in their amino acid sequence, which is determined by the nucleotide sequence of the species' genes and usually results in protein folding into a specific 3D structure that determines its activity and function. There are roughly seven different types of proteins in living things. These are structural proteins, storage proteins, hormonal proteins, contractile proteins, transport proteins, enzymes, and antibodies. Structural proteins are building blocks of the cells and bodies of living things. The most known structural protein is collagen which is found in bones and skin. Storage proteins keep amino acids and some metal ions in reserve for the body until they are needed. For instance, ferritin is a type of storage protein which stores iron. Some proteins are used in the form of hormones which are chemical signalling molecules. Such hormonal proteins are secreted from cells that regulate and control the physiological states of an organism such as metabolism, reproduction and growth. Oestrogen, testosterone, growth factors or cortisol can be examples of this type of proteins. Muscle contraction and movement are controlled by contractile proteins. The most popular sample case is actin-myosin proteins. Transport proteins are carrier proteins that move materials like oxygen from one organ to another in body. Enzymes are really special group

of proteins. Life without enzymes is not possible. These proteins are catalysts of all biochemical reactions in anabolism and catabolism of all living things. Lastly, antibodies are highly specialized proteins that protect the body from pathogens. They are used to identify and defend against disease causing guests such as bacteria, viruses and fungi which are noticed by the immune system due to their movement abilities in the circulatory system. When antibodies encounter any antigen, they immobilize and neutralize them allowing the white blood cells to destroy these antigens.

In addition to all these, proteins can be structurally divided into two main groups: fibrous and globular. This distinction here is based on whether the protein has a fibre like structure and insoluble in water or it has a globular structure and soluble in water. Fibre-like proteins which are mostly found in structural areas such as hair, nails, skin, muscles and connective tissues, are insoluble in water. These types of proteins have structural, binding and protective functions in body. Keratins, collagen, myosin, and elastin can be examples of fibrous and structural proteins. Globular proteins, the other major type, are soluble in water. When the peptide chains of these type of proteins are folded, they take on an almost globular, spherical shapes. Most of enzymes, serum albumin, haemoglobin can be given as examples of globular and soluble type of proteins [18].

Proteins are highly sensitive to their environment. This sensitivity of proteins can cause structural changes and functional deficiency that is called protein denaturation. Denaturation is the disturbance of many of the weak linkages or bonds (e.g., hydrogen bonds) within a protein molecule that are highly ordered, folded native protein molecules [19]. Denatured proteins have a looser, more random structure and are mostly insoluble and inactive. Proteins can be denatured in many ways such as being exposed to heat, UV radiation, extreme pH conditions, organic solvents like ethyl alcohol and urea, detergents, vigorous shaking, salts of heavy metals such as mercury, silver, and lead. In some cases, if the effect that causes denaturation is removed from the environment, it can be observed that the proteins return to their natural folded (native) state. This process is called renaturation. However, due to the differences in the structures of proteins, denaturation process can be irreversible. This means that even if the effect that causes denaturation is eliminated, the protein cannot return to its native form and perform its function. The encapsulation process can be a method that can prevent proteins from denaturing under difficult conditions.

1.2.1. Enzymes

An enzyme, which is a substance that works as a catalyst in living organisms, controls the rate at which chemical reactions occur without changing itself. All enzymes, except for a small subsection of catalytic RNA molecules [20], are proteins. Catalytic activity of enzymes is determined by the stability of their native protein structure. Biological activities of all living creatures involve chemical reactions, and most of them are controlled by enzymes. Many of these anabolism and catabolism reactions would not occur at all if enzymes were not present. All stages of cell metabolism are catalysed by enzymes. This involves food digestion, which breaks down biomacromolecules (such as proteins, carbohydrates, and lipids) into its monomers; chemical energy conservation and transformation (ATP synthesis and breaking down); and the creation of cellular macromolecules from building blocks of these biological polymers (condensation reactions for macromolecules). Many hereditary human disorders, such as albinism [21] and phenylketonuria [22], are caused by a lack of a specific enzyme. Enzymes are also useful in industrial and medicinal applications. Enzymes are used in medicine to destroy disease-causing microbes, promote wound healing, and diagnose certain disorders [23]. A complex protein enzyme molecule is made up of one or more polypeptide chains of amino acids. The amino acid sequence determines the distinctive folding patterns of the protein structure, which is required for enzyme specificity. Each enzyme activates a different biochemical reaction, and each enzyme has a different folding pattern, so this means different enzymes have different substrates. This is defined by the specificity of the enzyme. If the enzyme is exposed to changes, such as temperature or pH variations, the protein structure may lose its integrity (denaturation) as well as its enzymatic capacity. The denaturation can be reversed in some cases, but not always.

The protein component of such an enzyme is referred to as the apoenzyme or apoprotein. Cofactors and coenzymes bind to the apoenzyme and activate the enzyme, thus enabling the formation of a complete enzyme, the haloenzyme, thus initiating enzymatic activity of the enzyme. A cofactor can be an organic substance like a vitamin and/or an inorganic metal ion, as certain enzymes require both. A prosthetic group is a coenzyme or metal ion that is extremely strongly or even covalently attached to the enzyme protein. A

haloenzyme is a whole, catalytically active enzyme that includes its bound coenzyme and/or metal ions.

An enzyme-catalysed reaction is distinguished by the fact that it takes place in a pocket on the enzyme known as the active site. The molecule called substrate is a molecule that is affected by the enzyme by binding to the active site. The active surface of an enzyme is covered with amino acid residues that bind to the substrate and catalyse the chemical change of the substrate. These amino acid residues on the active site can show changes in different enzyme groups. An enzyme, substrate and product scheme can be seen in the image below (Figure 1.1). A simple enzymatic reaction can be written as

$$\mathrm{E} + \mathrm{S} \rightleftarrows \mathrm{ES} \rightleftarrows \mathrm{EP} \rightleftarrows \mathrm{E} + \mathrm{P}, \tag{1.1}$$

where E, S, P represent enzyme, substrate, and product, respectively. ES and EP are transient complexes of the enzyme with the substrate and with the product, respectively.

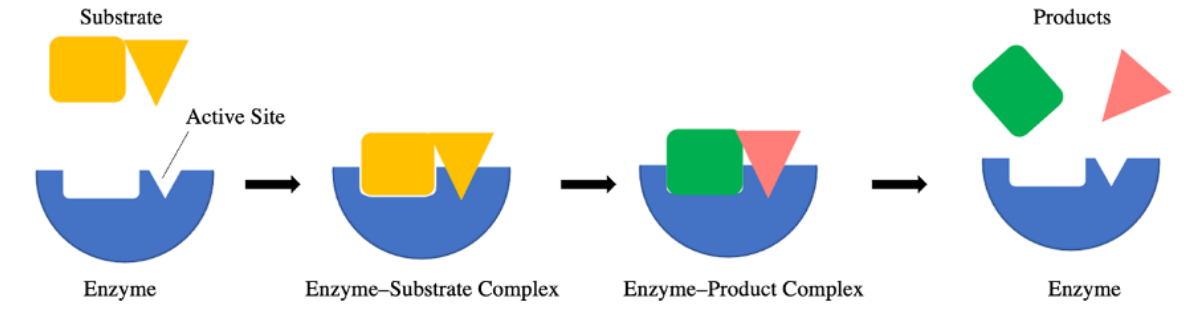


Figure 1.1. Enzyme-substrate interaction scheme.

Most biochemical reactions have an energy barrier that must be overcome for the reaction to take place in living things' metabolism. These barriers can prevent the assembly of amino acids and nucleotides necessary for life to occur, or the spontaneous degradation of complex polymers such as proteins and nucleic acids. However, when metabolic changes are required in a cell, monomers must be combined, a certain number of complex polymers must be broken down, and this energy barrier must be overcome. Heat may give the additional energy, meaning; the activation energy required, but it would also destroy the cell.

The only remaining option is to utilize a catalyst to reduce the activation energy level, which is the function of enzymes means biocatalysts. They react with the substrate to generate an intermediate complex called “transition state” that takes less energy to proceed with the reaction. The unstable intermediate chemical swiftly degrades into reaction products and freeing the active part of the enzyme for react with new substrate molecule. During all these processes, there is no deterioration in the folding of the enzymes that is the structure of the enzyme is preserved, and the enzyme can be used again for a new reaction.

1.3. Protein Encapsulation

Encapsulation of proteins and other biomacromolecules such as nucleic acids or carbohydrates is a rapidly developing and interesting field of research, making these biological substances more useful in drug delivery, personal care products, food industry and biocatalysts. When the literature is examined, there are many methods for encapsulation [10]. However, not all methods are suitable for protein encapsulation. This is because proteins are biopolymers that are highly sensitive to their environment. Fluctuations in pH, temperature, or the usage of an organic solvent may cause the protein to lose its function, as it may change the folding behaviour and lead to denaturation. Even if an enzyme is encapsulated, this degradation may change the shape of the active site of the enzyme, thus preventing the enzyme from binding to the substrate and the activity of the enzyme may be damaged by this process.

Since proteins cannot be encapsulated by every encapsulation method, extensive colloidal transport systems have been developed. Micro-emulsions, which are generally formed by hydrophilic forces, can be an example for this group. However, this encapsulation method is not suitable method for all proteins except hydrophobic ones. This is because many proteins are too hydrophilic to attach to the hydrophobic interior of the micro-emulsion particle [24]. Most emulsion and nano-emulsion methods are also unsuitable for protein encapsulation. For instance, the oil droplets on the inside of the emulsion particles are highly hydrophobic, and the water part on the outside of these particles will cause the adsorption of proteins and these capsules will not protect the proteins from any change in the environment [24].

Liposomes, which are another type of encapsulation, are composed of two layers of phospholipids like in cell and organelle membranes. The heads of phospholipids in these layers are hydrophilic and face both the outside and inside of the liposome. The hydrophobic tails are turned towards each other. Hydrophilic substances can be encapsulated inside the hydrophilic water core of liposomes while amphiphilic or lipophilic substances can be encapsulated within the hydrophobic lipid bilayers. Liposomes can encapsulate both hydrophilic proteins and proteins with huge hydrophobic domains. The main disadvantage of liposome-based delivery methods is that they have a low encapsulation efficiency [24,25].

According to the literature, two main concerns can be identified regarding the encapsulation processes in general. The first of these concerns is the use of organic solvents in the encapsulation method, which can denature proteins by disturbing their function, folding and structure. The second is the low loading capacity or low encapsulation efficiency. Most of these methods, particularly liposomes, and hydrogels suffer from low encapsulation efficiency and typically require the use of organic solvents that may destabilize and disturb the encapsulated material such as proteins [26]. Although polymer microspheres can be used to increase effectiveness of protein encapsulation, using organic solvents can cause protein denaturation. Proteins encapsulated in this manner are tend to aggregation, denaturation, unfolding, and cleavage [27].

As mentioned above in the first section, encapsulation has many advantages and disadvantages. Many methods for encapsulation were mentioned above. Below is a visual about some of these methods (Figure 1.2).

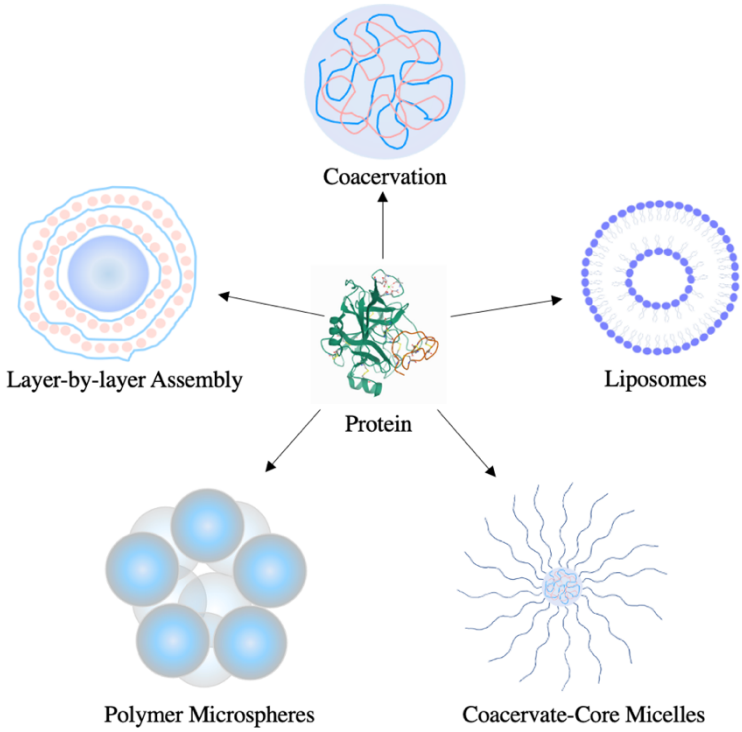


Figure 1.2. Encapsulation methods.

Complex coacervation is also a method used in encapsulation. Since organic solvents are not used in complex coacervation, a.k.a., liquid-liquid phase separation, it is a much more efficient method for proteins than other methods. With the widespread use of liquid-liquid phase separation method, stabilization of many biomolecules such as proteins and RNA can be achieved [28,29]. In contrast to other protein carriers such as hydrogels and microparticles, coacervates are formed by mixing oppositely charged macroions and taking advantage of spontaneous phase separation, and they do not require exposure to heat, organic solvents, protein modification, or additional chemical reagents that may result in protein denaturation [8].

Protein encapsulation is a natural phenomenon. For instance, protein shells are used by viruses to protect viruses' DNA or RNA (genetic material) before transferring it into host cells. Starting from this natural example, it can be suggested to use cage-like proteins to encapsulate some active materials such as drugs. Furthermore, since some amino acids have a wide range of hydrophilicity, the local environment may be suitable for the appropriate

drug. For example, the human immunodeficiency virus (HIV) which causes acquired immunodeficiency syndrome (AIDS) has a capsule made up of proteins and lipids. As can be seen in the examples herein, proteins can be used both as encapsulating material and as the carrier material [30].

1.4. Coacervation

Coacervation is a method for producing microdroplets in a suspension that is based on the separation of two liquid phases, i.e., coacervate and dilute equilibrium phase. The coacervate phase, which is made of condensed liquid-like droplets, is the concentrated dense component. The dilute phase which is visible following a centrifugation is known as the supernatant phase. There are two different types of coacervation: simple and complex coacervation. A single type of polymer is used in a simple coacervation method. This system is created by the addition of tiny molecules such as salts or organic solvents like alcohols. Organic solvents or salts added to the medium can trigger polymer collapse by enhancing intramolecular hydrophobic interactions. The increment in hydrophobic forces can cause droplet-like structures of this single type of polymer. These droplet-like structures provide simple coacervates. Electrostatic attraction between two oppositely charged particles, on the other hand, is one of the driving factors for complex coacervation. These opposite charges can be found on two separate molecules, such as two polymers or two different inorganic compounds, or on a single polymer, which is known as a polyampholyte. Among the numerous examples of coacervation that have developed in recent years are peptides, nucleic acids, lipids, tiny inorganic compounds linked with longer biopolymers, and other synthetic polymers [31,32].

Formation of complex coacervates is primarily influenced by a mixture of three major forces: electrostatic attraction, entropically favourable counterion rearrangement, and non-specific interactions such as hydrogen bonding and hydrophobic interactions between materials that can generate coacervate droplets. (1) When anion-cation pairings cause complex coacervation, the basic idea is that molecules with oppositely charged charges attach to one other via electrostatic interactions, resulting in soluble or insoluble complexes. (2) On the surfaces of polyelectrolytes, there exists oppositely charged counter-ions like sodium or chloride. When polyelectrolytes attract one other, these tiny, bounded counter-

ions from the polyelectrolyte surface are released into the solution medium. This is an entropically favourable molecular rearrangement. (3) Furthermore, weak energy interactions such as hydrophobic forces and hydrogen bonding can contribute significantly to complex formation [33].

Complex coacervation can be influenced by a variety of parameters such as polymer molecular weight, charge density, and chain stiffness, or component concentration or ionic strength, pH, and medium temperature [34]. Furthermore, utilizing various polymers such as homopolymers or block copolymers [31], many different forms of complex coacervation may be seen. By adding a hydrophobic or hydrophilic part to the polymer or changing the charged part of polyelectrolytes, oppositely charged homopolymers form bulk coacervates, whereas oppositely charged block copolymers form different structures such as coacervate core micelles, coacervate corona micelles, and coacervate core vesicles [31]. As can be seen here, different structures can be formed by the coacervation method. The hydrophobic or hydrophilic residues added to the main polymers are the most important point of these differences.

Meanwhile, complex coacervation has a wide range of applications. It can, for example, be used as a drug delivery platform [35], biosensors [36], or in the encapsulation of active components such as food flavour [37] or enzymes [38]. Furthermore, complex coacervation has been proposed as the foundation of membranelles organelles in the cell [39]. These separate compartments are formed by the complexation of biomolecules such as proteins rather than closed by lipid membranes.

Charged biomacromolecules can be utilized as components for encapsulating, targeting, and delivery of functional chemicals in many industrial areas to build complex coacervation. Investigations of encapsulation have received a considerable attention in various research. The encapsulation process refers to the trapping of an active substance. These active compounds, such as medications and enzymes, may be protected from various environmental conditions by applying this encapsulating method.

As an encapsulation method, many kinds of enzymes, proteins and biological polyelectrolytes can be used for complex coacervation. Xia et al. studied enzymes bovine

trypsin and horse liver alcohol dehydrogenase (ADH) which can produce complex coacervate droplets with a strong, synthetic homopolymer, positively charged polyelectrolyte poly (diallyldimethylammonium) chloride (PDADMAC) [40]. In that study, bovine trypsin and ADH produce coacervate droplets with PDADMAC at pH above the isoelectronic point (pI) of these enzymes. On the other hand, Lindhoud et al. studied encapsulation of lysozyme into the complex coacervate core micelles [34]. The system composed of negatively charged diblock copolymer poly (acrylic acid)-block-poly (acryl amide) ($PAA_{42}PAAm_{417}$), positively charged homopolymer poly (N, N-dimethylaminoethyl methacrylate) ($PDMAEMA_{150}$), and positively charged globular protein lysozyme.

As an enzyme-carrier strategy, polyelectrolyte-protein complexation may create changes in the thermal stability of the enzyme. In another research, which was studied with lysozyme, folding, unfolding, and refolding behaviour of the protein after complex formation with a double hydrophilic block copolymer methoxypoly (ethylene glycol)$_{5K}$-block-poly (L-aspartic acid sodium salt)$_{10}$ ($mPEG_{5K}$-b-PLD_{10}) was observed. According to that work, native state of lyzosyme transformed to a new partially unfolded state when it formed a complex with $mPEG_{5K}$-b-PLD_{10}. The native form of lysozyme was compared to its complex state, and I it was found that secondary and tertiary structure of lysozyme could be changed by this complexation. After this treatment, a significant unfolding and refolding behaviour was observed, while a decrease in the thermostability of the lysozyme was detected. However, the lysozyme-polyelectrolyte complex could unfold but not refold when the temperature was increased. After the heating, cooling and reheating processes, the free protein showed unfolding and refolding properties, while the complex protein did not show the refolding feature.

In the literature, the activity of enzymes inside protein-polyelectrolyte coacervates has also been studied. Xia et al. also investigated the activity of bovine trypsin and ADH enzymes when complexed with PDADMAC [40]. They found that these two enzymes were still active when they were in a complex state. The activity of enzymes can be evaluated in non-identical way because their substrates and the reaction pathways are very different from each other. The velocity of NAD^+ to NADH conversion was used to calculate ADH activity. Catalytic hydrolysis of the N-benzoyl-DL-arginine-p-nitroaniline (BAPNA) resulted in trypsin activity. They also plotted Lineweaver-Burke graphs for free trypsin and trypsin-

PDADMAC coacervate droplets. K_m and V_{max} values can be obtained very easily by using these graphs. According to the results of the study, it was found that K_m values of free and complex trypsin were close to each other. For this reason, they were able to comment that there was no deterioration in the active part of the enzyme. In addition to that, Tsuyoshi et al. investigated the influence of pH on the hydrolysis of BAPNA for native and complexed papain also known as papaya protease [41]. It was discovered that the molecular weight of the polyelectrolyte used in complexation was a crucial factor for papain activity because activity loss was approximately 20% for complexes with low molecular weight-sodium poly (styrene sulfonate) (NaPSS-7) and about 50% for complexes with high molecular weight (NaPSS-50).

