Trypsin Encapsulation within Pectin-Poly(diallyldimethylammonium Chloride) Complex Coacervates

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Abstract

Active substances like pharmaceuticals and enzymes can be shielded from a variety of unfavourable environmental conditions, including high pH, organic solvents, and chaotic agents, by employing complex coacervation as an encapsulation strategy. One of the goals of this research is to form complex coacervate droplets using pectin, a carbohydrate present in plant cell walls, and poly(diallyldimethylammonium chloride), a synthetic homopolymer. Electrostatic interactions between the positive charges in PDADMAC and the negative charges in pectin are the main factors for coacervation to assemble. Encapsulation of the trypsin enzyme within complex coacervates made of PDADMAC and pectin was examined as a function of mixing order of these polyelectrolytes and at various salt concentrations to learn more about protein encapsulation within complex coacervates. There were three different mixing orders used. We examined the effect of ionic strength on pectin-PDADMAC system by turbidimetric titrations at six different salt concentrations. Light microscopy allowed us to observe the formation of coacervate microdroplets at these salt concentrations. In order to determine the degree of ionization of pectin at the optimal pH for trypsin, which is 7.5, potentiometric titration studies were carried out on pectin. The most efficient mixing order is chosen for the subsequent experiments. The impact on enzyme encapsulation was also investigated by varying the amounts of trypsin and polyelectrolytes. Enzyme activity was assessed following the selection of the most efficient encapsulation technique. Circular dichroism studies were used to determine if the secondary structure of trypsin altered during encapsulation.

1. INTRODUCTION

Encapsulation is a technique for coating or entrapping a substance or a mixture of materials inside a different material or system. In this immobilization method, encapsulated materials such as drugs or enzymes constitute the core or payload phase. The material that forms the
coating is called membrane, capsule, carrier material or exterior phase\(^1\). Encapsulation is primarily used to protect core material from the external environment due to various factors such as high temperatures, organic solvents, and extreme pH levels. Aside from preservation of encapsulated material against harsh environment, other purposes of encapsulation include releasing key ingredient under controlled conditions, increasing the activity of the substance being encapsulated, masking offensive tastes and odours, solidifying liquid droplets, using toxic and harmful materials safely, and reducing the flammability and evaporative loss of liquids \(^2\).

The encapsulation technique has gained extensive interest in different industries and used in wide range of application areas such as pharmaceuticals \(^3\), food \(^4\), cosmetics and healthcare products \(^5\) and agriculture \(^6\). The encapsulation strategy, which finds use in several disciplines, offers various advantages. The benefits of the encapsulation approach, according to the literature, may be appreciated from the examples provided, particularly in the domains of the food industry \(^7\) and enzyme activity studies \(^8\). In the food industry, encapsulation is widely used to shield the components from heat, moisture and other harsh circumstances stated above and can increase the component’s stability and vitality.

Examining the outcomes of enzyme experiments reveals that encapsulation provides several benefits. The protection against hazardous external surroundings, increased enzyme endurance, shortened reaction time, and the fact that the enzyme remains in the reusable form are all the benefits of the encapsulation approach. The encapsulation technique has numerous advantages, but there are some drawbacks as well. For instance, certain active compounds can become unstable, some medications or enzymes might become inactive, or their efficacy can be diminished after encapsulation. Besides this, the process of isolating, purifying, and recovering active substances like proteins and drugs can be expensive.

General techniques for formation of microcapsules are classified into three different categories: physical methods, chemical methods, and physicochemical methods \(^10\). Physical procedures for formation of microcapsules involve mechanically combining the core and the coating materials by methods such as spray drying, spray cooling, freeze drying, and extrusion. Meanwhile, inclusion complexation and interfacial polymerization are examples for chemical methods of encapsulation of active agents into wall materials. In addition, coacervation, a.k.a. liquid-liquid phase separation, and liposome entrapment are the most used methods among the various physicochemical methods.
The process of coacervation, which is a type of liquid-liquid phase separation, is used to form microdroplets in a solution. The concentrated dense component is the coacervate phase, which consists of condensed liquid-like droplets. The supernatant phase is the diluted phase that is visible after a centrifugation. Simple and complex coacervations are the two distinct forms of coacervation. Simple coacervation process is driven by addition of small molecules, such as salts or organic solvents, into polymer solutions. In the presence of organic solvents or salts, the intramolecular hydrophobic interactions inside the polymer increase, leading to the collapse of the polymer. On the other hand, one of the driving factors of complex coacervation is electrostatic interactions between two oppositely charged particles. Aside from electrostatic interaction, entropically favourable counterion rearrangement, and non-specific interactions including hydrogen bonding and hydrophobic forces all play a vital role in the formation of complex coacervates \cite{13}. Components of coacervates so far involve polypeptides, nucleic acids, dendrimers, lipids, small inorganic molecules, and synthetic or biological polyelectrolytes \cite{11,12}. Numerous factors including macroion concentration, ionic strength, pH, and temperature of the medium, as well as polymer molecular weight, charge density, and chain stiffness, can affect the formation of complex coacervation \cite{14}.

