Chitosan–poly(acrylamide-co-maleic acid) composite synthesis, characterization, and investigation of protein adsorption behavior

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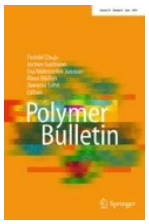

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Abstract

A new environmentally friendly superabsorbent chitosan–poly(acrylamide-co-maleic acid) (chitosan/PAA-MA) was prepared by blending biocompatible chitosan and poly(acrylamide-co-maleic acid) in the polymerization medium. Poly(acrylamide-co-maleic acid) was obtained by direct polymerization of the acrylamide and the maleic acid. The prepared chitosan/PAA-MA superabsorbent composite was characterized by swelling ratio, Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), and scanning electron microscopy (SEM). The lysozyme was separated using superabsorbent

composite. Lysozyme adsorption optimum conditions (such as pH, lysozyme concentration, temperature, and ionic strengths) were determined. The highest lysozyme adsorption was obtained at pH 9 with a maximum adsorption capacity of 220 mg g^{-1}. The desorption of lysozyme and the reusability of the chitosan/PAA-MA composite were also tested. As a result, this biodegradable polymer-based superabsorbent material will become an alternative for the adsorption of various proteins.

Introduction

Lysozyme is an enzyme, commonly found in cells and secretions of vertebrates, and called the human antibiotic. It is a compound commonly found of egg white, sweat, tears, digestive system of ruminants, papaya milk, horse, donkey and camel milk, insect larvae, and other animal and vegetable sources [1]. Due to some positive effects, lysozyme is widely used in various industrial areas. In addition, this enzyme prevents bacterial growth in foodstuffs (vegetables, milk, fish, and meat) and is used in the food industry as a food preservative. Also, it is widely used in wound healing creams, eye drops, and anticancer drugs in pharmacology [2, 3]. The use of lysozyme in various applications in

different industrial areas has increased the importance of the purification of this enzyme [4].

Superabsorbent polymers are petroleum-based materials that contain many hydrophilic groups and have high water retention. It has common usage areas such as agriculture, construction, food industry, biomedical applications, and adsorption. However, the most significant problems of petroleum-based superabsorbent polymers are not economical and environmentally friendly. In recent studies, these problems have been tried to overcome by using biodegradable, biocompatible, environmentally friendly, economical, and non-toxic carbohydrate polymers [5,6,7]. In this context, improved mechanical properties of superabsorbent materials are synthesized by the modification of eco-friendly biopolymers.

Chitosan is a biocompatible, physiologically inert, non-toxic, antimicrobial, and easy modifying polymer with a high affinity for proteins. However, the poor mechanical properties and low active adsorption surface areas of pure chitosan limit its repeated use as an adsorbent. There are many studies with the modification of chitosan (magnetite chitosan composite, chitosan membranes, and chitosan beads) to improve the protein adsorption capacity of chitosan in the literature [8,9,10,11,12,13,14]. The main purpose of these studies was to increase the protein-binding capacity of polymer. Chitosan has many functional groups (such as hydroxyl and amino groups), being important in increasing the adsorption capacity of proteins [15]. This case is an advantage both in adsorption studies and in modification. Xu et al. [16] synthesized chitosan/human hair fiber beads functionalized with citric acid (CA-CS/HHF) for the adsorption of lysozyme and found the lysozyme adsorption capacity of the CA-CS/HHF beads with increased mechanical strength to be 42.1 mg g^{-1}. Sun et al. [17] were able to purify the lysozyme successfully from an aqueous solution using carboxymethyl chitosan magnetic nanoparticles.

Conventional techniques such as ultrafiltration, dialysis, precipitation, adsorption, and chromatography have been used in lysozyme purification in studies in the literature [18,19,20,21,22,23]. The application of the adsorption method used in this study is cheaper and simpler than other methods, and it is not complicated and takes a shorter time.

In this study, firstly, chitosan modification by poly(acrylamide-co-maleic acid) (PAA-MA) copolymer was successfully prepared. The prepared composite (chitosan/PAA-MA) was characterized by swelling ratio, Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), and scanning electron microscopy (SEM). Later, by using chitosan/PAA-MA composite, optimum conditions were determined for parameters such as lysozyme concentration, pH, ionic strengths, and temperature, which have an effect on lysozyme adsorption. Lysozyme was successfully separated from the egg white solution using chitosan/PAA-MA composite under the optimum conditions. The high adsorption performance of the chitosan/PAA-MA composite shows that this composite would be useful adsorbent for biological molecules. The desorption of lysozyme and the reusability of the PAA-MA-modified chitosan composite were also tested. This study presents an interesting and eco-friendly approach to lysozyme adsorption using the chitosan/PAA-MA composite with satisfactory results.

Materials and methods

Apparatus

Maleic acid (MA), acrylamide (AA), ammonium peroxodisulfate APS ($H_8N_2O_8S_2$), 4-(2-pyridylazo), N,N′-methylenebisacrylamide, N,N,N′,N′-tetramethylethylenediamine (TEMED), resorcinol (PAR), arsenazo III (disodium salt), and lysozyme powder (from chicken egg white) were obtained from Sigma–Aldrich (Steinheim, Germany). Chitosan from crab shells [poly-(1,4-β-d-glucopyranosamine)] was purchased from Sigma–Aldrich. All other chemicals were of analytical-grade purity.

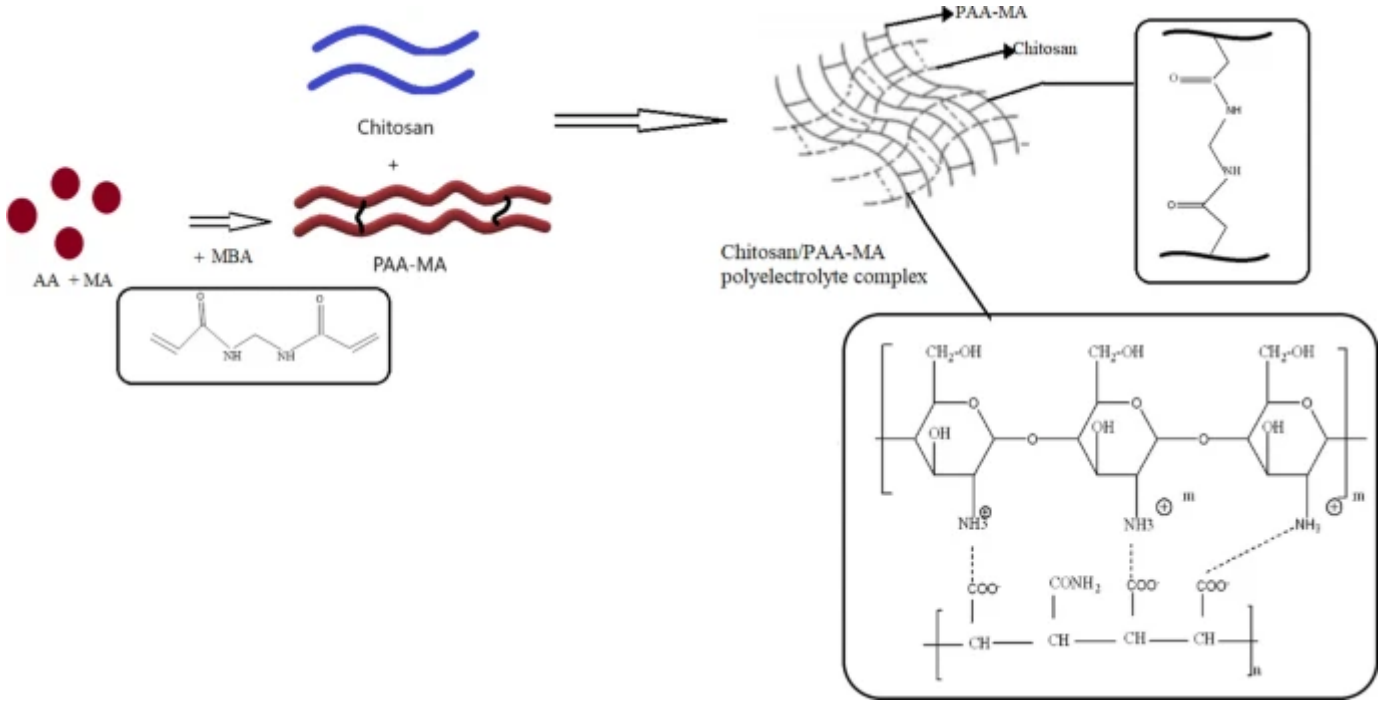
Preparation of chitosan-poly(acrylamide-co-maleic acid) composite

