Crosslinked Chitosan/Casein Polyelectrolyte Multilayers for Drug Delivery

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Abstract. Polyelectrolyte multilayers (PEMs) are widely used as drug delivery systems, but still remain challenging for their small drug immobilizing capacity. One way to increase the immobilized drug amount may be crosslinking of the PEMs, which stabilize them and increase their porosity. The aim of the present study is fabrication and characterization of chitosan/casein PEMs, which are crosslinked with different crosslinking agents – glutaraldehyde, sodium tripolyphosphate, CaCl₂ and combinations of two of them. XPS method was used to prove the PEMs crosslinking. SEM was used to observe film morphology and its variation due to cross-linking. Water capacity of PEMs in 100 % relative humidity was investigated. Release of model drug Benzydamine Hydrochloride was monitored spectrophotometrically at 306 nm. The crosslinking improves the PEMs stability and causes formation of porous surface. After crosslinking the amount of the immobilized drug increased several times.

1. Introduction

Controlled drug release materials are widely used in biomedical implants, tissue engineering, and targeted delivery devices [1]. Over the past decade polyelectrolyte multilayer films (PEMs) produced by the layer-by-layer (LbL) assembly technique [2, 3] find an ever wider application as drug delivery systems [4]. The layer-by-layer method is based on the electrostatic attraction between polyanions and polycations, hydrogen bonding or covalent bonding. The advantage of this is the possibility to structure PEMs with nanometer thickness and the ability to tune their internal properties [5] These films can contain a large variety of internal structures, depending on the used assembly conditions (pH and ionic strength) [6] during the deposition process or the type of used polyelectrolytes. LbL-assembly PEMs can be further chemically crosslinked to increase their stability or decrease the permeability [7].

The present research reports chemically crosslinked casein/chitosan PEMs as drug delivery systems. Some structural properties and drug upload capacity are commented.

2. Materials and methods

2.1. Materials and samples preparation
2.1.1. Pads formation. Biodegradable pads were prepared from polyactic acid (PLA) with ester end groups and intrinsic viscosity 0.55 - 0.75 dL/g purchased from Lactel Absorbable Polymers (USA). The PLA pads were cast from 2 % w/v PLA solution in chloroform and dried at 35 °C for 48 hours. Then the PLA pads were kept in a desiccator, at room temperature, and 54 % relative humidity (RH). Before the deposition process, the pads were charged in a corona discharge system, consisting of a corona electrode (needle), a grounded plate, and a metal grid placed between them. Positive 5 kV voltage was applied to the corona electrode. 1kV positive voltage was applied to the grid. The samples were charged under standard room conditions (\( T = 21\text{÷}23 \, ^\circ\text{C} \) and \( RH = 50\text{÷}60 \, % \)) for 1 min.

2.1.2. Polyelectrolyte multilayers deposition. Casein (molecular mass 24000 Da) and low molecular mass chitosan (molecular mass 127000 Da) were purchased from Sigma-Aldrich. They were used without further purification. The layer-by-layer (LbL) deposition technique was applied for multilayer build-up. For the LbL assembly 1% w/v casein solution in phosphate buffer (containing sodium dihydrogen phosphate and disodium hydrogen phosphate) with pH 8 and ionic strength 100 mM, and 1% w/v chitosan solutions in acetate buffer (containing acetic acid and sodium acetate) with pH 4 and ionic strength 100 mM, were prepared. The deposition was done by the dip-coating process. The first built-up layer was casein, which possesses opposite to the pad electric charge. Each polyelectrolyte deposition step was followed by rinsing in distilled water.

