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Academician Bogdan C. Simionescu (1948–2024)*

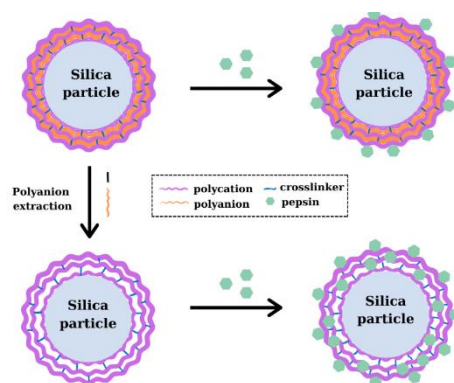
OPTIMIZATION OF PEPSIN SORPTION ON CORE-SHELL COMPOSITE MATERIALS FOR ENHANCING ENZYME RETENTION

Larisa-Maria PETRILA,* Florin BUCATARIU and Marcela MIHAI

“Petru Poni” Institute of Macromolecular Chemistry, 41 A Grigore Ghica Voda Alley, Iasi, Roumania

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Protein separation is a critical subject in the biological sciences, influencing applications such as purification, drug delivery or enzyme immobilization. Core-shell composites are efficient sorbents for separating proteins as they possess increased mechanical stability and can be easily reused in multiple separation cycles. Additionally, the surface chemistry of core-shell composites can be tailored to enhance the sorbent/protein affinity, increasing separation efficiency. This study investigates the main parameters influencing protein separation by sorption, using core-shell composites fabricated by layer-by-layer assembly of poly(ethyleneimine) and poly(acrylic acid) or poly(sodium methacrylate), as selective sorbents for proteins. Pepsin was selected as a model protein, testing key parameters influencing the sorption of the enzyme, including the characteristics of the composite sorbent such as the polyanion type, the stabilization of the composite by polyanion extraction, the sorbent's hydrophobicity, the protein concentration and pH. Optimal conditions for pepsin sorption were identified, observing an important influence of the pH and enzyme concentration. Enhanced sorption capacity was observed for the tested composite microparticles, better results being achieved with composites featuring increased hydrophobicity and those modified by removing the polyanion from their organic shells.



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INTRODUCTION

Protein separation is extremely important for applications such as chromatography and separation, drug delivery, enzyme immobilization and biomaterial fabrication. Among the methods applied for the separation of proteins, sorption is one of the simplest and most economical techniques, being generally applied in both medical and industrial fields. The mechanism of protein sorption is governed by the formation of weak

interactions between the protein and the sorbent, including electrostatic interactions, hydrogen bonding and hydrophobic interactions.¹ Protein sorption is influenced by various factors, including pH, protein concentration, surface chemistry or ionic strength.

Of comparable importance in the separation of proteins is the design of efficient and selective sorbents that allow the separation of the target compounds in various environmental conditions. Common sorbents used for protein separation

* Corresponding author: larisa.petrila@icmpp.ro

include ion-exchange resins,² affinity sorbents functionalized with specific ligands,^{3,4} porous microparticles such as silica,⁵ polymer beads,⁶ or composite materials.⁷ A particular type of porous composite sorbents that can be used for protein separation is represented by core-shell composites. From a structural point of view, core-shell composites present a multi-structured architecture, typically consisting of an inorganic (“hard”) core, covered by an organic (“soft”) shell. Such materials have drawn increasing interest due to their unique properties. The inorganic core imparts the composites with enhanced mechanical stability or even magnetic properties.⁸ At the same time, the organic shell plays a key role in the potential applications of the composites, improving the flexibility of the material, but also introducing functional groups that can enhance its chemical and physical properties. Additionally, carefully selecting the outer layer material can impart biocompatibility, biodegradability, or improved chemical stability to the composite.⁹

Core-shell composite materials can be obtained by a plethora of methods, including emulsion polymerisation,¹⁰ layer-by-layer (LbL) assembly¹¹ or sol-gel processing.¹² Among the methods established for the fabrication of core-shell composites, LbL has been imposed as an effective method to obtain highly organized architectures of the external organic shell. The LbL method consists of the alternating deposition of oppositely charged polyelectrolytes on a support matrix, with the formation of multilayered architectures with various properties.¹³ The layer thickness, stability and properties can be tuned by the careful selection of the building blocks and depositing conditions. LbL assembly depends on multiple parameters such as the molar mass, charge, ionic strength or concentration of the selected polyelectrolytes.¹¹ The precision-tailored multilayered architecture and the multifunctionality of the core-shell composites make them ideal candidates for various applications requiring stability, selectivity and increased efficiency.¹⁴ Their applications range from water decontamination by sorption processes,¹⁵ drug encapsulation and controlled release,^{16,17} enzyme immobilization or protein sorption and separation.¹⁸ Among the potential applications of such materials, the development of efficient sorbents for the separation of proteins holds significant promise as core-shell composites can successfully be used for the selective sorption of proteins in various conditions. The sorption capacity of core-shell composite materials is governed by the presence of reactive functional groups on the material's outer

