Research Article

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Fabrication and Bioapplications of Magnetically Modified Chitosan-based Electrospun Nanofibers

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Abstract: The fabrication of magnetically modified electrospun nanocomposite fibers based on a naturally-derived biocompatible and biodegradable polysaccharide chitosan (CS) and the hydrophilic and biocompatible poly(vinylpyrrolidone) (PVP) is reported herein. The anchoring of magnetic nanoparticles (MNPs) onto the surfaces of the electrospun PVP/CS fibers was carried out by a post-magnetization process based on chemical coprecipitation, *via* immersing the produced fibrous mats in an aqueous solution containing Fe(II) and Fe(III) salts at appropriate molar ratios, followed by the addition of a weak base to yield MNPs. Electron microscopy revealed the presence of continuous micron and submicron fibers surface-decorated with MNPs. The magnetically modified PVP/CS fibers exhibited superparamagnetic behavior at ambient temperature. The magnetic fibrous nanocomposite carrier was employed for the immobilization of *Saccharomyces cerevisiae* cells and their use for sucrose hydrolysis, and *Candida rugosa* lipase and its use for artificial substrate hydrolysis.

Keywords: Magnetically responsive polymer-based fibrous nanocomposites; electrospinning; post-magnetization; chitosan; polyvinylpyrrolidone; superparamagnetism; enzymes and cells immobilization

1 Introduction

Electrospinning is one of the most popular and versatile fiber fabrication techniques used for the production of synthetically or naturally-derived fibrous materials, enabling the expansion of their properties through blending and surface functionalization [1]. Fibers exhibit a range of unique features and properties such as extremely long length and thin diameters resulting in a high surface-to-volume ratio, a high pore density as a result of fiber entanglement, good mechanical properties especially in the case of fibrous nanocomposites, high flexibility, low weight and cost [2, 3]. These outstanding properties render electrospun polymeric fibrous materials good candidates in many fields including biomedical (tissue engineering, drug delivery [3]), environmental (filtration, water remediation [4]) and catalytic [5] applications. Due to their high porosity and interconnectivity, nanofibrous supports are very useful carriers for enzyme immobilization using both surface attachment and encapsulation [6]. Yeast cells can be also successfully incorporated into electrospun nanofibers maintaining their viability [7].

Despite the use of electrospinning in producing fibers of synthetic origin, the fabrication of electrospun fibers based on biopolymers and their derivatives attracts high attention due to the fact that materials derived from renewable resources are highly abundant and exhibit biocompatibility and biodegradability [8, 9].

Chitosan, which is a chitin-derived biocompatible and biodegradable polysaccharide exhibiting also antibacte-

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rial properties [10], has been employed in fiber fabrication via electrospinning [11]. Chitosan-containing electrospun fibers have been used among others in wound dressing [12, 13], tissue engineering [14], drug delivery [15, 16], enzyme immobilization [17, 18], protein delivery [19], as well as components in filtration membranes used in water remediation processes [20].

Magnetically modified (nano)fibers can be successfully used in various areas of medicine, biosciences and biotechnology [21]. It has been recently demonstrated that the addition of magnetic nanoparticles (MNPs) in composite nanofibrous films could induce a significantly higher proliferation rate and faster differentiation of osteoblast cells. Therefore, scaffolds containing MNPs may provide great potential in bone regenerative medicine. That is why magnetically responsive nanofibers have been utilized to immobilize various cells [22, 23]. Magnetically responsive nanofibrous composites have also been tested as potential drug delivery systems [24]; several drugs including indomethacin or aspirin [25] and acetaminophen [26] have been successfully immobilized within such functional fibrous matrices. Electrospun biodegradable chitosan nanofibers with embedded MNPs have been also tested for hyperthermia when exposed to an alternating magnetic field. The heat produced by the magnetically responsive electrospun mats has resulted to a temperature increase in the medium to 45°C, thus demonstrating the potency of these materials in endoscopic magnetic hyperthermia treatment of malignant tumors [27, 28].