Four different crosslinking procedures to crosslink the chitosan and/or the casein in the PEMs were used:

- crosslinking of chitosan by glutaraldehyde (GA) – after each chitosan layer the PEMs were immersed in 0.1 % w/v glutaraldehyde for 20 min;
- crosslinking of chitosan by two agents – glutaraldehyde and sodium tripolyphosphate (GA + Na TPP) - after each chitosan layer the PEMs were immersed consequently in 3% sodium tripolyphosphate for 20 min and 0.1 % w/v glutaraldehyde for 20 min;
- crosslinking of casein by CaCl\(_2\) (CaCl\(_2\)) – after each casein layer the PEMs were immersed in 1 % w/v CaCl\(_2\) for 20 min;
- crosslinking of chitosan by glutaraldehyde and casein by CaCl\(_2\) (GA + CaCl\(_2\)) - after each chitosan layer the PEMs were immersed in 0.1 % w/v glutaraldehyde for 20 min; then after each casein layer the PEMs were immersed in 1 % w/v CaCl\(_2\) for 20 min.

After each chitosan layer (after crosslinking) the PEMs were immersed in 1% solution of model drug Benzydamine hydrochloride (BH) and hold there for 30 min. The procedure was repeated until obtaining 8 layers casein/chitosan. After the deposition of the last layer the film was dried in hot air at temperature 60 °C for 30 min. The ready samples were stored in desiccator at room temperature and relative humidity 54 %.

2.2. Polyelectrolyte multilayers characterization

2.2.1. The X-ray photoelectron spectroscopy (XPS). XPS studies were performed in a VG Escalab II electron spectrometer using AlK\(_\alpha\) radiation with energy of 1486.6 eV under base pressure 10–7 Pa and a total instrumental resolution 1 eV. The binding energies (BE) were determined utilizing the C1s line (from an adventitious carbon) as a reference with energy of 285.0 eV. The accuracy of measuring the BE values was 0.2 eV. The C1s, Si2p, Na1s, P2p, Cl2p, Ca2p, N1s and O1s photoelectron lines were recorded and corrected by subtracting a Shirley-type of background and quantified using the peak area and Scofield’s photoionization cross-sections. The XPSPEAK41 software was used for deconvolution of recorded spectra, where was necessary.

2.2.2. Scanning electron microscopy (SEM). The general morphology of the obtained polyelectrolyte multilayers films was revealed by means of SEM. A scanning electron microscope Lyra 3 XMU (Tescan) was employed. The working voltage was 8.1 kV. Prior to the measurements, the samples were covered with a thin film of gold (about 30 nm).
2.2.3. Moisture absorption. The moisture absorption test was carried out to assess the physical stability of the films at high humid conditions. In the present study the moisture absorption capacity of the films was determined gravimetrically. Before the test the samples were stored at 25 °C in desiccator with relative humidity 54 % for 72 hour. Then the films (2x2 cm, n=3) were weighed accurately and replaced into a desiccator containing distilled water (100 % RH) at 25 °C. The samples were left to equilibrate for 72 hours before new weight measurement was done. Moisture absorption was calculated as the increase in weight, expressed as a percentage.

2.2.4. Drug content. Benzylamine hydrochloride (BH) loaded films (3 samples of each type) were placed into 20 mL phosphate buffer saline (pH 6.8) and stirred continuously for 72 hours on a magnetic stirrer. Then, the samples were sonicated for 5 minutes and filtered using Chromafil® syringe filter (0.45 µm). The amount of BH was determined using UV/Vis spectrophotometer (Evolution 300, Thermo Fisher Scientific, USA), monitoring the band at \( \lambda_{\text{max}} 306 \text{ nm} \) [8]. The drug concentration was calculated from a standard calibration curve of BH in phosphate buffer saline (pH 6.8).

3. Results and discussion

3.1. XPS investigations

Three different agents were used to achieve crosslinked PEMs: GA and Na TPP, which interact with the amine groups and CaCl\(_2\), which forms ionic bonds with the carboxylate ions. To verify the crosslinking process XPS elemental analysis was employed. The XPS spectra of the non-cross-linked and cross-linked PEMs were compared.

The binding energy of C, O, and N atoms would change during the interaction between the protonated amine group and the Na TPP. The change of XPS spectra of N atom before and after cross-linking by Na TPP was clearly visible due to the high electrondonating effect of O\(-\) ion shifting the XPS signal to higher energies, as shown in figure 1. Before crosslinking only 1 peak shows up at 399.3 eV indicating the existence of amine groups connected to the carbon chain. After crosslinking, in addition to the peak at around 399 eV, a new peak appears at about 402 eV belonging to the N atom of amide group [9]. The similar behavior was observed by others [10].