layer and is strongly influenced by the architecture of the organic shell, the number and distribution of the functional groups as well as by the concentration of the sorbate.^{19,20} The characteristics of these materials, including their tailored surface chemistry, stability, binding capacity towards charged compounds and their reusability, make them ideal candidates for the selective sorption and separation of proteins, compared with traditional materials used for such purposes. The proposed materials offer improved binding capacity, stability, and reusability, as well as reversible protein binding under mild conditions, which is a significant advantage in preserving protein structural integrity, justifying their use as selective sorbents for proteins. This study delves into the sorptive properties of core-shell composite materials with a functional polyelectrolyte shell and tunable selectivity for charged molecules, obtained by the LbL deposition on silica particles (SP) of poly(ethylene imine) (PEI) and poly(acrylic acid) (PAA) or poly(sodium methacrylate) (PMANa) and their potential use as novel sorbents for the selective sorption and separation of proteins. Following the optimization of the sorption of pepsin (PEP), selected as a model negatively charged protein, the proposed materials demonstrate their potential to act as innovative sorbents for proteins, emphasising a new direction of application for core-shell composite microparticles.

RESULTS AND DISCUSSIONS

The sorbents used for protein separation must respond to the need for an increased affinity for proteins without affecting their structure or biological function, allowing their separation as well as their further release in controlled conditions. The sorbents used in this study were obtained by the LbL deposition of PEI as polycation (PC) and PAA or PMANa as polyanions (PA) on silica microparticles, using a method already established.¹⁵ The choice of the PAs used for the deposition of the organic shell imparts the composites with different surface morphologies and hydrophobicity, properties that influence the sorption capacity of the composite material, as reported elsewhere.²¹ The LbL deposition of the polyelectrolyte pairs was conducted until the formation of materials with 0.5, 1.5, 2.5 and 4.5 bilayers PC/PA and the samples were named according to the number of bilayers and the used PC/PA pair, namely (SP/(PEI/PAA)_x or SP/(PEI/PMANa)_x). Additionally, the prepared composites were subjected to a stabilization

treatment consisting of the extraction of the weakly bound PA chains, as presented in the materials and methods section. The samples subjected to this treatment were coded SP/(PEI)_x-(PAA) or SP/(PEI)_x-(PMANa), to emphasise the extracted PA.

Pepsin is a negatively charged protease recognized as one of the major enzymes involved in the digestion of proteins. The enzyme is highly active at low pH values, a strong increase in pH potentially causing enzyme denaturation.²² From a biomedical point of view, PEP is extremely valuable in the obtaining of bioactive peptides,²³ the separation and analysis of peptides²⁴ or the purification of collagen,²⁵ evidencing a wide range of applications of interest in the medical field. Due to its negatively charged surface above its isoelectric point (pH > 2), PEP could potentially be separated by sorption on the surface of positively charged microparticles such as the ones presented in this study. Its sorption behaviour is highly sensitive to the properties of the sorbent, including surface charge density, availability of the oppositely charged functional groups and accessibility of these groups, providing valuable insights into the

mechanism of interaction between the sorbent and the target molecule. One of the main parameters influencing the sorption of compounds in/on support hybrid materials with core-shell structures is the number of functional groups capable of forming interactions, but also the accessibility of these target functional groups. In the case of the core-shell composites used in this study, the deposition of successive layers of PEI on the silica support drives a significant increase in the number of -NH₂ functional groups that could potentially interact with PEP. At the same time, the increase in the number of layers deposited on the silica core increases the packing density of the PE chains, decreasing the probability of interaction between the functional groups in the lower layers and the molecules of interest. To study the influence of the deposited number of bilayers of polyelectrolytes on the sorption capacity of composite materials toward PEP, the sorption of the enzyme was studied using composite materials with 0.5, 1.5, 2.5 and 4.5 PC/PA bilayers. Figure 1 shows the variation in the amount of sorbed enzyme depending on the number of PC/PA bilayers of the composite material.