Complex coacervation, meanwhile, has many different applications. For instance, it may be used in biosensors \cite{15}, medication delivery platforms \cite{16}, and the encapsulation of active ingredients like food flavours \cite{17} or enzymes \cite{18}. Furthermore, it has been suggested that the structure of membrane-bound organelles in the cell is based on complex coacervation \cite{19}. Instead of being sealed by lipid membranes, these distinct compartments are formed by the complexation of biomolecules like proteins. Compared to the encapsulation strategies such as hydrogels or liposomes, coacervation is advantageous since it does not require the use of organic solvents or other chemical reagents or heating of any ingredients \cite{8}.

In this study, pectin, a semi-flexible polysaccharide, is used as the anionic component of complex coacervates. Pectin is composed of (1 → 4) α-D-galacturonosyl and methyl ester units, and is found in the intermediate lamella and cell walls of plants. Apples and citrus fruits are frequently used to extract pectin. Natural pectin has an esterification level between 75 and 80%. The degree of esterification is defined as the proportion of methyl-esterified galacturonic acid groups to the total galacturonic acid groups \cite{23}. In solution, non-esterified carboxyl groups can dissociate, resulting in the formation of a negative charge. Pectin has a charge spacing of 6.5 Å.
and a persistence length of 7.5 nm \cite{24,25}. Meanwhile, poly(diallyldimethylammonium chloride (PDADMAC), is chosen as the cationic polyelectrolyte for complex coacervation with pectin. PDADMAC is a linear polyelectrolyte with a persistence length of 3 nm \cite{26} and charge spacing of 6.2 Å. PDADMAC is utilised as a flocculant in water facilities. Its ability to flocculate is a result of high charge density, which encourages the agglomeration of suspended particles. This feature makes PDADMAC particularly successful in flocculating, decolouring, eliminating algae, and removing organics like humus.\cite{27}.

In this study, trypsin, a serine protease, was immobilized as the model enzyme in pectin-PDADMAC complex coacervates, and we examined how this encapsulation procedure influenced the activity of the enzyme. Experiments were conducted at various salt concentrations to determine the effect of ionic strength. We also investigated how the addition order of the polyelectrolytes and the enzyme affect the efficiency of encapsulation. Lastly, the secondary structure of the encapsulated trypsin was determined by circular dichroism.

2. MATERIALS & METHODS

a. Materials

Trypsin from porcine pancreas (Mw 23.8 kDa) was purchased from Sigma Aldrich (Schnelldorf, Germany) as lyophilized powder. N-α-Benzoyl-DL-arginine 4-nitroanilide hydrochloride was purchased from Sigma Aldrich as a substrate of trypsin. P-nitroaniline was purchased from Sigma Aldrich as a product of the trypsin’s activity mechanism. Poly (diallyldimethylammonium chloride) (PDADMAC, Molecular weight = 100,000 – 200,000 g/mole as supplied by the manufacturer) was purchased from Sigma Aldrich (Schnelldorf, Germany). The dynamic viscosity of PDADMAC was supplied by the manufacturer as 110 cp. Potassium salt of pectin from citrus fruit (Molecular weight = 82 kDa) with a degree of esterification 61% and galacturonic acid composition of 87% (as supplied by Sigma, the manufacturer) was graciously donated by late Prof. Paul Dubin. Sodium phosphate monobasic monohydrate (NaH₂PO₄·H₂O) was purchased from VWR Life Science (Ohio, Germany) and di-sodium hydrogen phosphate (Na₂HPO₄) was purchased from Merck (Darmstadt, Germany). 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.
b. Preparation of Solutions