Proteins and enzymes are sensitive to environmental factors such as pH extremes (all values that are far from the optimum pH), temperatures, and the presence of organic molecules. Moreover, the coacervation process of biomolecules is affected by physicochemical parameters such as ionic strength, polyelectrolyte type, amount and concentration, salt type, and so on. As Lindhoud et al. studied complexes of lysozyme with $PAA_{42}PAAm_{417}$ and $PDMAEMA_{150}$, they investigated whether the stability of the structures of the micelles changed in compliance with the ratios of the materials used [34]. According to the results, changes were observed in the stability and the structure of micelles with the changing concentrations of polymers and lysozyme. Kaibara et al. investigated the influence of polyelectrolyte charge density on coacervation formation [42]. They used poly (2-methacryloxy-ethyl trimethyl ammonium chloride) (PMETAC) and PDADMAC system. The linear charge density of PMETAC is double that of PDADMAC. The findings indicate that the amount of polymeric charged sites per bovine serum albumin (BSA) protein affects coacervate production. The complexation process was unaffected by structural changes between PDADMAC and PMETAC, as well as the density of charged sites among these polymeric chains.

Since electrostatics is one of the primary driving forces in the coacervation process, the stability and formation of complexes are affected by the ionic strength of the medium. Kaibara et al., who investigated the effect of charge density of polyelectrolytes on complexation, also examined the effect of ionic strength on coacervation by determining the pH at which coacervate droplets were formed [42]. The outcomes show that, below 30 mM

NaCl, pH_θ (onset of coacervate droplet formation) was independent of ionic strength but above 30 mM NaCl, ionic strength contributes to a change in pH_θ. Also, Lindhoud et al. performed NaCl titrations to determine the resistance of micelles to salt solutions [34]. As mentioned above, for the lysozyme/homopolymer system, the charge density of system decreased as a function of the NaCl salt concentration. Therefore, they concluded that the electrostatic interactions were weaker when the salt concentration increased due to the salt screening effect. They also discovered that increasing the concentration of the homopolymer in the micelle increased the micelle's salt resistance Since the interaction strength of micelles will increase as the homopolymer concentration increases, more durable micelles can be obtained when the ionic strength increases.

In another article, Lindhoud et al. investigated how numerous factors, such as salt concentration, could influence enzyme activity by measuring lipase activity in polyelectrolyte complex micelles [34]. The outcome shows that lipase activity in both the free and incorporated states decreased at higher salt concentrations. They do, however, imply that at higher salt concentrations, lipase activity in the presence of polyelectrolytes is greater than free lipase. As a result, it was concluded that the presence of polyelectrolytes improved the enzyme activity.

Many studies have been carried out on the effect of temperature on coacervate formation. In one of these studies, the effect of temperature on the coacervation process of the BSA-PDADMAC system was investigated by Kaibara et al. [42]. They found that electrostatic interactions dominated the interaction between BSA and PDADMAC; nonetheless, if hydrophobic interactions had contributed to this system, it should have been visible via temperature dependency of the phase behavior. Hydrophobic interactions, as is widely known, have a considerable temperature dependency. Experiments at various temperatures revealed that the role of hydrophobic contacts on BSA-PDADMAC coacervation was insignificant.

1.5. Materials Used in Experiments

Pectin, a negatively charged and semi-flexible polysaccharide, is used for complex coacervation. Pectin is made up of chains of (1 → 4) α-D-galacturonosyl units and their

methyl esters with variation in composition, structure, and molecular weight. Pectin which is natural heteropolysaccharide present in plant cell walls and middle lamella. Due to its structural and chemical properties, pectin can interact with many biological and chemical materials [43]. Pectin is a versatile polysaccharide that has been used in the food and beverage industry for many years. Pectin is used especially as a gelling agent and thickening agent in these areas. In addition to this, pectin is also used as and emulsifier and stabilizer. Additionally, pectin is a good source of dietary fibre and even has pharmaceutical properties [44,45]. Citrus fruits and apples are commonly used to extract pectin. Natural pectin has a greater degree of esterification, ranging from 75 to 80%. The ratio of methyl esterified galactronic acid groups to total galactronic acid groups is known as the degree of esterification. Pectin molecules are categorized according to their degree of esterification, also known as degree of methoxylation [43].

In solution, non-esterified carboxyl groups can dissociate, resulting in the formation of a negative charge. It has a charge spacing of 6.5 Å and a persistence length of 7.5 nm [46,47]. The monomer structure of pectin is shown in the picture below.

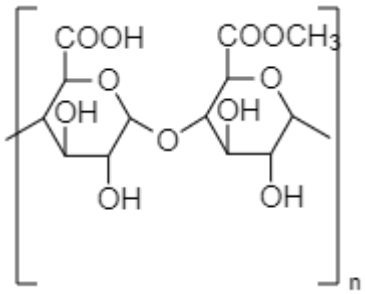


Figure 1.3.Monomeric Unit of Pectin.

PDADMAC is a positively charged synthetic homopolymer. PDADMAC is also a very flexible linear polyelectrolyte with a persistence length of 3 nm [48]. It has several applications including water purification and as controller of disturbing materials in paper industry [49]. PDADMAC's charge spacing is extremely close to that of pectin 6.2 Å, and its persistence length is 3 nm. The image below illustrates the monomer structure of PDADMAC.

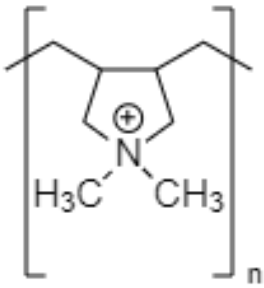


Figure 1.4. Monomeric unit of PDADMAC.

The enzyme trypsin is primarily synthesized in the pancreas in an inactive form. This inactive form is called trypsinogen. After trypsinogen secreted into the first region of the small intestine, it turns into its active form trypsin. After activation of trypsin enzyme that begins protein digestion by breaking down the complex amino acid chains into smaller fragments. This enzyme, which is found in digestive system of many vertebrates, belongs to serine protease family. Trypsin breaks down peptide bonds on the carboxyl side of the amino acids lysine and arginine [50]. Trypsin catalyses a reaction like other serine protease enzymes. Histidine- 57, aspartate- 102, and serine- 195 amino acids form the catalytic triad in such serine protein enzymes. The catalytic triad is usually the ternary amino acid groups found in active part of the enzymes and enabling the reaction to take place [51].

The name of the substrate used for the trypsin enzyme in this study is N-Benzoyl-L-Arginine-p-Nitroanilide (BAPNA). Trypsin cleaves the bond between arginine and p-nitroaniline to release free p-nitroaniline, which is yellow and easily quantified using a colorimeter. P-nitroaniline is the product of the enzymatic reaction of trypsin and detectable at 410 nm by using UV/VIS spectrophotometer. The speed of the enzyme can be found by measuring the product released at the end of this reaction. With this method, it is possible to find the velocity of the enzyme in different conditions and compare them with each other. To find the rate of these reactions we needed a calibration curve of the p-nitroaniline product. In the figure below, the chemical structure of the substrate BAPNA and the products formed in the reaction between the substrate and trypsin are given.

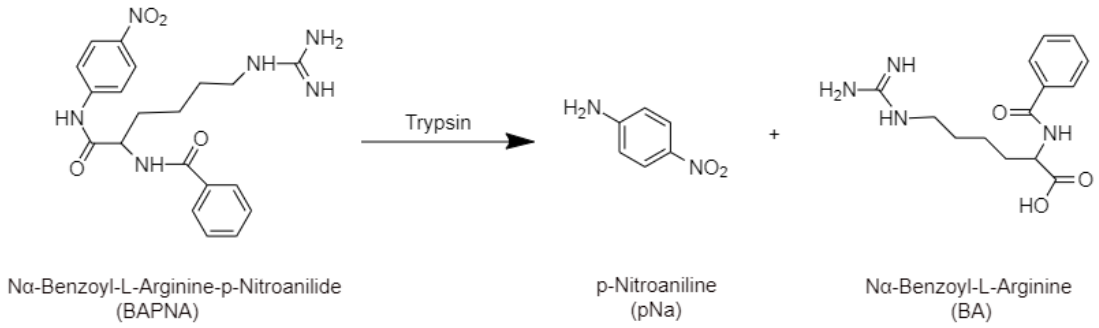


Figure 1.5. Reaction between BAPNA and trypsin.

Sodium phosphate monobasic monohydrate ($NaH_2PO_4.H_2O$) was purchased from VWR Life Science (Ohio, USA) and di-sodium hydrogen phosphate (Na_2HPO_4) was purchased from Merck (Darmstadt, Germany) to make phosphate buffer. NaCl is selected as salt to use in required experiments. 0.1 N HCl, 1 N HCl and 0.1 N NaOH, 1 N NaOH were purchased from ISOLAB (Wertheim, Germany). Sodium chloride (NaCl) was purchased from Merck. Milli-Q water with a resistivity of 18.2 MΩ·cm was used in all experiments.

2. AIM OF THE STUDY

In this work, trypsin which is a member of serine protease enzymes was encapsulated in pectin-PDADMAC complex coacervates, and how this encapsulation process affected the activity of the enzyme was investigated. Initially, it was examined whether or not complex coacervate droplets could occur between pectin and PDADMAC. Our objective was to generate pectin-PDADMAC coacervates within trypsin enzyme's optimal pH range. Because salt concentration effects coacervate formation, experiments were performed at different salt concentrations to see which concentrations lead to coacervation. After coacervate droplets were achieved in the optimum pH range of the trypsin enzyme, the maximum encapsulation efficiency was investigated. The effect of the order of addition of the polyelectrolytes and enzyme at the predetermined NaCl concentrations on encapsulation efficiency was also investigated.

Activity experiments were performed for trypsin enzyme in the experimental conditions where the highest encapsulation efficiency was observed. These results of these experiments were compared for both free trypsin and encapsulated trypsin in pectin-PDADMAC coacervates. Thus, it was investigated how this encapsulation process affected the activity of trypsin enzyme.

Finally, to examine how pectin-PDADMAC coacervate system could provide stability for the trypsin enzyme, activity experiments were conducted for free and encapsulated trypsin enzyme. Additionally, circular dichroism was used to observe free and encapsulated secondary structure of trypsin.

3. EXPERIMENTAL

The two oppositely charged polyelectrolytes in this work are an anionic polysaccharide, pectin, and a cationic synthetic polyelectrolyte, PDADMAC. Turbidity experiments were performed to study the complex coacervation of the pectin-PDADMAC system. The interactions of polymers with each other will be investigated in these studies at various pH values (ranging from 1.7 to 9.0 and from 9.0 to 1.7) and salt concentrations (10, 25, 50, 100, 200, and 400 mM NaCl). Our goal was to achieve complex coacervation of pectin/PDADMAC system at trypsin enzyme's optimal pH value of 7.5. In this study, the molecular weight of potassium salt of pectin from citrus fruit in this study is 82 kDa, and the degree of esterification is 61% and galacturonic acid composition 87% (as supplied by Sigma, the manufacturer) was donated by late Prof. Paul Dubin (University of Massachusetts Amherst). PDADMAC (Molecular weight = 100.000 – 200.000 g/mole as supplied by the manufacturer) was purchased from Sigma Aldrich (Schnelldorf, Germany). Trypsin from porcine pancreas (M_w 23.8 kDa) was purchased from Sigma Aldrich (Schnelldorf, Germany) as lyophilized powder. N-Benzoyl-L-Arginine-p-Nitroanilide (BAPNA) and p-nitroaniline were purchased from Sigma Aldrich (Schnelldorf, Germany).

Light microscopy was used to detect generation of coacervate microdroplets during turbidity experiments. Pictures from light microscopy pictures were collected at different salt concentrations with 10 mM phosphate buffer (0, 10, 25, 50, 100, 150, 200, 400 mM NaCl with 10 mM phosphate buffer) and at trypsin enzyme's optimal pH value of 7.5.

A potentiometric titration test was also conducted for pectin. The reason for this experiment was to determine at which pH the charge state of pectin was negative. Furthermore, the potentiometric titration is also an experiment that allows one to determine the isoelectric point (pI) of trypsin where the positive and negative charges are equal.

Protein encapsulation studies were carried out after identifying the appropriate ionic strength for coacervation at pH 7.5. Many factors influence encapsulation efficiency, including ionic strength, protein and polyelectrolyte concentrations, and the order in which

these components are combined. All these parameters were examined to determine the most effective encapsulation approach. After the desired degree of encapsulation efficiency is attained, the activity of the trypsin enzyme within oppositely charged polyelectrolyte complex coacervates was measured and compared to the activity of free trypsin. In addition, the influence of additional parameters (substrate concentration, polyelectrolyte concentration, protein concentration, and coacervate quantity) on trypsin enzyme activation were investigated.

3.1. Preparation of Materials

Impurities were removed from PDADMAC using dialysis. 5 mL of PDADMAC solution is mixed for 30 minutes with 125 mL of Milli-Q water to produce one gram of PDADMAC dialysis. For three days in Milli-Q water, this diluted PDADMAC solution is inserted into the Snakeskin Plated Dialysis Tubing (10.000 MWCO). For three days, Milli-Q water needs to be changed every day. The PDADMAC solution obtained from the Snakeskin is put into centrifuge tubes after dialysis process and stored at -20°C for two to three days. To obtain solid frozen polymer, lyophilize solutions in centrifuge tubes for two days. The salt and buffer solutions were prepared first. Because its buffer range is ideal for the optimal pH value of the trypsin enzyme, phosphate buffer is selected as the suitable buffer solution. Na_2HPO_4 and $NaH_2PO_4.H_2O$ salts were used to prepare the buffer solution. NaCl was chosen as the salt in the experiments where salt was used other than the buffer. In the produced buffer and salt solutions, pectin and PDADMAC polymers, as well as trypsin protein, were dissolved. Polymers required a minimum mixing duration of 2 hours, while proteins required 30 minutes. The final pH of all solutions was fixed to 7.5. A stock solution of the substrate (BAPNA) and p-nitroaniline were prepared as 2 mM in dimethlyl sulfoxide (DMSO) while stirring for 30 minutes. For activity experiments, BAPNA was dilute to 0.1, 0.2, 0.4, 0.8 mM concentrations by diluting it in the following solvents: 10 mM phosphate buffer, 50 mM NaCl and 10 mM phosphate buffer, and 100 mM NaCl and 10 mM phosphate buffer. A calibration curve was needed to determine the calibration curve for p-nitroaniline. Thus, P-nitroaniline was diluted to 0.01, 0.05, 0.1, 0.2, 0.5, 0.75, 1.0, and 1.5 mM concentrations in the three NaCl/buffer solutions mentioned above. Polymers, protein, and other materials were filtered by cellulose acetate filter with the pore size 0.45 μm

(Labmarker) (İstanbul, Turkey) once they have been dissolved. All experiments were repeated at least three times to obtain more accurate and precise results.

3.2. Potentiometric Titration Experiments

Potentiometric titration in NaCl solutions was used to determine the degrees of ionization of pectin and isoelectric point of trypsin. 0.5 mg/ml pectin and 0.25 mg/ml trypsin solutions were prepared in NaCl solutions. 0.1 – 1 N NaOH and 0.1 – 1 N HCl were used to modify the pH of the pectin solution to pH 2.0 and the pH of the trypsin solution to pH 4.0. The pH of the polymers and blank (salt) solutions were raised by adding 0.1 N NaOH with a micro burette (Gilmont Instruments) (Illinois, USA). pK_a and pI values were calculated using the pHs corresponding to the midpoint of delta V. Delta V is the difference in total volume of NaOH between the polymer experiments and the blank (salt) experiment. Degrees of ionization value for pectin were calculated by the expression of

$$pK_a = pH + log\frac{(1-\alpha)}{\alpha}, \tag{3.1}$$

where α is degree of ionization and pK_a is negative log of acid dissociation constant value [52].

3.3. Turbidimetric Titration Experiments

Salt solutions for dissolving 1.0 mg/ml pectin and 1.0 mg/ml PDADMAC polymers were prepared first. In this experiment, six different NaCl concentrations were prepared. These concentrations were as follows: 10, 25, 50, 100, 200, and 400 mM NaCl. After mixing the polymer solutions for a minimum of two hours, the pH of each polymer solution was adjusted to 1.7. The pH of the mixture was evaluated again after the polymers had been mixed in a 1:1 (by w/v) ratio. If the pH fell below 1.7, it was adjusted again. The turbidity (100% - transmittance) of a mixture of oppositely charged polyelectrolytes were measured using a colorimeter after titrating the mixture up to pH 9.0 with various concentrations of 1 N and 0.1 N NaOH. The turbidity experiments were repeated from pH 9.0 to pH 1.7 to see

whether the titration was reversible. In these studies, we adjusted the pH with 1 N and 0.1 N HCl.

A light microscope (Leica DM6000M) was used to visualize the coacervate formation between pectin and PDADMAC. While preparing the samples for light microscopy, phosphate buffer was used in addition to the salt so that the pH value would not change from 7.5. Polymer solutions were prepared using the required salt and buffer at the same concentrations as in the turbidity experiments. The coacervate droplets were very mobile in the experimental medium. Thus, vaseline was applied between the slide and the lamella, allowing the sample to remain in the form of a droplet, and an image was taken.

3.4. Encapsulation Efficiency Experiments

After the best conditions for coacervation were found from the turbidity studies, encapsulation experiments were begun. 10 mM phosphate buffer solution in the absence and presence of various concentrations of NaCl (10, 25, 50, 100, 150 mM) was used for encapsulation experiments. The purpose of utilizing buffer is to maintain the pH constant while mixing polymers and proteins. To determine encapsulation efficiency, the influence of mixing order of macromolecules was investigated initially. These experiments were done following three different approaches. The first of these approaches involves mixing the polyanion (pectin) and protein (trypsin) for 30 minutes before adding the polycation (PDADMAC). The second approach involves mixing the polycation and protein for 30 minutes before adding the polyanion and mixing for another 30 minutes. The third approach involves first preparing a coacervate suspension between the two polyelectrolytes (pectin and PDADMAC), followed by the addition of protein to that suspension. Concentrations of these macroions after mixing were 0.5 mg/ml, 0.5 mg/ml and 0.25 mg/ml for pectin, PDADMAC, and trypsin, respectively. The mixtures were then centrifuged at 4000 rpm for 60 minutes to separate the coacervate and supernatant phases in all three procedures. To evaluate the quantity of encapsulated protein in the coacervate phase, coacervate must be broken, which was accomplished by adding 2 M NaCl salt to the coacervate phase. Protein absorbance was then measured in both the coacervate and supernatant phases. The protein concentration obtained for coacervate was corrected for the addition of 2 M NaCl. A wavelength of 280 nm is used for UV absorbance measurements because aromatic amino

acids like tyrosine and tryptophan have a high absorption at this wavelength. Following these measurements, the relevant protein concentration was read from a calibration curve. Protein concentrations of 0.05, 0.1, 0.2, 0.5, 1.0, and 2.0 mg/mL were used to create the calibration chart. Thus, by determining which of these three protein encapsulation procedures was the most effective, we were able to prepare the samples for enzyme activity assays.

