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Preparation and characterization of polymer-coated mesoporous silica nanoparticles and their application in Subtilisin immobilization

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Abstract−The preparation and characterization of polymer-coated mesoporous silica nanoparticles (MSNs) and their application in Subtilisin (Alcalase®) immobilization were investigated. For the synthesis of polymer-coated MSNs, acrylic acid (AA) and chitosan (CS) mixture were blended as poly(acrylic acid) (PAA) and CS polymer layer onto MSNs via in-situ polymerization technique. Then, both uncoated MSNs and polymer-coated mesoporous silica nanoparticles (CS-PAA/MSNs) were characterized by taking into account properties such as morphologic pattern, size distribution, surface charge of the particles as well as thermogravimetric stability with SEM, TEM, Zetasizer and TGA analyses. Subtilisin was immobilized onto polymer-coated mesoporous silica nanoparticles via adsorption technique. For optimizing the enzyme immobilization process, the percent enzyme loading depending on the matrix amount, immobilization time and pH were investigated. Then, the activity values of immobilized enzyme and free enzyme were compared at various pH and temperature values. The maximum enzyme activity was achieved at pH 9.0 for both immobilized and free enzyme. Immobilized enzyme showed more stability at higher temperatures compared with free enzyme. Furthermore, the operational and storage stability of immobilized enzyme were determined. The activity of immobilized enzyme was reduced from 100% to 45.83% after five repeated uses. The storage stability of immobilized enzyme was found to be higher than that of free enzyme. The activity of immobilized enzyme was reduced from 100% to 60% after 28 days of storage time. We concluded that the polymer-coated MSNs were a suitable matrix for Subtilisin immobilization compared to uncoated MSNs.

Keywords: Nanobiocatalysis, Silica Nanoparticles, Chitosan, Poly(acrylic) Acid, Enzyme Immobilization, Subtilisin, Optimal Conditions

INTRODUCTION

The global enzymes market is expected to grow on average 4.6% until 2020 to $7.2 billion from $5.78 billion in 2015, considering the enzymes used in industrial applications such as food, beverages, cleaning products, biofuel production, animal feed and etc. and their applications in the biotechnological and research area [1]. Proteases are assumed to have 60% of the total enzyme market with $1.5-1.8 billion annual sale per year. Looking at the sales of alkaline protease, the most wide use is in cleaning industry [2,3]. Among the protease enzymes, Subtilisin is the key player as a detergent additive to remove protein deposits and stains. This enzyme is used in dish washer and laundry detergents in powder and/or liquid form. Subtilisin (<10%) is also used as catalyzer in processes such as protein hydrolyzation, leather treatment as well as in the textile and cosmetics industry [4]. The lack of the operational stability of the enzymes has resulted in low efficiency in the industrial applications. High temperatures or non-convenient pH values may lower enzyme activity, causing a reaction rate decrease. Since its advantages like flexibility for the process conditions and increased stability of enzyme, the immobilization process is the key solution to overcome these obstacles [5,6].

Nanotechnology has led to the development of nanobiocatalysis in which enzymes are embodied with nanomaterials include nanoporous media, nanofibers, carbon nanotubes and nanoparticles. Nanobiocatalysis systems have shown great efficiency in the manipulation of the nanoscale structure of the enzyme by developing activity and stability of enzymes [7]. MSNs have great potential as nanocarriers for biomolecules because of their biocompatibility, low toxicity, large surface area, high porosity, modifiable pore diameter, and adjustable surface properties. Surface functionalized particles with reactive groups are required to increase the percent enzyme loading and stability [8,9].

Natural and synthetic polymers are commonly used for the functionalization of mesoporous nanoparticles. Feng et al. [10] proposed a simple layer-by-layer self-assembly technique to construct MSNs as an agent for a pH-responsive drug delivery system with extended efficiency and biocompatibility. In this system, biocompatible polyelectrolyte multilayers of alginate/chitosan are coated on MSN's surface to obtain pH-responsive nanocarriers. Peng et al. [11] studied the synthesis of pH-responsive nanocarrier MSNs-PAA, having MSNs cores and PAA shell-layers. The MSNs-PAA was investigated as drug carrier at various pH values. The results showed that the PAA layers on the surface of MSNs-PAA achieved opened-closed states at different pH values, which can provide controlled drug delivery. Haghighizadeh et al. [12] investigated the immobilization of noble metal nanoparticles on silica microspheres treated by chitosan. Increased chitosan concentration caused amor-

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phous structure, and the shell became unclear. Higher metal loading was also observed when concentration of CS is lower.

Popat et al. [13] proposed an effective and facile method for coating chitosan onto phosphonate-functionalized MSNs to generate pH sensitive nanocarriers. Controlled-release of ibuprofen was studied, and it was found that ibuprofen released under mild acidic conditions because of dissolution of chitosan layer, and a decreased release was achieved at pH above the isoelectric point of chitosan. This work validated the potential of chitosan based inorganic-polymer blends as next generation delivery agents for biomedical applications.

Tang et al. [14] studied pH-responsive polymer, chitosan/poly (methacrylic acid), CS-PMAA-coated MSNs, through "the facile in situ polymerization method." The release of a cancer treatment drug was studied and the results showed pH-responsive release characteristics. Yuan et al. [15] studied the preparation of PAA grafted mesoporous silica nanoparticles through a facile "graft onto" strategy. By means of the covalent graft of hydrophilic and pH-sensitive PAA, the particles were well-dispersed in aqueous solution, which is a favorable property of drug carriers to build a pH-responsive and adjustable drug delivery system.

On the other hand, for an efficient enzyme immobilization, the surface functionalized particles with reactive groups are required to increase the enzyme loading and stability [16]. CS has great potential as enzyme immobilization support because it is non-toxic, inexpensive and, user-friendly. The main reason for this great potential is the amino and hydroxyl groups in the chemical structure of CS [8]. This multifunctional biopolymer has many applications in immobilization, and adsorption processes in the literature either itself or combined with silica.