In the first part of this study, PAA-MA copolymer was synthesized by bulk copolymerization of AA and MA using N,N′-methylenebisacrylamide as the cross-linker. APS and TEMED were used as an initiator and an activator, respectively. The solution containing AA (4 g), MA (0.36 g), N,N′-methylenebisacrylamide (1.2 g), APS (4 mL), and TEMED (800 µL) was prepared in 40 mL water mixture.

For preparation adsorbent (chitosan/poly(acrylamide-co-maleic acid), 5.0 g of chitosan in acetic acid (1% v/v) was stirred for 15 min and added to the polymerization medium. Monomer solutions of maleic acid (10 mL 0.135 g maleic acid) and acrylamide (20 mL 1.5 g acrylamide) were added to the suspension and stirred for 4 h. After adding N,N′-

methylenebisacrylamide (1.2 g) and ammonium persulfate (50 mg) as the cross-linking agent and the initiator, respectively, into the 10 Ml suspension, N,N,N′,N′-tetramethylethylenediamine (200 µL) was added to proceed the polymerization. After the polymerization was completed, the composite was washed with distilled water. Chitosan/PAA-MA composite was dried at ambient temperature and stored in polypropylene containers after grinding. The formation scheme of the composite is shown in Fig. 1.

Fig. 1



The proposed mechanism for the formation of the chitosan/PAA-MA composite

The $-NH_2$ groups in the chitosan polymeric chain are protonated in acetic acid, giving the chitosan molecules a cationic character [24]. It is predicted that an intercomposite is formed due to the electrostatic interactions of the oppositely charged groups, being the negatively charged $-COO^-$ groups of poly(acrylamide-co-maleic acid) molecules and the positively charged chitosan (Fig. 1).

Swelling ratio

One gram of dry composites and components was placed in distilled water and kept at a constant temperature of 25 ± 0.5 °C to determine swelling behavior. Swollen composites

and components were removed and weighed using an electronic balance on a regular basis. The weight ratio of dry and swollen samples was determined. The water content of the swollen composites and components was calculated as Eq. (1):

$$ {\text{Swelling}}\,{\text{ratio\,}}\left(\% \right) = \left[{\left({{\text{Wf}}{-}{\text{Wo}}} \right)/{\text{Wo}}} \right] \times 100 $$

(1)

where Wo and Wf represent the weights of the composites and components before and after swelling, respectively.

Scanning electron microscopy (SEM)

The surface morphology of the composite was examined using scanning electron microscopy (SEM). SEM was obtained by a model of TESCAN MIRA3 XMU. The composite was coated with a thin layer of gold under reduced pressure, and its SEM images were taken.

FTIR analysis of composite

The prepared chitosan/PAA-MA composite was characterized by FTIR spectroscopy. Measurements were taken using a spectrophotometer (Bruker mode: Tensor II).

X-ray diffraction (XRD)

X-ray diffraction analysis of the composite was carried out in a Bruker brand D8 Advance instrument using Cuk α probe ($\lambda = 1.5406$ A^{o}). Scans were made between 2 and 30°.

Adsorption studies of lysozyme

For the adsorption studies of lysozyme on chitosan/PAA-MA composite, 5.0 mL of lysozyme solution (1.0 mg/mL) was incubated with 0.1 g composite and the adsorption was carried out at 25 °C for 2 h. Adsorption of lysozyme was studied at various pHs, in phosphate buffer (pH 6.0–8.0). The effects on adsorption capacity of adsorption method parameters (such as initial concentration of lysozyme, temperature, pH, and ionic strength) were investigated. The adsorbed lysozyme concentration was carried out using

a UV 1601 model UV–VIS spectrophotometer. Solution absorbances were measured at 280 nm. Adsorption capacity was calculated with Eq. 2:

$$ Q = \left({{\text{Co}} - C} \right) \times V/m $$

(2)

where Q is the adsorption capacity, Co and C the initial and final concentration of lysozyme (mg/ml), respectively, V the medium volume (ml), and m the amount of adsorbent (g).

Reusability experiment

After adsorption and desorption processes, the chitosan/PAA-MA composite resorption reuse number was determined in the desorption agent solution (1.0 M NaCl). Ten resorption experiments were carried out.