Following the selection of the preparation process, the effects of polymer and protein concentration on encapsulation were investigated. To understand the effect of protein concentration, four different trypsin concentrations of 0.25, 0.40, 0.50, and 0.75 mg/mL were used where concentrations of PDADMAC and pectin concentration were each kept constant at 0.5 mg/ml. In order to understand the effect of polymer concentration, the following five different combinations of concentration were used as: 0.25 mg/ml pectin and 0.5 mg/ml PDADMAC, 0.5 mg/ml pectin and 0.50 mg/ml PDADMAC, 0.75 mg/ml pectin and 0.5 mg/ml PDADMAC, 0.5 mg/pectin and 0.25 mg/ml PDADMAC, 0.5 mg/ml pectin and 0.75 mg/ml PDADMAC. In all these five experiments, 0.25 mg/ml trypsin was used. In each experiment, the encapsulation efficiency percentage was calculated as

$$Encapsulation\ Efficiency\ \% = \frac{mass\ of\ total\ protein - mass\ of\ protein\ in\ supernatant\ phase}{mass\ of\ total\ protein}. \quad (3.2)$$

3.5. Trypsin Activity Experiments

At room temperature, trypsin's enzymatic activity was measured using N-Benzoyl-L-Arginine-p-Nitroanilide (BAPNA) as the substrate. Trypsin is a serine protease that cleaves peptide links in proteins. Breaking the peptide links in proteins produces two distinct molecules. p-Nitroaniline (pNa) and N-Benzoyl-L-Arginine (BA) are generated by breaking the peptide link in the N-Benzoyl-L-Arginine-p-Nitroanilide (BAPNA) with trypsin [53]. After determining the optimal encapsulation technique, the activity of encapsulated trypsin was evaluated using UV/VIS spectrophotometry. As the stock solution, substrate solutions with various concentrations (0.1, 0.2, 0.4, 0.8 mM) in phosphate buffer with 0, 50, 100 mM NaCl were prepared. These varying concentrations of substrate stock solutions were then added to both free and encapsulated trypsin solutions (0.25 mg/ml). The concentrations of trypsin (0.25 mg/ml) and polyelectrolytes (0.5 mg/ml) were kept constant. A UV/Vis

spectrophotometer (Molecular Devices SpectraMax i3) was used to measure the change in optical density at 410 nm caused by the absorption maximum of p-Nitroaniline (pNa). Activity experiments were performed in a 96-well plate (TPP Tissue Culture Test Plate, Trasadingen, Switzerland). Kinetics was measured for one hour for free trypsin and for two hours for encapsulated trypsin. Due to the optimal pH of the trypsin enzyme, all studies were performed at pH 7.5.

3.6. Circular Dichroism Experiments

Trypsin samples at 1 mg/mL were prepared for circular dichroism (CD) experiments to investigate the secondary structure of trypsin. Firstly, coacervate microdroplets consisting of 0.5 mg/mL pectin and 0.5 mg/mL PDADMAC were prepared. Then, CD spectra of ellipticity, θ(mdeg), versus wavelength (nm) were obtained for 1 mg/mL native trypsin and encapsulated trypsin. For encapsulated trypsin, the CD spectra of the suspension before centrifugation and the coacervate phase were examined separately. To investigate the effect of temperature on free and encapsulated trypsin, CD spectra for free and encapsulated trypsin samples were examined at both room temperature and 45°C. All measurements were made between 250 and 190 nm. All samples were prepared as described above.

4. RESULTS AND DISSCUSSION

4.1. Potentiometric Titration

PDADMAC is a polyelectrolyte that remains fully positively charged at all pH values. In order to create electrostatic attraction to form complex coacervate microdroplets, a polyanion must attract the polycation in the same medium. In this study, a biological polyelectrolyte, pectin, which is a negatively charged but heteropolysaccharide, was chosen. Pectin chains contain both esterified carboxyl groups (-$COOCH_3$) and non-esterified carboxyl groups (-COOH). This non-esterified carboxylic groups may show acidic properties in some environments depending on the pH. This means that at pH > pK_a, non-esterified carboxylic acid groups lose their proton, gains a negative charge and becomes a negatively charged group (-COO^-).

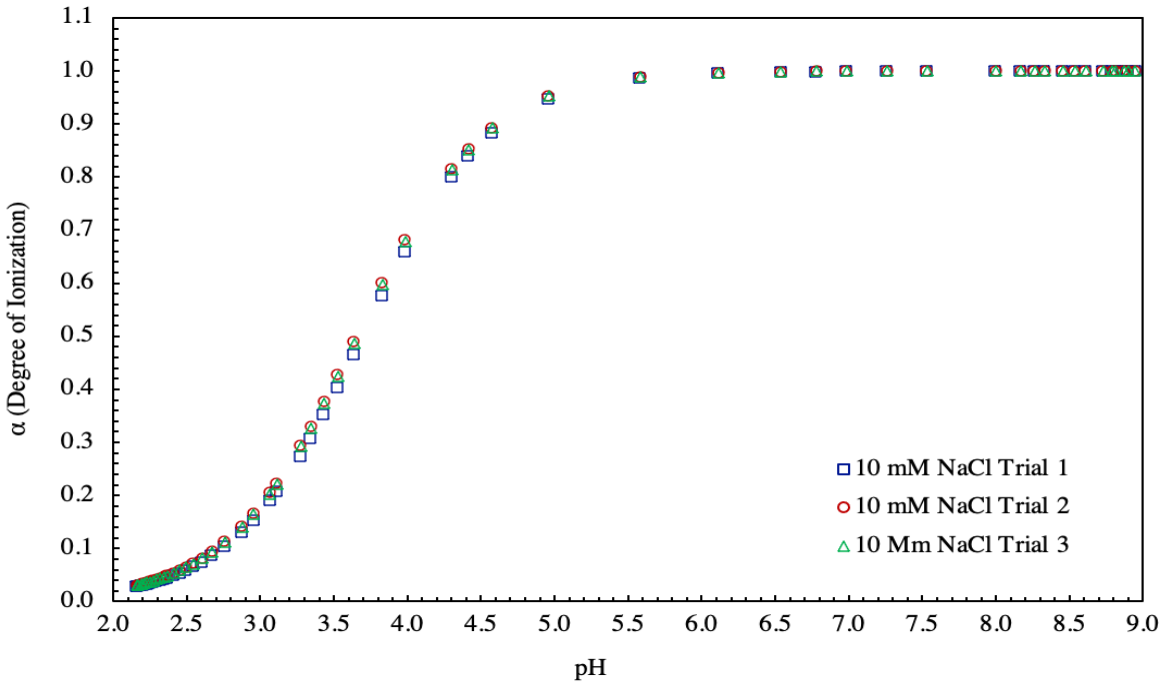


Figure 4.1. pH titration profile of pectin at 10 mM NaCl.

Potentiometric titration experiments were carried out to measure how much of the carboxylic acid groups of pectin lost protons and became negatively charged at pH = 7.5, which is the optimum pH value of trypsin. Therefore, degree of ionization (α) for pectin was necessary to determine the charge of the polysaccharide. Figure 4.1 shows the results of potentiometric titration experiments for pectin at 10 mM NaCl. The ionization level results of the potentiometric titration experiments for 0 mM and 150 mM NaCl are shown in Appendix A. Degree of ionization (α) was determined by using Equation (3.1).

As a result of these experiments, the pK_a value found for pectin was found as 3.67 ± 0.02. According to this value, the degree of ionization was found for each pH value. Since pectin does not give away protons in acidic environments, it is in charged state at neutral pH. Thus, at pH = 7.5, the pectin must be negatively charged. According to Figure 4.1, the α value of pectin at pH 7.5 is 1. An α value of 1 means that this polyelectrolyte is completely ionized at that medium. Based on this information, it can be said that all non-esterified carboxyl groups in pectin polyelectrolyte give off protons to the medium. Pectin can interact with PDADMAC at the desired pH in a fully charged state to form complex coacervate droplets.

The potentiometric titration experiments for trypsin were performed with the same method to find the isoelectric point of the protein. In Figure 4.2, ΔV versus pH graph was obtained as a result of a potentiometric titration of trypsin in 10 mM NaCl. The pH value corresponding to the midpoint of the ΔV axis gives the isoelectric point value for trypsin. The pH value at which the number of protein's negative and positive charges is equal is known as the isoelectric point. Identification of this point gives information about the protein's charge at the pH under study.

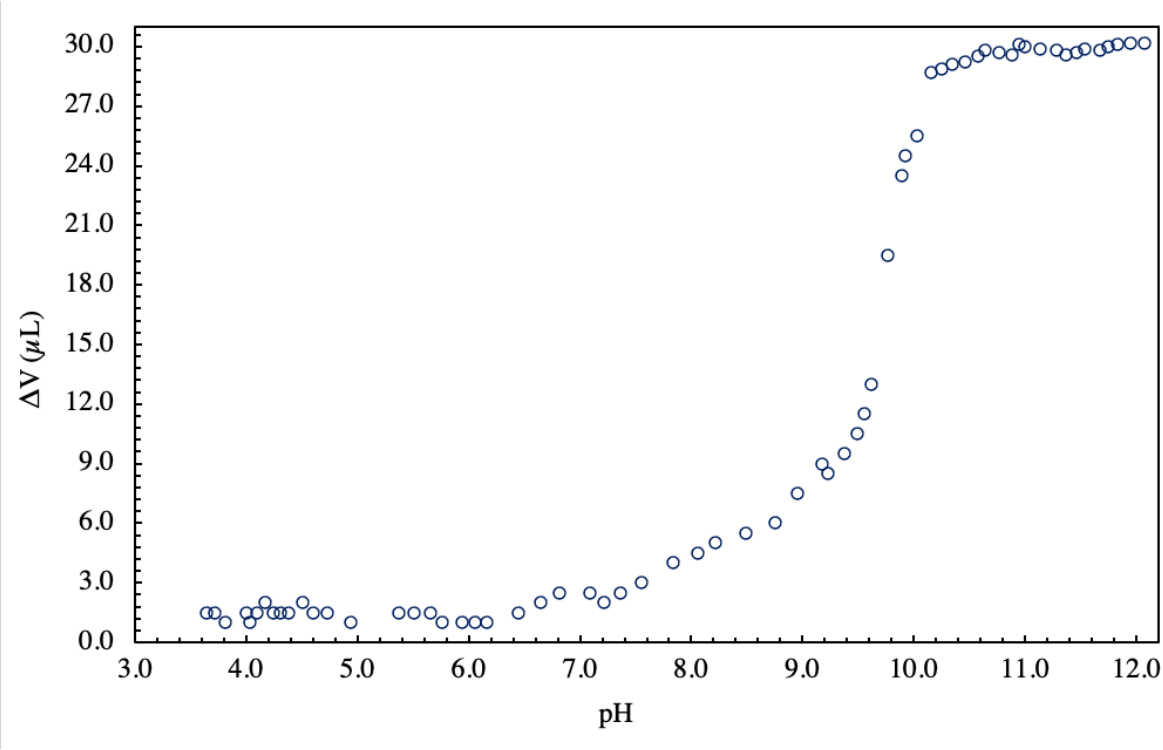


Figure 4.2. ΔV versus pH graph at 10 mM NaCl.

The isoelectric point for trypsin was determined as 9.8 ± 0.10 in 10 mM NaCl. In experiments performed at 150 mM NaCl concentration, the isoelectric point was determined to be approximately 9.5 ± 0.10. Graphs showing the results of the other potentiometric titration experiments can be viewed in the Appendix A. As a result of all these experiments, it was observed that the salt concentration had a small effect on the isoelectric point of trypsin. Proteins have a net positive charge at pH levels below their pI and a net negative charge at pH levels above their pI. Using this information, experiments were performed at pH 7.5 in this study and it can be seen that the protein has a predominantly positive charge at this pH value. This does not mean that only positive charges remain on protein, i.e., both positive and negative charges continue to be present, but it can be understood that there are greater numbers of positive charges. As a result, fully positively charged PDADMAC, fully negatively charged pectin and positively charged trypsin were available at the optimum pH value of trypsin.

4.2. Turbidimetric Titration and Light Microscopy

This section presents the results of turbidity experiments performed for pectin-PDADMAC mixtures at six different NaCl concentrations. Before colorimetric titration experiments were done on pectin-PDADMAC system, turbidity tests were performed on pectin and PDADMAC separately. This is because between pH 1.7 and 9.0, it was not known whether these polymers went through aggregation, precipitation, or some other behaviour. In the turbidity tests, titration was done both from acidic to basic pH and from basic to acidic pH. As a result of the experiments with a single polyelectrolyte, a turbid solution was not obtained, and the turbidity values were close to 0 in 10 mM and 400 mM NaCl. The results of those experiments are presented in Figures A.4 and A.5 of the Appendix A. The 0 value means that at the beginning and end of the turbidity experiments, polymer solutions are clear and do not exhibit an aggregation or precipitation behaviour in this pH range. Thanks to these results, turbidity tests were performed at 10, 25, 50, 100, 200 and 400 mM NaCl concentrations when the two polyelectrolytes were mixed. The aim of these experiments was to observe and obtain the formation of coacervate microdroplets at pH 7.5, the optimum pH value for trypsin. In Figure 4.3, the turbidimetric titration results for the pectin-PDADMAC mixture from pH 1.7 to 9.0 at different salt concentrations (10, 25, 50, 100, 200, and 400 mM NaCl) are given. In Figure 4.4, the turbidimetric titration data of pectin-PDADMAC mixture titrated with 0.1 N and 1.0 N HCl from pH 9.0 to 1.7, are presented for different salt concentrations.

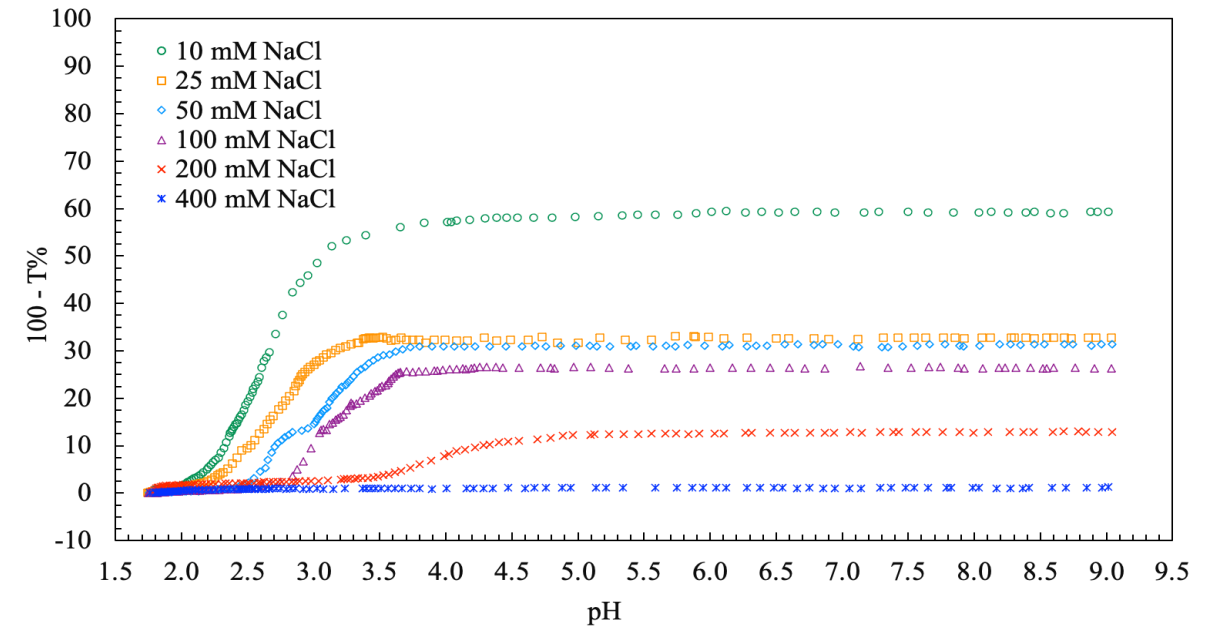


Figure 4.3. Turbidimetric titration graph for pectin-PDADMAC system from acidic to basic pHs at different salt concentrations.

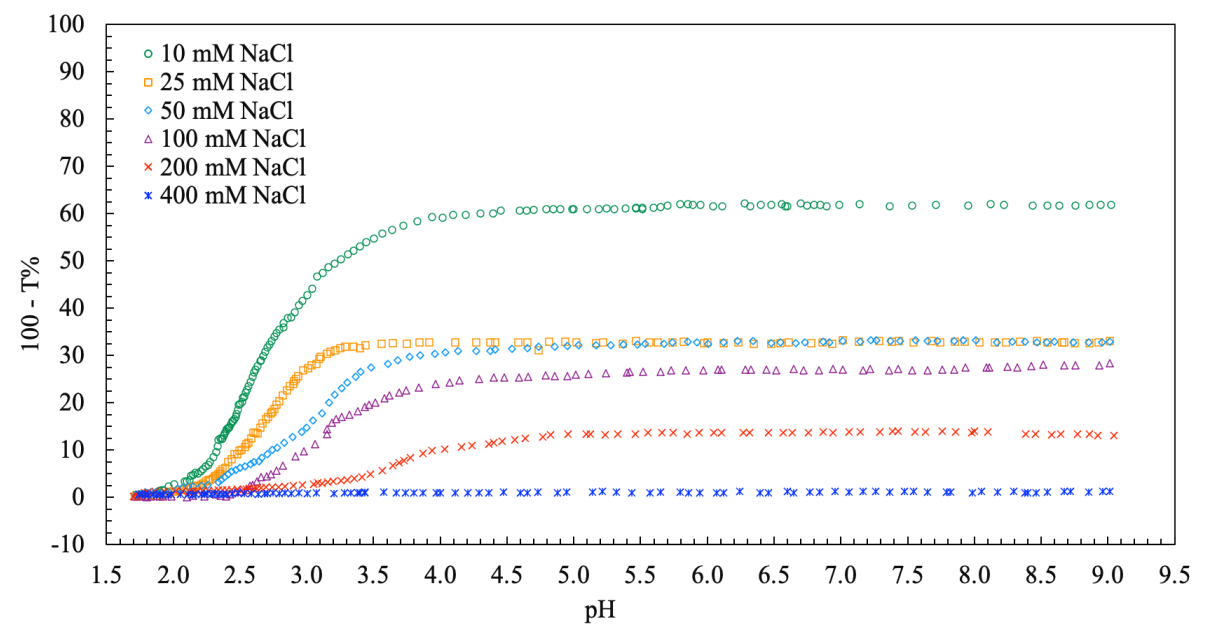


Figure 4.4. Turbidimetric titration graph for pectin-PDADMAC system from basic to acidic pH at different salt concentrations.

The only common point in the experiments carried out with two different methods is that the turbidity values are close to zero at the highest salt concentration, i.e., 400 mM NaCl. The formation of complex coacervate structures depends on the properties of the polymers as well as the properties of the environment (e.g. pH, ionic strength, and salt concentration) [31]. The salt concentration and the ionic strength do not always have the same value because the term called ionic strength depends on the number of ions in the salt and their valence. Since the only salt used in the turbidimetric titrations is NaCl and it is a monovalent salt, the ionic strength and salt concentration are always the same for NaCl solutions. Due to the fact that the complex coacervation is driven by the release of counter-ions to the medium and electrostatic interactions, the amount of salt ions in the medium has a crucial effect on the formation of complex coacervate droplets. Complex coacervation is thought to be sensitive to the total concentration of salt ions present because of this essential entropic dependence on the release of counter-ions [31]. Liquid-liquid phase separation becomes unfavourable, as high concentration of salt weakens the entropic driving force. This is called the salt screening effect. In other words, in high salt concentrations, polyelectrolytes do not get rid of the counter-ions around them, and the surrounding salt ions are attracted by these counter-ions, reducing the interaction of the two polymers with each other and coacervate formation is not observed [32]. This described situation might have occurred for pectin-PDADMAC in 400 mM NaCl. For this reason, no coacervate droplet was formed and the turbidity data always remained close to zero.

Lower salt concentrations, on the other hand, promote the formation of complex coacervate, increasing the electrostatic interaction between oppositely charged polyelectrolytes. Decreasing salt concentrations has a favourable effect on the formation of complex coacervate. For example, turbidity values increase as the salt concentration decreases. At the same time, this situation was observed visually during the experiment, i.e., white colour of the pectin-PDADMAC mixture observed at low salt concentrations disappeared gradually as the salt concentration increased.