Singh et al. [17] studied the immobilization of procerain B, a novel cysteine protease, on glutaraldehyde-activated CS beads via covalent attachment. The CS-immobilized procerain B has been reported as with extended pH and thermal optima. The effects of substrate concentration and operational stability of immobilized beads were also studied and nearly 50% of the original activity was retained until the tenth use.

Tang et al. [18] studied the immobilization of neutral proteinase on CS nanoparticles prepared by ionization gelation method. The range of the optimum pH, and temperature was extended. The thermal, operational and storage stability values were found to be better for immobilized enzymes on CS nanoparticles. Kasavi and Güvenilir [19] studied the immobilization of protease enzyme on chitin and CS by covalent binding method and on alginate and zeolite by physical adsorption method. Chitin and CS were treated with glutaraldehyde for cross-linking.

Kalkan et al. [16] studied the preparation of CS-coated magnetite nanoparticles and usage of these particles for immobilization of laccase enzyme. Immobilized laccase activity had a better stability against pH and temperature compared with soluble enzyme. Pastor et al. [20] studied protein delivery via uncoated and CS-coated MSNs. Lei and Bi [8] studied a strategy for the preparation of the silica-coated CS support using layer by layer method. The results showed that the pectinase immobilized on the silica-coated CS support had better stability against thermal and pH denaturation.

Although, for the case of immobilization process, CS has the disadvantage of having a highly hydrophilic and swelling structure in aqueous media due to its high water content, which causes the polymer structure to lose its mechanical strength. To eliminate this, CS was used as a component of the polymer blend structure to obtain more strong and efficient matrices [21]. Due to the electrostatic interaction, the negatively charged acrylic monomers align along the CS molecules and a polymer blend can be obtained [22]. It is known that blending PAA and CS provides increases mechanical strength and pH stability. Also, linear polymer chains (such as PAA) grafted onto the surface of MSNs show pH-dependent behavior [23].

Dai et al. [24] studied preparation of PAA-blended CS (CS/PAA) hydrogel beads by one-step method. It was found that GLA cross-linked CS/PAA beads had higher mechanical strength and better stability in lower pH solutions than GLA cross-linked CS beads. Wu et al. [25] studied a preparation method of CS-PAA polymer magnetic microspheres with high Fe_3O_4. This study, which was performed on the release of the entrapped ammonium glycyrrhizinate in polymer magnetic microspheres, showed potential applications of microspheres for drug delivery system.

To sum up, MSNs have great potential as nanocarriers, but are not surface functionalized particles which are required to increase the percent enzyme loading and stability. Chitosan has great potential as enzyme immobilization support because of the amino and hydroxyl groups in its chemical structure [8]. However, high water content causes the polymer structure of chitosan to lose its mechanical strength [21]. Blending chitosan with poly(acrylic acid) provides increases mechanical strength and pH stability [23]. AA and CS polymer blend has not been studied as a coating layer onto MSNs in the literature so far. Therefore, in the present study, AA and CS were blended onto MSNs via in-situ polymerization technique to investigate their application on the enzyme immobilization. For this purpose, Subtilisin (Alcalase®) was used for the immobilization studies.

EXPERIMENTAL

1. Materials

CS (Coarse ground flakes and powder) with the deacetylation degree >75% was purchased from Sigma-Aldrich. Acrylic acid (AA) (Sigma-Aldrich) was distilled under reduced pressure before use. Tetraethyoxysilane (TEOS), cetyltrimethylammonium bromide (CTAB), sodium hydroxide (NaOH) and glutaraldehyde were obtained from Sigma-Aldrich. Subtilisin (Alcalase®) enzyme produced from *Bacillus licheniformis* was obtained from Novozymes.

2. Preparation of MSNs

MSNs were prepared according to the sol-gel method [26,27]. Briefly, 2 g CTAB and 0.56 g NaOH were mixed in 960 mL deionized water. The solution was stirred vigorously at 80 °C for 30 minutes, and 10 mL TEOS was added to be stirred for another 2 hrs. Then, as-prepared product (MSN-CTAB) was washed with ethanol for three times and redispersed in ethanol solution of NH_4NO_3 (800 mL, 10 mg/mL) at 80 °C to remove the organic template of CTAB. The mixture was refluxed for 6 hrs, collected by centrifugation and washed with ethanol repeatedly. Finally, the white powder of MSNs was obtained after drying process.

3. Preparation of Polymer-coated MSNs

For the methodology, the study of Tang et al. [14] was taken as a reference. They used CS/PMAA as polymer coating layer for MSNs. 0.215 g CS was dissolved in 30 mL of AA solution at the molar ratio of 1 : 2 ([glucosamine unit] : [AA]). The amount of AA was maintained constantly at 3 mmol in all experiments. Then, 0.16 g MSN was added to the mixture and sonicated for 20 mins by ultrasonic probe. The solution was stirred under nitrogen environment for 30 mins while being heated to 80 °C. When the temperature was kept constant, the initiator (Potassium persulfate: $K_2S_2O_8$) of the polymerization reaction was added to the reaction mixture. The concentration of potassium persulfate in the reaction mixture was about 4.0×10^{-3} mole/L. The reaction was continued at 80 °C under a nitrogen atmosphere for another 2 hrs. During polymerization reaction, white color of the reaction mixture turned to light yellow. At the end of 2 hrs, the temperature of the system was lowered to 50 °C. After the system reached a stable condition, 0.1 mL glutaraldehyde (0.25 wt%) was added to the system to start cross-linking reaction. The cross-linking reaction was continued for 2 hrs, and the reaction solution became dark yellow. The sample was separated from solution by centrifugation. Finally, the sample obtained was washed three times by deionized water.

4. Enzyme Immobilization

Subtilisin immobilized polymer-coated MSNs were prepared by dissolving Subtilisin in 2 mL of 0.1 M sodium phosphate buffer solution at pH 7.0, and then well-dispersed particles (10 mg) in 2 mL of 0.1 M sodium phosphate buffer at pH 7.0 were added to enzyme solution (0.2 mg/mL). The final volume of the immobilization solution was 4 mL. The experiments were at 25 °C and a stirring rate of 250 rpm. To optimize the immobilization process, the pH, matrix amount and immobilization time effects on the percent enzyme loading were investigated.