Results and discussion

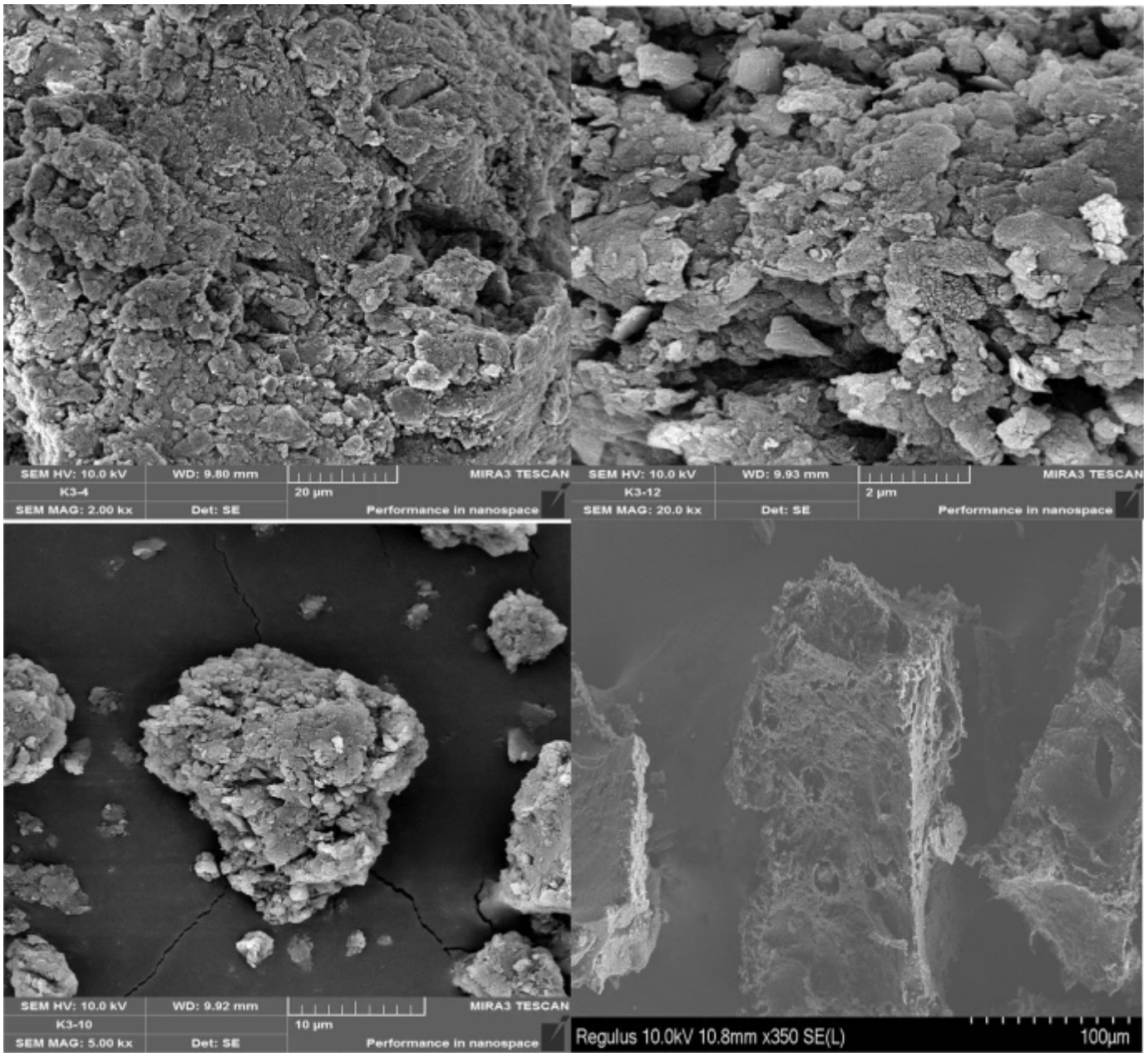
Swelling ration

The maximum swelling ratio of composite was found as 224%. It is obvious that the swelling of a composite is caused by the electrostatic repulsion of its network's ionic charges.

SEM

The chitosan/PAA-MA composite surfaces appear rough, granular, and homogeneous (Fig. 2). SEM images of chitosan/PAA-MA composite, which have high protein adsorption, show that they have more pores and surfaces. Because of their large inner surface area, these large pores (Fig. 2 bottom right is taken after lyophilized material) reduce diffusional resistance and facilitate mass transfer.

Fig. 2

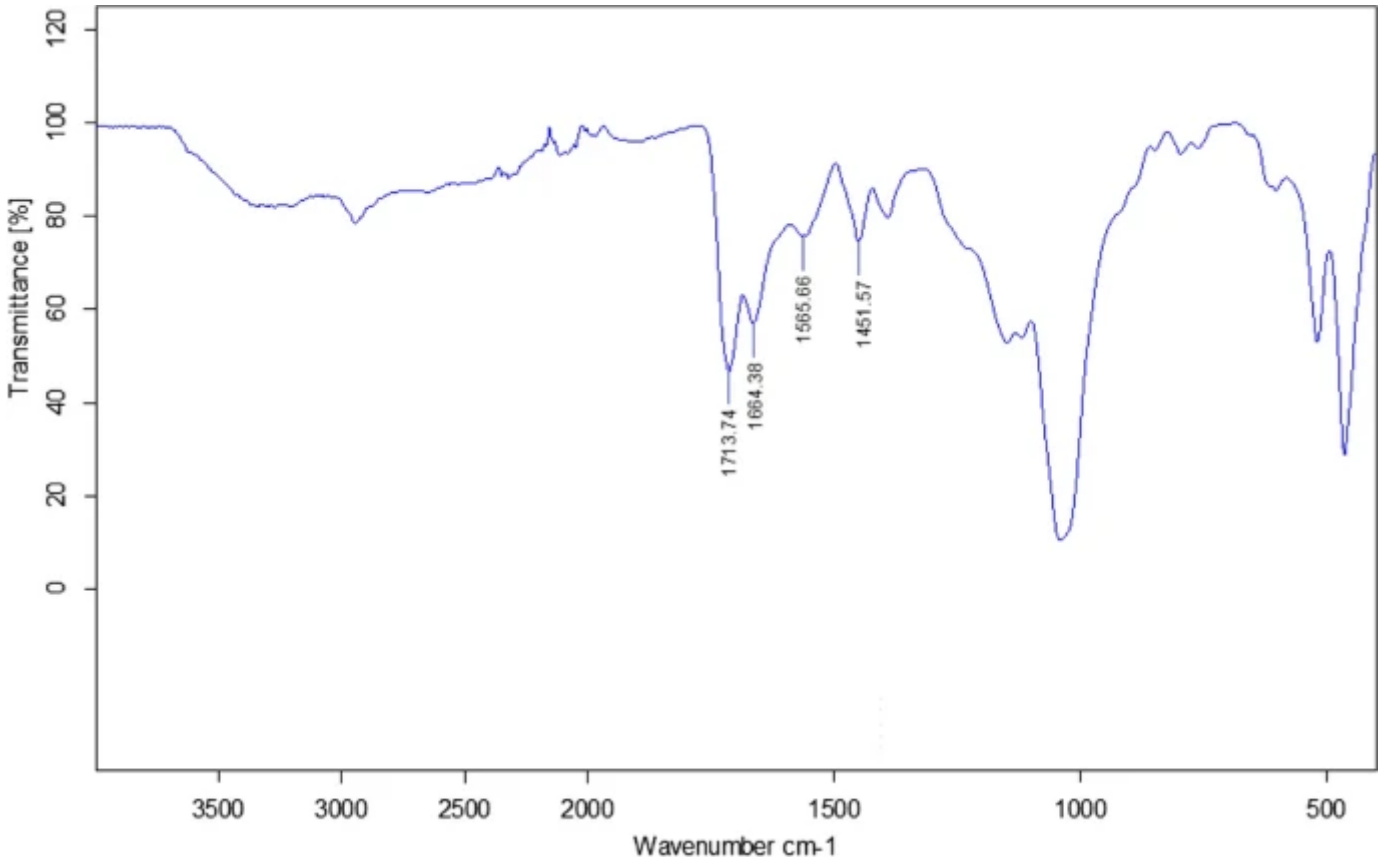


SEM images of composite

FTIR analysis

The FTIR spectrum of the chitosan/PAA-MA composite is shown in Fig. 3. The characteristic wave numbers compiled from the literature for the composite components chitosan and PAA-MA are presented in Table 1.