In these experiments, we determined conditions for coacervation by doing both acidic to basic and basic to acidic pH titrations. It has already been explained above why there is no coacervate formation in 400 mM NaCl. A clear solution between pH 1.7 and 2.5 and a turbid, cloudy solution above pH 2.5 were observed while going from acidic to basic pHs in

200 mM NaCl. However, at lower salt concentrations of 10, 25, 50, 100 mM NaCl, precipitation was observed above pH 2.5. Below this pH, there was a clear solution indicating that pectin and PDADMAC did not interact with each other due to the absence of negative charges on pectin. When precipitation occurred above pH 2.5, visible and floating white particles were found in the medium.

In the experiments carried out by going from basic to acidic pHs at 10, 25, 50, 100, 200 mM NaCl, a turbid solution was obtained between pH 9.0 and 2.6. This turbid solution was considered as the proof for coacervation. Precipitation was observed between pH 2.6 and 2.4, and below pH 2.4 a clear solution was obtained indicating that the polymers did not interact at all.

According to the literature, interaction between charged macromolecules can be explained by sets of specific pH values that correspond to the boundaries of various regions of phase behaviour as the pH value increases [42]. According to Kaibara et al., the results of the phenomenological analysis were parametrized by defining two crucial pH values, i.e., pH_{crit} and pH_{θ} [42]. At pH_{crit}, primary complex formation while at pH_{θ} coacervate droplet formation begins.

These two critical pH values of salt concentrations used in the experiment are given in Table 4.1.

Table 4.1. pH_{crit} and pH_{θ} of pectin-PDADMAC at six different NaCl concentrations.

NaCl Concentrations (mM)	pH_{crit} primary complexes	pH_{θ} coacervate formation
10	1.87 ± 0.01	2.17 ± 0.01
25	1.91 ± 0.01	2.24 ± 0.03
50	2.31 ± 0.02	2.61 ± 0.01
100	2.51 ± 0.02	2.77 ± 0.03
200	2.88 ± 0.01	3.48 ± 0.02
400	-	-

According to these results, soluble complexes form at more acidic pHs than coacervate droplets. As the salt concentration increases, both pH_{crit} and pH_{θ} increases. This is because, as explained above, salt ions of Na^{+} and Cl^{-} in the environment reduce the strength of electrostatic interaction between pectin and PDADMAC.

Samples were examined under the light microscope in the presence of 10 mM phosphate buffer at different salt concentrations (10, 25, 50, 100, 150, 200, 400 mM NaCl) in order to observe whether the coacervate droplets was formed at pH 7.5, which is the optimum pH for trypsin. Light microscope images in 10 mM phosphate buffer with no NaCl, 10 mM NaCl and 400 mM NaCl are given in Figure 4.5, Figure 4.6 and Figure 4.7, respectively. Light microscope images for all other NaCl concentrations are presented in the Appendix A.

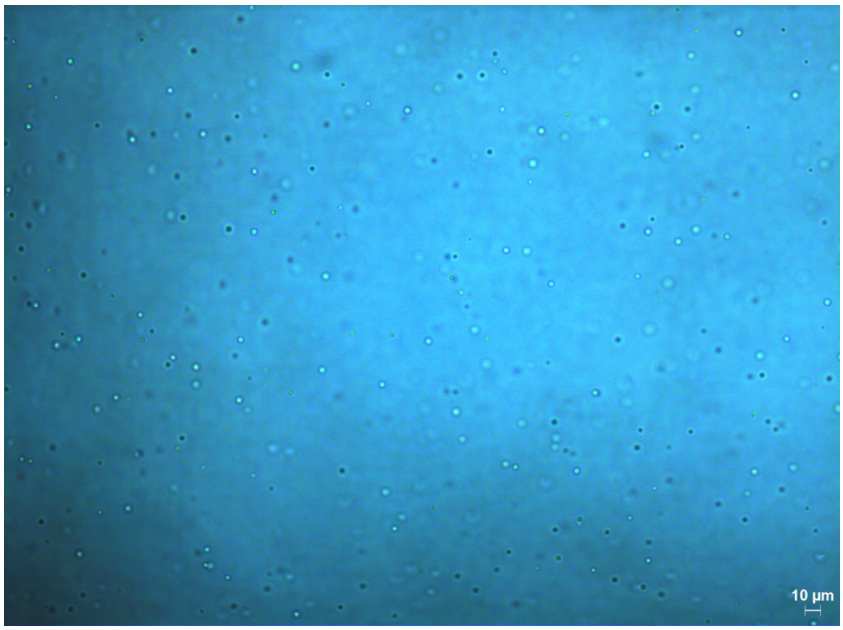


Figure 4.5. Microdroplets of 0.5 mg/mL pectin - 0.5 mg/mL PDADMAC mixture at pH: 7.5 in 10 mM phosphate buffer, magnification: 20X.

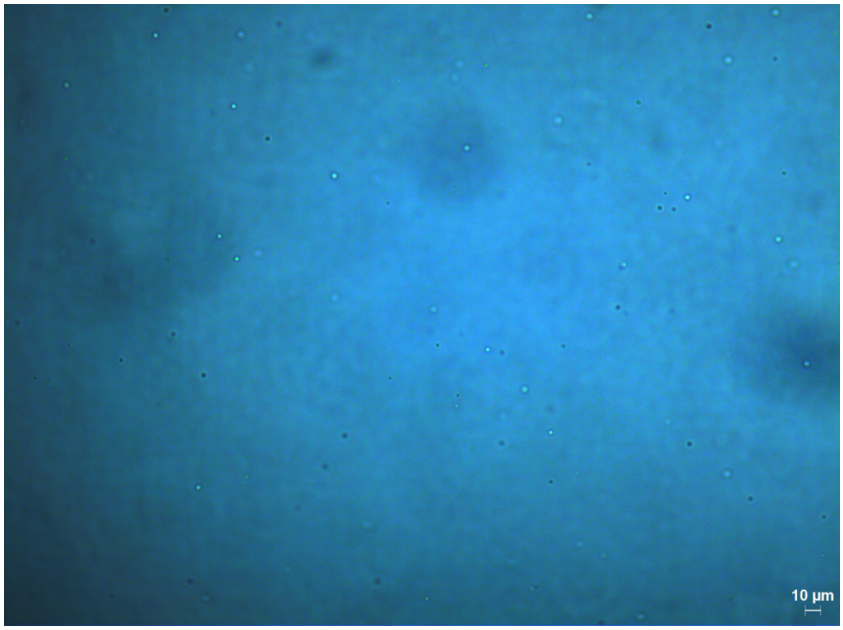


Figure 4.6. Microdroplets of 0.5 mg/mL pectin - 0.5 mg/mL PDADMAC system at pH: 7.5 in 10 mM NaCl with 10 mM phosphate buffer, magnification: 20X.

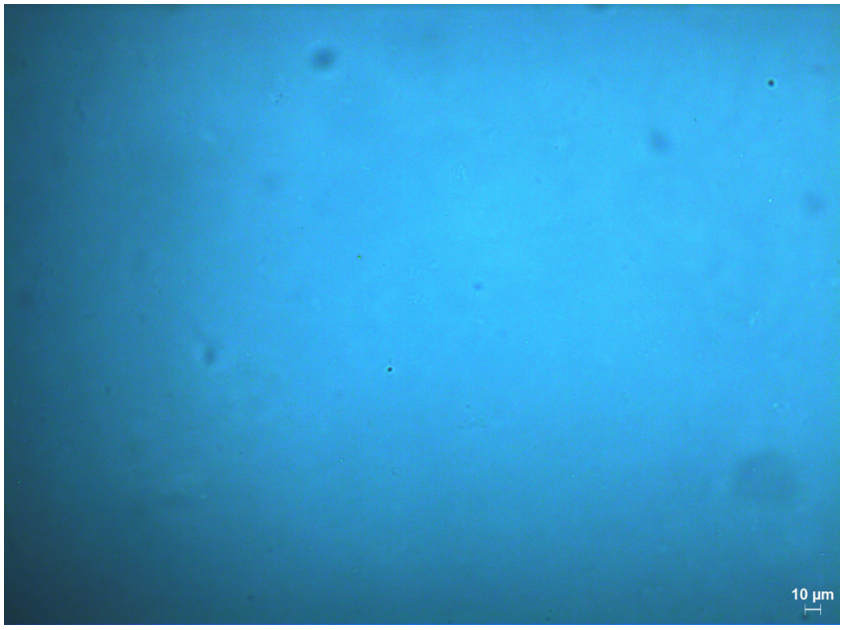


Figure 4.7. Microdroplets of 0.5 mg/mL pectin – 0.5 mg/mL PDADMAC system at pH: 7.5 in 400 mM NaCl with 10 mM phosphate buffer, magnification 20X.

The reason for using phosphate buffer in light microscopy experiments is to ensure that the desired pH value remains stable while the microscope image is taken. As can be seen from these images, there is a noticeable decrease in the number of coacervate droplets as a result of adding salt to the medium. All turbidimetric titration experiments and light microscopy images show that coacervate microdroplets formed by pectin- PDADMAC system can be used to encapsulate trypsin at its optimum pH. Since the desired turbidity values and the number of coacervate microdroplets could not be obtained at 200 and 400 mM NaCl concentrations, these salt concentrations were not included in the next steps of the study.

In addition, Table 4.2 shows the average size data of the coacervate micro droplets imaged at different salt concentrations. There is a decreasing trend in the size of coacervate droplets formed as the salt concentration increases. The salt screening effect may be behind this trend. Due to the increased number of salt ions, the interaction of two differently charged polymers may have decreased and the size of the coacervate droplets may have decreased.

Table 4.2. Coacervate micro droplet size data.

Experimental Condition	**Coacervate Micro Droplet Size (µm)**
10 mM NaCl with 10 mM phosphate buffer	5.76 ± 1.43
10 mM NaCl with 25 mM phosphate buffer	5.68 ± 1.45
10 mM NaCl with 50 mM phosphate buffer	4.87 ± 0.82
10 mM NaCl with 100 mM phosphate buffer	4.81 ± 0.89
10 mM NaCl with 150 mM phosphate buffer	4.64 ± 0.70
10 mM NaCl with 200 mM phosphate buffer	3.40 ± 0.94
10 mM NaCl with 400 mM phosphate buffer	3.09 ± 0.83

4.3. Encapsulation Efficiency

To understand how trypsin would be more efficiently encapsulated in pectin-PDADMAC coacervates, experiments were performed by changing parameters such as mixing order, total protein concentration, and polyelectrolyte concentration. At the selected pH of 7.5, pectin is fully negatively charged, PDADMAC and trypsin are positively charged. Although trypsin has a net positive charge, it still has both positive and negative charges at this pH [54]. Thus, trypsin can interact with both pectin and PDADMAC chains. Figure 4.8 shows the electrostatic charge distribution of the porcine trypsin enzyme found in the study by Gorfe et al. [54].

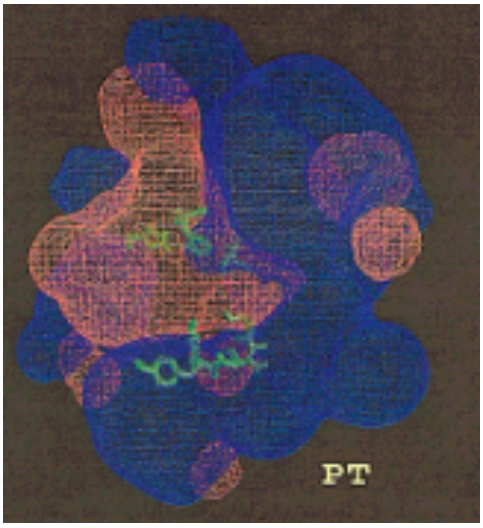
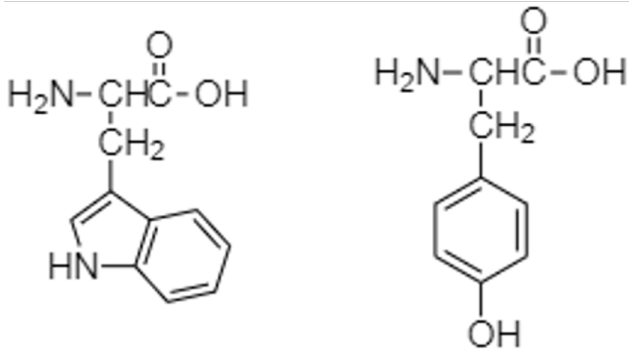


Figure 4.8. Electrostatic surface potential of porcine trypsin presented in 3D. Red and blue colours show negative and positive charges respectively. (Reprinted with permission from [Gorfe et al, 2000]. Copyright (2022) Wiley).

Considering the electrostatic potential distribution of this enzyme given in Figure 4.8, the outer surface of the porcine trypsin is mostly positive charged, and the pocket part where the active part is located, is negatively charged.

In the experiments to measure encapsulation efficiency, absorbance of trypsin was measured at 280 nm in order to detect the presence and amount of the protein. The reason

for this is that the tryptophan and tyrosine in proteins are aromatic amino acids and can be detected at 280 nm due to their electron movement. In order to determine the protein concentrations in the experiments, a calibration curve was first drawn for trypsin. Figure 4.9 shows the chemical structure of tryptophan and tyrosine amino acids.



(a) (b)

Figure 4.9. Structures of amino acid residues of (a) tryptophan and (b) tyrosine.

4.3.1. Effect of Mixing Order on Encapsulation Efficiency

The experiments were performed by mixing the three macromolecules in different orders to optimize encapsulation. Three different orders are given as follows: (i) mixing pectin with trypsin first and adding PDADMAC solution on it (pectin-trypsin/PDADMAC); (ii) firstly mixing PDADMAC and trypsin, then adding pectin solution on it (PDADMAC-trypsin/pectin; (iii) by adding trypsin into the previously formed pectin-PDADMAC mixture (pectin-PDADMAC/trypsin).

Figure 4.10 shows the results of the experiments performed at six different salt conditions. Encapsulation efficiency % was calculated according to Equation (3.2). All experiments were performed in the presence of 10 mM phosphate buffer to keep the pH constant at 7.5.

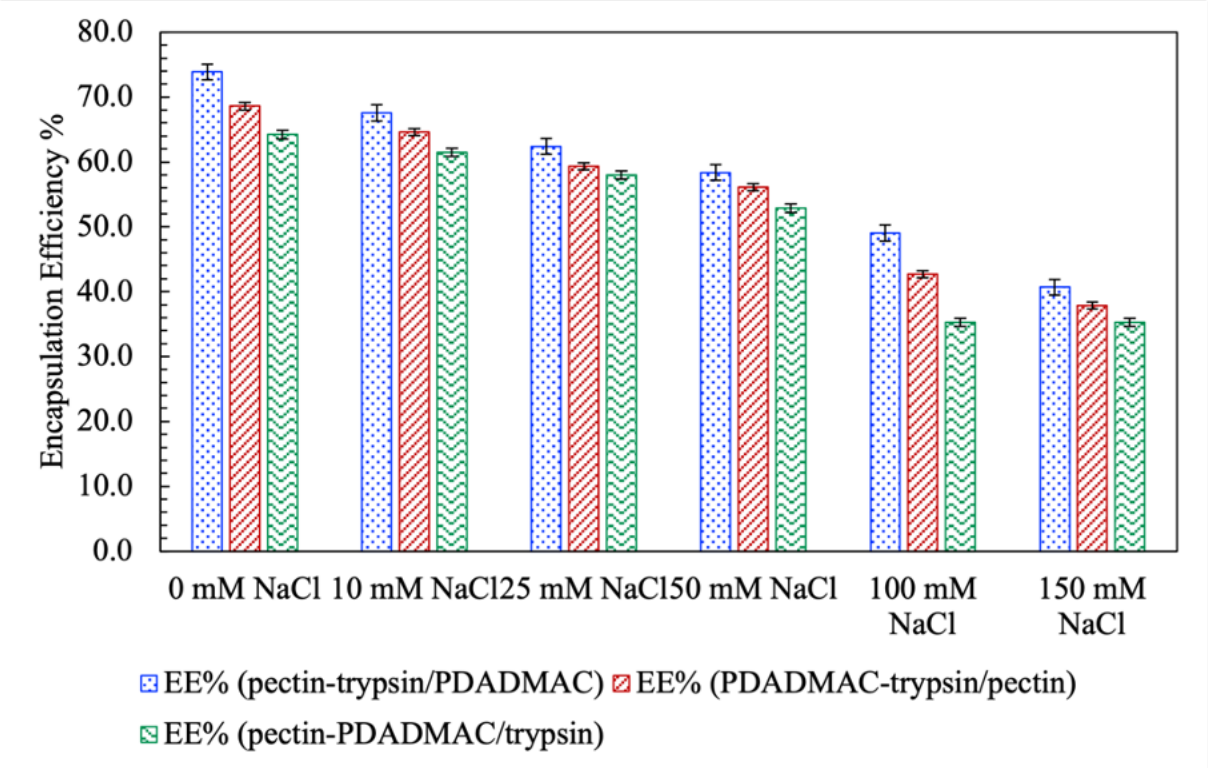


Figure 4.10. Encapsulation efficiency % according to different mixing order at different salt concentrations.

According to the results, the encapsulation efficiency decreased as the salt concentration increased. This is a result of reduced number of coacervate droplets (see Figure 4.5 and Figure 4.7) because of the salt screening effect. As the number of coacervate droplets formed at high salt concentration decreases, the amount of encapsulated trypsin also decreases. For this reason, as the salt concentration increases, the encapsulation efficiency decreases. Table A.1 in the Appendix A section provides all encapsulation data in the mixing order experiments.

As seen in Figure 4.10, the mixing order has a significant effect on the encapsulation efficiency. Mixing of net positively charged trypsin with negatively charged pectin (pectin-trypsin/PDADMAC) resulted in higher trypsin encapsulation efficiency than trypsin-PDADMAC/pectin and pectin-PDADMAC/trypsin complexes. Since the electrostatic attraction of opposite charges between negatively charged pectin and net positively charged trypsin is greater than the electrostatic attraction between cationic PDADMAC and net

positively charged trypsin, a greater amount of soluble complex formation is expected, which could increase the encapsulation efficiency. On the other hand, forming pectin-PDADMAC coacervates before addition of trypsin made it difficult for trypsin to enter into pectin-PDADMAC complexes, so it had the lowest encapsulation efficiency. Our results were consistent with Zhao et al., where BSA protein with an isoelectric point of 4.7, positively charged poly (allylamine hydrochloride) (PAH) and negatively charged poly (acrylic acid) (PAA) were used [8]. In their study, experiments are carried out at pH 7.4, where BSA is net negatively charged. According to the results, higher efficiency was obtained when BSA and positively charged PAH were mixed first. Meanwhile, the lowest encapsulation yield was observed when PAH-PAA coacervates were formed first. This study also confirms that encapsulation efficiency is higher when soluble complexes are formed from opposite charges.

4.3.2. Effect of Protein Concentration on Encapsulation Efficiency

For these experiments, samples were prepared after choosing the mixing order with the highest encapsulation efficiency. In other words, in all experiments, trypsin and pectin were mixed first and then PDADMAC was added to the intermediate complex solution. Here, the goal was to determine how total trypsin concentration affected the encapsulation efficiency. Experiments were performed at four different trypsin concentrations (0.25, 0.40, 0.50, and 0.75 mg/mL) at pH 7.5 in 10 mM phosphate buffer with varying concentrations of salt (0, 10, 25, 50, 100, and 150 mM NaCl). In Figure 4.11, the results of the encapsulation efficiency experiments are given. Encapsulation efficiency % was calculated according to the Equation (3.2) and detailed data are given in Table A.2 in Appendix A.