PDADMAC was dialyzed for three days in SnakeSkin plated dialysis tubing with 10.000 MWCO, Thermofisher, USA), and then dialyzed before use. Solutions of PDADMAC, pectin and trypsin were prepared in 10 mM phosphate buffer or in 10 mM phosphate buffer with 10, 25, 50, 100, 150, 200 or 400 mM NaCl at pH 7.5 for encapsulation efficiency and activity experiments. The pH of solutions was adjusted with 0.1 N, 1 N HCl or 0.1 N, 1 N NaOH. For the turbidimetric and potentiometric titrations, polymers were prepared in 10, 25, 50, 100, 200, 400 mM NaCl solutions. For experiments where encapsulation efficiency and activity were measured, polymer and trypsin solutions were prepared in 10 mM phosphate buffer with required NaCl solutions (0, 10, 25, 50, 100, 150 mM). For enzyme activity reactions, the substrate N-Benzoyl-L-Arginine-p-Nitroanilide (BAPNA) and the product of this reaction p-nitroaniline were dissolved in 2 mM dimethyl sulfoxide (DMSO) while stirring for 30 minutes. All polymer solutions were mixed for a minimum of two hours, while protein solutions were mixed for at least 30 minutes. For activity experiments, BAPNA was prepared in 0.1, 0.2, 0.4, 0.8 mM concentrations by diluting it in the following salt solutions: 10 mM phosphate buffer, 10 mM phosphate buffer/50 mM NaCl, 10 mM phosphate buffer/100 mM NaCl. A calibration curve was prepared for p-nitroaniline with concentrations of 0.01, 0.05, 0.1, 0.2, 0.5, 0.75, 1.0 and 1.5 mM in the three NaCl/buffer solutions mentioned above. All solutions were filtered by cellulose acetate disposable syringe filters of pore size 0.45 μm (Labmarker, Istanbul, Turkey). All experiments were repeated at least three times to obtain reproducible results.

c. Potentiometric Titrations

The degree of ionization of pectin was determined by potentiometric titrations in 10 and 150 mM NaCl, using Mettler Toledo pH electrode Inlab Expert Pro and Thermo Scientific temperature probe under inert gas (nitrogen). The initial pH of pectin and blank solutions was adjusted to 2.0 and the end point of the titration was pH 9.0. The concentration of the polymer was 0.5 mg/mL. Potentiometric titration was also used to find the isoelectric point (pI) of trypsin in 10 and 150 mM NaCl solutions. The concentration of trypsin was 0.25 mg/mL. Before the titration, the pH of the trypsin and blank (protein-free) solutions was brought to 3.5 with 0.1 N HCl and 1 N HCl. Using a microburette (Gilmont Instruments) (Illinois, USA), the pH of the polymer and blank solutions was raised to 12.0 by adding 0.1 N NaOH. pKₐ and pI values were
determined from the pHs corresponding to the midpoint of \( \Delta V \) (\( V \): volume of added NaOH, \( \Delta V = V_{\text{polymer}} - V_{\text{blank}} \)). Degrees of ionization values (\( \alpha \)) were calculated by Henderson-Hasselbalch equation which is shown below in Equation 1:

\[
pK_a = p\text{H} + \log \frac{1-\alpha}{\alpha}
\]

(1)

d. Turbidimetric Titrations

Turbidity measurements were made at 420 nm with a Brinkmann PC 950 probe colorimeter with a 2.0 cm path-length fiber optics probe. Turbidity (\( \tau \)) is expressed as 100 - T\%, where T is the transmittance. Samples for turbidimetric titrations were prepared by mixing 1.0 mg/mL PDADMAC stock solution into 1.0 mg/mL pectin solution which were prepared in 10, 25, 50, 100, 200 and 400 mM NaCl. After the polymers were mixed in a 1:1 (by w/v) ratio, the initial pH of mixture was adjusted to 1.7 by using 1 N HCl and the experiment was finished at pH 9.5. The turbidity experiments were repeated from pH 9.0 to 1.7 to check whether the titration was reversible. The transmittance was recorded at every 60 s after 0.1 N NaOH addition. All experiments were repeated three times for reproducibility.

e. Light Microscopy

The coacervate formation between pectin and PDADMAC was observed using a light microscope (Leica DM6000M). Pectin/PDADMAC coacervates were prepared in phosphate buffer with added salt (NaCl) to keep the pH of the medium constant at 7.5. Polymer solutions were prepared using the same concentrations of salt and buffer as in the turbidity experiments. Since the coacervate droplets were very mobile, vaseline was placed between the slide and the lamella before a picture was captured.