Fig. 3



FTIR analysis of chitosan/PAA-MA composite

Table 1 FTIR bands of chitosan and PAA-MA with assignments [25, 26]

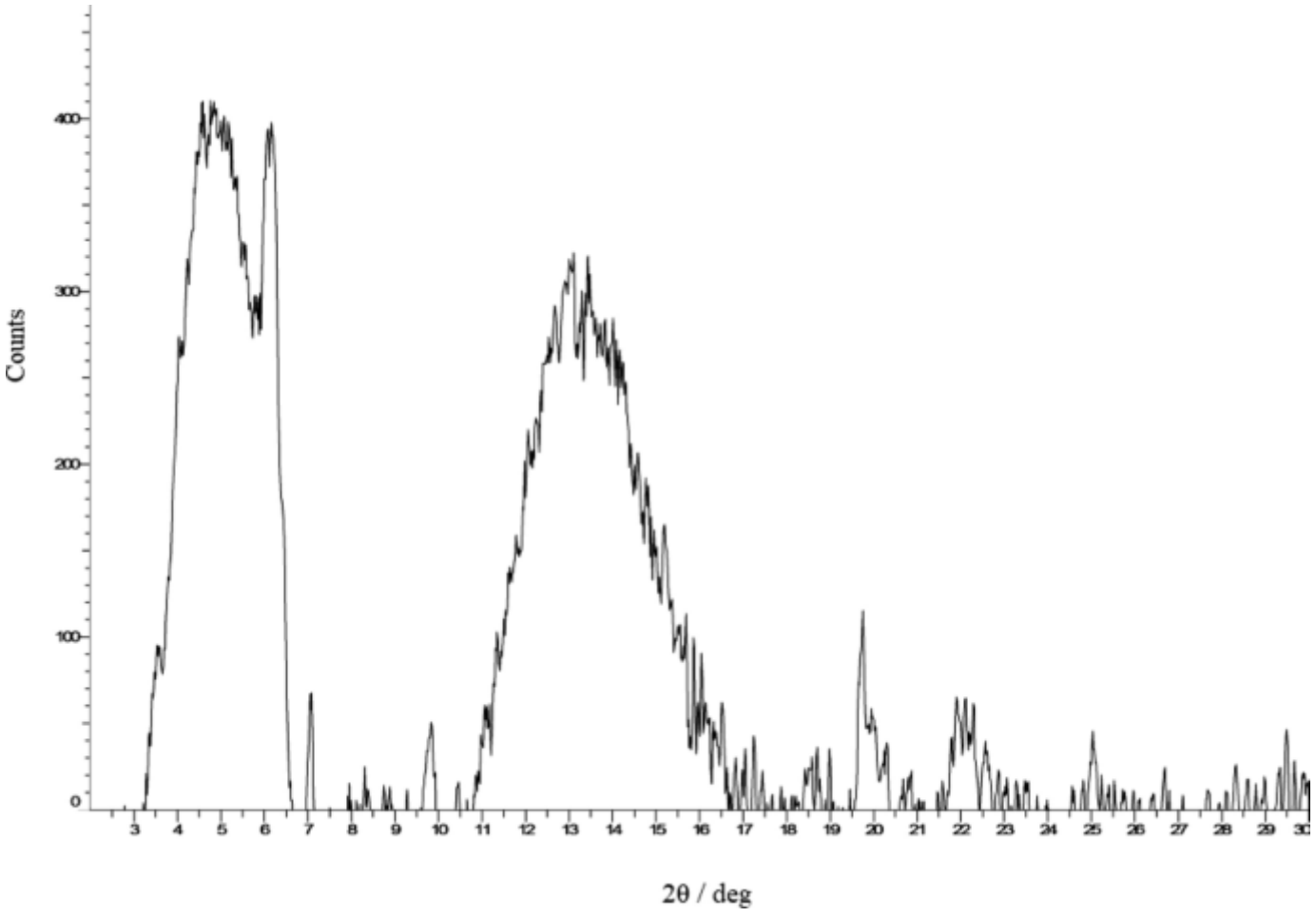
The characteristic NH absorption band of chitosan has shifted to the band at 1565.66 cm^{-1} due to the protonated amine (δNH_3^+) [24]. It is seen that the CH_2, C=O, and carbonyl group bands shift to 1451 cm^{-1}, 1664.38 cm^{-1}, and 1713.74 cm^{-1}, respectively, depending on the increasing electron density in the composite material.

X-ray diffraction (XRD) analysis

X-ray diffraction is used for the qualitative identification of crystalline compounds. The XRD device creates an opportunity to examine the properties of the crystal structure with the diffraction of X-rays in the crystal structure. The X-ray diffraction method is based on the principle that each crystal refracts X-rays in a characteristic pattern depending on the specific atomic arrangement of the phase. These diffraction profiles for each crystal phase identify that crystal, sort of like a fingerprint. Figure 4 shows the XRD analysis of

the composite. Pure chitosan is a polysaccharide being semi-crystalline structure (approximately 2θ = 20° strong peaks and 2θ = 8° weak peaks) [27,28,29]. The reason for having the regular structure and crystalline property of chitosan is intramolecular and intermolecular hydrogen bonds [28]. The PAA-MA copolymer is amorphous because the suspended acid and amide fragments cause less chain packing and significantly inhibit regular macromolecular packing [30]. According to the information in the literature, it is seen in Fig. 4 that the composite formed between cationic chitosan and anionic PAA-MA molecules has a crystal structure. The presence of ionic interaction between chitosan and PAA-MA caused the crystal regions to increase and some of the amorphous components to be lost [30].