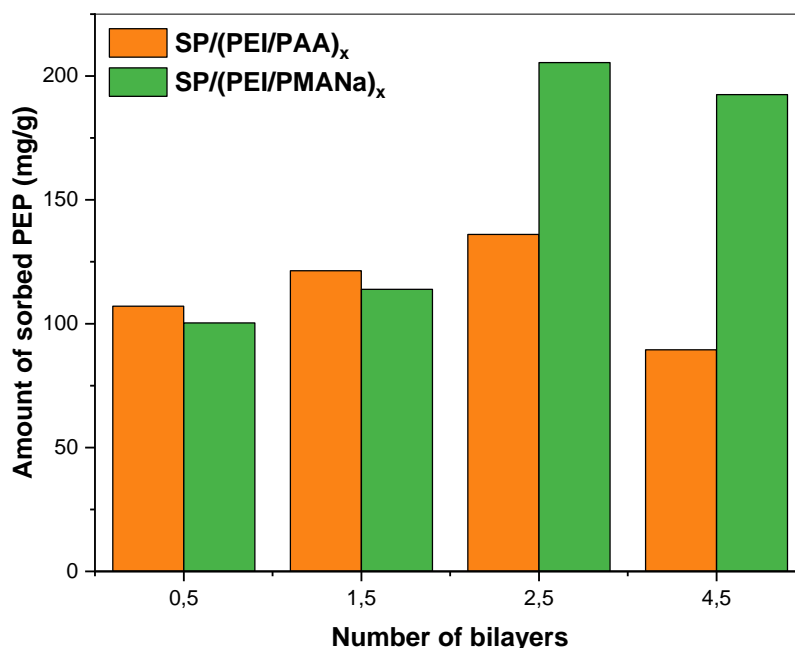


Fig. 1 – Influence of the number of bilayers on the sorption of PEP, using a PEP solution of 1 mg/mL prepared at pH = 4.5.

Two patterns can be observed regarding the effect of the number of deposited bilayers on the sorption capacity of the composites tested. For the composite materials fabricated by the deposition of PEI/PAA polyelectrolyte pair, the sorption capacity seems rather stable, independent of the number of bilayers (or independent of the amount of organic

material deposited on the silica core), suggesting that when the PAA is not extracted from the organic shell, the sorption of the protein takes place mostly at the surface of the material. This behaviour is rather expected considering that the deposition of the PEI/PAA polyelectrolyte pair leads to the formation of a thick organic shell.²¹ On the other

hand, the composite microparticles obtained by the deposition of the PEI/PMANa pair exhibit an increase in the sorption capacity with the increase in the deposited number of bilayers. This behaviour is most likely the result of the presence of PMANa chains in the structure of the material, which favours the formation of hydrophobic interactions with amino acid residues in the enzyme, leading to increased sorption on the composite surface. It can also be observed that, in the case of materials with 2.5 and 4.5 bilayers, the amount of sorbed enzyme does not largely varied, most likely because as the number of bilayers increases the polymer network becomes denser, blocking the access of the enzyme to the lower layers of the material, the sorption of the enzyme being carried out mainly on the surface of the material. Based on the information obtained in these studies, it can be assumed that composite materials with 2.5 and 4.5 bilayers offer a satisfactory sorption capacity for PEP.

An efficient method to increase the flexibility of the polymeric network and to enhance the number of accessible functional groups of core-shell

composites is the partial extraction of the PA from the polymeric shell. As previously reported,²¹ a strong acid/ strong base washing protocol will only extract the weakly bound polyelectrolyte chains, preventing the delamination of the material upon use, thus increasing the porosity of the material and enhancing the accessibility of the functional groups, which are essential in sorption processes. Generally, such treatment will predominantly extract the PA chains, which are not crosslinked and only desorb the weakly bound PC chains, which are more stable due to their chemical crosslinking and will not affect the overall chemical stability of the polymeric shell. To determine the influence of the PA extraction on the sorption capacity of the composite materials, the sorption of PEP on composite materials with 2.5 and 4.5 bilayers, obtained before and after the extraction of PA and weakly bound PC chains, was studied. Figure 2 shows the amounts of PEP sorbed on composite materials SP/(PEI/PAA)_{2.5}, SP/(PEI/PAA)_{4.5}, SP/(PEI/PMANa)_{2.5}, and SP/(PEI/PMANa)_{4.5}, using a PEP solution of 1 mg/mL prepared at pH = 4.5.