f. Encapsulation Efficiency Experiments

Stock solution of trypsin (2 mg/mL) were prepared in 10 mM phosphate buffer or buffer with 10, 25, 50, 100, 150 mM NaCl at pH 7.5. To encapsulate trypsin in PDADMAC-pectin coacervates, three different mixing orders were investigated: (a) trypsin solution was first added into PDADMAC solution, with 30-minute stirring to form trypsin-PDADMAC complexes,
followed by addition of pectin solution into trypsin-PDADMAC mixture with further stirring for 30 minutes to prepare trypsin-PDADMAC/pectin coacervates; (b) trypsin solution was added into pectin solution, with magnetic stirring for 30 minutes to form trypsin-pectin complexes, followed by addition of PDADMAC solution into trypsin-pectin mixture with further stirring for 30 minutes to prepare trypsin-pectin/PDADMAC coacervates; (c) PDADMAC solution was added into pectin solution to form PDADMAC-pectin coacervates with magnetic stirring for 30 minutes, followed by the addition of trypsin stock solution into PDADMAC-pectin mixture with further stirring for 30 minutes to prepare PDADMAC-pectin/trypsin coacervates. 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.

Effects of different concentrations of PDADMAC (0.25, 0.50, 0.75 mg/mL), pectin (0.25, 0.50, 0.75 mg/mL) and trypsin (0.25, 0.40, 0.50, 0.75 mg/mL) on encapsulation efficiency were investigated by using UV-Vis spectroscopy. 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. All experiments were conducted in triplicate.

**g. Measuring the Concentration of Trypsin**

All coacervate samples were centrifuged (Hermle Z206 A) for 1 h at 4000 rpm. After centrifugation, the supernatant phase was removed using a micropipette and the coacervate phase was left in the bottom of the centrifuge tubes. Mass of the coacervate phase as well as the volumes of the supernatant and coacervate phases were measured. UV-Vis spectrophotometer (Shimadzu UV-1700) was used to determine the trypsin content in both the supernatant and the coacervate phases. To directly measure the amount of protein in the coacervate phase, the coacervates were broken up with 2 M NaCl and the trypsin was released. Measurements were carried out at 280 nm. The encapsulation percentage of trypsin in the pectin-PDADMAC coacervates was calculated according to Equation 2 and Equation 3.
Encapsulation % = \frac{\text{total mass of the protein−mass of the protein in the supernatant phase}}{\text{total mass of the protein}} \quad (2)

Encapsulation % = \frac{\text{mass of the protein in the coacervate phase}}{\text{total mass of the protein}} \quad (3)

h. Activity Experiments of Free and Encapsulated Trypsin

Enzymatic activity of trypsin was measured using N-Benzoyl-L-Arginine-p-Nitroanilide (BAPNA) as the substrate at 25°C. Trypsin is a serine protease that cleaves peptide links in proteins. p-Nitroaniline (pNa) and N-Benzoyl-L-Arginine (BA) are generated when by trypsin catalyzes the hydrolysis of the amide link in N-Benzoyl-L-Arginine-p-Nitroanilide (BAPNA)\textsuperscript{[28]}. After determining the optimal encapsulation technique, the activity of encapsulated trypsin was evaluated using UV/Vis spectrophotometry. As the stock solution, substrate (BAPNA) 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.

Due to the optimal pH of the trypsin enzyme, all experiments were performed at pH 7.5. A multiplate UV/Vis spectrophotometer (Molecular Devices SpectraMax i3) was used to measure the change in optical density at 410 nm due to the absorption maximum of p-Nitroaniline (pNa). Yellow colour formation was observed at the end of activity experiments. Lineweaver-Burke plots were drawn to calculate $V_{\text{max}}$ and $K_m$ values of the native and encapsulated trypsin enzymes. Activity experiments were performed in a 96-well plate (TPP Tissue Culture Test Plate, Trasadingen, Switzerland). Optical density (OD) of p-nitroaniline, which is the product of the reaction, was measured every 25 seconds for one hour for the free trypsin and every 25 seconds for two hours for the encapsulated trypsin. A calibration curve was prepared for p-nitroaniline to convert ODs to concentrations (mM). The slope of the plot for the concentration of the product (p-nitroaniline) versus reaction time at time zero was determined to find the initial velocity ($V_0$) at four different substrate concentrations (0.2, 0.4, and 0.8 mM BAPNA).
Lineweaver-Burke plots were then drawn in order to determine the maximum velocity \( (V_{\text{max}}) \) and Michaelis constant \( (K_m) \) values for the free trypsin and the encapsulated trypsin. This plot was drawn separately for each BAPNA concentration according to the expression below

\[
\frac{1}{V_0} = \left( \frac{K_m}{V_{\text{max}}} \right) \left( \frac{1}{[S]} \right) + \left( \frac{1}{V_{\text{max}}} \right)
\]

where \([S]\) is the concentration of substrate.