Fig. 4



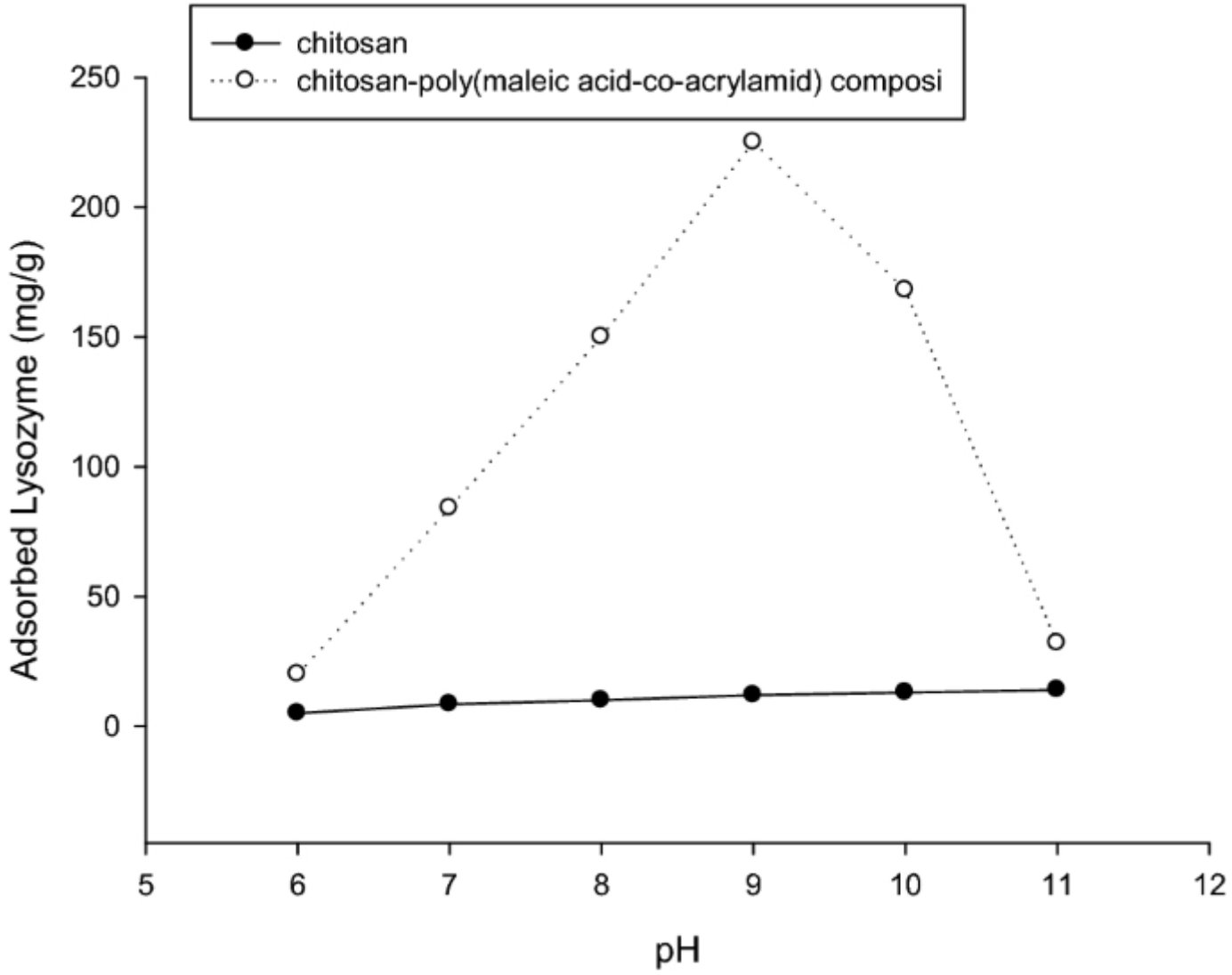
XRD pattern of chitosan/ PAA-MA composite

Adsorption studies

Effect of solution pH on adsorption

One of the most important parameters that significantly affect the adsorption performance is the solution pH. The effect of pH on the amount of lysozyme adsorbed on chitosan and chitosan/PAA-MA composite was examined between pH 6.0 and 11.0, and the results are given in Fig. 5. Lysozyme adsorption in chitosan adsorbent is quite low. As a result of the weak electrostatic interactions between the chitosan (which is negatively charged above pKa 6.5) [31] adsorbent and the lysozyme molecule (positively charged), nonspecific adsorption was performed on the chitosan adsorbent. Zeng and Ruckenstein reported that chitosan is positively charged at values below pH 7.0 due to protonation of the amine group. So, chitosan can act as a weak anion exchanger and adsorb negatively charged proteins [32]. In the chitosan/PAA-MA composite, the amount of adsorbed lysozyme increased up to pH 9.0 and decreased at higher pH values. The high adsorption capacity at pH 9.0 indicates a strong interaction between composite and lysozyme. Lysozyme molecules will be cationic below pH 11.2, as their isoelectric point value (pI) is 11.2 [33]. The carboxylic groups of the composite ionize in the range of $6 < pH < 11$, and interaction increased between the positive lysozyme molecules. The lysozyme molecules have a net positive charge of up to a pI value of 11.2 in this pH range. Due to decreasing the positive charges of the lysozyme after pH 9.0, the interaction between adsorbent and protein decreased.