After the immobilization process, the immobilized enzymes were separated by centrifuge from free enzymes. Lowry method was used to determine the amount of free enzymes. The sample was taken from supernatant and diluted with 25 mM potassium phosphate buffer at pH 7.0. The absorbance values were measured at 750 nm by spectrophotometer. Blank sample was prepared by using 1 mL buffer instead of 1 mL sample. For determination of the enzyme concentrations using the absorbance values, the calibration curve was prepared using Bovine Serum Albumin (BSA). The equation obtained for the calculation of protein concentration was given as below:

C (g protein/L)=0.4212 x (Absorbance value)

The statistical values, regression coefficient (R^2) and standard deviation (σ), were calculated as 0.9867 and 0.0427, respectively.

5. Enzyme Activity Assay

Enzymatic activity of Subtilisin was determined via spectrophotometric analysis by means of the density of orange color existing as a result of the reaction at which azocasein breaks down into sulfanilamide groups at the end of the incubation. Azocasein is a modified protein with orange colored sulfanilamide groups. The procedure modified by Tomarelli et al. [28] was used for the enzyme activity assay. The calibration curve was prepared by reading absorbance values at various enzyme units. The samples with different enzyme concentrations were reacted with azocasein. The absorbance values were measured at 440 nm by spectrophotometer. The equation obtained for the calculation of enzyme activity versus absorbance value was given as below:

Enzyme activity (Au/L)=3.2894 x (Absorbance value)

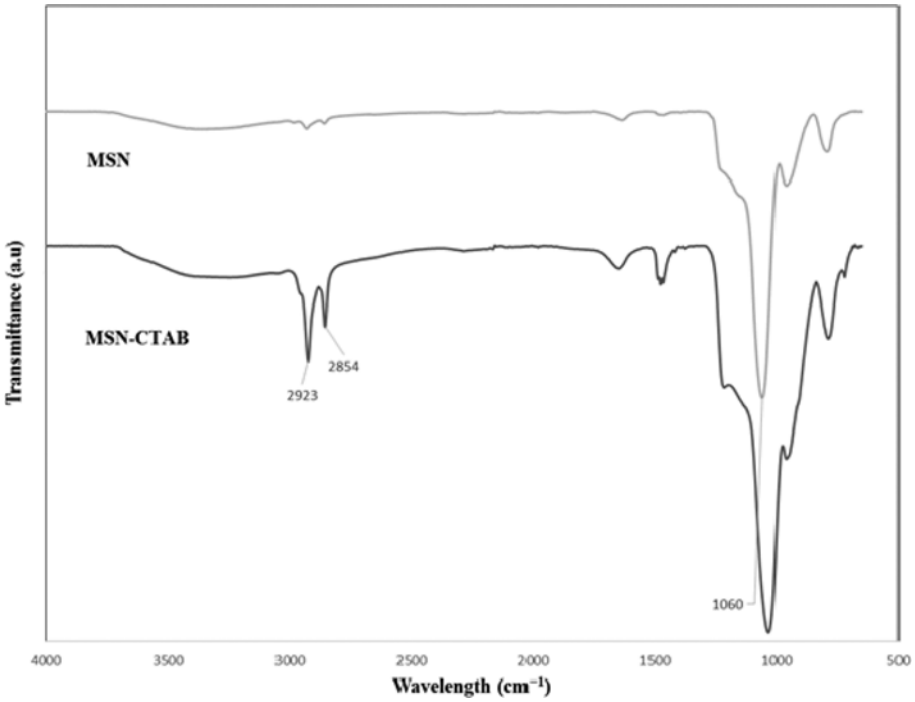

Fig. 1. FTIR spectra of MSN and MSN-CTAB.

The statistical values, regression coefficient (R^2) and standard deviation (σ), were calculated as 0.9885 and 0.0399, respectively.

6. Characterization Studies

For the characterization of uncoated and polymer-coated MSNs, the following devices were used: Transmission electron microscopy (TEM) images were obtained using a JEOL 2100 JEM HRTEM (High resolution transmission electron microscope) at TUBITAK (The Scientific and Technological Research Council of Turkey) Materials Institute. The mean size and size distribution of the particles were measured via dynamic light scattering by Zetasizer (Malvern, Zen 3600) in aqueous solution at pH 4.5 and 7.4. The zeta potential of the Particles was measured on a Zetasizer Nano-ZS (Malvern) at 25 °C. FTIR spectra were measured on a Perkin Elmer-Spectrum 100. Thermogravimetric analysis of the particles was performed using SII Nanotechnology-SII6000 Exstar TG/DTA 6300 with a heating rate of 20 °C/min at a nitrogen environment.

RESULTS AND DISCUSSION

1. Characterization of MSNs

MSNs were prepared according to the sol-gel method, and chemical structure was determined via FTIR spectra; porous structure and molecular size was analyzed with SEM and TEM micrographs.

As can be seen in Fig. 1, the characteristic absorption band was

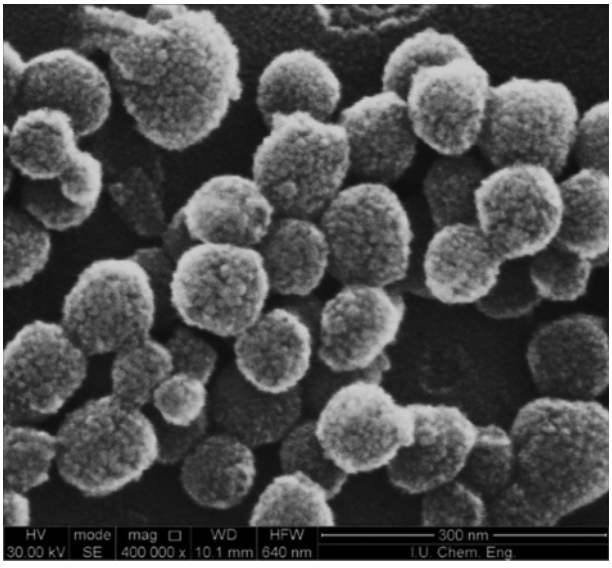

Fig. 2. SEM image of MSNs.