Activity measurements at various time points \((0, 1, 3, 7, 14, \text{ and } 30 \text{ days})\) were performed on samples prepared on the same day. The samples were kept at 4°C until the analysis time, and were allowed to reach room temperature before the activity measurements. All experiments were conducted in triplicate.

i. Circular Dichroism Experiments

Trypsin samples at 1 mg/mL were prepared for circular dichroism (CD) experiments to investigate the secondary structure of trypsin. Firstly, CD spectra of ellipticity, \( \theta (\text{mdeg}) \), versus wavelength (nm) were obtained for coacervate microdroplets consisting of 0.5 mg/mL pectin and 0.5 mg/mL PDADMAC, and then obtained for 1 mg/mL free trypsin and encapsulated trypsin. For encapsulated trypsin, the CD spectra of the suspension (before centrifugation) and the coacervate phase (after centrifugation) 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 25°C and 45°C. All measurements were made between 250 and 190 nm. All samples were prepared as described above.

3. RESULTS AND DISCUSSION

a. Potentiometric Titration

In this work, PDADMAC and pectin were chosen to make complex coacervate droplets. Pectin is a biological heteropolysaccharide that includes both esterified (-COOCH₃) and non-esterified carboxyl groups (-COOH). Depending on the pH of the medium, these non-esterified carboxylic groups may carry negative charges. Potentiometric titration experiments were performed to
determine the degree of ionization (α) of the pectin. Figure 1 shows the dependence of α on pH at 0, 10, and 150 mM NaCl. The pK_a value for pectin was determined to be 3.42 ± 0.08, 3.67 ± 0.02 3.33 ± 0.04 for 0, 10, 150 mM NaCl respectively. According these results, pectin was found to be 100% deprotonated at pH = 7.5, which is the optimum pH for trypsin.

Figure 1: Degree of ionization values for pectin at different salt concentrations: (A) 0 mM NaCl, (B) 10 mM NaCl, (C) 150 mM NaCl.

The potentiometric titration experiments for trypsin used the same procedure as for pectin to determine the isoelectric point of trypsin enzyme. Figure 2 shows the ΔV versus pH graph, where ΔV corresponds to the volume difference for titration with 0.1 N NaOH between blank (protein-free) and trypsin solutions in 10 and 150 mM NaCl. The isoelectric point for trypsin is determined as 9.8 ± 0.10 in 10 mM NaCl and 9.5 ± 0.10 in 150 mM NaCl. Thus, it can be concluded that that the salt concentration very slightly affects the isoelectric point of trypsin. At pH 7.5, trypsin has a positive net charge, which will contribute favourably to its interaction with positively charged PDADMAC.
b. Turbidimetric Titrations and Light Microscopy

Turbidity (100 - Transmittance, T, %) at different pHs allow us to distinguish no-interaction regions, where turbidity is close to zero, from regions of interaction, where turbidity is greater than zero. First we present turbidity vs. pH plots for pectin and PDADMAC separately in 10 mM and 400 mM NaCl (Figure 3A & B). Since the turbidity values were nearly zero for these polyelectrolytes regardless of the salt concentration, it was concluded that neither pectin nor PDADMAC aggregated within the pH range from 1.7 to 9.0.

Figure 3: Turbidity (100-T%) versus pH plots. (A) Turbidity plots for pectin and PDADMAC separately from low to high pH at 10 mM NaCl, (B) Turbidity plots for pectin and PDADMAC separately from low to high pH at 400 mM NaCl, (C) Turbidity plots for pectin-PDADMAC
mixtures from low to high pH at different salt concentrations, (D) Turbidity plots for pectin-PDADMAC mixtures from high to low pH at different salt concentrations.

Then turbidity experiments were also carried out after mixing the two polyelectrolytes at 10, 25, 50, 100, 200, and 400 mM NaCl (Figure 3 C and D). Regardless of performing the titration from low to high pH or from high to low pH, turbidity values were almost zero at 400 mM NaCl. This result was expected since salt ions contribute to charge screening of the polyelectrolytes, and weakened the electrostatic interaction between them. On the other hand, lower salt concentrations favour the formation of complex coacervates, enhancing the electrostatic interaction between polyelectrolytes with opposing charges. For example, as pH was increased from 1.7 till 2.5 at 200 mM NaCl, a clear solution was observed, which was followed by a milky, turbid solution above pH 2.5. Meanwhile, pectin/PDADMAC mixture precipitated at pH ≥ 2.5 and salt concentrations of 10, 25, 50, and 100 mM NaCl. The clear solution observed below this pH indicated that pectin and PDADMAC did not interact with one another as pectin was only 10 ± 2 % deprotonated (charged) at pH 2.5. When the turbimetric titrations were done by changing the pH from pH 9.0 to 2.6 at 10, 25, 50, 100, and 200 mM NaCl, a turbid solution was formed. This cloudy solution turned into a precipitate between pH 2.6 and pH 2.4. Below pH 2.4, a clear solution was observed indicating dissolution of pectin/PDADMAC precipitates.