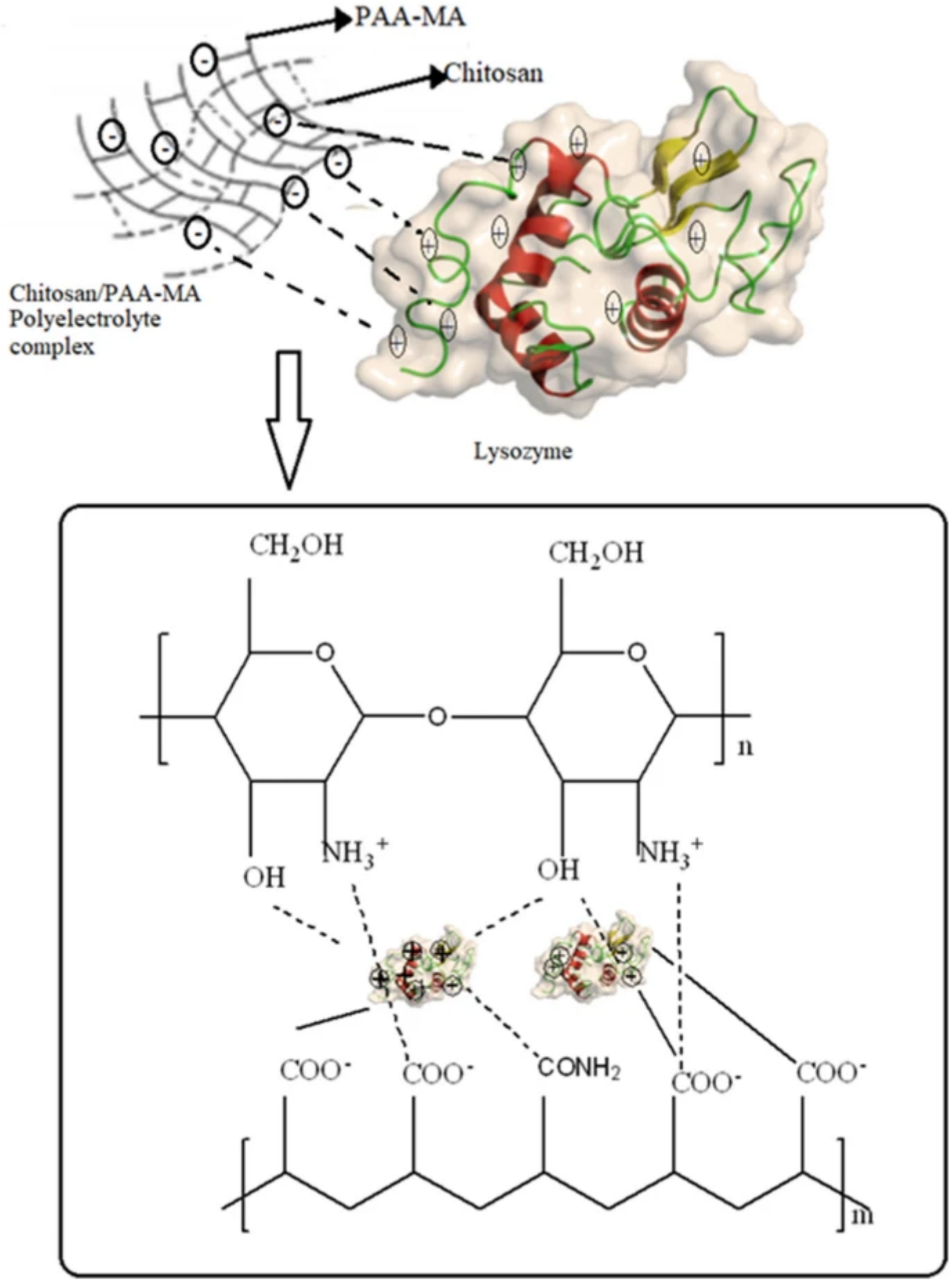
Fig. 5



Effect of pH on adsorption capacity [lysozyme concentration: 1.0 mg/mL, $T = 25\ °C$, NaCl = 0]

In summary, when the pH was higher than 7, but lower than 11, increased electrostatic attraction between negatively charged chitosan/PAA-MA composite (due to $-COO^-$ groups in PAA-MA macromolecular chains) and positively charged lysozyme causes increasing lysozyme adsorption (Fig. 6).

Fig. 6

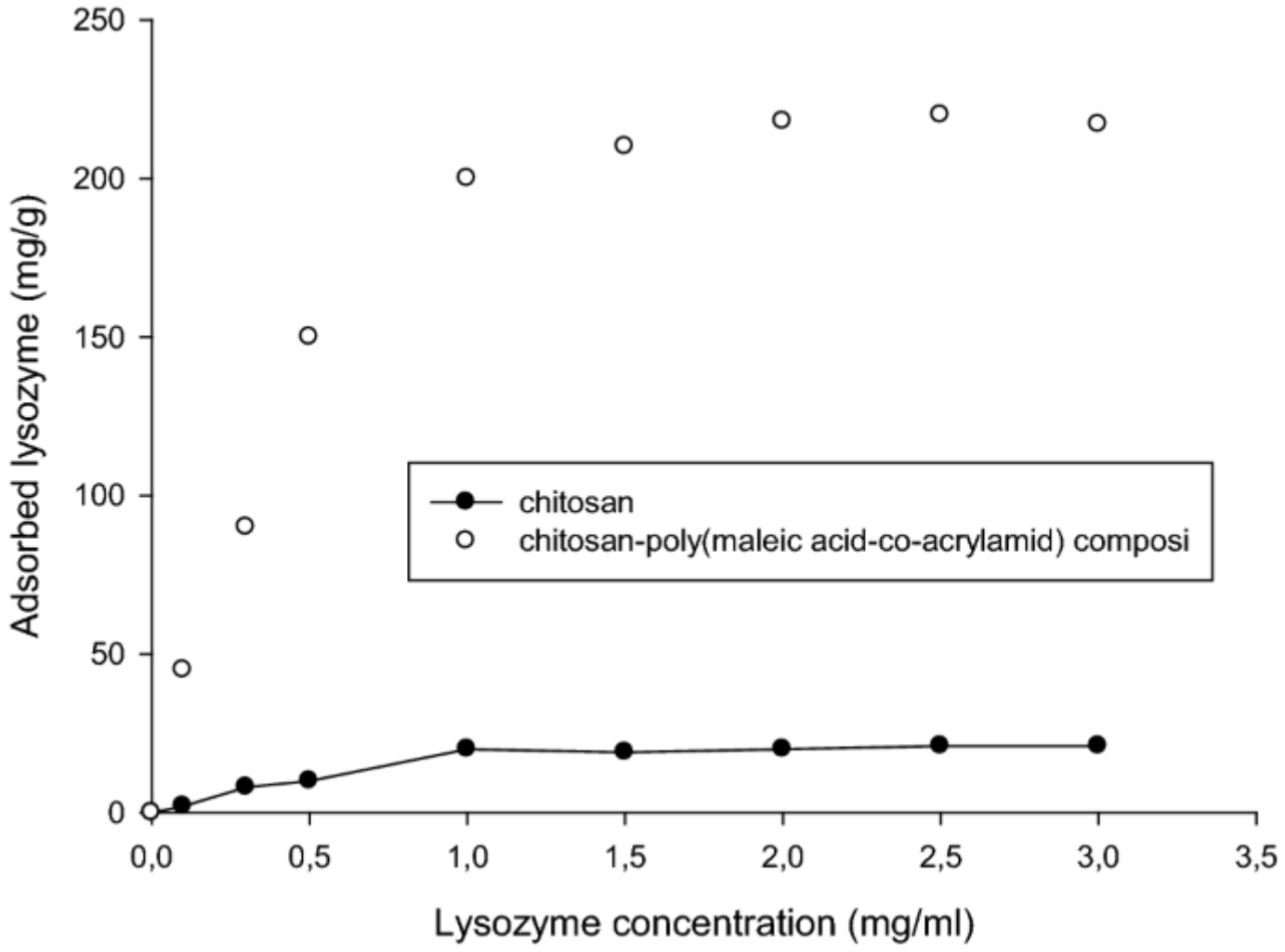


Mechanism of lysozyme adsorption by chitosan/PAA-MA

Effect of concentration lysozyme on adsorption

The adsorption results of lysozyme using chitosan and chitosan/PAA-MA composite adsorbents are presented in Fig. 7. With the increase in lysozyme concentration, the amount of protein adsorbed to the composite increased. As shown in Fig. 5, the adsorption capacity of chitosan adsorbent was linear at initial lysozyme concentrations below 1.0 mg/mL. This can be attributed to the saturation of the ion exchange adsorbent with lysozyme molecules after a certain concentration and the slowing of the adsorption rate. The maximum lysozyme adsorption capacity was determined as 220 mg/g (Fig. 7). The high affinity of the composite to the lysozyme molecule increased the adsorption capacity. Arıca and Bayramoğlu [34] reported that the lysozyme adsorption capacity of Reactive Blue-4 and Reactive Red-120 dye ligand-retained PHEMA/chitosan membranes was 17.9 and 35.7 mg/mL, respectively.

