Pepsin and Lysozyme Immobilization onto Daisogel Particles Functionalized with Chitosan Cross-linked Multilayers

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One type of cross-linked multilayer, based on chitosan (CHI) has been prepared using 3,3',4,4'-benzophenonetetracarboxylic-dianhydride (BTCDA) and glutaraldehyde (GA) as cross-linkers. CHI was adsorbed from acetic acid aqueous solution onto Daisogel microparticles. X-ray photoelectron spectroscopy showed a regular increase of the cross-linked multilayers onto Daisogel microparticles. The immobilization of pepsin and lysozyme onto the functionalized Daisogel surface, via GA, has been tested. The amount of immobilized enzymes significantly depended on isoelectric point of enzyme.

Keywords: chitosan, cross-linked multilayers, pepsin, lysozyme, silica microparticles

Hybrid microparticles provide suitable supports for biomolecules and active agents. In biomedical diagnostic, the immobilization of biomolecules (proteins, nucleic acids, enzymes, bacteria, viruses) is of tremendous importance [1, 2]. One of the most versatile and efficient technique for the functionalization of a variety of solid surfaces is the layer-by-layer (LbL) deposition of polyelectrolyte multilayers [3]. The building blocks for creation of biointerfaces are based mainly onto polysaccharides, synthetic polyelectrolytes, proteins, enzymes, antibodies, etc. [4-7]. Chitosan (CHI) is one of the most used cationic polyelectrolyte in the construction of LbL multilayers. The chitosan properties (nontoxicity, solubility in dilute acids, complexation with anions) recommend its utilization in pharmaceutical, medical fields and other industrial areas [8-11]. The presence of amine and hydroxyl functionalities in the CHI structural unit gives the possibility to carry out chemical reactions such us acetylation, quaternization, reactions with aldehydes, chelation of metals, complexation with other polyelectrolytes, etc. to provide a variety of new products [12]. The enzymes immobilization onto solid surfaces modified with polyelectrolytes is an intensively studied process [13-16].

By alternate deposition of poly(vinyl amine) (PVAm) and poly(acrylic acid) (PAA) onto silica microparticles and silicon wafers, we have obtained single component cross-linked multilayers by thermal treatment [17, 18]. Other studies reported the construction of single polyelectrolyte cross-linked multilayer based on poly(vinyl amine) (PVAm), using 3,3',4,4'-benzophenonetetra-carboxylic-dianhydride (BTCDA) and glutaraldehyde (GA) as cross-linkers [19, 20].

In this study, chitosan (CHI), a polycation positively charged under *p*H = 6.3, two cross-linkers, BTCDA and GA, and Daisogel microparticles have been used to investigate the formation of single CHI cross-linked multilayer. The immobilization of pepsine (PEP) and lysozyme (LYS) onto single polycation cross-linked multilayer deposited onto Daisogel microparticles, has been carried out.

Experimental part

Materials and methods

One type of silica microparticles was used, i.e. Daisogel with the particle diameters between 40 – 60 μm and pore size of about 100 nm, purchased from Daiso co. Japan. The CHI (Mv ≈ 400000 g/mol), was purchased from Aldrich and used without further purification. BTCDA, purchased from Merck, Darmstadt, Germany, and glutaraldehyde (GA) (25% solution in water, w/v) from Sigma-Aldrich, were used as received. Chemical structures of the main compounds used in this study are presented in figure 1.

LYS, from chicken egg white (70000 units/mg) and PEP, from porcine gastric mucosa (920 units/mg) were purchased from Fluka and Sigma, respectively, and used without further purification.

Strategy for the single CHI cross-linked multilayer formation

A Daisogel sample (4 g), was suspended in 200 mL CHI aqueous acidic solutions (c = 10^{-2} mol/L, *p*H ≈ 4). During the adsorption (1 h), the suspension was gently shaken at room temperature. In order to cross-link CHI chains deposited onto Daisogel microparticles, the cross-linking reaction with BTCDA was carried out in acetone. The polyelectrolyte adsorption and cross-linking steps were repeated until three polyelectrolyte layers were deposited, sample obtained being noted Daisogel//$(CHI\text{-}BTCDA)_n$.

Enzymes immobilization onto CHI modified Daisogel microparticles

The Daisogel//$(CHI\text{-}BTCDA)_{2.5}$ modified microparticles were suspended in 1% (w/v) aqueous solution of GA. The GA treatment, besides the suplimentary cross-linking of

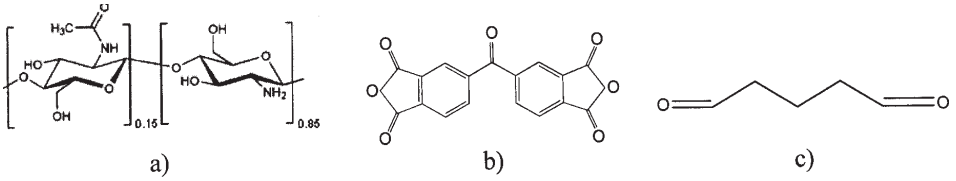

Fig. 1. Structures of CHI (a), BTCDA (b), and GA (c) used to produce single polyelectrolyte cross-linked multilayers.