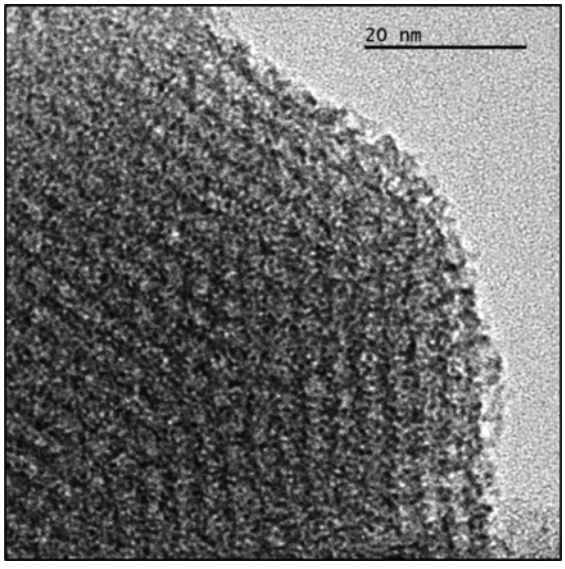

Fig. 3. TEM image of MSNs.

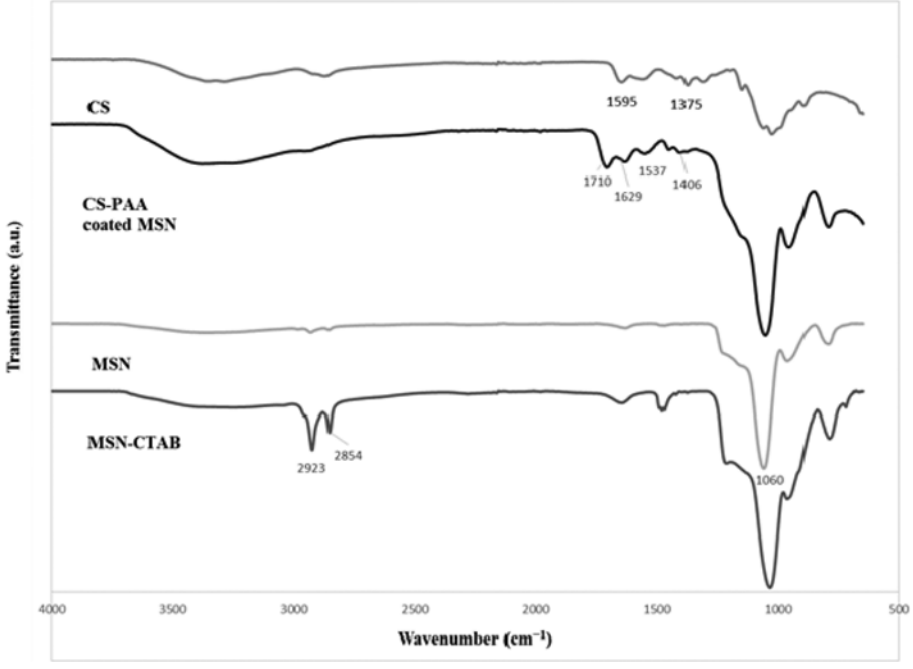

Fig. 4. FTIR spectra of MSN-CTAB, MSN, CS-PAA/MSN and CS.

at 1,060 cm^{-1}, which could be assigned to the absorption of Si-O in the spectrum of MSN. C-H stretch (2,854 cm^{-1} and 2,923 cm^{-1}) peaks from CTAB surfactant disappeared after the CTAB removal (reflux section) process, which showed that there was no residual CTAB in the structure of MSNs, so the effect of the toxicity from organic template was eliminated, as pointed out by Tang et al. [14].

Fig. 2 shows the SEM image of MSNs. The MSNs were spherical with the particle size in the range of 60-100 nm with the mean value of 80±10 nm (Fig. 2). The porous structure of the nanoparticles can also be seen clearly from the TEM image of MSNs as given in Fig. 3.

2. Characterization of CS-PAA/MSNs

As can be seen from Fig. 4, CS revealed typical peaks of 1,595 cm^{-1} (amide II) and 1,375 cm^{-1} (amide III) [29]. In the spectrum of CS-PAA/MSNs, when compared with those of CS and MSN, the characteristic absorption bands were found at 1,710 cm^{-1}, 1,629 cm^{-1} and 1,060 cm^{-1}, respectively, which could be assigned to the absorption of carboxyl groups of PAA [30], protonated amino groups of CS [31], and Si-O of MSN [32].

The bands at 1,537 and 1,406 cm^{-1} could be assigned to asymmetric and symmetric stretching vibrations of COO- anion groups, which verified that PAA were dissociated into COO- groups and complexed with CS through electrostatic interaction to form polyelectrolyte complexes during polymerization. Similar reaction was achieved by Tang et al. [14] with CS and PMAA acid.

To investigate the structural difference between CS-PAA and CS-PAA coated MSNs, CS and AA were mixed without MSNs and gel formation was observed. FTIR spectrum (Fig. 5) of this gel structure lacks a peak at 1,060 cm^{-1} (absorption of Si-O of MSN) in contrast to that of CS-PAA/MSN.

Fig. 6 shows the TEM images of MSNs and CS-PAA/MSNs. In Fig. 6(a), MSNs have uniform-porous-spherical structure with a diameter of ~100 nm. When the polymerization occurred on the surface of MSNs, the porous surface was covered by a smooth-gray outer layer which proving that the presence of organic moieties and, the diameter was increased to the range of 100-200 nm.