The present study focuses on the fabrication of biocompatible electrospun biopolymer-based fibers composed of chitosan (CS), a naturally-derived biocompatible and biodegradable polysaccharide and poly(vinylpyrrolidone) (PVP), that is a hydrophilic and biocompatible synthetic polymer of low cost. The successful modification of the external surfaces of these fibers with magnetic iron oxide nanoparticles (MNPs) by following a post-magnetization route based on chemical coprecipitation resulted to the generation of magnetoactive fibers. For the first time in this study, these fibers were further used as carriers for the immobilization of *Saccharomyces cerevisiae* cells and their use in sucrose hydrolysis, and the immobilization of *Candida rugosa* lipase and its use in artificial substrate hydrolysis.

2 Materials and Methods

2.1 Materials

Baker's yeast (*Saccharomyces cerevisiae* cells) was purchased in a local supermarket. Glutaraldehyde (GA), *Candida rugosa* lipase (EC 3.1.1.3), 4-nitrophenyl butyrate, poly(ethyleneimine) (PEI, P3143), polyvinylpyrrolidone (PVP, average molecular weight = 1 300 000 g mol⁻¹), chitosan (50,000-190,000 g mol⁻¹; 75-85% deacetylated), 25% ammonium hydroxide solution, iron(II) chloride tetrahydrate, iron(III) chloride hexahydrate, glycine and methanol (analytical grade) were obtained from Sigma-Aldrich (St. Luis, MO, USA). Glucose GOD kit was from Pliva-Lachema (Brno, Czech Republic). Sucrose, D-glucose, NaCl, K₂HPO₄ and KH₂PO₄ were supplied by Lachner (Neratovice, Czech Republic). All compounds were used without further purification.

2.2 Preparation of electrospun chitosan-based fibers (PVP/CS)

The fabrication of PVP/CS fibers was accomplished by means of the electrospinning technique according to the following procedure. Firstly, an appropriate amount of commercially available PVP was dissolved in methanol, at a 15% w/v polymer solution concentration. Afterwards the as-received CS was added in the PVP solution in a 30% wt. concentration in respect to the total polymer mass and it was left to stir at room temperature until a homogeneous suspension was obtained. Immediately after preparation, the prepared suspension was loaded into a glass syringe (10 mL volume) and then electrospun. All electrospinning experiments were performed at ambient temperature. Systematic parametric studies were carried out by varying the applied voltage, the needle-to-collector distance, the needle diameter and the flow rate, so as to determine the optimum experimental conditions for obtaining bead-free fibrous materials. Equipment included a controlled-flow, four channel volumetric microdialysis pump (KD Scientific, Model: 789252), syringes with specially connected spinneret needle electrodes, a high-voltage power source (10-50 kV) and a custom-designed, grounded target collector, inside an interlocked Faraday enclosure safety cabinet.

2.3 Membrane crosslinking

Insoluble electrospun PVP/CS fibrous mats were generated *via* gradual thermal crosslinking (3-step process), upon placing the electrospun mats in an oven initially at 100°C (3 hrs), 140° (3 hrs) and finally at 170°C (3 hrs) [29].

2.4 Preparation of electrospun magnetic chitosan-based fibers (M-PVP/CS)

The thermally crosslinked PVP/CS fibrous mat was placed in an aqueous solution containing a mixture of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (0.50 g, 1.85 mmol) and $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ (0.18 g, 0.925 mmol) salts (molar ratio 2:1) that was prepared in deionized water (10 mL) and kept under nitrogen atmosphere. This was followed by the addition of NH_4OH solution (1.5 mL) resulting to the immediate formation/precipitation of Fe_xO_y nanoparticles. The resulting magnetically-modified fibers (M-PVP/CS) were extensively washed with deionized water (5x10 mL) to remove any unbound Fe_xO_y , unreacted iron salts and reaction by-products (e.g. NH_4Cl) from the fibers' surface. The modified material was left to dry inside the chemical fume hood at room temperature.