Fig. 7

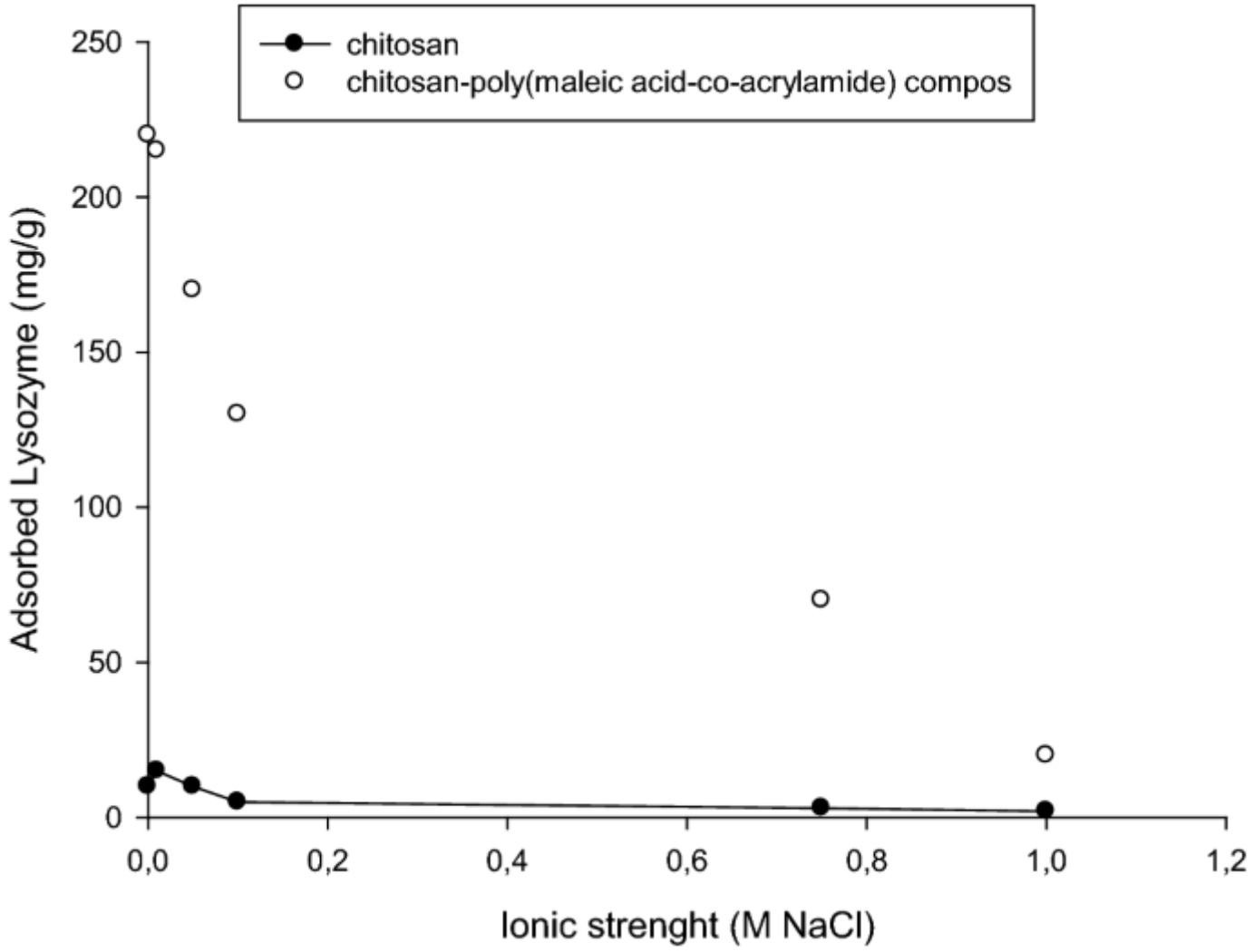


Effect of initial concentration of lysozyme on adsorption capacity [pH: 9.0, T = 25 °C, NaCl = 0]

Effect of ionic strength on adsorption

The effect of ionic strength was investigated by changing the NaCl content of the phosphate buffer between 0.0 and 1.0 M. It was observed that the adsorption capacity decreased from 220 to 20 mg/ml with the increase in ionic strength (Fig. 8). This decrease in adsorption capacity was due to the reduction in electrostatic interactions between lysozyme and composite molecules. As the ionic strength increases, the charge density of the electrical double layer around the molecules would change, and the protein surface charge would be blocked [19, 22].

Fig. 8

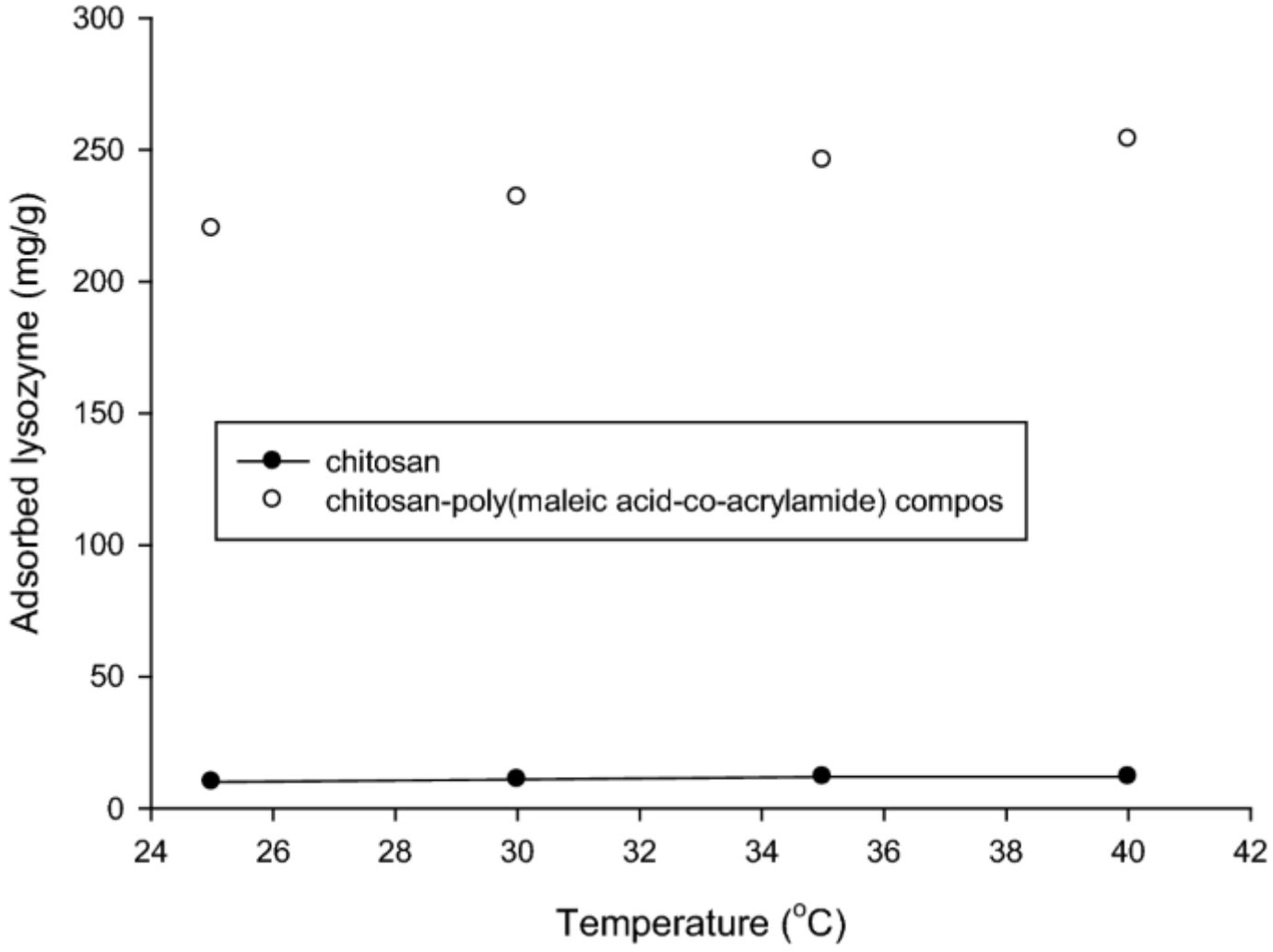


Effect of ionic strength on adsorption capacity [lysozyme concentration: 1.0 mg/mL, pH: 9.0, T = 25 °C]