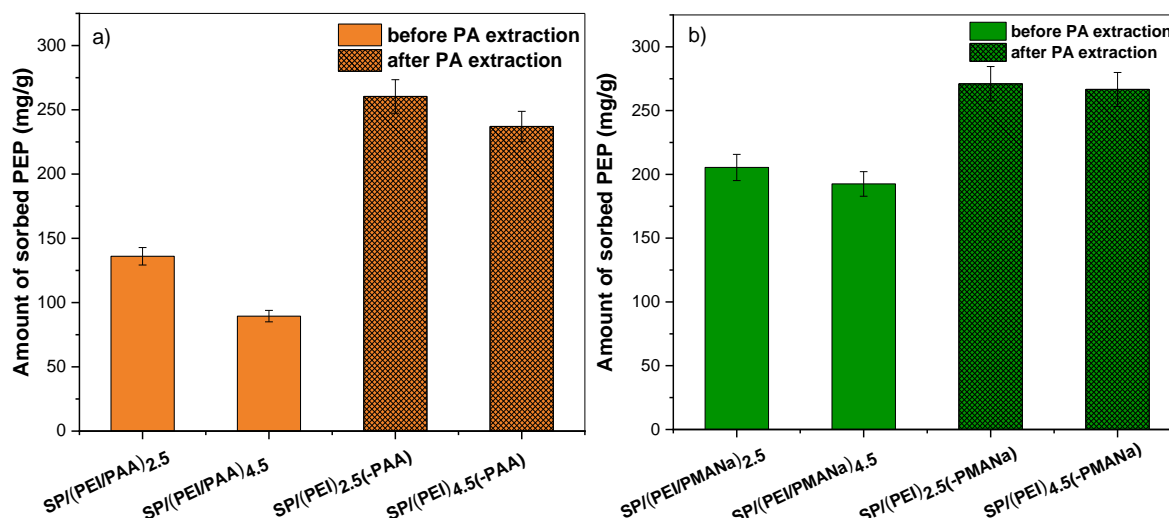


Fig. 2 – Influence of the extraction of the PA on the sorption of PEP, studied using a PEP solution of 1 mg/mL prepared at pH = 4.5.

Accordingly, in the case of composite materials obtained with PEI and PAA (Fig. 2a), a strong influence of the PAA extraction treatment can be observed. Thus, the amount of sorbed enzyme on the composites containing PAA is small both due to the reduced number of available $-NH_2$ functional groups and to the electrostatic repulsions between PEP and PAA chains. However, the extraction of PAA chains leads to an increase in the flexibility of the organic shell and to an increase in the number of functional groups available for interaction with PEP, so that the amount of sorbed enzyme on the

composites subjected to PA extraction is significantly higher. In the case of composite materials obtained with PEI and PMANa (Fig. 2b), the behaviour is similar, with the extraction of PA chains from the polymeric network leading to an increase in the amount of sorbed enzyme.

The high affinity of the composites was additionally confirmed by calculating the amount of sorbed PEP, from the weight loss of the materials before and after PEP sorption, for materials with PA or after the extraction process, determined from the thermogravimetric analysis (Fig. 3).

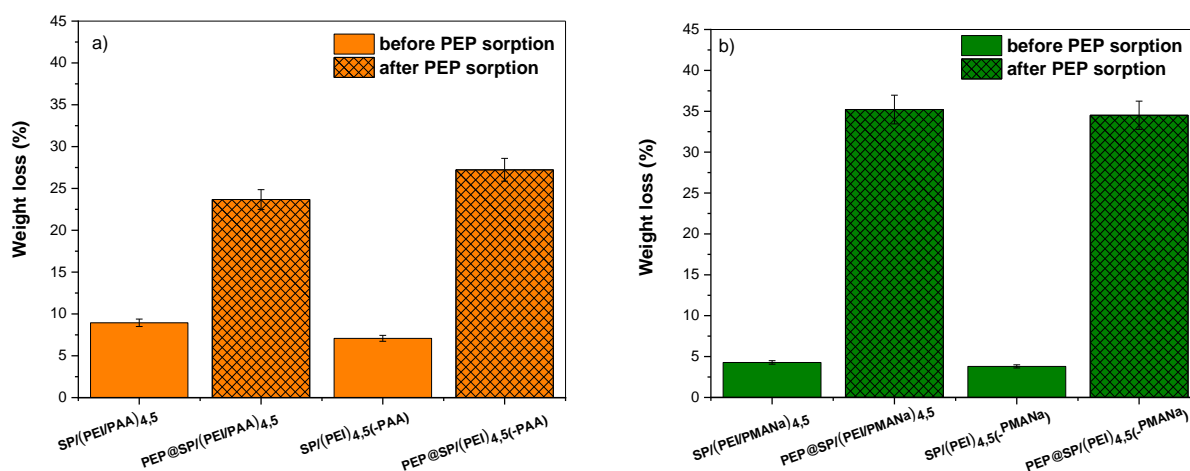


Fig. 3 – Mass loss of the composite materials with PA or after the extraction process, before and after the sorption of PEP, studied using a PEP solution of 1 mg/mL prepared at pH = 4.5.