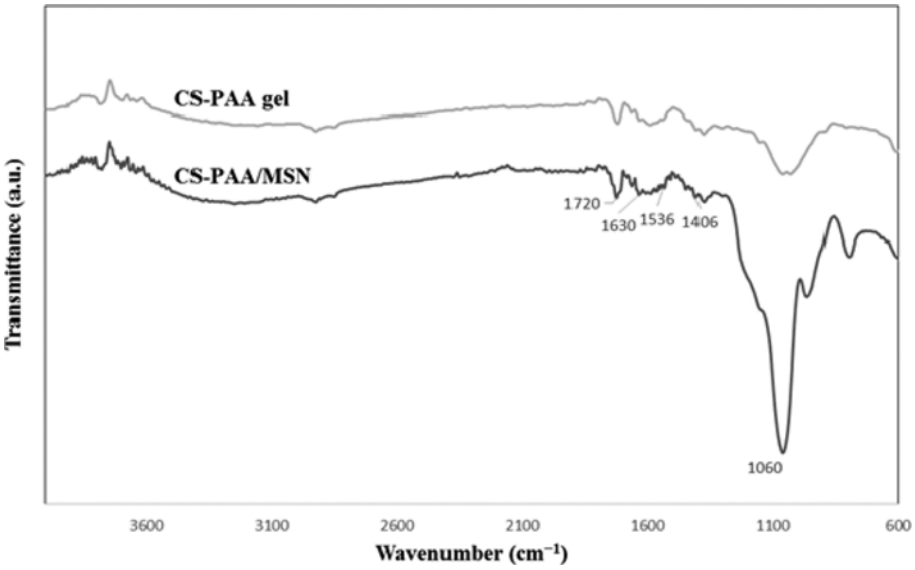

Fig. 5. FTIR spectra of CS-PAA gel and CS-PAA/MSN.

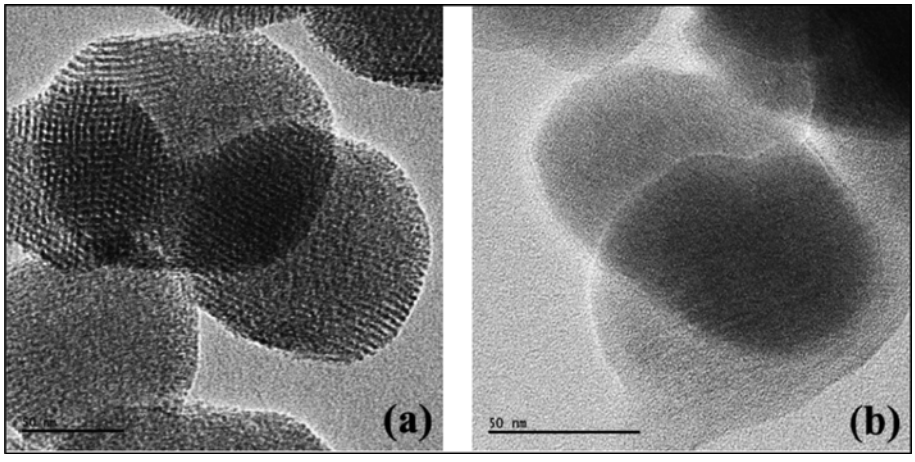

Fig. 6. TEM micrographs of (a) MSNs and (b) CS-PAA/MSNs.

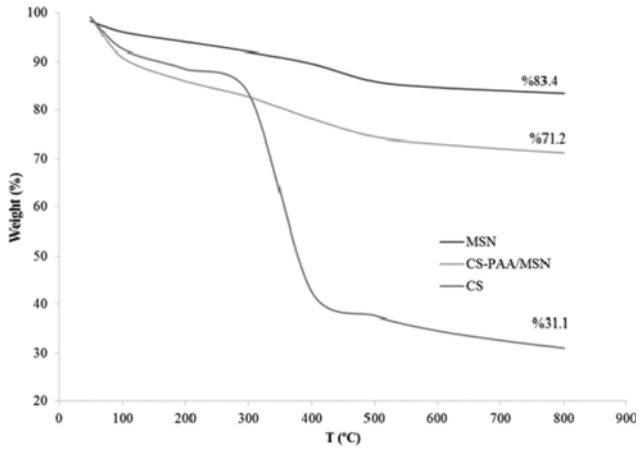

Fig. 7. TGA curves of MSN, CS-PAA/MSN and CS.

The shell layer seemed to cause the uniform spheres to lose their standard shape and size. As can be seen from Fig. 6(b), the particles stick together with irregular shape. Pore size can be read as 4-5 nm from TEM micrographs.

CS-PAA coated MSN composite particles were measured through the TGA runs in the condition of nitrogen atmosphere at the heating rate of 20 °C/min. The TGA curves at Fig. 7 show that the weight loss of MSN was 16.6%, while that of CS-PAA/MSN was 28.8%. The weight loss of pure CS was 68.9%. Thus, the content of CS-PAA blend was about 23.4 wt% in CS-PAA/MSNs. For CS-PAA/MSNs, the weight loss below 200 °C was quite small (7%) because of the removal of absorbed physical and chemical water. The structure of CS-PAA began to decompose from ~230 to 500 °C, and there was no significant weight loss from 500 to 900 °C, as also found by Wu et al. [25].

Zeta potential analysis is a technique for determining the surface charge of nanoparticles dispersed in solutions (colloid). Nanoparticles have a surface charge that pulls toward a thin layer of ions of opposite charge to the nanoparticle surface. This attraction causes the formation of a double-layer of ions on the surface. This double layer moves with the nanoparticle as it spreads throughout the solution. The electric potential at the boundary of the double layer is known as the zeta potential of the particles and has values that typically between +100 mV and −100 mV [33].

As pointed out by Malkoç [34], silica nanoparticles have high colloidal stability in aqueous medium. Thus, the hydroxyl groups in silica shift zeta potential of MSNs to negative region at neutral pH.

The effect of pH values on the zeta potential of the CS-PAA/MSNs is plotted in Fig. 8. The changes in the zeta potential values with pH change of the medium are an image of the charge density of the particles. The zeta potential of CS-PAA/MSNs decreased from −11.3 to −26.4 mV as the pH value increased from 4.5 to 7.4 for 2 : 1 ratio of PAA : [NH_2] because of the decrease of CS ionization and increase of PAA ionization. Zeta potential difference increases to negative side, showing the increase of the negatively charged particles. When PAA/[NH_2] ratio was increased, zeta potential values decreased for both pH values due to increase of PAA amount in the polymer layer. More PAA amount means more ionization and more negative charge density.