2.5 Characterization of native and magnetically modified electrospun fibers

2.5.1 Morphological characterization

The morphological characteristics of the native and magnetically modified fibers were determined by scanning electron microscopy (SEM) (Vega TS5136LS-Tescan). The samples were gold-sputtered (~30 nm) (sputtering system K575X Turbo Sputter Coater-Emitech) prior to SEM inspection. Fiber diameters were determined from the acquired SEM images using Digimizer image analysis software.

High resolution transmission electron microscopy (HRTEM) investigations of the M-PVP/CS were performed by using TECNAI F30 G2 S-TWIN microscope operated at 300 kV equipped with energy dispersive X-ray spectrometer (EDX). Samples were placed into a double copper grid (oyster) to be visualized by TEM.

2.5.2 Thermal analysis

Thermal gravimetric analysis (TGA) measurements were carried out on a Q500 TA instrument. Samples (6 ± 0.2 mg) were placed in ceramic crucibles. An empty ceramic crucible was used as a reference. Samples were heated from ambient temperature to 600°C in a 50 mL min^{-1} flow of argon (Ar) with a heating rate of $10^\circ\text{C min}^{-1}$.

2.5.3 Magnetic characterization

The magnetic properties, full magnetization curve and hysteresis loop of the magnetically responsive textile were measured with a Vibrating Sample Magnetometer - Model 880 from ADE Technologies USA at 300 K, in the applied magnetic field range of 0 kA m^{-1} to $\pm 950 \text{ kA m}^{-1}$.

2.5.4 Immobilization of yeast cells

Approximately 6 mg of PVP/CS and M-PVP/CS were activated with 10 mL of 3% GA. The mixture was incubated on a rotator mixer for 3 h and then thoroughly washed with distilled water to remove remaining GA. The cell suspension (2 g of baker's yeast in 30 mL of saline) was then added to the fibers and mixed on a rotator mixer at room temperature for 24 h. The unbound cells were washed with saline and unreacted aldehyde groups were blocked with excess of 0.5% glycine solution.

2.5.5 Sucrose hydrolysis by immobilized yeast cells

Invertase activity of yeast cells immobilized on PVP/CS and M-PVP/CS after reuse was studied. Ca. 2 mg of PVP/CS and M-PVP/CS with immobilized yeast cells were shaken in 15 mL of 20% sucrose for 30 min (at 150 rpm). Subsequently, 20 μL of sample was mixed with 2 mL of reaction kit (Glucose GOD) solution and incubated in the dark for 30 min. Absorbance of sample was measured at 498 nm using a UV/VIS spectrophotometer (Cintra 20, GBC Scientific Equipment, Australia) and the amount of formed glucose was calculated from the following formula:

$$\text{glucose (mM)} = \frac{C_{\text{gluc}} * A_{\text{sample}}}{A_{\text{gluc}}} \quad (1)$$

where C_{gluc} is the concentration of standard glucose solution (mM), A_{sample} is the absorbance of the measured sample and A_{gluc} is the absorbance of standard glucose solution.

Both systems (PVP/CS/yeast and M-PVP/CS/yeast biocatalysts) were washed for several times with saline before each cycle. All experiments were performed in duplicate and each sample was measured twice; average values are presented.

2.5.6 Immobilization of lipase

PVP/CS and M-PVP/CS were cut into small pieces (0.5×0.5 mm). Two types of covalent immobilization procedures were tested for both samples. In the first procedure, the fibers were modified by 5% (v/v) GA solution (approx. 10 mg of material was shaken in 2 mL of GA for 3 h at room temperature on automatic rotator, 24 rpm). Then, the GA was removed and the activated materials were thoroughly washed with water. In the second procedure, the fibers were modified by PEI (15 g L⁻¹ in water; approx. 10 mg of material was shaken in 2 mL of PEI overnight at room temperature on automatic rotator, 24 rpm). Subsequently, the free PEI was removed and the modified materials were thoroughly washed with water. Amino groups of PEI-modified materials were activated by GA solution, as described previously.