Turbidity vs. pH plots in Figure 3 can be used to understand the phase behaviour of pectin/PDADMAC system. The pH where turbidity deviates from zero in the low turbidity range of 0-5% is called the pH for the onset of soluble (primary) complex formation; i.e. pHcrit. Meanwhile, the pH where turbidity shows a sharp increase in the larger turbidity range such as 0-100%, is called the pH for the onset of phase separation, a.k.a. pHθ[29]. Figure 4 represents the results for the pectin/PDADMAC system at different salt concentrations. At all salt concentrations, pHcrit is observed at more acidic pHs than pHθ. The narrow pH range between pHcrit and pHθ indicates the high charge complementarity between pectin and PDADMAC. Both pHcrit and pHθ with the salt concentration, which leads to the conclusion that both soluble complex formation and phase separation are driven by long-range electrostatic interactions.
Figure 4: $pH_{\text{crit}}$ and $pH_\theta$ for pectin-PDADMAC system at different NaCl concentrations.

In order to determine if coacervate droplets formed at pH 7.5, which is the optimum pH for trypsin, samples were examined under a light microscope in the presence of 10 mM phosphate buffer at various salt concentrations including 10, 25, 50, 100, 150, 200, and 400 mM NaCl (Figure 5). As can be seen from these pictures, adding salt to the medium causes an observable reduction in the quantity of coacervate droplets. Consequently, light microscopy and turbidimetric titration results point out to the fact that coacervate microdroplets are readily formed via pectin-PDADMAC system to encapsulate trypsin at its optimum pH. 200 and 400 mM NaCl concentrations were omitted in the following experiments on enzyme activity since the turbidity values and the quantity of coacervate microdroplets were low at these salt concentrations.
Figure 5: Microdroplets of 0.5 mg/mL pectin - 0.5 mg/mL PDADMAC mixture at pH: 7.5 in 10 mM phosphate buffer with (A) 0 mM NaCl, (B) 10 mM NaCl, (C) 25 mM NaCl, (D) 50 mM NaCl, (E) 100 mM NaCl, (F) 150 mM NaCl, (G) 200 mM NaCl, (H) 400 mM NaCl, magnification: 20X.

Figure 6 also provides information on the average size of the coacervate microdroplets imaged by light microscopy at different salt concentrations. As the salt concentration was increased from 10 mM phosphate buffer in the absence of NaCl to 10 mM phosphate buffer in the presence of 400 mM NaCl, a drop in the size of the droplets was observed from $5.76 \pm 1.43 \mu m$ to $3.09 \pm 0.83 \mu m$. This result is also attributed to the charge screening effect.
c. Encapsulation Efficiency

Experiments at different mixing orders, trypsin concentrations, trypsin:PDADMAC weight ratios were carried out to determine the highest encapsulation efficiency according to Eqn. 2 in pectin-PDADMAC coacervates (Figure 7). To maximize the encapsulation efficiency, experiments were conducted by combining the three macromolecules in three different orders: (i) mixing the PDADMAC and trypsin solutions first, and then adding the pectin solution on the PDADMAC-trypsin mixture (PDADMAC-trypsin/pectin); (ii) combining PDADMAC and trypsin solutions first, and then adding the pectin solution on the PDADMAC-trypsin mixture (PDADMAC-trypsin/pectin); (iii) introducing the trypsin solution into the already mixed pectin-PDADMAC system (pectin-PDADMAC/trypsin). The results in Figure 7A show that when the NaCl concentration was increased, the encapsulation efficiency dropped. Since the size and number of droplets decreased with salt, the amount of encapsulated trypsin also got lower.
Regarding the mixing order, the trypsin-PDADMAC/pectin and pectin-PDADMAC/trypsin ternary complexes did not encapsulate trypsin as effectively as pectin-trypsin/PDADMAC complexes at pH 7.5 at all salt concentrations (Figure 7A). At this pH, pectin is 100% negatively charged (Figure 1) while PDADMAC is positively charged since the latter has quaternary amine groups. Meanwhile, pH 7.5 is around two pH units lower than the isoelectric point of trypsin (Figure 2), indicating an overall positive charge for this protein. The higher electrostatic attraction between fully anionic pectin and net positively charged trypsin compared to that between PDADMAC and trypsin would lead to a greater amount of coacervation, which could increase the encapsulation efficiency.