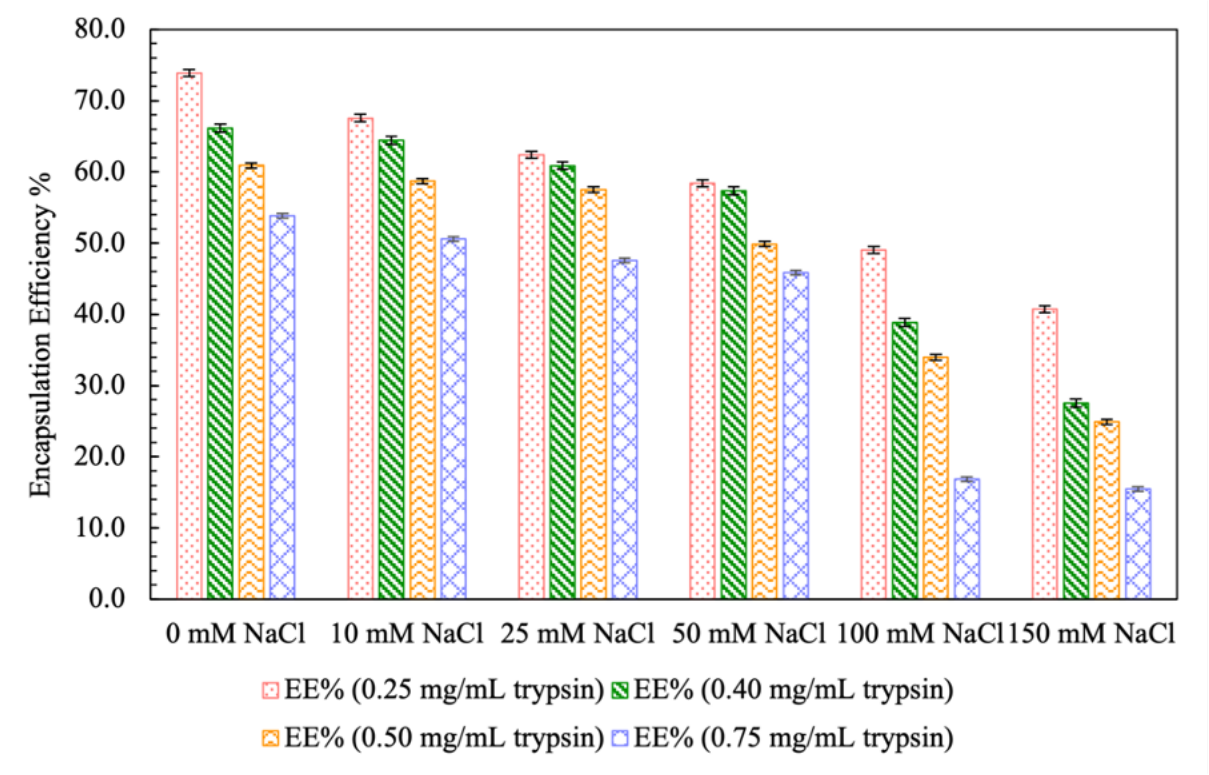


Figure 4.11. Encapsulation Efficiency % results according to trypsin concentration.

Salt screening effect was observed again in Figure 4.11. As the salt concentration increased, the encapsulation efficiency % decreased due to the scarcity of the number of coacervate droplets because counterions formed a cloud around the polyelectrolytes, screened their charges and weakened their interaction with each other. Therefore, the number of coacervate droplets decreased.

As seen from Figure 4.11, the increase in trypsin concentration decreased the encapsulation efficiency, i.e., as the trypsin concentration increases, there is a drop in the amount of trypsin remaining in the coacervate phase, and an increase in the amount of trypsin in the supernatant. Also, the amount of trypsin in the supernatant phase was found to be very close to the amount of trypsin in the coacervate phase. For example, in 10 mM phosphate buffer (no NaCl), 0.25 mg/mL trypsin, corresponding to 2.856 mg of trypsin in total, was distributed into coacervate and supernatant phases after centrifugation. As a result, 2.056 mg trypsin was found in the coacervate while 0.765 mg trypsin was present in the supernatant. Again in 10 mM phosphate buffer (no NaCl), of 0.75 mg/mL trypsin, corresponding to a

total of 12.0 mg of trypsin, only 6.078 mg remained in the coacervate phase while 5.449 mg went into the supernatant phase. Considering these results, for high initial trypsin concentration, the amount of trypsin remaining in the coacervate droplets decreased to around 50% in the absence of NaCl and to 13% in 150 mM NaCl. The amount of trypsin found in the supernatant and coacervate parts for each condition is given in Table A.3 in the Appendix A.

These results can be explained as follows: Electrostatic attraction between pectin and trypsin is weaker than the attraction between pectin and PDADMAC because trypsin contains negative charges although its overall net charge is positive. Therefore, at high concentrations of trypsin, a certain amount of trypsin molecules will be repelled by the pectin and pass into the supernatant phase.

4.3.3. Effect of Polyelectrolyte Concentration on Encapsulation Efficiency

In order to maximize the encapsulation efficiency, both the mixing order and the trypsin concentration were varied, and a suitable method was selected as described above. The effect of the concentrations of polyelectrolytes is examined in this section. To reach high encapsulation efficiency, the order of addition was to mix pectin and trypsin first and then add PDADMAC last. In addition, the trypsin concentration was chosen as 0.25 mg/mL. The same salt concentrations used in the previous experiments were used in the presence of 10 mM phosphate buffer. In this section, the effect of polyelectrolyte concentrations on the encapsulation efficiency was investigated in five different groups: (i) 0.25 mg/mL pectin and 0.50 mg/mL PDADMAC; (ii) 0.75 mg/mL pectin and 0.50 mg/mL PDADMAC; (iii) 0.50 mg/mL pectin and 0.50 mg/mL PDADMAC; (iv) 0.50 mg/mL pectin and 0.25 mg/mL PDADMAC; (v) 0.50 mg/mL pectin and 0.75 mg/mL PDADMAC. The polyelectrolyte concentrations and encapsulation efficiency % measured for these five groups are shown in Figure 4.12.

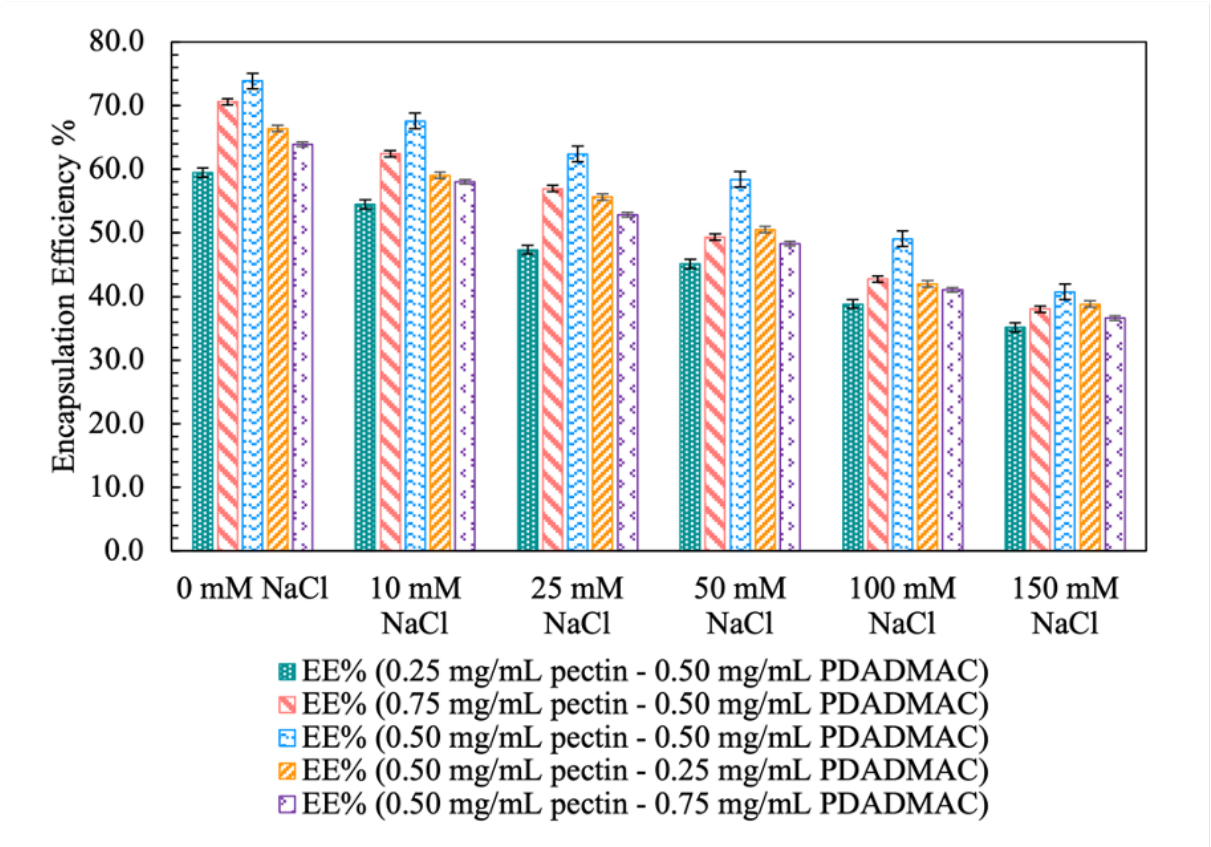


Figure 4.12. Encapsulation Efficiency % at five different groups of polyelectrolyte concentration at pH 7.5 in 10 mM phosphate buffer with 0, 10, 25, 50, 100, and 150 mM NaCl.

According to these experiments, concentration of polyelectrolytes with the highest encapsulation efficiency % was 0.5 mg/mL pectin and 0.5 mg/mL PDADMAC. One of the reasons why a concentration ratio of 1:1 (w/v), corresponding to $[-]/[+] = 0.81$ in charge ratio, between pectin and PDADMAC mixtures has the best efficiency is that the distance between the neighbouring charges on the polymers, a.k.a., the charge spacing value, is close to each other; i.e., the charge spacing is 6.5 Å for pectin and 6.2 Å for PDADMAC [47,48]. The fact that these charge spacings are close to each other is the reason why the ratio of 1:1 gives the highest efficiency because each negative charge is opposite to a positive charge. Since the effect of polyelectrolyte concentration on the formation of coacervate droplets is determined, next, turbidity was measured for these five different polyelectrolyte groups without trypsin. The results of the turbidity measurements are shown in Table 4.3. Since

activity experiments were only carried out for 10 mM phosphate buffer with 0, 50, and 100 mM NaCl, turbidity was measured only under these conditions.

Table 4.3. Turbidity (100-%T) data for pectin-PDADMAC mixtures at pH 7.5.

	10 mM phosphate buffer	50 mM NaCl with 10 mM phosphate buffer	100 mM NaCl with 10 mM phosphate buffer
0.25 mg/mL pectin-0.50 mg/mL PDADMAC	3.750 ± 0.100	2.535 ± 0.315	1.505 ± 0.265
0.75 mg/mL pectin-0.50 mg/mL PDADMAC	9.810 ± 0.040	8.655 ± 0.105	7.150 ± 0.090
0.50 mg/mL pectin-0.50 mg/mL PDADMAC	19.770 ± 0.110	18.560 ± 0.210	16.490 ± 0.280
0.50 mg/mL pectin-0.25 mg/mL PDADMAC	7.655 ± 0.005	9.550 ± 0.033	8.340 ± 0.175
0.50 mg/mL pectin-0.75 mg/mL PDADMAC	4.685 ± 0.135	6.725 ± 0.175	4.735 ± 0.185

According to Table 4.3, the group with the highest turbidity is 0.5 mg/mL pectin and 0.5 mg/mL PDADMAC. A higher turbidity value suggests a higher number of coacervate droplets, which might be the reason for higher encapsulation efficiency. The encapsulation efficiency % for the other groups is also consistent with the turbidity data, e.g., the group having lowest encapsulation efficiency also has the lowest turbidity. All the results of the experiments performed according to the polyelectrolyte concentration are given in the Appendix A in Table A.4.

As a result of all these experiments, the polyelectrolyte concentrations with the highest encapsulation efficiency % were used for the activity experiments; concentrations were 0.25 mg/mL and 0.50 mg/mL for trypsin and both polyelectrolytes, respectively.

In Figure 4.13, light microscope image for pectin-trypsin/PDADMAC coacervate droplets in the presence of 10 mM phosphate buffer at pH 7.5 is given which was prepared at the optimum conditions giving the highest encapsulation efficiency %.

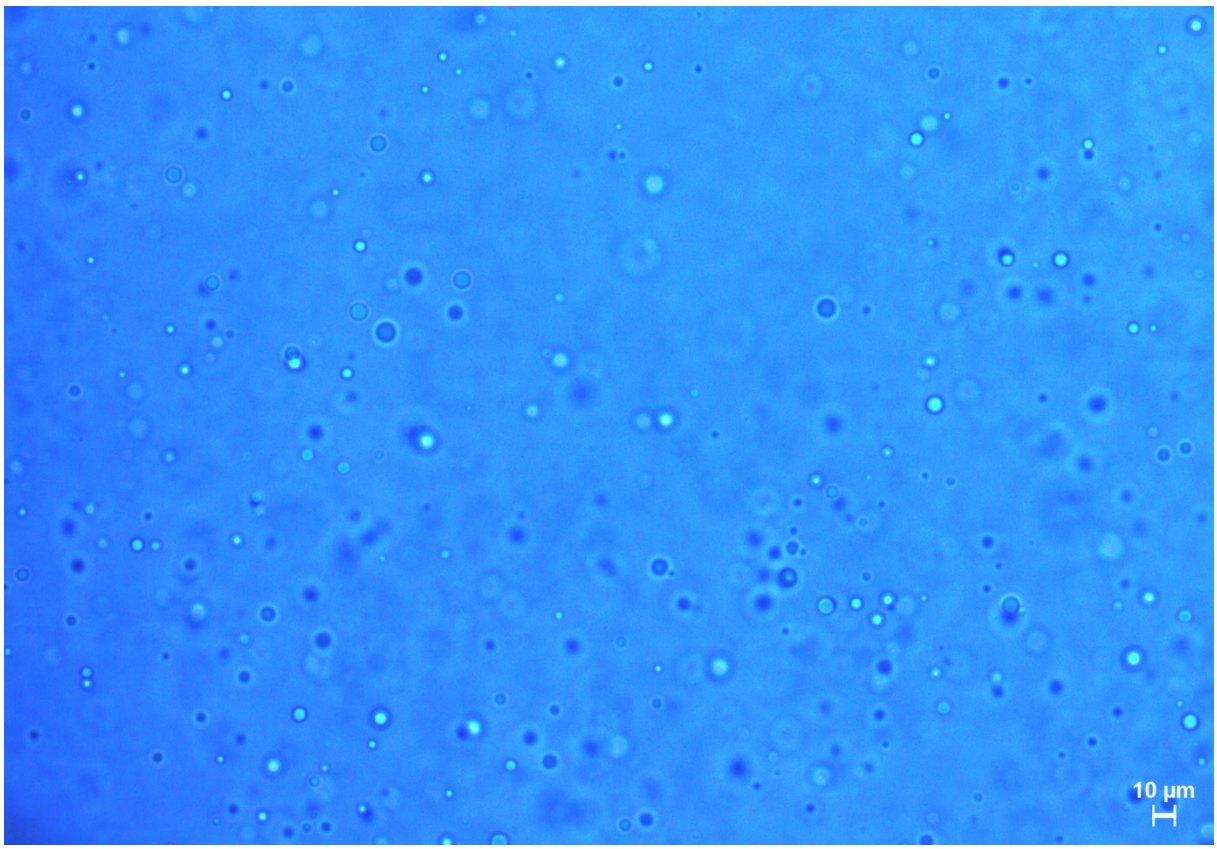


Figure 4.13. Light microscopy image of pectin-trypsin/PDADMAC coacervate suspension at pH: 7.5 in 10 mM phosphate buffer, magnification: 20X.

4.4. Trypsin Activity

In this part of the study, activity experiments were performed for both free trypsin and encapsulated trypsin in accordance with optimum conditions. The enzyme trypsin breaks the

peptide bonds from carboxylic side of the amino acids of lysine and arginine from serine protease group. Here, lyophilized trypsin enzyme obtained from porcine pancreas was used. Its optimum pH value is 7.5 [54], and this has been noted in previous experiments. Trypsin is a tertiary-structured globular enzyme consisting of a polypeptide chain of 223 amino acids.

For activity measurements, a molecule called N-Benzoyl-L-Arginine-p-Nitroanilide (BAPNA) was used as the substrate. At the end of the reaction of this molecule with trypsin, a visible yellow product, p-nitroaniline, is produced the absorbance/optical density of which can be measured at 410 nm. Figure 4.14 shows the reaction between BAPNA and trypsin. The amide bond broken in BAPNA by catalysis with trypsin is shown with a blue circle. Following bond breakage, two different products are formed, i.e., p-nitroaniline (pNa) and Nα-Benzoyl-L-Arginine (BA).

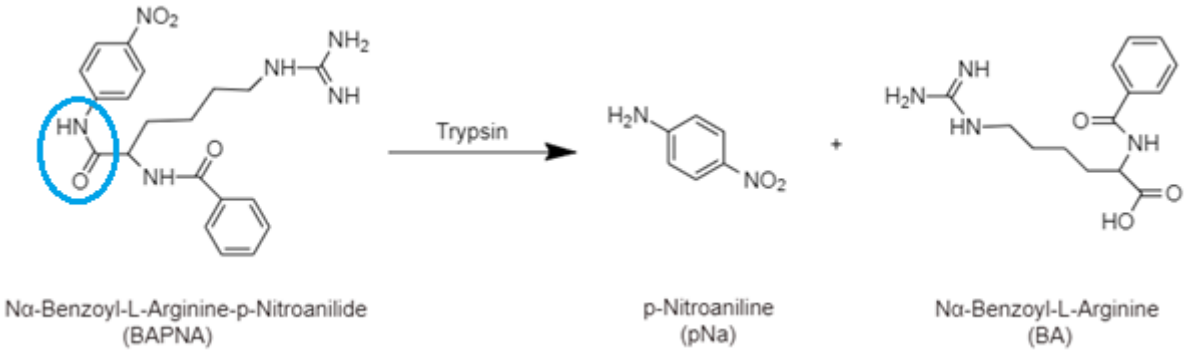


Figure 4.14. Reaction scheme between BAPNA and trypsin.

Here, we also examined the effect of salt concentration on catalytic activity of encapsulated trypsin. For the activity experiments, four different substrate concentrations were selected, i.e., these concentrations were 0.1, 0.2, 0.4, and 0.8 mM BAPNA. Activity was measured for both free and encapsulated trypsin. Experiments were done in 10 mM phosphate buffer with 0, 50, and 100 mM NaCl. Therefore, results can be compared as a function of salt concentrations.

The experiments were measured in optical density (OD) unit, but in order to find the velocity of the enzyme-catalysed reaction, time-dependent concentration change of p-

nitroaniline (the product) had to be drawn. Also, a calibration curve was prepared for p-nitroaniline to convert ODs to concentrations.

As a representative of graphs obtained for activity measurements, the results for free trypsin enzyme at 0.1 mM BAPNA are given in Figure 4.15.

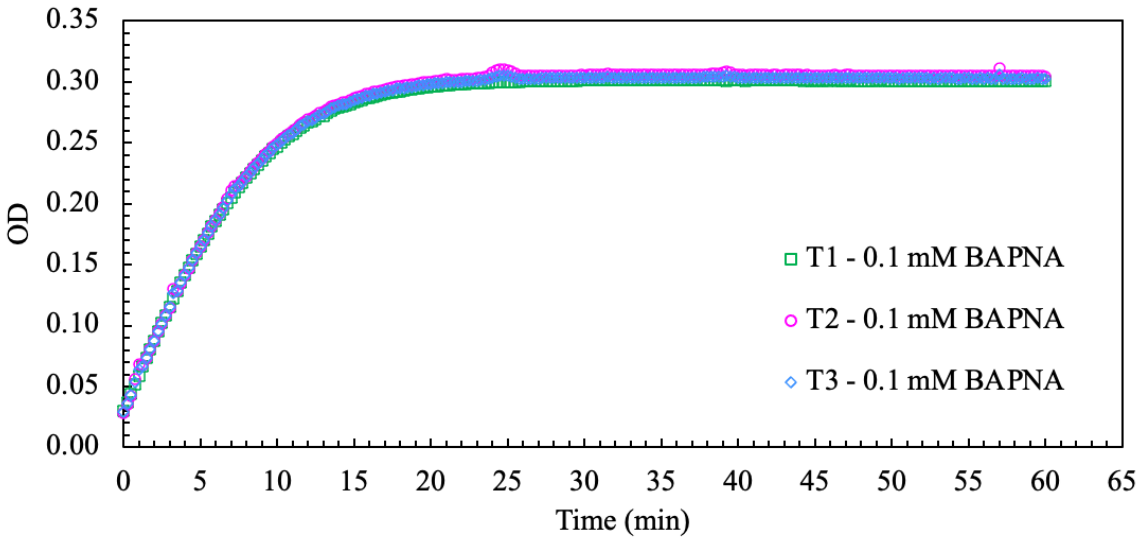


Figure 4.15. OD vs. time plot for free trypsin using 0.1 mM BAPNA at 10 mM phosphate buffer. T1-T3 represent different runs.