The final step of the enzyme immobilization procedure was performed by incubation of GA-activated materials (native and PEI-modified nonmagnetic or magnetic CS-based fibrous mats) with enzyme solution (2 mL of lipase solution in 50 mM of potassium phosphate buffer pH 7.5; 1 mg mL⁻¹; gentle shaking overnight at 10°C, 20 rpm). The supernatant with the unbound enzyme was then removed and all materials were repeatedly washed with buffer until zero enzyme activity detection in the washing buffer. Unreacted aldehyde groups were blocked with excess of 0.5% glycine solution. Samples with immobilized enzyme were stored in buffer in a fridge.

2.5.7 Lipase activity assay

Activity of lipase immobilized on PVP/CS or M-PVP/CS was determined by spectrophotometry after the reaction with the artificial substrate 4-nitrophenyl butyrate in 50 mM potassium phosphate buffer pH 7.5. The increasing amount of the yellow-colored reaction product 4-nitrophenol was measured at 405 nm.

2.5.8 Operational stability of immobilized lipase

Operation stability of lipase immobilized on PVP/CS or M-PVP/CS was evaluated according to the residual enzyme activity after the 10 repeated reaction cycles of samples with the substrate. Residual activity after each reaction cycle was determined in%, after comparison with the initial activity in the first cycle (100%). Between the reaction cycles, samples were washed with phosphate buffer.

3 Results and discussion

3.1 Preparation of electrospun chitosan-based textile

Initially, a homogeneous suspension of PVP/CS was prepared in methanol. The obtained suspension was then electrospun under specific electrospinning conditions to yield fibrous electrospun mats. The successful generation of fibrous membranes requires the determination of the optimal processing parameters that include the concentration of the polymer solution, the applied voltage, the delivery rate of the solution towards the collector, the diameter of the needle, and the distance between the tip of the needle and the collector. The optimum experimental conditions employed for the fabrication of continuous, bead-free PVP/CS electrospun fibrous textiles were as follows: needle diameter 16 G; flow rate 1.5 mL h⁻¹; voltage 23 kV; needle-to-collector distance 25 cm.

3.2 Magnetic modification

The chemical co-precipitation of ferrous and ferric ions at high pH is one of the most widely used principles for the synthesis of magnetite particles in the nanometer range [30]. The chemical co-precipitation reaction employed in the present work is given as follows:

The anchoring of the produced magnetic iron oxide nanoparticles onto the surface of the PVP/CS fibers was realized by a post-magnetization process involving the occurrence of the above-mentioned chemical co-precipitation reaction in the presence of the fibers, as schematically depicted in Figure 1.

Figure 2 provides a photograph of the M-PVP/CS nanofibrous mat attracted by a permanent magnet, thus

Figure 1: Schematic presentation of the post-magnetization process followed for the preparation of the magnetically-modified (M-PVP/CS) fibers.

Figure 2: Magnetic separation of the M-PVP/CS nanofibrous mat from water solution.

demonstrating the possibility of the magnetic-field assisted recovery of these materials from a liquid medium.

3.3 Characterization of PVP/CS electrospun fibers and their magnetic derivatives

3.3.1 Morphology

The morphological characteristics of the PVP/CS were determined by SEM. In Figure 3 characteristic SEM images of the PVP/CS before (Figure 3(a)) and after (Figure 3(b)) thermal crosslinking are provided, demonstrating that the thermal crosslinking process employed did not lead to any alteration in the fibers' morphology. The average diameter and standard deviation of the heat-treated PVP/CS determined from the acquired SEM images by using the Digimizer image analysis software was calculated to be 690 ± 215 nm.

In Figure 4, SEM and TEM images of the M-PVP/CS are provided. By comparing the SEM images of the magnetically-modified fibers with those shown in Figure 3(b) corresponding to the non-modified analogue, it can be clearly seen that upon hydration of the fibers during the post-magnetization step, some morphological changes were observed, *i.e.* fiber swelling, due to the presence of the highly hydrophilic PVP. These results are in line with the study of Bhattarai *et al.* on PEO/chitosan electrospun membranes [31] and with our previous study on PEO/PLLA electrospun fibers employed as adsorbents for radioactive metal ions in aqueous media [32].