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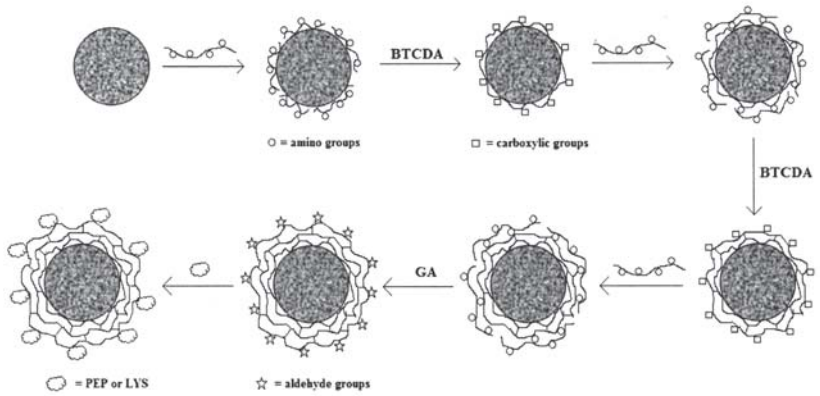

Fig. 2. The formation of $(CHI\text{-}BTCDA)_{2.5}$-GA cross-linked multilayer onto Daisogel and pepsin (PEP) and lysozyme (LYS) immobilization onto modified microparticles.

the formed multilayer, activate the hybrid surface for PEP and LYS immobilization. Finally, the washed samples were named as follows: Daisogel//$(CHI\text{-}BTCDA)_{2.5}$-GA-PEP and Daisogel//$(CHI\text{-}BTCDA)_{2.5}$-GA-LYS.

Streaming potential was performed employing an Electrokinetic Analyzer EKA (Anton Paar, Aus-tria). The *p*H-dependent measurements started from neutral *p*H of a KCl solution ($c = 10^{-3}$ mol·L^{-1}). The zeta-potential values were calculated from the measured streaming potentials by the Smoluchowski equation [21].

X-ray photoelectron spectroscopy (XPS) was used to determine the chemical composition of Daisogel modified microparticles. XPS measurements were carried out with an AXIS ULTRA photoelectron spectrometer (KRATOS ANALYTICAL, Manchester, England). The spectrometer was equipped with a monochromatic Al Kα (h . n = 1486.6 eV) X-ray source of 300 W at 15 kV. Quantitative elemental compositions were determined from peak areas using experimentally determined sensitivity factors and the spectrometer transmission function.

Results and discussions

The silanol groups from the surface of Daisogel microparticles ionize in the presence of water and in a wide range of *p*H the solid surface is negatively charged. The primary amino groups of CHI are protonated by hydronium ions from the acetic acid, resulting in positively charged groups. Therefore, the adsorption of CHI onto Daisogel microparticles is driven by electrostatic interactions and hydrogen bonds.

In order to stabilize CHI onto Daisogel surface, the cross-linking of polycation chains with BTCDA was carried out. The BTCDA could have two important effects: (i) cross-linking the polycation chains adsorbed onto Daisogel microparticles, and (ii) covering the microparticles with carboxylic groups. A new polycation layer was adsorbed after the cross-linking step. Before the enzymes immobilization, the multilayer was activated with GA. The schematic representation of single polyelectrolyte cross-linked multilayer buildup onto Daisogel microparticles and enzyme immobilization is illustrated in figure 2.

After each adsorption/cross-linking step, the streaming potential measurements of the modified Daisogel microparticles were performed (fig. 3).

Electrokinetic results allow the study of the charge formation at the solid surface. The *p*H value where zeta-potential (z) is zero was defined as the isoelectric point (*iep*). The protonation of primary amino groups from CHI chains in acidic environment, results in a positive net surface charge, supported by the positive values of z. When *p*H value of aqueous solution increases, the HO^- ions

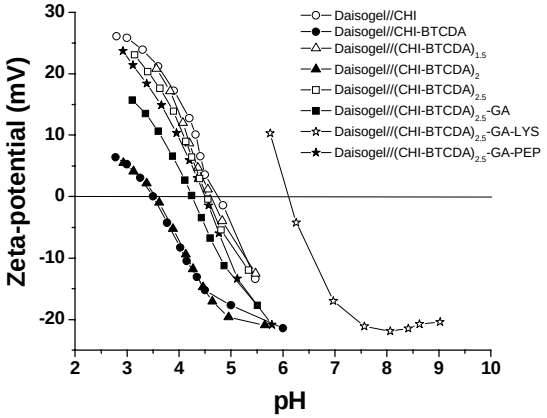

Fig. 3. Zeta-potential values of Daisogel hybrid microparticles in dependence on pH of the aqueous KCl solution: opened symbols – measurements after CHI adsorption and LYS immobilization; closed symbols – measurements after BTCDA/GA cross-linking, and PEP immobilization.