The OD vs. time plot in Figure 4.15 was converted to concentration (mM) versus time plot using the previously drawn calibration curve for p-nitroaniline (Figure 4.16.) The same method was used for every different concentration of BAPNA used for kinetics experiments of free and encapsulated trypsin.

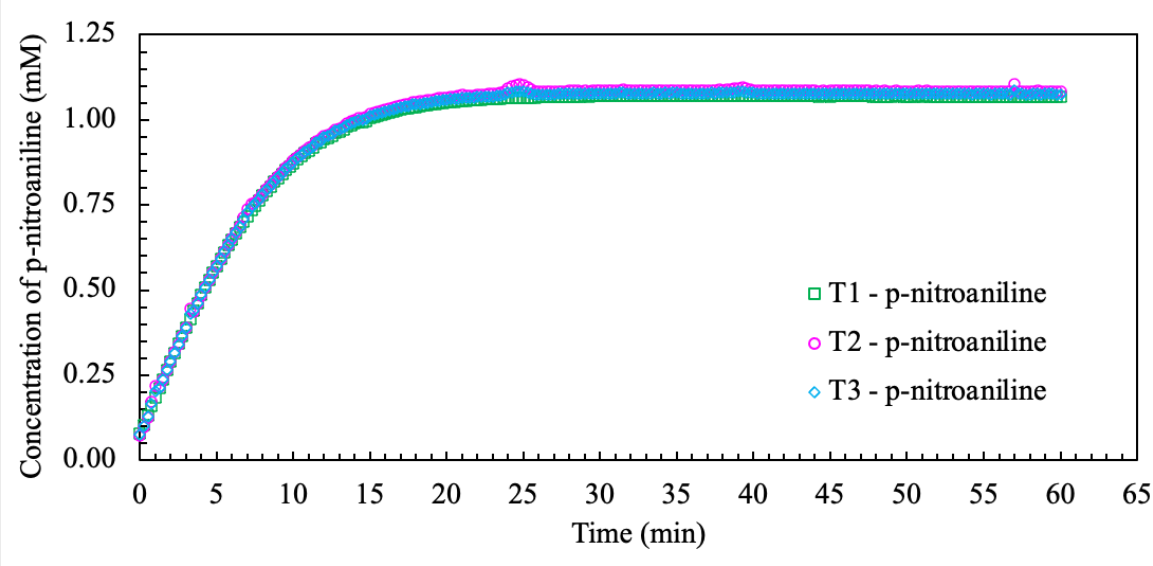


Figure 4.16. Concentration of p-nitroaniline (mM) vs. time for free trypsin using 0.1 mM BAPNA at pH 7.5 and 10 mM phosphate buffer.

After obtaining the plot of product concentration versus reaction time in Figure 4.16, initial velocity (V_0) for the enzyme reaction was determined. In order to find the V_0 value, all three trials were drawn separately and their slopes at time zero were determined by drawing a line fitting the data upwards from the starting point of the experiment (Figure 4.17).

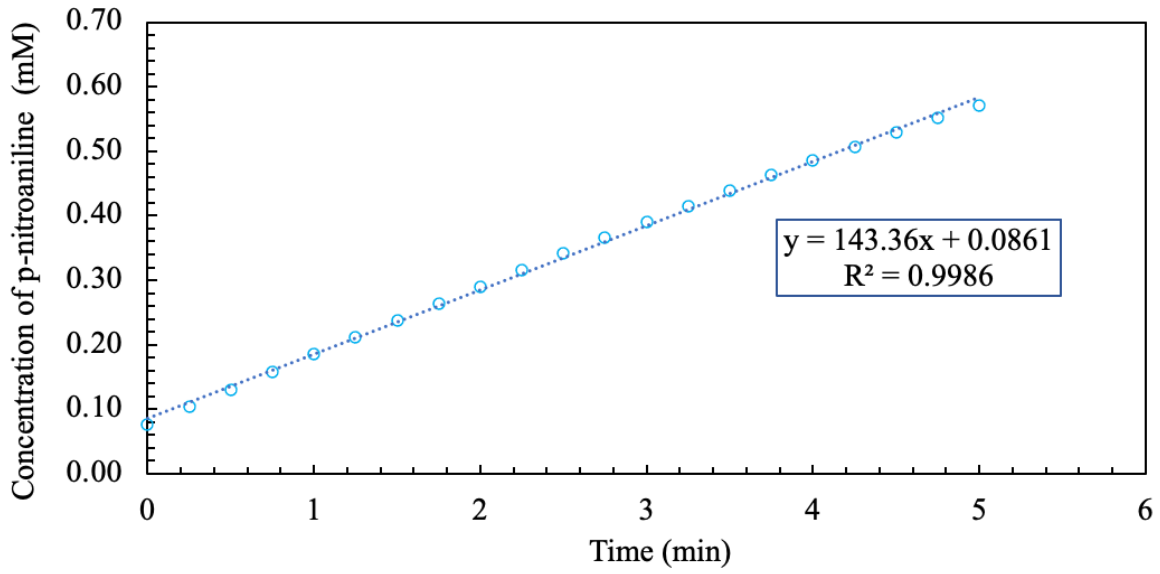


Figure 4.17. Product (p-nitroaniline) concentration versus time plot for free trypsin at 10 mM phosphate buffer in the presence of 0.1 mM BAPNA. The dotted line is a linear fit to the data. R2 is the square of the correlation coefficient. The equation represents the dotted line.

The slope of the linear line drawn in Figure 4.17 shows the V_0 value of the trypsin enzyme under the given conditions. Since it is desired to obtain the maximum velocity (V_{max}) and Michaelis constant (K_m) values for the trypsin enzyme, it is necessary to perform kinetics experiments with also 0.2, 0.4, and 0.8 mM BAPNA under the same pH and salt conditions and find the V_0 values for each substrate concentration.

In Figure 4.18, concentration of p-nitroaniline versus time plots are given at four different substrate concentrations. In Table 4.4, the V_0 values obtained for each BAPNA concentration are given.

Table 4.4. V_0 values for free trypsin at 10 mM phosphate buffer at pH 7.5.

Substrate (BAPNA) Concentration (mM)	V_0 Values (millimole/L per min)
0.10	143.06 ± 0.01
0.20	251.14 ± 0.02
0.40	364.03 ± 0.01
0.80	388.28 ± 0.02

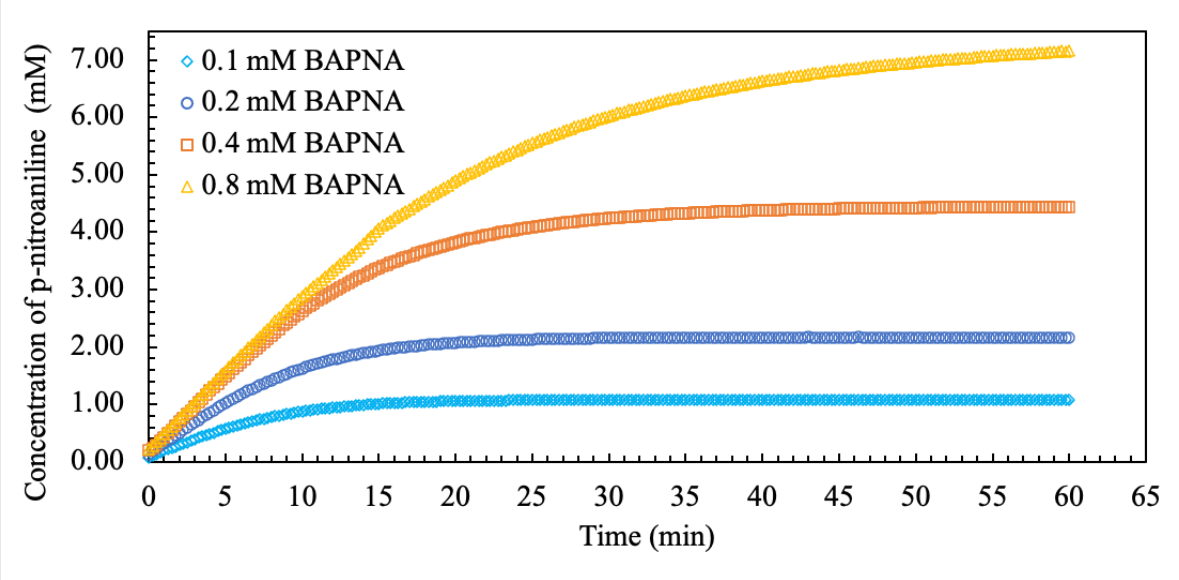


Figure 4.18. Product concentration vs time at pH 7.5 at 10 mM phosphate buffer.

In order to compare the activity of free trypsin with the encapsulated one, Lineweaver-Burke plots were needed to be drawn so as to obtain V_{max} and K_m values. This plot was drawn separately for each BAPNA concentration according to the expression of

$$\frac{1}{V_0} = \left(\frac{K_m}{V_{max}}\right)\left(\frac{1}{[S]}\right) + \left(\frac{1}{V_{max}}\right), \tag{4.1}$$

where K_m is Michaelis constant, V_{max} is maximum velocity of trypsin enzyme, V_0 is the initial rate, and [S] is the concentration of substrate. Since this equation is a linear

equation, a plot of $1/V_0$ versus 1/[S] should be drawn, which is called Lineweaver-Burke plot. Table 4.5 gives [S], 1/[S], V_0 and $1/V_0$ values for free trypsin in 10 mM phosphate buffer.

Table 4.5. 1/V0 and 1/[S] values for free trypsin at pH 7.5 and in 10 mM phosphate buffer.

Substrate (BAPNA) Concentration [S] (mM)	1/[S] (1/mM)	V_0 (millimole/L per min)	$1/V_0$ (L/millimole per min)
0.10	10.00	143.06	0.0070
0.20	5.00	251.14	0.0040
0.40	2.50	364.03	0.0027
0.80	1.25	388.28	0.0026

According to the data given in Table 4.5, a Lineweaver-Burke plot is drawn as shown in Figure 4.19.

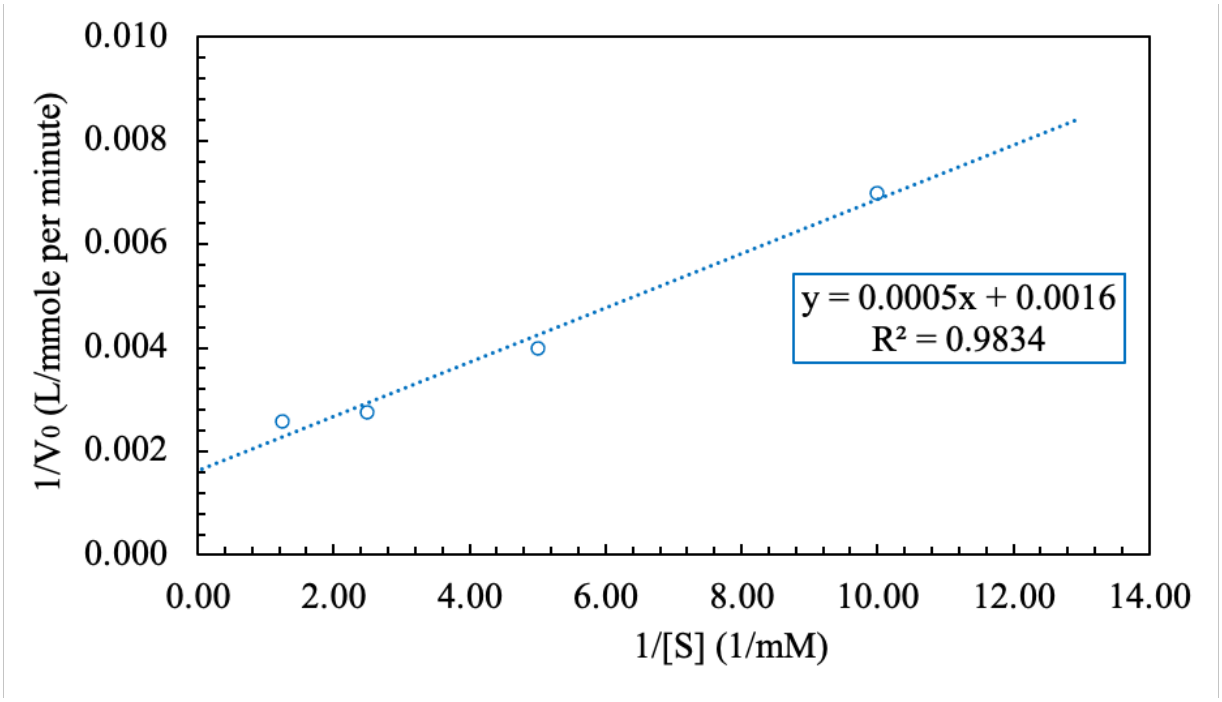


Figure 4.19. Lineweaver-Burke plot for free trypsin at pH 7.5 in 10 mM phosphate buffer.

In these plots, the vertical intercept gives the $1/V_{max}$ value, and the slope gives the K_m/V_{max} value. Thus, these values were calculated for each condition. In addition, if this linear line is extrapolated to the x-axis, the horizontal intersect gives $-1/K_m$ value. For Figure 4.19, the plot that has both horizontal and vertical intercepts as shown in Figure A.11 in the Appendix A. For the experiment given here, that is free trypsin in 10 mM phosphate buffer, $1/V_{max}$ value was 0.0016, and the V_{max} value, which gave the maximum velocity of this enzyme, was 625.00 millimole/L per min. The K_m/V_{max} obtained from the Lineweaver-Burke plot was 0.0005 for trypsin under these conditions. Since the V_{max} is known, the K_m was easily calculated as 0.3125 mM or free trypsin. In Table 4.6 given below, the V_{max} and K_m values of free and encapsulated trypsin enzymes at different salt concentrations calculated from Lineweaver - Burke plots are shown.

Table 4.6. V_0 and Km values for free and encapsulated trypsin.

	V_{max} (mmole/L per min)	K_m (mM)
Free trypsin in 10 mM phosphate buffer	625.0 ± 0.1	0.3125 ± 0.0012
Free trypsin in 10 mM phosphate buffer with 50 mM NaCl	476.2 ± 0.2	0.3333 ± 0.0025
Free trypsin in 10 mM phosphate buffer with 100 mM NaCl	500.0 ± 0.1	0.3000 ± 0.0019
Encapsulated trypsin in 10 mM phosphate buffer	120.5 ± 0.1	0.1927 ± 0.0014
Encapsulated trypsin in 10 mM phosphate buffer with 50 mM NaCl	86.2 ± 0.3	0.0948 ± 0.0017
Encapsulated trypsin at in mM phosphate buffer with 100 mM NaCl	80.6 ± 0.2	0.0565 ± 0.0016

K_m values indicate the binding strength between an enzyme and a substrate molecule. According to Xia et al., a drop in K_m value might a change in the active part of an enzyme [40]. In their study, trypsin enzyme was also used and its complexation with PDADMAC

was investigated. According to their results, when trypsin interacted with PDADMAC, its active part was not affected. Therefore, the K_m values were really close to each other.

In our study, active part of the free trypsin enzyme was not affected by the increasing salt concentrations since all K_m values were near to 0.3150 mM. However, K_m and V_{max} values were lowered for the encapsulated trypsin, so the encapsulation process seems to have affected the active part of the enzyme. The decrease in the maximum rate of the enzyme suggests an effect on the binding of the enzyme with the substrate, leading to a loss of activity. Meanwhile, a decrease in K_m values with salt concentration was observed for encapsulated trypsin. This can be explained by the fact the increase in salt concentration affects electrostatic interactions between the enzymes and the polyelectrolytes.

As mentioned above, V_{max} values are decreased due to the encapsulation process. Xia et al., also observed a decrease in trypsin's activity when trypsin formed a complex with PDADMAC [40]. They this decreases in V_{max} with a shift of the optimum pH value of trypsin due to complexation [40]. This explanation might also apply to our results although pH value and salt concentrations were different in our case. A second explanation might be such that negative amino acid residues in the active part of trypsin [54] might interact with the positively charged PDADMAC. This might inhibit the binding of the enzyme with the substrate.

Stability of the free and encapsulated trypsin enzyme were investigated by measuring activity at different time intervals (0, 1, 3, 7, 14, and 30 days). Activity tests were performed on these samples prepared from the same stock solutions prepared at the same day. Samples were kept at 4°C till the experiment day and was left to reach room temperature before the activity experiments. The V_{max} and K_m values are given in Table 4.7.

Table 4.7. V_{max} and K_m values for free and encapsulated trypsin at different time intervals.

	V_{max} (mmole/L per min)	K_m (mM)
0^{th} Day – Free Trypsin	625.2 ± 0.3	0.3125 ± 0.0016

Table 4.7. V_{max} and K_m values for free and encapsulated trypsin at different time intervals. (cont.)

	V_{max} (mmole/L per min)	K_m (mM)
1st Day – Free Trypsin	625.0 ± 0.2	0.3125 ± 0.0015
3rd Day – Free Trypsin	625.0 ± 0.2	0.3125 ± 0.0017
7th Day – Free Trypsin	588.2 ± 0.1	0.2941 ± 0.0019
14th Day – Free Trypsin	555.6 ± 0.2	0.1666 ± 0.0020
30th Day – Free Trypsin	454.5 ± 0.3	0.0454 ± 0.0014
0th Day – Encapsulated Trypsin	120.5 ± 0.1	0.1927 ± 0.0017
1st Day – Encapsulated Trypsin	120.5 ± 0.1	0.1924 ± 0.0014
3rd Day – Encapsulated Trypsin	120.4 ± 0.2	0.1920 ± 0.0013
7th Day – Encapsulated Trypsin	119.4 ± 0.1	0.1899 ± 0.0011
14th Day – Encapsulated Trypsin	115.8 ± 0.1	0.1756 ± 0.0012
30th Day – Encapsulated Trypsin	111.1 ± 0.3	0.1444 ± 0.0014

According to Table 4.7, there is a decrease in the activity of encapsulated trypsin with time. However, the drop in the activity of encapsulated trypsin enzyme is much slower than the decrease in the activity of free trypsin enzyme over time. Thus, we can conclude that encapsulation via complex coacervation can preserve the activity of the enzyme up to 30 days despite its lower starting activity.

4.5. Circular Dichroism Experiments

Circular dichroism is the most widely used device to predict the secondary structure of proteins. The stimulation of electronic transitions in amide groups is the primary mechanism used in the far UV circular dichroism (CD) spectroscopy of proteins and peptides. All measurements at this step were made between 190 and 250 nm. CD data also provides information about the alpha helix, beta sheet or random coil structures of proteins [55]. In this section, the effect of the encapsulation process on the secondary structure of trypsin was investigated with CD.

All the samples examined contain the same amount of trypsin, i.e., samples with trypsin, except for the pectin-PDADMAC coacervate sample, have 1 mg/mL trypsin. CD

data of the empty pectin-PDADMAC coacervates, without trypsin, was taken as a control. Between 250-190 nm, pectin-PDADMAC mixtures did not have any distinct peaks or troughs. On the other hand, the CD data of free trypsin was very intense. The intensity of the data for the encapsulated trypsin samples was reduced due to scattering by coacervate droplets. Encapsulated trypsin enzymes were studied in two different ways. The first was by carrying out of the CD process in suspension, and the second was by performing the CD process of the coacervate remaining at the bottom by centrifugation of this suspension.