As observed in the case of all tested materials, regardless of their chemical composition and eventual stabilization, after the PEP sorption, the weight loss is higher than that of the corresponding composite material before sorption, confirming the increased sorption capacity of the materials after PA extraction. Furthermore, by calculating the amount of organic material lost in the case of composites with and without sorbed enzyme, the amount of PEP can be determined by a direct method, the obtained values being similar to those obtained by the indirect quantification method, namely by UV-Vis spectrophotometry and presented in Fig. 2.

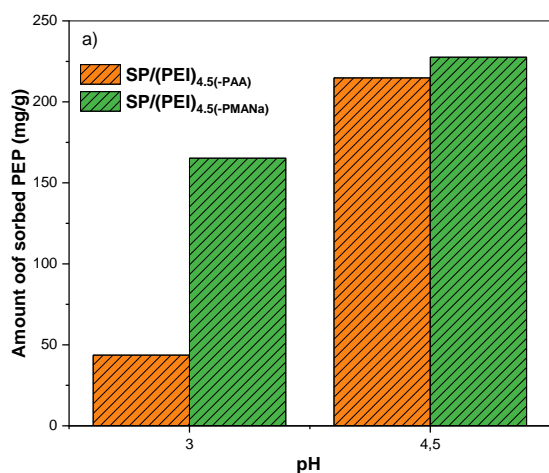
As observed, the composite material used as sorbent plays an important role in the sorption of PEP. While all the tested composites were able to sorb PEP in the tested conditions, better results were obtained for the composites with a higher number of bilayers (namely 4.5), and for the composites that were subjected to the extraction of the PA, which led to better accessibility of the $-NH_2$ functional groups. These results suggest that the composites SP/(PEI)_{4.5}(-PAA) and SP/(PEI)_{4.5}(-PMANa) are ideal candidates for the separation of PEP by sorption and were selected for further tests. In what concerns the nature of the PA, the results obtained for the sorption of PEP confirm previous literature observations^{21,26} regarding the increased affinity of more hydrophobic supports towards proteins. Such materials are better sorbents for proteins, being able to form both electrostatic and hydrophobic interactions with proteins.

While the role of the sorbent is primordial in the success of the sorption process, other parameters can also influence the amount of protein separated, such as their pH and concentration (Fig. 4). The pH

strongly impacts the separation of PEP as it can influence the conformation and the surface charge of the enzyme, as well as the stability and properties of the sorbent. To assess the influence of the pH on PEP sorption, tests were performed at pH = 3 and pH = 4.5, to ensure adequate ionization of the functional groups on the composite material without affecting its stability. While lower pH values would favour the preservation of the catalytic activity of the enzyme, a decrease in pH could cause an alteration of the stability of the deposited PE multilayers, with a strong protonation of the PEI chains and deprotonation of the PAA ones. The sorption tests were performed using the composite microparticles which ensured the highest sorption affinity, namely SP/(PEI)_{4.5}(-PAA) and SP/(PEI)_{4.5}(-PMANa), using an enzyme solution of 1 mg/mL, as in the previously performed tests.

By evaluating the amounts of the sorbed enzyme as a function of pH (Fig. 4a), it can be noted that in the case of both types of support microparticles, the sorption is favoured at pH = 4.5. In the case of the SP/(PEI)_{4.5}(-PMANa) composite, the influence of the pH seems less pronounced, suggesting the importance of the hydrophobic interactions in the sorption process, as well as the better stability of the polymeric shell in acidic media. In contrast, in the case of the SP/(PEI)_{4.5}(-PAA) composite, the influence of the pH is stronger, with the PEP sorbed amount at pH = 3 being less than 25% of the amount sorbed at pH = 4.5. In addition to the effect it has on the enzyme structure, the variation of pH can cause changes in the stability of the composite materials, especially by modifying the degree of ionization of the polyelectrolytes on the organic shell, which can lead to a weaker or stronger interaction with the

enzyme. Thus, it is expected that at low pH values, the stability of the composite materials would be lower, with some of the polyelectrolyte chains being desorbed from the silica microparticles. In this situation, the number of functional groups of the composite material that can form electrostatic interactions with the enzyme is low, reducing the chances of PEP sorption on the material surface.



However, the effect is less evident in the case of SP/(PEI)_{4.5}(-PMANa) composites which are more prone to form hydrophobic interactions with the enzyme, leading to increased sorption even when the formation of electrostatic interactions is not favoured. Based on these results, it was concluded that PEP sorption is optimal at pH = 4.5, a value at which further tests will be performed.