produce negatively charged surfaces. After the adsorption of the first CHI layer, the *iep* of the Daisogel//CHI composite is ≈ 4.7. This value, located in the acidic region of *p*H, showed that the CHI chains are adsorbed only onto the external surface of Daisogel microparticles, the 100 nm pores being inaccesible to the streched CHI chains ($M_v \approx$ 400000 g/mol). The BTCDA linked two amino groups from different or the same CHI chains, resulting in the formation of amide bonds and carboxylic groups. After the first BTCDA cross-linking reaction, the *iep* of Daisogel//CHI-BTCDA hybrid microparticles shifted to 3.5, lower than *iep* corresponding to Daisogel//CHI (fig. 3). The cross-linking reaction took place in acetone, which is non-solvent for CHI. Therefore, the adsorbed CHI chains colapsed onto the Daisogel surface before cross-linking reaction. After the first cross-linking reaction, a new CHI layer was deposited onto the negatively charged Daisogel//CHI-BTCDA. Deposition process was carried out in the same manner as described for the first adsorbed CHI layer. The *iep* of Daisogel//$(CHI\text{-}BTCDA)_{1.5}$ had nearly the same value, 4.7, like for Daisogel//CHI, that means a new thin CHI layer was adsorbed. After the second BTCDA cross-linking, the *iep* of Daisogel//$(CHI\text{-}BTCDA)_2$ was around 3.5, which is the same like *iep* obtained after the first cross-linking. The third deposited layer of CHI was cross-linked with GA in excess to create free aldehyde groups. The *iep* of Daisogel//$(CHI\text{-}BTCDA)_{2.5}$-GA was situated at 4.2, lower with approximately 0.5 *p*H units than before GA cross-linking, due to the reaction of GA with primary amino groups of

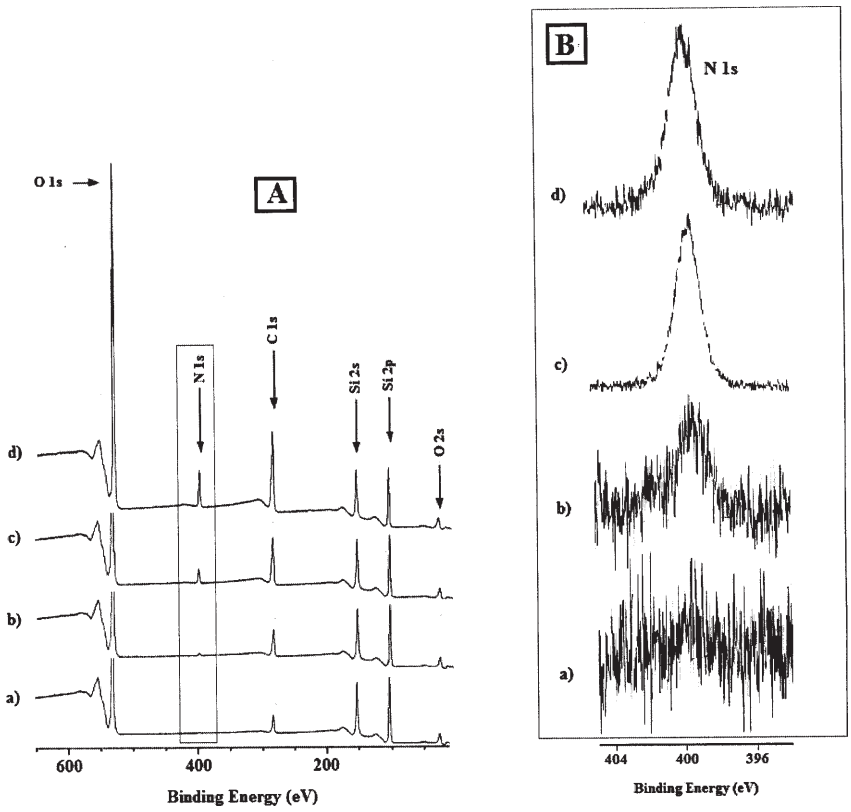

Fig. 4. Wide-scan (A) and N 1s (B) XPS spectra of Daisogel//CHI (a), Daisogel//(CHI-BTCDA)$_{2.5}$-GA (b), Daisogel//(CHI-BTCDA)$_{2.5}$-GA-PEP (c), and Daisogel//(CHI-BTCDA)$_{2.5}$-GA-LYS (d) composite microparticles.