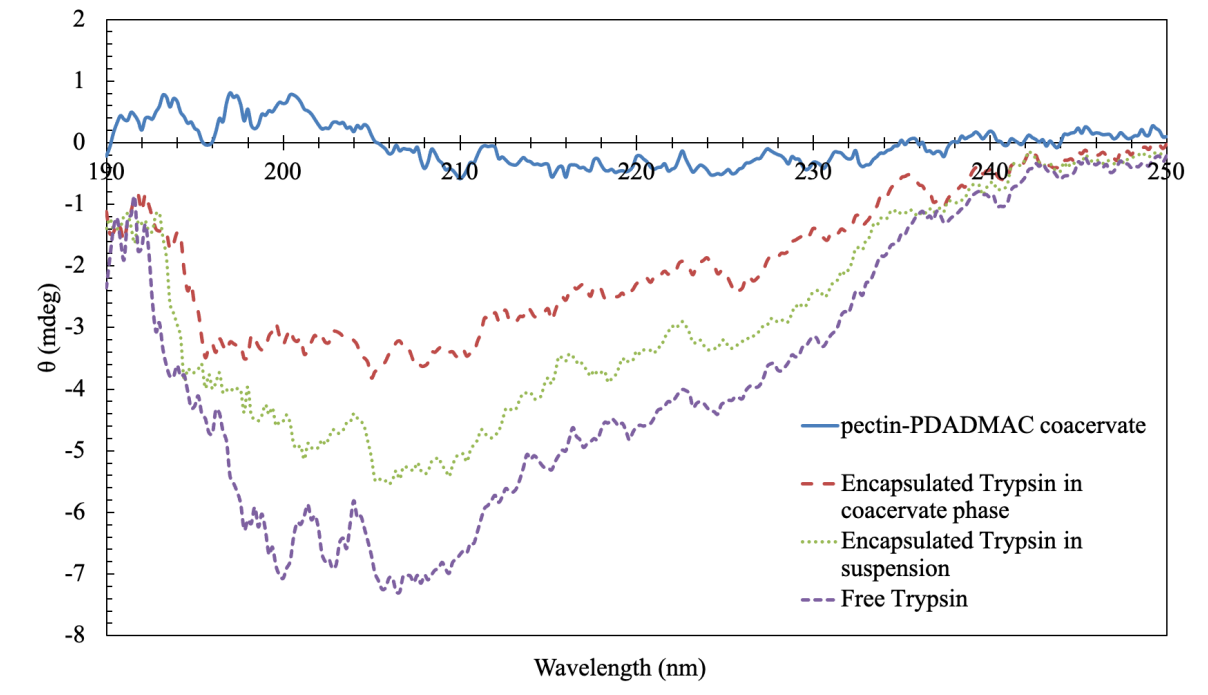


Figure 4.20. CD spectra for coacervates and trypsin samples at pH 7.5 in 10 mM phosphate buffer.

Referring to Figure 4.20, the secondary structures of encapsulated trypsin were affected by this encapsulation process. In fact, the intensity values being less than the free state can be explained by the reflection of light from the CD by the coacervate droplets formed [8]. The higher intensity of the suspension than the coacervate phase alone in Figure 4.20 may be due to the greater amount of free trypsin enzyme in the suspension sample. According to the CD, pectin-PDADMAC coacervate droplets change the secondary structure of trypsin enzyme but do not denature the protein. The fact that pectin-PDADMAC

coacervates do not cause denaturation of the encapsulated trypsin can be seen as positive result for this study.

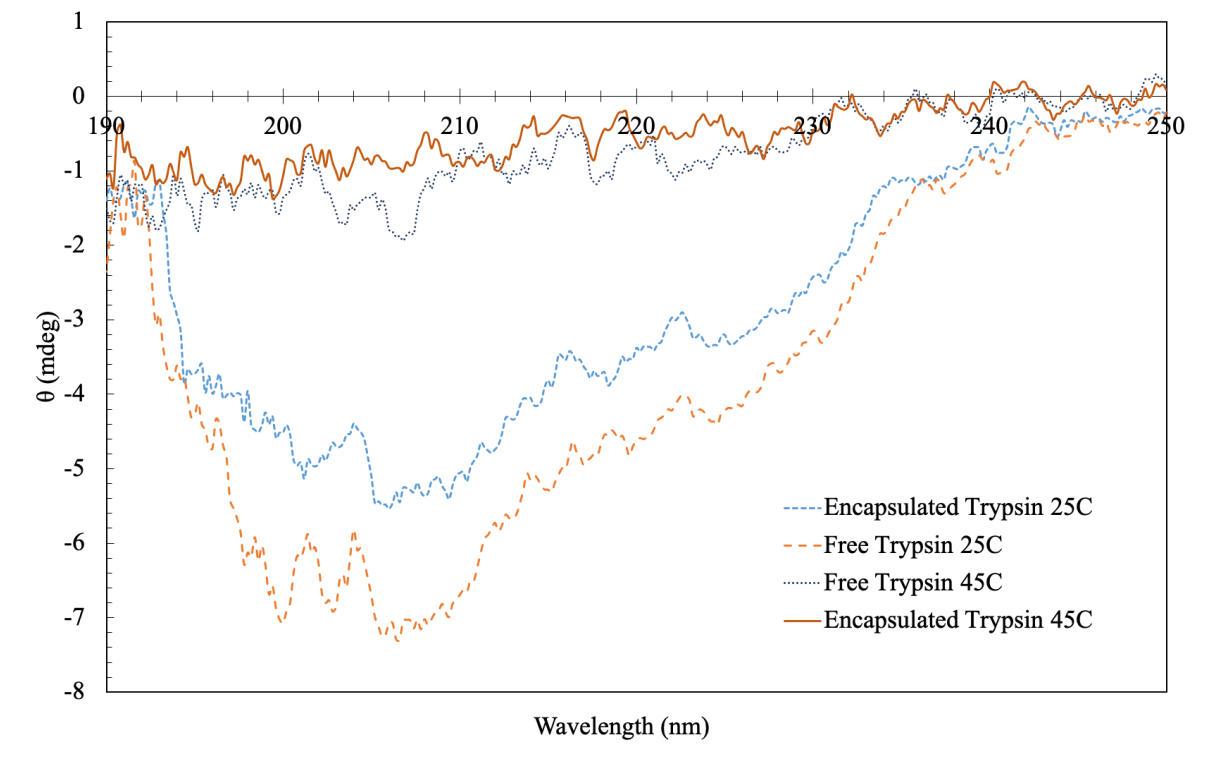


Figure 4.21. CD spectra for free and native trypsin samples at pH 7.5 in 10 mM phosphate buffer at 25ºC and 45ºC.

In Figure 4.21, the effect of temperature was examined. High temperature damages the natural secondary structure of trypsin and free trypsin data at the 25ºC and 45ºC can support this information. Here it can be said that the folded structure of the free trypsin changes completely when exposed to heat. The graphs for trypsin at these two different temperatures are quite different from each other. In addition, encapsulated trypsin exposed to high temperature could not preserve its secondary structure. It can be said that pectin-PDADMAC coacervates do not provide sufficient protection for trypsin enzyme at high temperature.; i.e., the data of encapsulated trypsin at 25°C and 45°C are different from each other. As seen in Zhao et al. study, the encapsulation process via coacervation is not a very effective method to protect proteins from heat [8]. In their study, PAA-PAH coacervate droplets could not be a very effective capsule for BSA protein.

5. CONCLUSION

This study aims to encapsulate the porcine enzyme trypsin, which has an optimum pH value of 7.5, into coacervate droplets formed between pectin, an anionic biopolymer, and PDADMAC, a cationic synthetic polymer. With potentiometric titration experiments, it has been shown that the pectin polymer is completely ionized at pH 7.5 at 0, 10, 150 mM NaCl, and all –COOH groups are negatively charged by donating protons. First, it has been proven that pectin-PDADMAC complex coacervates are formed at the desired pH value of 7.5. This was done both by turbidimetric titration experiments and by taking images with a light microscope. In addition, the effect of salt on coacervate droplets was observed with these methods. The salt screening effect was observed as the salt concentration increased. That is, as the salt concentration increased, both the number and size of coacervate droplets decreased.

After providing the formation of coacervate droplets, the encapsulation process was performed with the most suitable and high-capacity method. It was observed that the order of adding the materials, protein concentration, and concentration of the polymers affected encapsulation. The method made by first mixing pectin and trypsin and adding PDADMAC to the mixture gave the best encapsulation efficiency. The best encapsulation efficiency was observed with 73.9 % in the medium prepared by this addition method, containing 0.25 mg/mL trypsin and 0.50 mg/mL pectin-PDADMAC in 10 mM phosphate buffer. As salt is added to the medium, a decrease in encapsulation efficiency has been observed due to the salt screening effect.

The trypsin enzyme was prepared with the most efficient encapsulation method and the activity of the free trypsin enzyme was tested by using the BAPNA molecule as a substrate. Here, measurements were made based on the formation of the p-nitroaniline molecule. As a result of these experiments, it was observed that encapsulation decreased the activity of the trypsin enzyme and there was a decrease in K_m values. However, as a result of the activity experiments carried out on certain days, it was observed that the trypsin activity stayed constant upon encapsulation in comparison to the free enzyme.

Finally, circular dichroism experiments were carried out to see how the secondary structure of the protein was affected by the encapsulation process. According to the results, the secondary structure of the trypsin enzyme was affected by the encapsulation process and could not preserve its accessible conformation. In addition, as can be seen from the experiments at high temperature, coacervate droplets were not an effective method to protect the trypsin enzyme.

In this study, the encapsulation process was performed for porcine trypsin using the complex coacervation method, and how the enzyme reacted to this process functionally and structurally was investigated. For this reason, it has been a helpful study for those who want to encapsulate porcine trypsin.

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APPENDIX A: RESULTS OF OTHER EXPERIMENTS

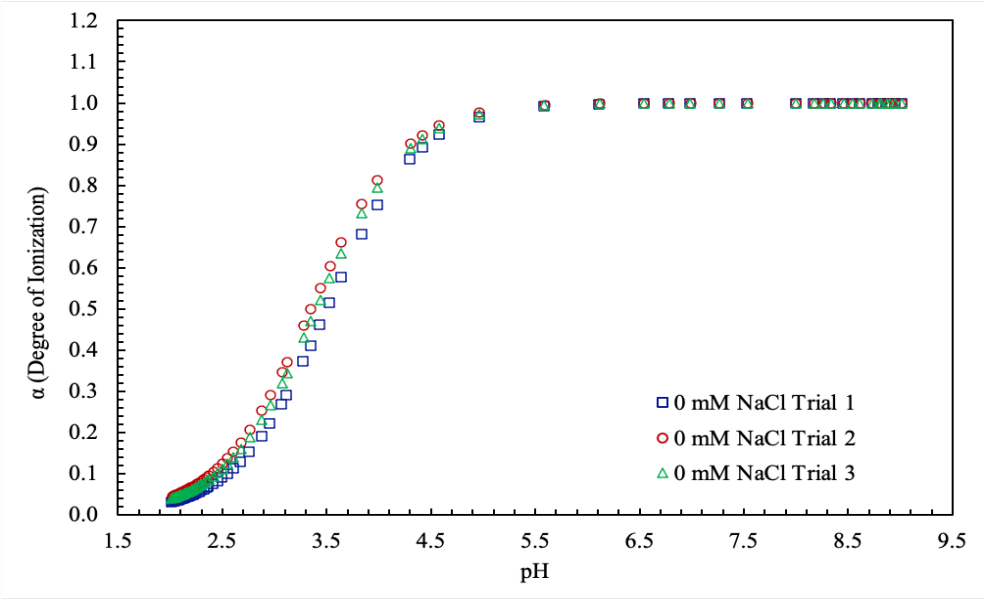

Figure A.1. pH titration profile of pectin at 0 mM NaCl.

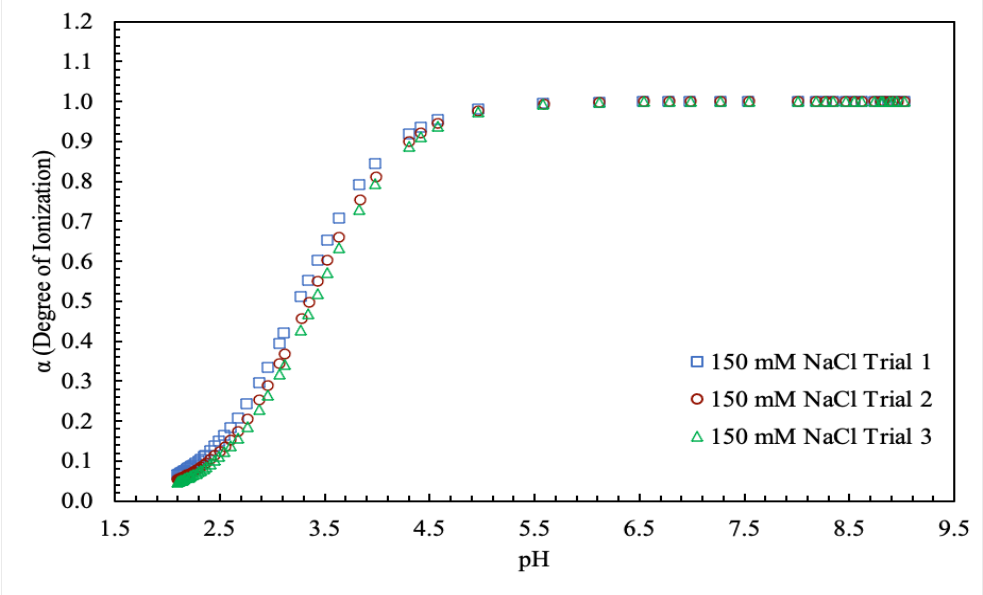

Figure A.2. pH titration profile of pectin at 150 mM NaCl.

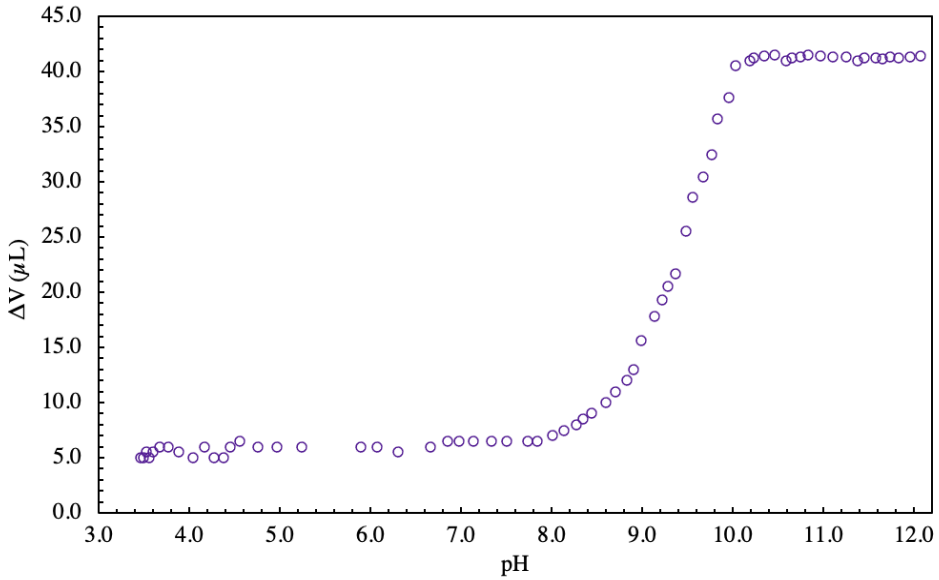

Figure A.3. Potentiometric titration plot for trypsin at 150 mM NaCl.

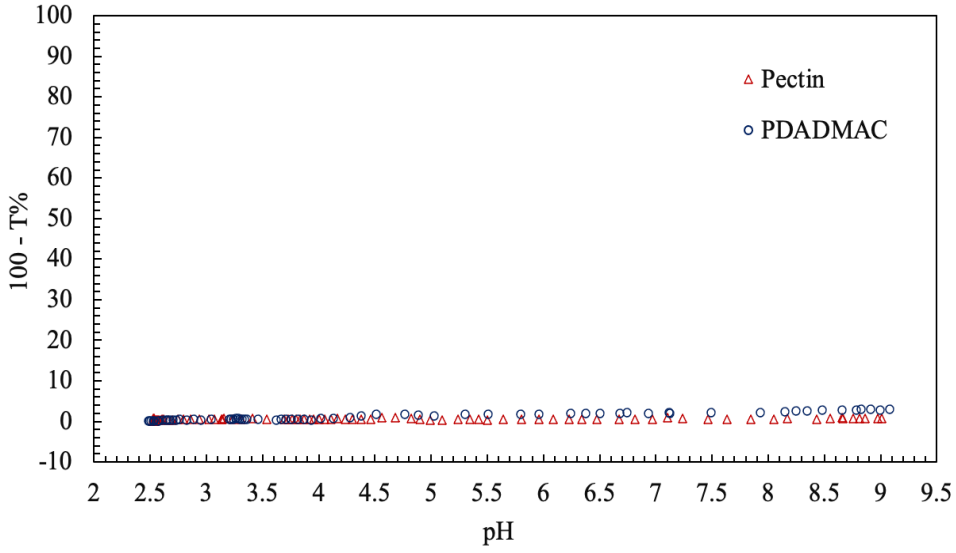

Figure A.4. Turbidimetric titration plots for pectin and PDADMAC separately at 10 mM NaCl.

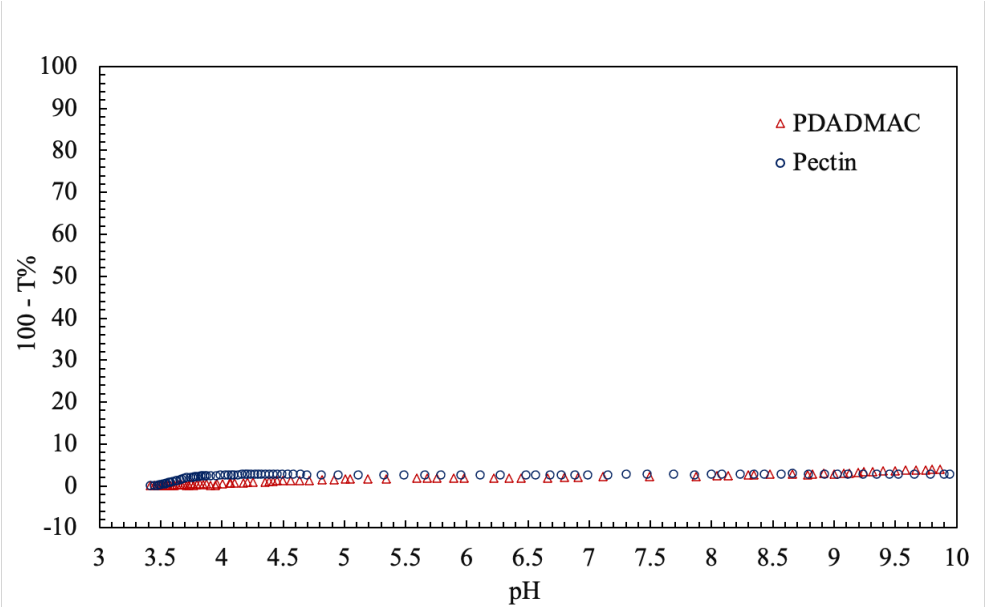

Figure A.5. Turbidimetric titration plots for pectin and PDADMAC separately at 400 mM NaCl.