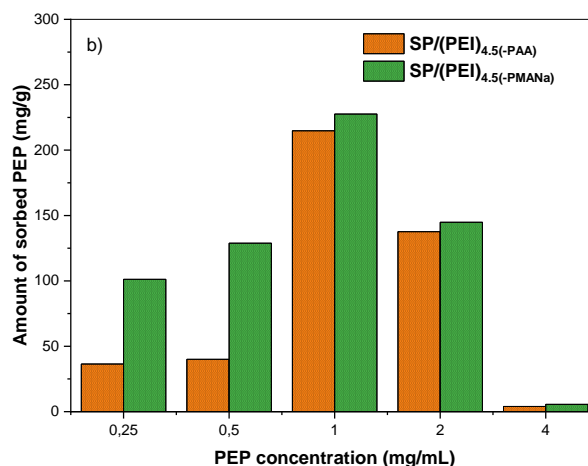


Fig. 4 – a) Influence of pH on the sorption of PEP from a 1 mg/mL solution; b) influence of concentration on PEP sorption at pH = 4.5, on the SP/(PEI)_{4.5}(-PAA) and SP/(PEI)_{4.5}(-PMANa) composites.

The concentration of the sorbate is another key parameter in the success of the sorption process. In the case of proteins, the use of a too-low protein concentration does not necessarily imply complete sorption due to its increased mobility in solution, while concentrations that are too high cause protein aggregation and diffusional limitations that will make the interaction between the support and the protein more difficult. Subsequently, the sorption of PEP was studied depending on the concentration of the used enzyme solution, as presented in Fig. 4b. By analysing the amount of sorbed PEP as a function of enzyme concentration it can be appreciated that the sorbed PEP amount increases with the increase in protein concentration up to an initial concentration of 1 mg/mL, while at higher initial concentrations surface saturation might occur. At lower PEP concentrations most of the enzyme is sorbed on the surface of the material, obtaining thus a higher distribution coefficient as compared to high concentrations of enzymes. The optimal results are obtained when the initial concentration of the enzyme solution is 1 mg/mL which guarantees a high enough amount of enzyme to interact with most of the support material's functional groups without producing secondary processes like the formation of enzyme aggregates

or the accumulation of the enzyme on the material's surface that can be readily desorbed during the washing steps. At the same time, it can be observed that the amount of sorbed PEP is higher for the SP/(PEI)_{4.5}(-PMANa) support material since it tends to form both electrostatic and hydrophobic interactions with the enzyme, a behaviour previously reported for hydrophobic materials. Based on these results, it can be concluded that the concentration of 1 mg/mL is optimal for PEP sorption at pH = 4.5.

The effect of the PA extraction and the successful sorption of PEP on the surface of the composite materials is additionally confirmed by scanning electron microscopy (SEM). As observed from the SEM micrographs in Figs. 5a,d, the deposition of the polyelectrolyte chains leads to the formation of a polymeric shell on the composite microparticles, which seems to cover the pores of the silica. Upon PA extraction in strong acid media, some of the organic material deposited is desorbed, leading to an increased porosity of the material, as evidenced by comparing Figs. 5a with b and d with e. Besides this modification in the porosity, no other phenomena associated with the degradation or delamination of the material could be observed, suggesting that the polymeric shell is rather stable.