The lowest encapsulation efficiency was observed for the pectin-PDADMAC/trypsin system since pectin and PDADMAC have similar linear charge densities and molecular weights, leading to a higher degree of charge complementarity. Therefore, it is less likely for the trypsin to bind to the neutral pectin-PDADMAC complex. According to the electrostatic potential distribution of porcine trypsin[30], the protein has a negative charge patch despite having a net positive potential distribution. Thus, encapsulation of trypsin inside pectin-PDADMAC...
complex was still possible due to electrostatic attraction with cationic PDADMAC and negative patch of trypsin.

The effect of protein (trypsin) concentration on the encapsulation efficiency was then examined for the ternary complex with the highest encapsulation efficiency according to the mixing order; trypsin-pectin/PDADMAC complexes. 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 were used in the experiments (0, 10, 25, 50, 100, and 150 mM NaCl). The results of the encapsulation efficiency studies are shown in Figure 7B. The encapsulation efficiency % dropped at higher NaCl concentrations due to charge screening.

Meanwhile, as the trypsin concentration was increased, the encapsulation efficiency dropped at all salt concentrations. In the absence of NaCl, at low trysin concentrations, the amount of trypsin was high in the coacervate phase compared to the supernatant phase. For example, the ternary complex with 0.25 mg/ml trypsin resulted as 74% (by mass) trypsin in the coacervate phase and 26% in the supernatant phase. less trypsin is found in the coacervate phase, and more trypsin is found in the supernatant. As trypsin concentration was increased, the amount of trypsin in the coacervate and supernatant phases approached each other. For example, trypsin at 0.75 mg/mL in 10 mM phosphate buffer (no NaCl) partitioned itself as 51% (by mass) in the coacervate and 46% in the supernatant. These results might be attributed to the fact that trypsin has a negative charge patch which would cause trypsin to repel the pectin chains at higher trypsin concentrations, leading to partitioning of the trypsin in the supernatant phase.

The effect of polyelectrolyte concentration on the encapsulation efficiency was also investigated in five different combinations of pectin and PDADMAC concentration while keeping the trypsin concentration constant at 0.25 mg/ml. According to the results given in Figure 7C, the highest encapsulation efficiency was achieved when PDADMAC and pectin was mixed as 1:1 w/v, i.e. 0.5 mg/ml PDADMAC and 0.5 mg/ml pectin. This result can be attributed to that fact that pectin and PDADMAC have similar charge spacings and this concentration ratio corresponds to a near-stoichiometric charge ratio of pectin to PDADMAC; i.e. $[-]/[+] = 0.81$. In fact, the 1:1 (w/v) pectin:PDADMAC pair in the absence of trypsin had the highest turbidity (100-%T), 19.77 % ± 0.11, suggesting a greater yield of coacervation for this system. Meanwhile, lower turbidity values contributed to the lower encapsulation efficiency:
pectin:PDADMAC pair (0.25 mg/ml pectin:0.50 mg/ml PDADMAC) which had the lowest encapsulation efficiency also had the lowest turbidity, 3.75 % ± 0.10.

d. Trypsin Activity

Enzymatic activity for both free and encapsulated (in pectin-trypsin/PDADMAC complexes) trypsin complexes were measured by catalysis of the substrate N-Benzoyl-L-Arginine-p-Nitroanilide (BAPNA) into p-nitroaniline. This product gives a visible yellow color and has an absorbance maxima at 410 nm. The $V_{\text{max}}$ and $K_m$ values of free and encapsulated trypsin enzymes at various salt concentrations, derived using Lineweaver-Burke plots, are presented in Figure 9 below.

Figure 9: (A) $V_{\text{max}}$ and (B) $K_m$ values for the free and encapsulated trypsin

$K_m$ values indicate the binding strength between an enzyme and a substrate. Since the $K_m$ values for the free trypsin were close to 0.3150 mM in 10 mM phosphate buffer with NaCl concentrations up to 100 mM, it can be concluded that the active part of the free enzyme is not affected by the increasing salt concentrations. Meanwhile, both the $K_m$ and $V_{\text{max}}$ values for the encapsulated trypsin were reduced, indicating a negative impact of coavervation-based encapsulation on the active site of enzyme, leading to the loss of its activity. These results are in line with Xia et al[31], where a reduction in trypsin activity was observed when it was complexed with PDADMAC. This result was attributed to a shift in the optimal pH of trypsin upon complexation, resulting in a drop of $V_{\text{max}}$. It is also possible that cationic PDADMAC binds to the negative charge patch of the trypsin in its active region, which might prevent the enzyme from attaching to the substrate. Lastly, the decrease in $K_m$ values at higher NaCl concentrations can be attributed to weaker electrostatic interactions between the enzyme and the polyelectrolytes due to charge screening.