The pKa value determines the effect of change in pH to the structure of polymer via affecting the degree of ionization. PAA becomes negatively charged when the pH of the solvent is above its pKa 4.7 due to the ionization of its -COOH groups and it has poor solubility at low pH values. The intrinsic pKa of the CS was reported as 6.5 by Ahn et al. [35].

Above pH 6.5, -COOH from PAA dissociates and -NH_2 from CS deprotonates, increase of pH leads to an increase of the ionization degree and charge density of the PAA molecules, which would reduce the electrostatic interactions and molecular linkages including hydrogen bounds between the CS and PAA chains, resulting in the increase of hydrodynamic size and the decrease of zeta potential simultaneously. Below pH 6.5, -NH_2 from CS would be protonated. With the decrease of pH value, the electrostatic repulsion of the protonated amino groups of the CS chains extends, the polymer layer swells and the zeta potential increases, which is consistent with the findings of Tang et al. [14].

Hydrodynamic diameter values are greater than that of the determined values from TEM images because of the hydrate layer formation on the surface of the particles in aqueous environment as also pointed out by Yuan et al. [15].

The effect of pH values on the particle size of the CS-PAA/MSNs is shown in Fig. 9. As can be seen, the particle size increased from

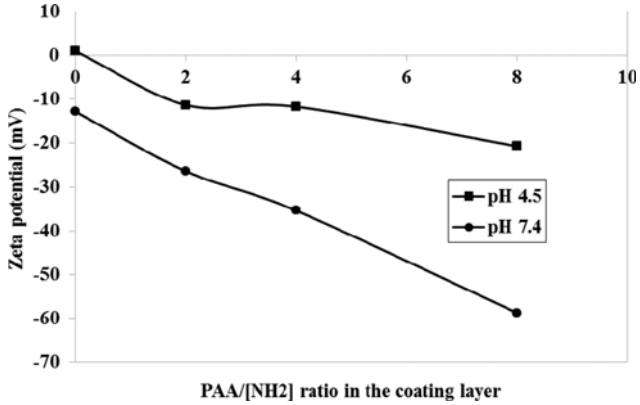

Fig. 8. The effect of PAA/[NH_2] ratio to the zeta potential at various pH values (ratio 0 nominates MSN without coating).

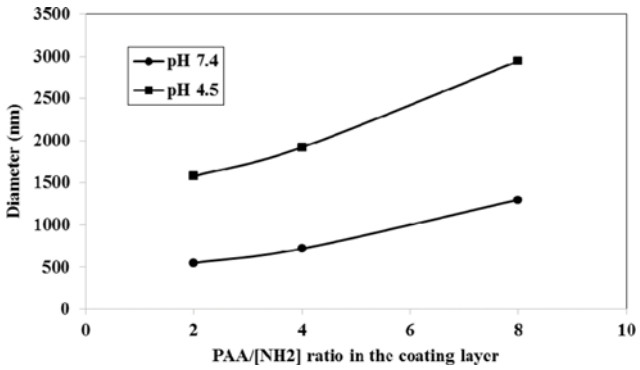

Fig. 9. Effect of PAA/[NH_2] ratio to the hydrodynamic diameter of CS-PAA/MSNs at pH values of 4.5 and 7.4.

550 to 1,578 nm for 2 : 1 ratio of PAA : [NH_2] with decreasing the pH value from 7.4 to 4.5 due to the extending polymer layer. Linear polymer chains of PAA are known to perform as pH-sensitive gatekeeper when grafted onto the surface of MSNs [34].

The polymer chain is open at high pH value by means of extending the polymer chain. As mentioned, the polymer layer below pH 6.5 swells progressively due to electrostatic repulsion of the protonated amino groups of the CS chains. Thus, since 4.5 is closer in value than 7.4 to the pKa value of 6.5, the diameter values are bigger at 4.5 due to swelling CS. If pH value was higher than 9-10, the values of hydrodynamic diameter would be close to values obtained at pH 4.5. When PAA/[NH_2] ratio was increased, hydrodynamic diameter was also increased at both pH values by means of increasing PAA amount in the layer.

According to the findings mentioned above, CS-PAA/MSNs were pH-sensitive, which means the surface of MSNs could have positive or negative charges due to the change of the medium pH.

3. Enzyme Immobilization Studies

One of the parameters affecting percent loading was the matrix amount, which was examined as 10, 15, 20 mg based on the studies in the literature. 10 mg was chosen as optimum matrix amount since percent enzyme loading/mg matrix is higher at this amount as can be seen in Fig. 10.

Another parameter affecting percent loading is the immobilization time. As can be seen in Fig. 11, maximum loading occurs in first 15-20 minutes, but then leaching starts immediately. However, there is no significant difference between 4 and 20 h. Thus, it was assumed that the system reached equilibrium in 4 h, and the immobilization time was determined as 4 h.

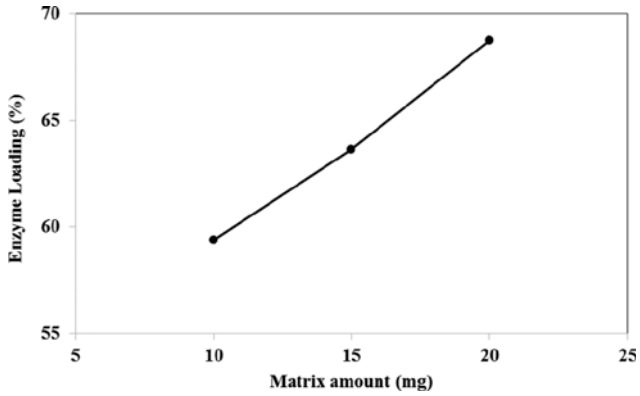

Fig. 10. Matrix amount effect on percent enzyme loading.

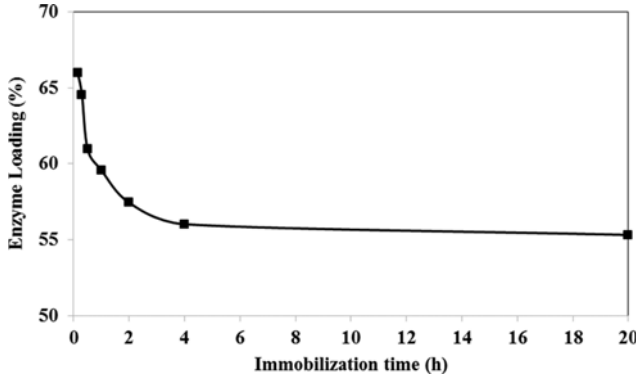

Fig. 11. Immobilization time effect on percent enzyme loading.