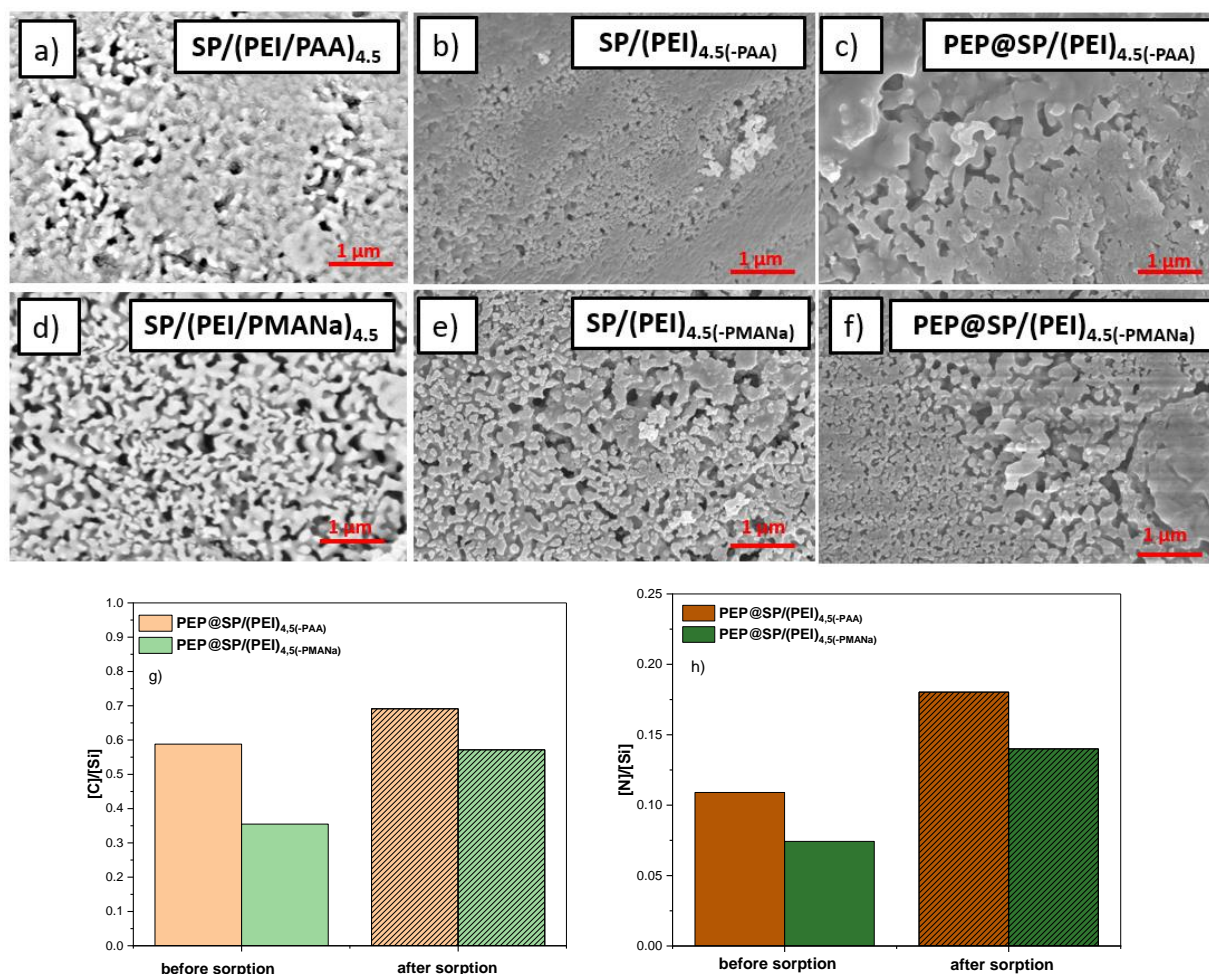


Fig. 5 – a-f) SEM micrographs of the composite materials before and after the acid/base treatment and after the sorption of PEP from a 1 mg/mL solution at pH = 4.5; g) [C]/[Si]; h) [N]/[Si] mass ratios before and after the sorption of PEP, calculated from energy-dispersive X-ray analysis

Additionally, the method can offer valuable information about the sorption PEP. The SEM micrographs (Figs. 5c,d) reveal a decrease in the apparent porosity of the composite materials upon enzyme sorption. As evidenced in Figs 5c,d, the sorption of PEP leads to the formation of a thin enzyme film that partially covers the surface of the material, suggesting that the sorption of the enzyme takes place mostly at the surface of the material. Using the energy-dispersive X-ray analyzer (EDX) coupled with the SEM microscope, the EDX analysis of the samples was performed for selected composites before and after PEP sorption. As concerns the chemical composition of the materials before and after PEP sorption, important information can be obtained by analysing the mass ratios [C]/[Si] and [N]/[Si] calculated from EDX, as presented in Figs. 5g,h. A significant increase in the two ratios can be observed with the sorption of the enzyme on the surface of the material, confirming the success of the sorption process. It can be noted

that the method confirms the chemical composition of the composite materials, observing lower [C]/[Si] and [N]/[Si] atomic ratios in the case of SP/(PEI)4.5(-PMANa) composite materials as compared to SP/(PEI)4.5(-PAA), confirming that the first type of composite has a thinner organic shell.

The performed optimisation experiments highlighted the fact that core-shell composites fabricated by LbL deposition of polyelectrolytes are efficient sorbents for PEP, being able to retain high amounts of protein from solution. The increased affinity for PEP of the composite microparticles was confirmed by the characterization methods employed, demonstrating an increased feasibility of protein separation by sorption.

EXPERIMENTAL

Materials: Branched PEI ($M_w = 25.000$ g/mol), PAA ($M_w = 100.000$ g/mol), PMANa ($M_w = 9.500$

g/mol), glutaraldehyde and PEP from porcine gastric mucosa were acquired Sigma – Aldrich, Germany. Silica microparticles of Daisogel type with pores of 200 nm and a mean diameter of 40–60 μm were obtained from Daiso Co., Japan.