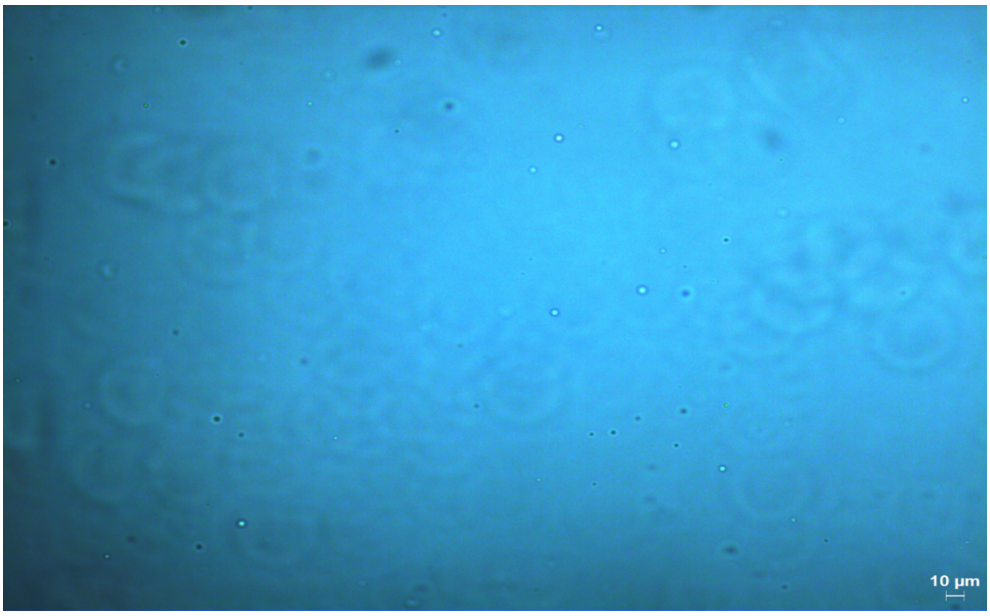

Figure A.6. Microdroplets of 0.5 mg/mL pectin – 0.5 mg/mL PDADMAC system at pH: 7.5 in 25 mM NaCl with 10 mM phosphate buffer, magnification 20X.

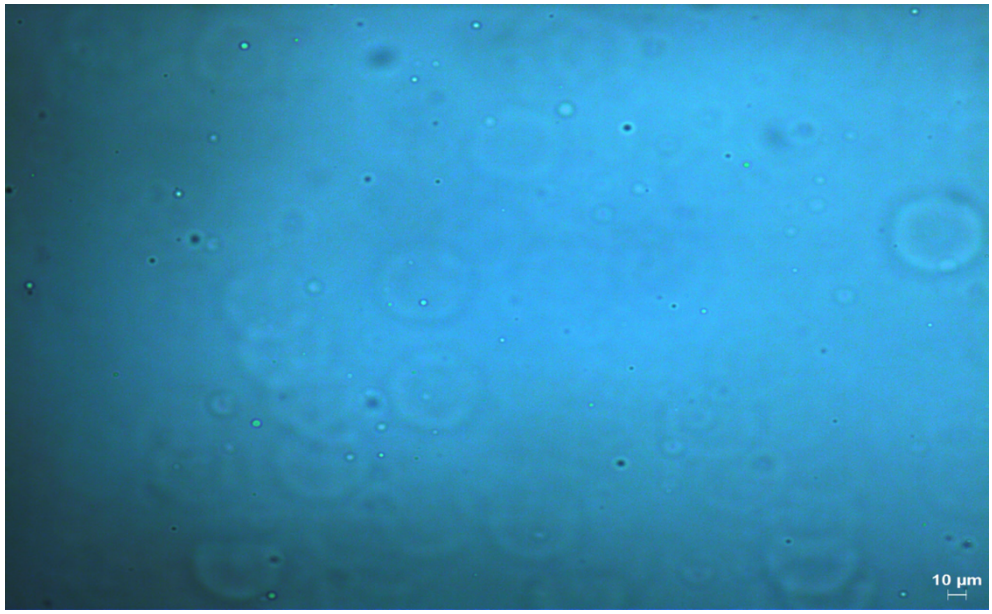

Figure A.7. Microdroplets of 0.5 mg/mL pectin – 0.5 mg/mL PDADMAC system at pH: 7.5 in 50 mM NaCl with 10 mM phosphate buffer, magnification 20X.

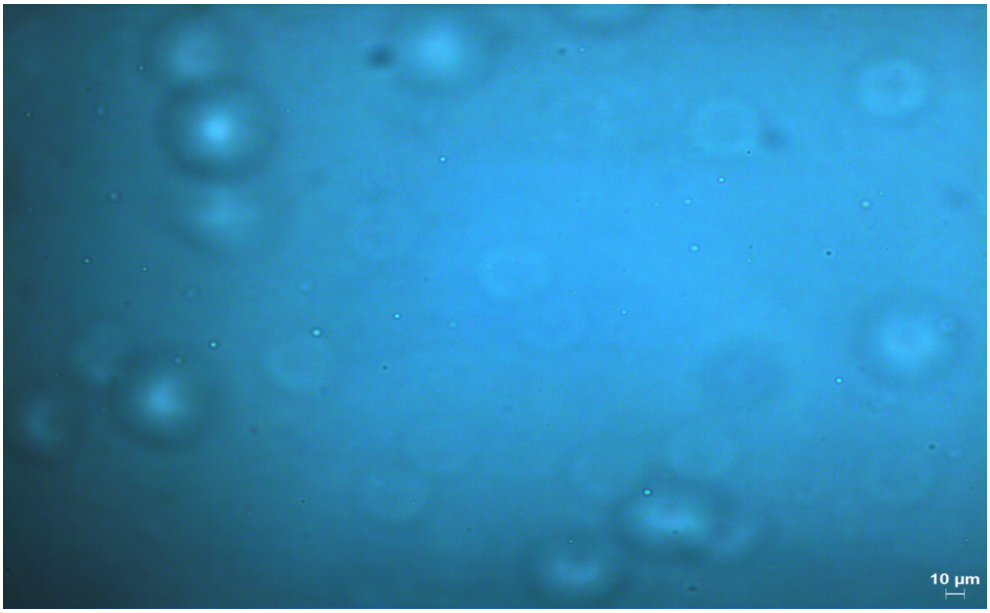

Figure A.8. Microdroplets of 0.5 mg/mL pectin – 0.5 mg/mL PDADMAC system at pH: 7.5 in 100 mM NaCl with 10 mM phosphate buffer, magnification 20X.

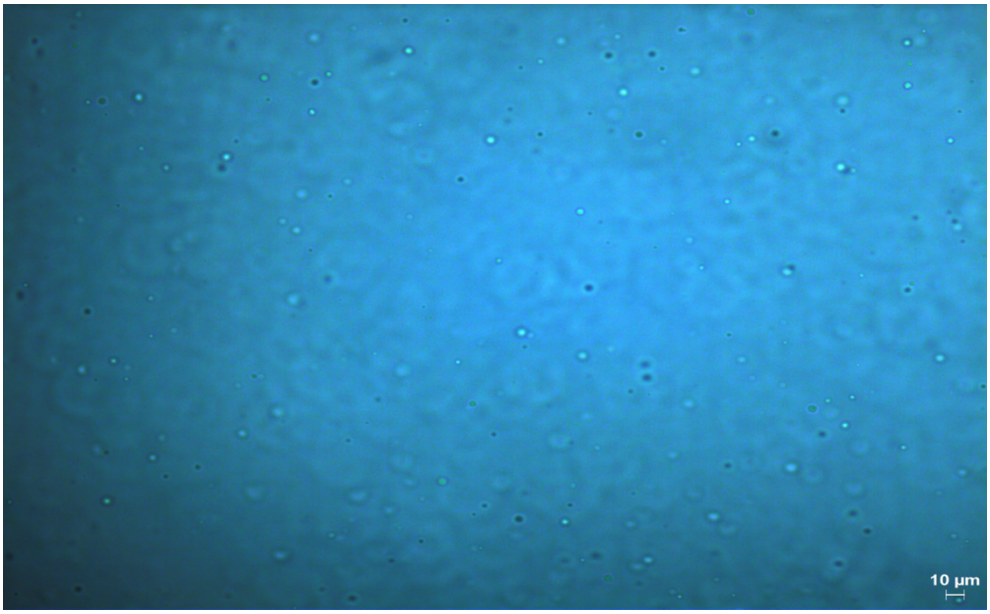

Figure A.9. Microdroplets of 0.5 mg/mL pectin – 0.5 mg/mL PDADMAC system at pH: 7.5 in 150 mM NaCl with 10 mM phosphate buffer, magnification 20X.

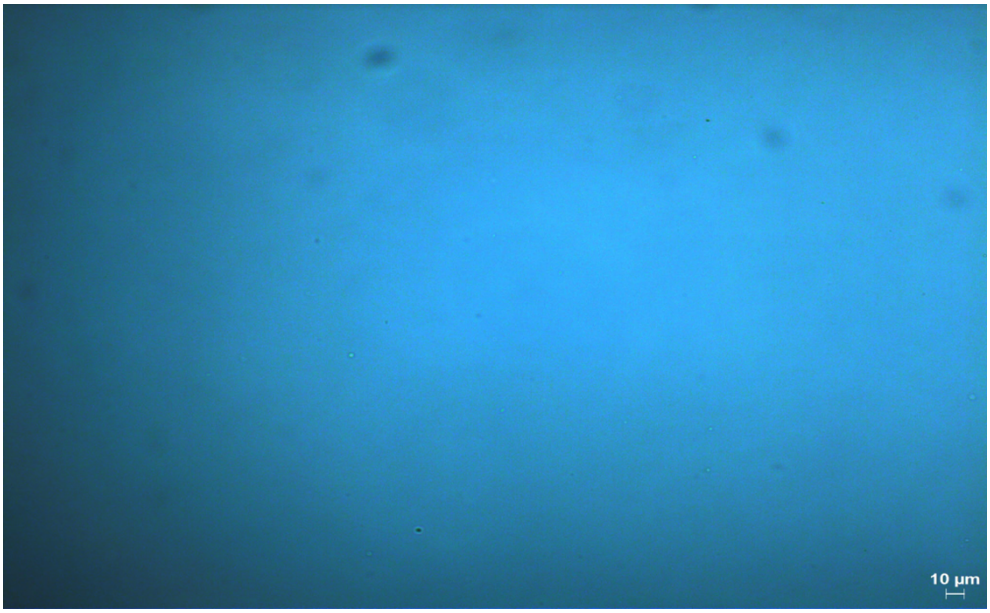

Figure A.10. Microdroplets of 0.5 mg/mL pectin – 0.5 mg/mL PDADMAC system at pH: 7.5 in 200 mM NaCl with 10 mM phosphate buffer, magnification 20X.

Table A.1. Encapsulation Efficiency percentages (EE %) at different mixing orders and salt concentrations.

Mixing Order EE%	0 mM NaCl	10 mM NaCl	25 mM NaCl	50 mM NaCl	100 mM NaCl	150 mM NaCl
Pectin-Trypsin/ PDADMAC	73.9 % ± 1.2	67.6 % ± 0.5	62.4 % ± 0.5	58.4 % ± 0.6	49.1 % ± 0.5	40.7 % ± 1.0
PDADMAC-Trypsin/ Pectin	68.6 % ± 0.5	64.6 % ± 0.8	59.4 % ± 0.4	56.1 % ± 0.5	42.7 % ± 1.0	37.9 % ± 0.5
Pectin-PDADMAC/ Trypsin	64.2 % ± 0.6	61.5 % ± 1.2	58.0 % ± 0.5	52.9 % ± 0.7	35.3 % ± 0.9	35.3 % ± 0.8

Table A.2. Encapsulation Efficiency percentages (EE%) at different trypsin concentrations and salt concentrations.

Protein Concentration EE%	0 mM NaCl	10 mM NaCl	25 mM NaCl	50 mM NaCl	100 mM NaCl	150 mM NaCl
0.25 mg/mL Trypsin	73.9 % ± 1.2	67.6 % ± 0.5	62.4 % ± 0.5	58.4 % ± 0.6	49.1 % ± 0.5	40.7 % ± 1.0
0.40 mg/mL Trypsin	66.2 % ± 0.6	64.4 % ± 0.6	60.9 % ± 0.7	57.4 % ± 0.4	38.9 % ± 0.3	27.5 % ± 0.5
0.50 mg/mL Trypsin	60.9 % ± 0.7	58.7 % ± 0.4	57.5 % ± 0.5	49.9 % ± 0.2	34.0 % ± 0.4	24.9 % ± 0.1

Table A.2. Encapsulation Efficiency percentages (EE%) at different trypsin concentrations and salt concentrations. (cont.)

Protein Concentration EE%	0 mM NaCl	10 mM NaCl	25 mM NaCl	50 mM NaCl	100 mM NaCl	150 mM NaCl
0.75 mg/mL Trypsin	53.9 % ± 0.5	50.6 % ± 0.3	47.6 % ± 0.2	45.9 % ± 0.3	16.9 % ± 0.2	15.5 % ± 0.2

Table A.3: Trypsin amount in coacervate and supernatant phases.

		0 mM NaCl	10 mM NaCl	25 mM NaCl	50 mM NaCl	100 mM NaCl	150 mM NaCl
0.25 mg/mL Trypsin	Total amount (mg)	2.856	2.856	2.856	2.856	2.856	2.856
	Coacervate phase (mg)	2.069 ± 0.008	1.550 ± 0.009	1.530 ± 0.010	1.343 ± 0.008	1.051 ± 0.008	0.921 ± 0.011
	Supernatant phase (mg)	0.746 ± 0.012	0.926 ± 0.008	1.074 ± 0.009	1.188 ± 0.005	1.455 ± 0.003	1.693 ± 0.004
0.40 mg/mL Trypsin	Total amount (mg)	5.000	5.000	5.000	5.000	5.000	5.000
	Coacervate phase (mg)	2.969 ± 0.004	2.560 ± 0.002	2.511 ± 0.001	2.314 ± 0.002	1.620 ± 0.002	1.222 ± 0.001
	Supernatant phase (mg)	1.691 ± 0.003	1.779 ± 0.002	1.956 ± 0.002	2.132 ± 0.004	3.058 ± 0.003	3.623 ± 0.002

Table A.3: Trypsin amount in coacervate and supernatant phases. (cont.)

		0 mM NaCl	10 mM NaCl	25 mM NaCl	50 mM NaCl	100 mM NaCl	150 mM NaCl
0.50 mg/mL Trypsin	Total amount (mg)	6.666	6.666	6.666	6.666	6.666	6.666
	Coacervate phase (mg)	3.654 ± 0.007	3.368 ± 0.006	3.241 ± 0.005	3.229 ± 0.005	1.579 ± 0.005	1.541 ± 0.006
	Supernatant phase (mg)	2.607 ± 0.008	2.753 ± 0.007	2.832 ± 0.007	3.341 ± 0.007	4.411 ± 0.006	5.005 ± 0.005
0.75 mg/mL Trypsin	Total amount (mg)	12.000	12.000	12.000	12.000	12.000	12.000
	Coacervate phase (mg)	6.122 ± 0.005	5.703 ± 0.003	5.489 ± 0.002	5.510 ± 0.001	1.453 ± 0.003	1.546 ± 0.002
	Supernatant phase (mg)	5.537 ± 0.005	5.930 ± 0.003	6.288 ± 0.005	6.494 ± 0.005	9.979 ± 0.001	10.142 ± 0.002

Table A.4. Encapsulation Efficiency % at different polyelectrolyte concentrations.

Polyelectrolyte Concentration EE%	0 mM NaCl	10 mM NaCl	25 mM NaCl	50 mM NaCl	100 mM NaCl	150 mM NaCl
0.25 mg/mL pectin-0.50 mg/mL PDADMAC	59.5 % ± 0.7	54.5 % ± 0.5	47.3 % ± 0.2	45.1 % ± 0.4	38.8 % ± 0.4	35.1 % ± 0.2

Table A.4. Encapsulation Efficiency % at different polyelectrolyte concentrations. (cont.)

Polyelectrolyte Concentration EE%	0 mM NaCl	10 mM NaCl	25 mM NaCl	50 mM NaCl	100 mM NaCl	150 mM NaCl
0.75 mg/mL pectin-0.50 mg/mL PDADMAC	70.6 % ± 0.5	62.5 % ± 0.4	56.9 % ± 0.4	49.4 % ± 0.5	42.8 % ± 0.4	38.0 % ± 0.4
0.50 mg/mL pectin-0.50 mg/mL PDADMAC	73.9 % ± 1.2	67.6 % ± 0.5	62.4 % ± 0.5	58.4 % ± 0.6	49.1 % ± 0.5	40.7 % ± 1.0
0.50 mg/mL pectin-0.25 mg/mL PDADMAC	66.4 % ± 0.5	59.0 % ± 0.6	55.6 % ± 0.2	50.5 % ± 0.5	42.0 % ± 0.4	38.8 % ± 0.4
0.50 mg/mL pectin-0.75 mg/mL PDADMAC	63.9 % ± 0.4	58.0 % ± 0.2	52.8 % ± 0.5	48.3 % ± 0.4	41.1 % ± 0.4	36.6 % ± 0.1

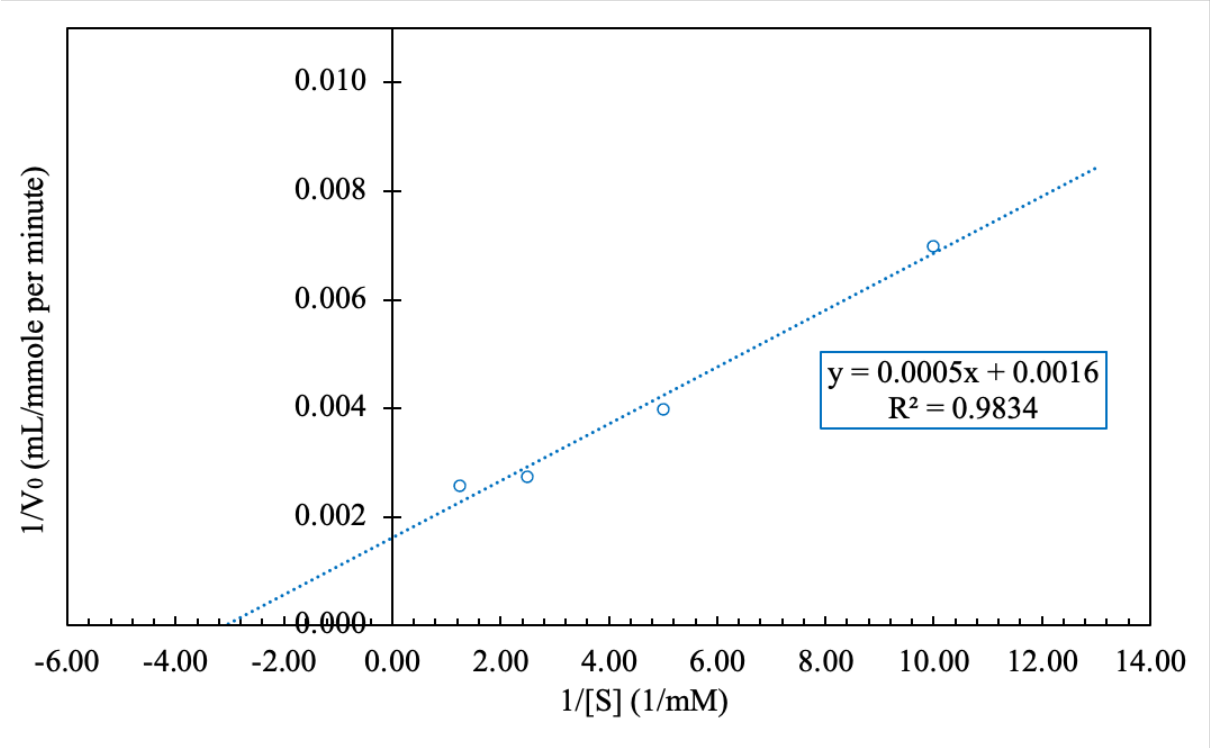

Figure A.11. Lineweaver-Burke plot with intercepts for free trypsin at pH 7.5 in 10 mM phosphate buffer.

APPENDIX B: PERMISSIONS

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