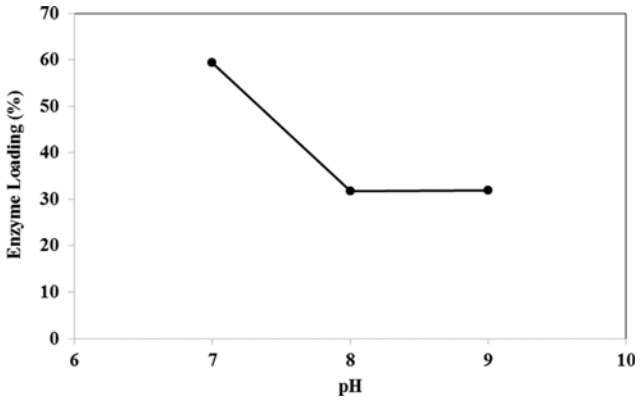

Fig. 12. pH effect on percent enzyme loading.

For determination of the percent enzyme loading as a function of immobilization time, various mathematical equations were examined. Among the models applied, Weibull empirical model, Eq. (1), was observed as the most appropriate one for the data because of the higher value for the coefficient of determination (R^2) and lower standard error (σ). The statistical values, R^2 and σ, were calculated as 0.9951 and, 0.4766, respectively. The estimated coefficients were found as 66.66, 11.38, 0.412, −1.043 for a, b, c, and d, respectively.

Weibull Model:

$$y=a-b\cdot\exp(-c\cdot x^{d}) \quad (1)$$

As can be seen in Fig. 12, the effect of pH of immobilization solution to percent enzyme loading was examined, taking the optimal pH interval of Subtilisin published in the literature by Novozymes (2014) into consideration [37]. At pH 7, percent enzyme loading equals twice that of pH 8 and 9. Most of the studies had been achieving in the range of pH values between 7 and 8, which had investigated Subtilisin immobilization.

The denaturation of the enzyme was higher when the pH value was lower. The isoelectric point (pI) of serine protease was ~6 as pointed out by Mehrnoush et al. (2012); thus, the enzyme had negative charge above pH 7-9 [38]. pKa of PAA was 4.7; when pH of the medium was increased, ionization of PAA increased. pKa value of CS was 6.5 as stated by Wu et al. (2006); above this value CS was neutral [25]. Above pH 7, a sharp decrease in the percent loading was observed. This can be explained by the tendency of the particles to repel each other since both of the matrix and enzyme were loaded with a negative charge.

4. Immobilized Enzyme Activity Studies

The optimal conditions for the immobilization process were determined at pH 7, temperature of 25 °C, immobilization time of 4 h, and mixing rate of 250 rpm. After optimizing the percent enzyme loading, the effects of temperature, pH, repeated use, and storage time on immobilized and free enzyme activity were investigated.

The effect of pH on free and immobilized Subtilisin enzyme activity was studied for azocasein hydrolysis at 40 °C. The effect of pH on free and immobilized enzyme preparations was investigated in

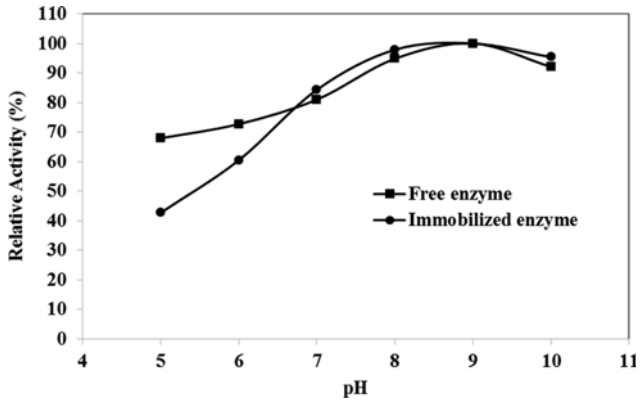

Fig. 13. pH effect on free and immobilized Subtilisin activity.

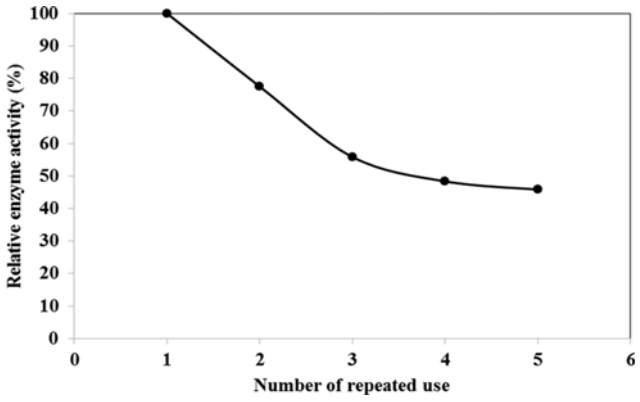

Fig. 15. Operational stability of Subtilisin immobilized onto CS-PAA/MSNs.

the pH range of 5.0 and 10.0 in sodium phosphate buffer, and the results were presented in Fig. 13.

The maximum enzyme activity was achieved at pH 9.0 for free Subtilisin and immobilized Subtilisin. The optimum pH value for free enzyme is consistent with that in the literature published by Novozymes [37]. The trends of the curves are similar for both cases. After immobilization process, the optimum pH interval for azocasein hydrolysis did not change. As pointed out by Erdem [39], at pH values lower than 8, the enzyme activity was inhibited for two reasons: i) the relative-narrow structure of pH-sensitive polymer chains at low-pH values, and ii) a possible change of the enzyme conformation due to an unfavorable charge distribution of the amino acid moieties that causes a further activity decrease.

It can be concluded that the change in pH of the reaction solution affected immobilized enzyme activity since the surface charge of the polymer layer of CS-PAA/MSNs was pH-dependent, and the structure of free enzyme was not stable at the values other than 6-10.