CHI. These aldehyde groups have been utilised to immobilize PEP and LYS. The *iep* of Daisogel//(CHI-BTCDA)$_{2.5}$-GA-PEP was found at 4.5, that means the interactions of PEP macromolecules (*iep* = 1.0) with CHI modified Daisogel were not electrostatically favored. Instead, LYS macromolecules with *iep* ≈ 10 were electrostatically favored to interact with Daisogel//(CHI-BTCDA)$_{2.5}$-GA. The composite microparticles Daisogel//(CHI-BTCDA)$_{2.5}$-GA-LYS had an *iep* at 6.2, higher than all previously modified Daisogel microparticles. Thus, LYS is immobilized in a higher amount than PEP onto the CHI modified Daisogel microparticles.

The alternate adsorption of CHI chains onto Daisogel microparticles, the cross-linking reaction of polycation layers with BTCDA and GA, and enzymes immobilization onto modified microparticles introduced considerable amounts of carbon and nitrogen on the solid Daisogel surface. Therefore, after each adsorption/cross-linking/immobilization steps, the qualitatively and quantitatively elemental analyses of the organic thin films were determined by XPS (fig. 4).

In the XPS spectra the signal of the Daisogel microparticles was considered the peak of Si 2p. The C 1s peak appeared from CHI, BTCDA, GA, PEP, and LYS, but N 1s peak appeared only from the CHI and enzymes (fig. 4). After each modification step, the atomic ratio [C]:[Si] and [N]:[Si] was considered a measure of the relative amount of the organic material deposited onto Daisogel (fig. 5).

The atomic ratios [C]:[Si] and [N]:[Si] increased after each CHI adsorption/crosslinking step. The contribution of N 1s photoelectrons in the XPS wide-spectra of the modified microparticles is very low because: (i) CHI adsorbed amount is low and (ii) the N atomic concentration in the chitosan structural unit is low (9.09%). From these atomic ratios it can be concluded that the amount of the film based on CHI increased with the number of single cross-linked CHI layers. After the PEP and LYS immobilization onto Daisogel//(CHI-BTCDA)$_{2.5}$-GA, the [C]:[Si] and [N]:[Si] atomic ratios showed that LYS was immobilized in a higher amount than PEP. This fact could be attributed to the

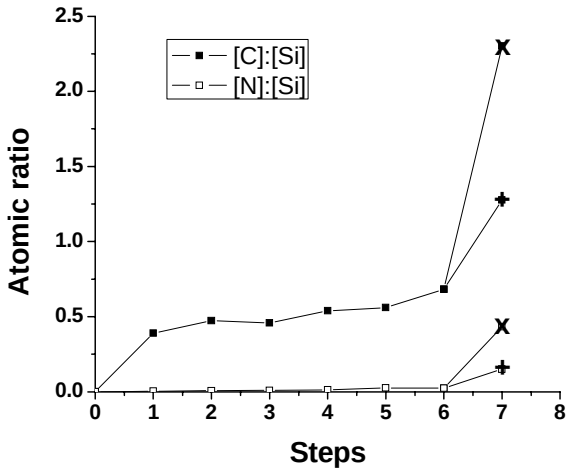

Fig. 5. Atomic ratios [C]:[Si] (closed symbols) and [N]:[Si] (opened symbols) calculated from XPS spectra of the Daisogel microparticles modified with CHI after each polycation adsorption (steps 1, 3, 5), cross-linking with BTCDA (steps 2, 4) and GA (step 6), and after PEP (step 7, marked with „+") and LYS (step 7, marked with „x") immobilization.

differences between the *iep* of each enzyme. Thus, LYS having the *iep* around 10 was favorized in the immobilization process by the electrostatic interactions with the organic cross-linked film (CHI-BTCDA)$_{2.5}$-GA with *iep* around 4.2. Because the *ieps* of PEP and composite microparticles were situated in the same acidic region of *p*H, the electrostatic repulsions between them were unfavorable for the immobilization process.

Conclusion

Using one single polyelectrolyte, CHI, and BTCDA and GA as cross-linkers, it was demonstrated the step-by-step construction of a single component polyelectrolyte multilayer on Daisogel microparticles. Using electrokinetic measurements and XPS method, it was shown that the cross-linked multilayer increased linearly on Daisogel. PEP

(*iep* ≈ 1) was immobilized onto Daisogel//(CHI-BTCDA)$_{2.5}$-GA in a lower amount than LYS (*iep* H" 10). This new type of CHI single component multilayers could be used for subsequent depositions layers or in the immobilization of bioactive compounds.

Abbreviations

BTCDA, 3,3',4,4'-benzophenone tetracarboxylic dianhydride; CHI, chitosan; GA, glutaraldehyde; LYS, lysozyme; PEP, pepsin; XPS, X-ray photoelectron spectroscopy.

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