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Measurements of activity at various time points (0, 1, 3, 7, 14, and 30 days) were performed to assess the stability of the free and encapsulated trypsin. Figure 10 provides the $V_{\text{max}}$ and $K_m$ values. $V_{\text{max}}$ of both the free and encapsulated trypsin decreased over time; i.e. 27% vs. 7.8% in 30 days, respectively. Meanwhile, $K_m$ of free trypsin decreased by 85.5% while that of encapsulated trypsin dropped by 25% in 30 days. Thus, despite the enzyme's decreased initial activity, encapsulation by complex coacervation can maintain its activity for up to 30 days.

**Figure 10:** $V_0$ and $K_m$ values for free and encapsulated trypsin at different time intervals

### e. Secondary structure of trypsin

CD was used to examine how encapsulation via coacervation affected the secondary structure of trypsin (Figure 11). CD spectra from the empty pectin-PDADMAC coacervates, without trypsin, showed no clear peaks or troughs between 250 and 190 nm. On the other hand, the CD signal for the free trypsin was quite strong. Due to scattering of the coacervate droplets, the signals for the encapsulated trypsin samples were decreased.

Encapsulated trypsin were run both in the suspension form (before centrifugation) and in the coacervate form (after centrifugation of the suspension). The higher CD signal for the suspension than the coacervate phase in Figure 11A may be due to the greater amount of free trypsin enzyme in the suspension. According to these results, it can be concluded that the pectin-trypsin/PDADMAC coacervate droplets change the secondary structure of the trypsin but do not denature the enzyme.
Figure 11: (A) CD spectra for coacervate alone, free and encapsulated trypsin samples at pH 7.5 in 10 mM phosphate buffer, (B) CD spectra for free and encapsulated trypsin samples at pH 7.5 in 10 mM phosphate buffer at 25°C and 45°C.

The effect of temperature on the secondary structure of trypsin was also examined as shown in Figure 11B. Even the native secondary structure of free trypsin was damaged when exposed to 45°C. In addition, encapsulated trypsin as pectin-trypsin/PDADMAC complex was unable to maintain its secondary structure when temperature was raised from 25°C to 45°C. This result is in line with literature [8], where BSA in PAA-PAH coacervates also couldnot preserve its structure when exposed to heat.
4. CONCLUSION

In this study, coacervate droplets between pectin, an anionic biopolymer, and PDADMAC, a cationic synthetic polymer, were prepared to encapsulate the porcine trypsin enzyme, which has an ideal pH value of 7.5. With potentiometric titration experiments, it has been shown that the pectin polymer is fully ionized at pH 7.5 and 0, 10, and 150 mM NaCl. The formation of pectin-PDADMAC complex coacervates at pH 7.5 were confirmed by both light microscopy imaging and turbidimetric titration experiments. These techniques were also used to understand how NaCl concentration affected the formation of coacervate droplets. As the salt concentration was increased, the quantity and size of coacervate droplets reduced due to charge screening.

Our results also showed that the protein and polymer concentrations, and the order of mixing the components all had an effect on encapsulation. The highest encapsulation efficiency was achieved as 73.9% by first combining 0.5 mg/ml pectin and 0.25 mg/ml trypsin, and then adding 0.5 mg/ml PDADMAC to the mixture in 10 mM phosphate buffer. With increasing concentrations of added NaCl, a reduction was observed in the encapsulation efficiency.

For the activity measurements, the BAPNA used as a substrate was catalyzed by trypsin encapsulated in the pectin-trypsin/PDADMAC complex, and the p-nitroaniline was formed. According to our results, encapsulation via coacervation reduced the enzymatic activity of trypsin; both $V_{\text{max}}$ and $K_m$ values decreased for encapsulated trypsin. However, it was shown that the trypsin activity remained constant within 7.8 % for encapsulated trypsin after 30 days.

Finally, experiments using circular dichroism were performed to determine how the encapsulation procedure affected the secondary structure of trypsin. CD signals were reduced with the encapsulated trypsin in comparison to the free enzyme. Additionally, coacervate droplets did not preserve the secondary structure of trypsin but prevented it from denaturation when exposed to heat.

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