The activities of free and immobilized Subtilisin were calculated by measuring the absorbance of the azocasein solutions at 440 nm. The temperature effect on the activity of free and immobilized enzymes is shown in Fig. 14, by means of relative activities. Free enzyme exhibited its highest activity at around 70 °C and this interval shifted to 70-80 °C for immobilized enzyme. It can be said that immobilized enzyme was more stable at higher temperatures compared with the free enzyme.

The activity of Subtilisin immobilized on CS-PAA/MSNs was determined after repeated use (operational stability) at the experimental conditions: pH 7, immobilization time of 4 h, mixing rate of 250 rpm. Then, the relationship between immobilized enzyme activity and number of repeated use was obtained. Immobilized enzyme activity was reduced from 100% to 45.83% after five repeated uses, as seen in Fig. 15. The reason for the decrease of the enzyme activity can be explained by the enzyme lost from CS-PAA/MSNs after each repeated use.

For determination of the immobilized enzyme activity as a function of repeated use, various mathematical equations were examined. Among the models applied, the MMF empirical model, Eq. (2), was observed as the most appropriate one for the data with higher values for the coefficient of determination (R^2) and lower standard error (σ). The statistical values, R^2 and σ, were calculated as 0.9999 and, 0.0547, respectively. The estimated coefficients were 44.12, 0.043, 102.39, −4.12 for a, b, c, and d, respectively.

MMF Model:

$$y=(a\cdot b+c\cdot x^d)/(b+x^d) \quad (2)$$

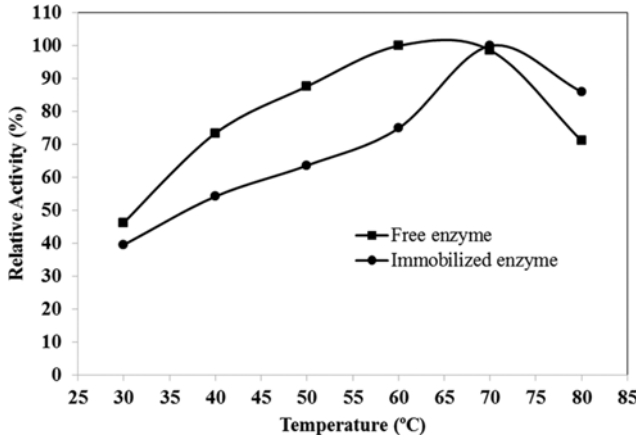

Fig. 14. Temperature effect on free and immobilized Subtilisin activity.

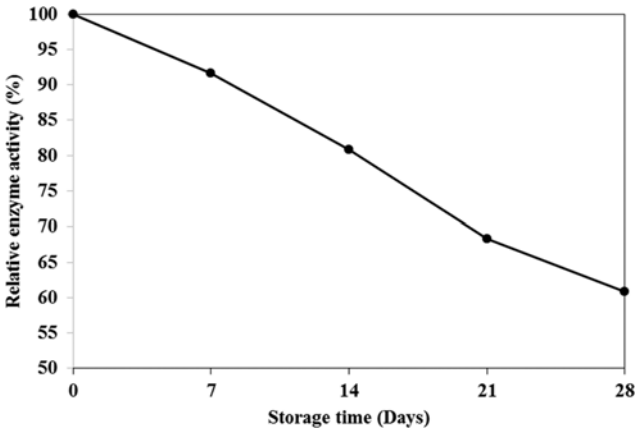

Fig. 16. Storage time stability of Subtilisin immobilized onto CS-PAA/MSNs.

Storage time stability of immobilized Subtilisin was examined after storing in 0.1 M sodium phosphate buffer (pH 7.4) at 4 °C for 28 days. The activity of immobilized enzyme was measured every seven days, and the residual activity was determined in comparison with the activity at the beginning of storage. Activity of immobilized enzyme was reduced from 100% to 60% after 28 days of storage time, as can be seen in Fig. 16. The data obtained from the present study was compared with the data taken from the study by Ferreira et al. [40] using the same free enzyme. In their study, the free Subtilisin enzyme activity remained nearly 20% after 14 days of storage time. In the present study, it remained as nearly 80% (which is four-times higher) for 14 days of storage time. It can be concluded that the immobilized Subtilisin presents an improved stability over its free form and may be an attractive biocatalyst for industrial purposes.

On the other hand, we focused on the differences between uncoated MSNs and polymer-coated MSNs (CS-PAA/MSNs) as matrices for the enzyme immobilization process. The enzyme loading data for both uncoated and polymer-coated MSNs were found as approximately 55% and 60%, respectively, at the optimum conditions obtained for immobilization process. The activity of Subtilisin immobilized on polymer-coated MSNs was found at 84.34%. But, uncoated MSNs showed lower enzyme activity at 1.55%. The possible explanation for this could be the blockage of the active sites of the enzyme during binding to the uncoated surface of MSNs.

CONCLUSIONS

The optimal process conditions for the synthesis of polymer-coated mesoporous silica nanoparticles (CS-PAA/MSNs) were investigated considering the properties such as morphologic pattern, the mean size, size distribution, surface charge of the particles as well as thermogravimetric stability with SEM, TEM, Zetasizer and TGA analyses. Subtilisin enzyme was chosen as a model to investigate the efficiency of the polymer-coated mesoporous silica nanoparticles as immobilization matrix. The effects of pH, temperature, storage time and repeated use on the immobilized enzyme activity were investigated. The findings showed that CS-PAA/MSNs were a suitable agent for enzyme immobilization. The results indicated that the activity of enzyme immobilized onto polymer-coated MSNs was increased up to 84.34% as compared to 1.55% obtained for the enzyme immobilized onto uncoated MSNs. But, covalent immobilization should be investigated to increase the stability and for efficient use of the enzyme. The synthesis part of the present study could also have an application for drug delivery systems, since the particles provided pH-sensitive behavior due to the zeta potential and hydrodynamic size analysis. Polymer-coated MSNs have been widely studied in the development of drug-delivery systems, because polymers can provide adjustable properties of structure, size, stability, biocompatibility and functionality.

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