The M-PVP/CS were also visualized by TEM. Figures 4(b) and 4(c) depict transmission electron micrographs and the EDX spectrum of the magnetically-modified nanofibers. The TEM bright field images verified the successful anchoring of the iron oxide nanoparticles

Figure 3: SEM images of the as-prepared PVP/CS fibers (a) and of the thermally-treated (crosslinked) analogues (b).

on the surface of the fibers. The nanoparticles that were homogeneously distributed along the fibers were found to be spherical in shape with average diameter of 5.3 nm.

The selected area electron diffraction (SAED) image from Figure 4(b) demonstrates that the monocrystalline compound is Fe_3O_4 . HRTEM imaging discloses the crystalline planes with 2.53 Å interplanar distance corresponding to crystalline planes with (311) Miller index of the Fe_3O_4 compound.

The EDX spectrum provided in Figure 4(c) shows the presence of Fe, O and C as the major elements in the sample (element Cu comes from the copper grid). The presence of minor Cl elements is attributed to mild sample contamination during the post-magnetization process.

3.3.2 Thermal properties

The degradation temperatures of the PVP/CS and the M-PVP/CS fibers were determined by means of thermal gravimetric analysis (TGA). The TGA traces of the thermally-

crosslinked PVP/CS electrospun fibers before the post-magnetization process are shown in Figure 5. Both materials have similar thermal degradation profiles with the first decomposition step appearing at $\sim 250^\circ\text{C}$, corresponding to the degradation temperature of CS [33] and the principal thermal decomposition step appearing at $\sim 400^\circ\text{C}$ corresponding to PVP [34]. The remaining residue observed in the case of the magnetically-modified system above 450°C is higher compared to that observed in the case of the native PVP/CS fibers, due to the presence of the inorganic iron oxide content in the former.

3.3.3 Magnetic properties

Investigation of the magnetic properties of the M-PVP/CS fibrous nanocomposites was carried out by vibrating sample magnetometer (VSM) at 300 K. Figure 6(a) shows the hysteresis loop, *i.e.* magnetization *versus* applied magnetic field ($M=f(H)$), corresponding to the magnetically-modified (M-PVP/CS) fibrous mat. The M-PVP/CS exhibits

Figure 4: (a) SEM and (b) TEM images and selected area electron diffraction (SAED) image and (c) Energy dispersive X-ray (EDX) spectrum of the M-PVP/CS.

a magnetization (M) value of $\sim 9 \text{ emu g}^{-1}$ at the highest value of the applied magnetic field ($H \pm 950 \text{ kA m}^{-1}$), whereas the sigmoidal shape of the plot and the lack of a hysteresis loop area demonstrate the superparamagnetic behavior of this material at ambient temperature. The sat-

uration magnetization of the sample (M_s) was evaluated in the quasi-saturation region of the magnetization curve ($H > 700 \text{ kA m}^{-1}$), where the contribution to the material magnetization is given by the magnetic particle interac-

Figure 5: TGA thermographs of the electrospun PVP/CS and the corresponding M-PVP/CS.

tions with the applied magnetic field. M_s was found to be approximately 10 emu g^{-1} .

In the linear region of small magnetic fields of static magnetization curve (Figure 6(b)), measured for the M-PVP/CS nanocomposite fibers, the initial magnetic susceptibility has been evaluated ($\chi_i = 0.36$). Together with the asymptotic variation of magnetization in intense magnetic fields, it forms the basic instruments of magnetogranulometric analysis in order to determine the mean magnetic diameter of the dispersed magnetic particles, $\langle D_m \rangle$. Assuming that the non-interacting embedded magnetic particles have a log-normal distribution of the magnetic diameters, a mean magnetic diameter of $\langle D_m \rangle = 3.59 \text{ nm}$ was found [35]. Considering the thickness of non-magnetic layer at the surface of magnetic nanoparticles [36] $\delta_m = 0.83 \text{ nm}$, a value of $\langle D_p \rangle = 5.25 \text{ nm}$ was found for the mean physical diameter, by employing Eq. 2, which is in an excellent agreement with the TEM results.

$$\langle D_p \rangle = \langle D_m \rangle + 2 \cdot \delta_m \quad (2)$$

3.4 Immobilized yeast cells

The overall structure of the native and the magnetically modified CS-based nanofibers was studied by optical microscopy, with the focus on the location of immobilized cells in the fibrous structure (Figure 7).