![Figure 1. XPS spectra of the N atom (1 s) of the non-cross-linked and cross-linked by Na TPP and GA PEMs.](image)

According to Öztop et al. [11] the crosslinking of chitosan with GA occurs by destruction of C=O group in the GA and formation of N=C bond between the GA and the N atom in the chitosan. The lack of the high energy binding at 289.259 eV in the XPS spectra of crosslinked by GA layers verifies the C=O destruction (figure 2).
Figure 2. XPS spectra of the C atom (1 s) of the non-cross-linked and cross-linked by GA PEMs.

After crosslinking of PEMs by CaCl₂, a Ca 2p line in the XPS spectrum could be observed, while for the non-crosslinked samples this line was in the range of noise. Calcium was bound to the casein since no chlorine was detected in the XPS spectrum.

3.2. SEM morphology

The surface morphology of the PLA pads and casein/chitosan PEMs cross-linked with different agents, are presented in figure 3.

Figure 3. SEM micrographs of PLA pad (a), non-crosslinked layers (b), layers crosslinked by CaCl₂ (c), layers crosslinked by GA (d), layers crosslinked by GA and CaCl₂ (e), layers crosslinked by GA and Na TPP (f).
It is important initially to study the morphology of the pad, since it largely determines the mode and the type of the deposited thereon thin layers. As it is seen from figure 3 a) PLA pad is characterized by homogeneous structure.

LbL deposition of the PEMs let to a gradual change of the morphology of the surface (figure 3b). The polyelectrolytes were adsorbed in separate morphological elongated entities with size of about several microns. This structure might be a reason of partial chitosan desorption during the rinsing process. The structure significantly changed after the crosslinking. It became denser and smoother. Crosslinking with one agent (GA) caused smoothening of the surface, but the morphological entities were retained (figure 3d). When 2 agents were used, the structure became denser due to more stable binding of the chitosan molecules (figure 3f). When the casein layer before the last one is crosslinked the surface became more homogeneous (figure 3e). In case of double crosslinking of both casein and chitosan (figure 3e), the structure is porous with pore size of approximately 1 micron.

3.3. Water content

As it was shown the crosslinking process let to formation of denser structure, which resulted in different water uptakeabsorption properties of the PEMs. The water content of crosslinking samples decreased several times in comparison to the non-crosslinked ones. The lowest water content is observed in case of double crosslinking, where it is less than 10 % - figure 4.

3.4. Drug content investigations

The amount of uploaded model drug BH strongly depends on the PEMs structure and its ability for water uptake. If the layers are too dense there is no enough space for drug molecules entrapment. On the other hand, in case of very loose and swollen structure the drug release process dominates the process of uploading. Hence porous structure with an optimal pore size should be achieved for maximum drug content.

The drug content of the layers is presented in figure 5. The lowest drug is gripped in the non-crosslinked PEMs and in those crosslinked by means of glutaraldehyde. The growth of the layers in this case is insular type (see figure 3 b, d) and probably their thickness and density are not suitable for gripping a drug. The highest drug amount is uploaded in PEMs wherein the chitosan is crosslinked by double agents – glutaraldehyde and Na TPP. The PEMs in this case are strongly bound to the pad and to each other and the water uptake is small, i.e. the air pore size is not too big. Similar structure and
water capacity possess PEMs crosslinked via glutaraldehyde and CaCl$_2$ where the drug amount is again high.

4. Conclusion

PEMs constructed from natural polymers casein and chitosan by layer-by-layer method on preliminary charged PLA pads were successfully crosslinked by glutaraldehyde, Na TPP, and CaCl$_2$. The PEMs crosslinking let to formation of stable and porous structure with less water capacity and higher potential for drug upload. The most effective crosslinking process is achieved by the usage of two agents - glutaraldehyde and Na TPP.

Acknowledgments

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