Effect of temperature on adsorption

Adsorption of lysozyme to chitosan composite was studied at different temperatures from 25 to 40 °C (Fig. 9). As shown in Fig. 9, the adsorption of lysozyme on chitosan/PAA-MA composite increased with increasing temperature. The surface of the composite and the lysozyme between the contact areas increased at high temperatures. Therefore, the binding sites of the lysozyme for the adsorbent were also increased [19, 35, 36].

Fig. 9



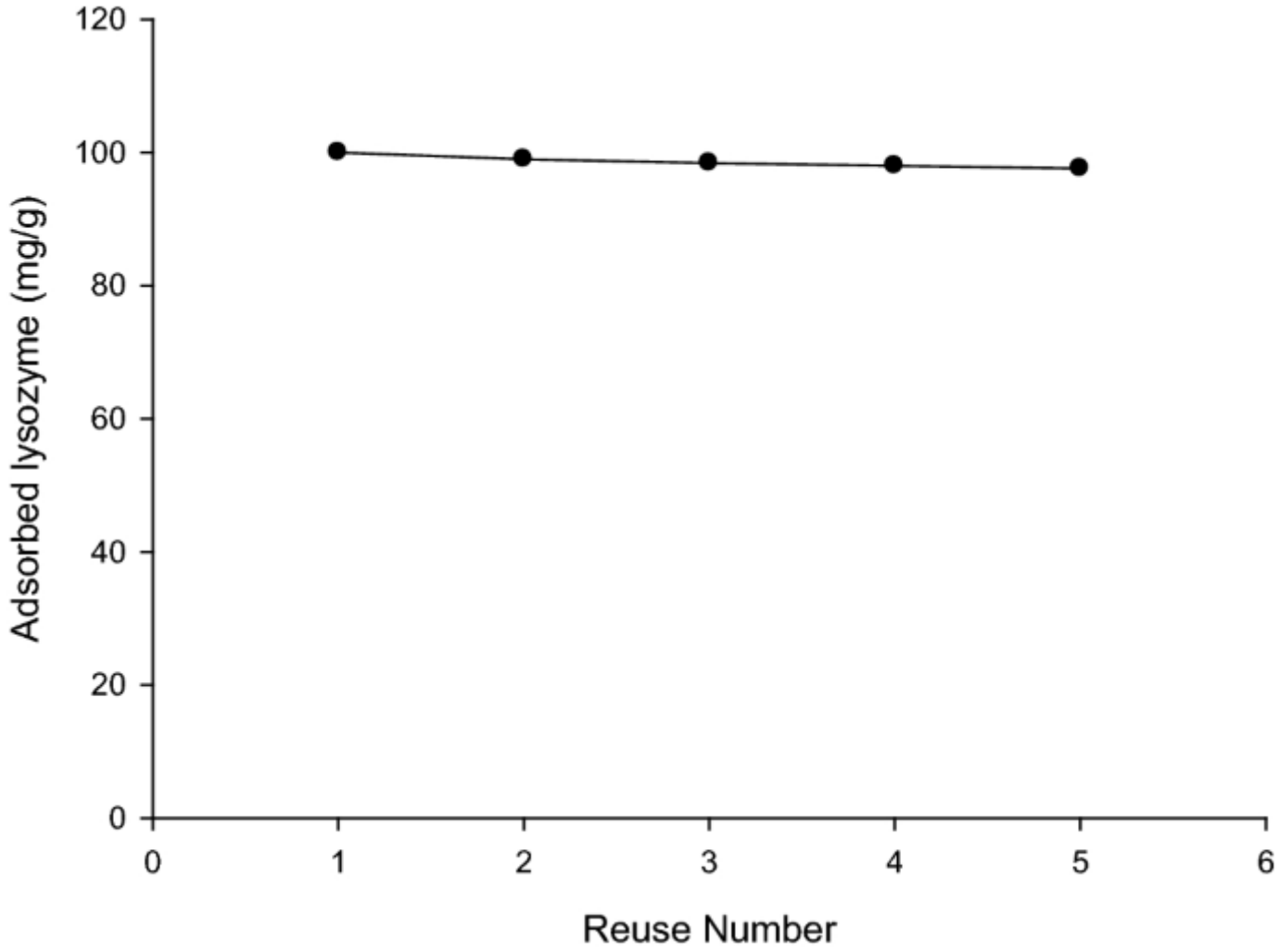
Effect of temperature on adsorption capacity [lysozyme concentration: 1.0 mg/mL, pH: 9.0, NaCl: 0.0]

Desorption and repeatability

Lysozyme-loaded chitosan/PAA-MA composite was put in a desorption solution at pH 9.0 (50 mM carbonate buffer medium) including NaCl. Ninety-seven percent of the lysozyme adsorbed was desorbed from the composite for 1 h (Fig. 10). As seen from Fig. 10, NaCl

was a suitable desorption agent for lysozyme from the chitosan/PAA-MA composite [37]. As a result of repeating the adsorption–desorption cycle with the same chitosan/PAA-MA composite ten times, the reusability of the composite was investigated. After the adsorption–desorption processes, there is no significant loss in adsorption capacity.

Fig. 10



Reusability of composite

Comparison with the literature

The lysozyme adsorption capacity of chitosan/PAA-MA composite was compared with some studies in the literature. According to Table 2, it is shown that the chitosan/PAA-MA composite has high adsorption capacity.

Table 2 Lysozyme adsorption capacities' comparison with the literature

Conclusion

In this study, the adsorption behavior of lysozyme on chitosan/PAA-MA composites was investigated under various reaction conditions. The medium pH has a significant effect on the adsorption of lysozyme, and there is good interaction between lysozyme and chitosan/PAA-MA at pH 9. The highest lysozyme adsorption was obtained at pH 9 with a maximum adsorption capacity of 220 mg g^{-1}. Lysozyme adsorption capacity decreased with increasing ionic strength in chitosan/PAA-MA composites. It was observed that the adsorbed lysozyme was desorbed up to 97% when 1.0 M NaCl at pH 9.0 was used as the desorption agent. In addition, the fact that chitosan/PAA-MA composites are easily, economically reproducible and can be used repeatedly in adsorption–desorption studies proves they are an alternative material to the literature. It can be seen that a high rate of lysozyme purification could be achieved; in the present study, it is thought it will also benefit the purification of other essential proteins from various biological sources.

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