PVP/CS and M-PVP/CS with immobilized *S. cerevisiae* cells were repeatedly tested for sucrose hydrolysis, the enzyme reaction that is extensively used for invert sugar production. As can be seen from Figure 8, the M-PVP/CS/yeast biocatalyst exhibited significantly higher enzyme activity after reuse than the native analogue; after the second use,

(a)

(b)

Figure 6: Hysteresis loop (a) and static magnetization curve (b) of the magnetically modified nanocomposite fibrous textile (M-PVP/CS).

Figure 7: Optical microscopy of PVP/CS/yeast biocomposite.

the invertase activity dropped to 93%, while in the next three cycles it remained around 70-76% and it then fell

to 65%. The enzyme activity of PVP/CS/yeast composite was still relatively high (84%) after the second use, but steeply fell down to 33% during next reuse cycles. The higher efficiency of the magnetic nanocomposite could be explained by the adsorption of cells onto the magnetic nanoparticles resulting in a stronger binding in the case of the magnetically-modified nanofibers. To conclude, both biocatalysts can be efficiently employed for invert sugar formation at least in two cycles.

Figure 8: Invertase activity of yeast cells immobilized on PVP/CS (O) and M-PVP/CS (□) after consecutive cycles.

3.5 Immobilized lipase

The biotechnologically important enzyme lipase was immobilized on the native and magnetically modified nanofibers. Two procedures of covalent immobilization were followed in this case. The first one involved the activation of the amino groups that were present onto the surfaces of both the native and the magnetically-modified nanofibers by GA with the subsequent immobilization of enzyme. In the second procedure, native and magnetic nanofibers were at first modified by the polymer PEI in order to increase the amount of amino groups on their surfaces followed by GA activation and enzyme immobilization. The stability of the immobilized biocatalysts was evaluated by testing the operational stability during ten repeated reaction cycles with an artificial substrate. Based on the experimental results, all tested samples of immobilized lipase retained high enzyme activity without significant losses; after the tenth cycle the activity was higher than 90% for all samples (see Figure 9). The negligible activity decrease could be attributed to losses of small amounts of material during the intermediate washing steps. This implies that the performed immobilization procedures can be successfully used for the bind-

Figure 9: Operational stability of lipase immobilized on PVP/CS (●), M-PVP/CS (Δ), PEI modified PVP/CS (◇) and PEI modified M-PVP/CS (■); initial activity in the first cycle is 100%.

ing of lipase onto the PVP/CS and M-PVP/CS electrospun nanofibers.

4 Conclusions

Novel biocompatible nanofibers comprised of chitosan (CS) and polyvinylpyrrolidone (PVP) were fabricated by means of the electrospinning technique. The successful anchoring of iron oxide (Fe_3O_4) magnetic nanoparticles onto the surfaces of the produced nanofibers was realized by following a post-magnetization process based on the chemical co-precipitation of iron salts in the presence of the fibrous mat. The resulting magnetically-modified nanofibers exhibited superparamagnetic behavior at room temperature and enhanced thermal stability. The PVP/CS nanofibers, in both their native and magnetic form were further evaluated as carriers for the immobilization of yeast cells and lipase. Invertase activity of PVP/CS and M-PVP/CS containing *Saccharomyces cerevisiae* cells was evaluated after biocatalyst reuse, indicating a significantly higher enzyme activity in the case of the magnetically-modified nanofibrous carrier. Lipase was immobilized on both nanofiber types using 2 different modification strategies, with negligible loss of enzyme activity after 10 reuse cycles.

To conclude, magnetically modified, chitosan-based electrospun nanofibers have been shown to be very good supports for enzyme and yeast cell immobilization due to their biocompatibility, large surface area, active groups enabling their activation and response to external magnetic field.

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