Fabrication of the composite microparticles:

The composite core-shell microparticles were constructed following a method previously published.¹⁵ Briefly, the composite microparticles were obtained by the alternating deposition of PEI from a 10⁻² M solution, with intermittent stirring at room temperature for one hour, followed by washing with distilled water to eliminate weakly bound PC chains. After washing, the PEI layer was chemically crosslinked with 2.5% glutaraldehyde at a 1:10 ratio between the crosslinker's carbonyl groups and the polycation's amino groups. After cross-linking, the microparticles were rewashed 3 times with distilled water, then the PA was deposited using PAA or PMANa solution, 10⁻² M. After another hour, the microparticles were decanted, washed 3 times with distilled water and the next layer of PC was deposited and crosslinked following the same protocol. At the end of the deposition process, composite materials with 0.5; 1.5; 2.5 and 4.5 PC/PA bilayers were obtained, where a bilayer is defined as a double layer formed by a layer of PC and a layer of PA, the last layer being always the PC one. The obtained samples were coded as follows: SP/(PEI/PAA)_x or SP/(PEI/PMANa)_x, where x corresponds to the number of bilayers.

To increase the stability and flexibility of the organic shell deposited on the SP microparticles, the weakly bound PA and PC chains were extracted in strong basic media (NaOH, 1M), and strong acidic media (HCl, 1 M). The samples obtained after the extraction treatment were coded as follows: SP/(PEI)_x(-PAA) or SP/(PEI)_x(-PMANa), where the (-PAA) or (-PMANa) subscript signifies the PA extracted from the organic shell.

PEP sorption studies: The capacity of the composite core-shell microparticles to serve as sorbents for the separation of proteins was assessed in various conditions using PEP as a model protein. The sorption of PEP was tested under various conditions in the sorption process, assessing the influence of factors such the pH, enzyme concentration, number of bilayers and extraction of the PA. For this, 50 mg of selected core-shell composite microparticles were put in contact with 20 mL of enzyme solution at room temperature, with intermittent stirring for 24 h. The

amount of sorbed enzyme was determined by UV-Vis spectrophotometry, using a SPECORD 200 Plus Analytic Jena (Germany) spectrometer, at λ = 278 nm,²⁷ using acetate buffer solution as blank. For this, the calibration curve of PEP was constructed, and the amount of sorbed enzyme was determined using eq. (1):

$$q_e = \frac{(C_0 - C_e) \cdot V}{m \cdot 1000} \quad (1)$$

where q_e – amount of sorbed enzyme (mg/g); C_0 – initial concentration of enzyme in solution (mg/L); C_e – equilibrium concentration of enzyme in solution (mg/L); V – volume of enzyme solution used for sorption ($V = 20$ mL); m – the amount of composite material used for sorption ($m = 50$ mg).

To study the effect of the pH, PEP solutions were prepared in acetate buffer at pH = 3 and pH = 4.5 and the effect of PEP concentration was studied using enzyme solutions of 0.25–4 mg/mL, the sorption tests being performed in similar conditions.

Characterisation of the composites with sorbed PEP: The successful sorption of PEP was followed using thermogravimetric analysis. The thermal degradation of the samples (~ 6 mg) was carried out in a temperature range of 20–700 °C, with a heating rate of 20 °C/min, flow rate of 40 mL·min⁻¹, under nitrogen atmosphere, using a Star System equipment (Mettler, Toledo).

The morphological analysis of the core-shell microparticles, before and after the acidic/basic treatment and after the sorption of PEP was performed using the Verios G4 UC SEM microscope (Thermo Fisher Scientific, Czech Republic) equipped with an EDX analyzer (Octane Elect Super SDD detector, USA) and a concentric backscattered electron detector. To reduce the electrostatic charge and increase the conductivity of the samples, they were coated with a 10 nm layer of platinum using a Leica EM ACE200 metallizer. The chemical composition of the samples was assessed using EDX analyses.

CONCLUSIONS

This study provides a comprehensive analysis of the main parameters that influence the sorption of PEP on core-shell composites, fabricated by LbL deposition of polyelectrolytes onto silica microparticles. Various parameters were assessed, including characteristics related to the protein, such as the pH and concentration of the PEP solution, but also the properties of the composite material,

including the number of deposited polyelectrolyte layers on the support silica, the nature of the PA used for the fabrication of the composites and the extraction of PA. The sorption of PEP was strongly influenced by the pH and the concentration of enzyme, the optimal sorption being observed at pH = 4.5 and a concentration of 1 mg/mL. In what concerns the characteristics of the material, a very good sorption capacity was observed for the tested composites, where higher sorption capacity was recorded when the PA chains were extracted from the organic shell of the composite, irrespective of PA structure. The study confirms that such core-shell composite microparticles can be successfully used for the sorption/separation of PEP. Further tests will evaluate potential applications of the PEP/composite systems in different processes of catalysis.

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