Current Trends in Polymer Science
Vol. 15, 2011

Original Communication

Lactate dehydrogenase immobilized in electrospun poly(vinyl alcohol) nanofibers as biosensor for lactate

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ABSTRACT

A method for the detection of lactate using the lactate dehydrogenase enzyme (LDH) non-covalently labelled with 8-anilino-1-naphthalene sulfonic acid (ANS) is described in this work. The enzyme was immobilized in poly(vinyl alcohol) (PVA) nanofibers using the electrospinning technique. The presence of the LDH-ANS system in the PVA fibers was confirmed by laser scanning confocal microscopy. According to scanning electron microscopy analysis, the PVA electrospun nanofibers containing the labelled enzyme showed a free defects morphology, with a diameter distribution in the range of 98-115 nm. The ANS-labelled LDH system shows a decrease in the fluorescence intensity upon binding lactate. This fluorescence quenching was used to detect the presence of lactate in the reaction medium. The immobilized LDH-ANS system shows sensibility toward lactate for concentrations as low as 22 µg/mL.

KEYWORDS: lactate dehydrogenase, nanofibers, lactate biosensor, polymers, electrospinning

INTRODUCTION

The detection of lactate has a considerable importance as analyte in the medical area [1, 2]. Elevated blood lactate concentration can predict multiple organs failures and death of patients with septic shock [3]. It is known that most tumors have high rates of lactic acid production [4]. The detection de lactate is used by the athletes as an indicator of the athletic performance [1, 5]. The need for continuous measurement of blood lactate still remains hindered by the time detection requires by the current techniques available in the laboratories [6-9]. Therefore, there is a need for specific, simple and effective methods for the detection of lactate. The enzymes surge as a promising alternative in the detection of this important analyte, due to the specificity, reliability and effectivity of its catalytic function [10]. However, the instable nature of the three dimensional proteinic structure of the enzymes has complicated its commercial use as catalysts for this potential application.

Electrospinning is a technique that has been proven to be effective in the immobilization of several biological substances, including viruses and enzymes [11-14]. In a previous publication, we have reported the successful encapsulation of lactate dehydrogenase in electrospun nanofibers of poly(vinyl alcohol) (PVA), where the enzyme was observed to maintain its activity for longer periods of time [15].

In this work, we propose the immobilization of a system, consisting of lactate dehydrogenase enzyme, non-covalently labeled with a 8-anilino-1-naphthalene sulfonic acid (ANS) compound, in poly(vinyl alcohol) (PVA) nanofibers obtained

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through the electrospinning technique. This system fluoresces due to the interaction of the ANS compound with the hydrophobic amino acid residues of the enzyme. The LDH-ANS system undergoes a fluorescence quenching upon binding lactate [2, 16]. This fluorescence quenching occurs without consumption of lactate.

MATERIALS AND METHODS

Materials

Poly(vinyl alcohol) (PVA) (98% hydrolyzed) (average Mw 126 kg/mol), poly(vinyl alcohol) (PVA) (87-89% hydrolyzed) (average Mw 13-23 kg/mol) and methanol were purchased from Aldrich. L-lactic dehydrogenase (LDH) type II from rabbit muscle (EC 1.1.1.27) and lactic acid were obtained from Sigma. The compound 8-anilino-1-naphthalene sulfonic acid (ANS) was purchased from Fisher Scientific.

Characterization

The morphology of the nanofibers was studied using a Field Emission Scanning Electron Microscope (FE-SEM, JSM-7401F). The UV-visible spectra were recorded using a SHIMADZU 2401 spectrophotometer. The electrospinning setup consists of a syringe with a flat-end metal needle bottom round cut, a high voltage dc power supply (Spellman CZE1000R), a syringe pump (Cole Parmer) and a grounded aluminum collector. A Zeiss Pascal 5 was used in the laser scanning confocal microscopy (LSCM). A spectrofluorometer (Perkin Elmer LS50B) was used to obtain the fluorescence spectra. For the immobilized LDH-ANS system the excitation wavelength used was of 370 nm, and the emission wavelength analyzed was of 460 nm.

Solutions

A phosphate buffer pH 7.3 was used in the preparation of the solutions. In all samples, the preparation procedure utilized was as follow: a LDH and ANS solutions with a concentration of 4 µM and 3 µM, respectively, were prepared accordingly to the literature [2]. A poly(vinyl alcohol) (PVA) solution of 8% w/w was used in the encapsulation process, 1:1 relation of both types of PVA was used to ensure a good morphology in the final nanofibers [15].

LDH-ANS system immobilization

A first solution was prepared by dissolving 178 µL of enzyme in one millilitre of the phosphate buffer; this first solution was added to 4 ml of polymeric solution. This blend was stirred during 40 minutes until a good dispersion of the enzyme in the polymeric solution was achieved. The operational parameters of the electrospinning process were: 20 kV of applied voltage, 10 cm gap between the electrode and the collector and a feeding rate of 0.33 mL/h.

Crosslinking process

The poly(vinyl alcohol)(PVA) nanofibers were crosslinked through immersion in a methanol bath during 24 hours. Afterwards the nanofibers were withdrawn from the methanol bath and left to dry for 30 minutes. In order to probe the effectiveness of the crosslinking process, the crosslinked and non crosslinked nanofibers were submerged in water for 3 days.

RESULTS AND DISCUSSION

Figure 1 shows FE-SEM images of the morphology of the PVA nanofibers before and after the enzyme immobilization process. The PVA nanofibers morphology before enzyme immobilization was a defect free morphology, without bead defects and showed uniform fibers (Figure 1a). The diameter distribution analysis shows a central value of 90 nm ±2 (R^2 = 0.9407) (Figure 1c). However, the electrospun nanofibers morphology after the LDH-ANS immobilization through the electrospinning technique underwent a dramatic change (Figure 1b). As we can see, there are groups of nanofibers joined together and almost with the same orientation (labelled with white arrows in the figure). A possible explanation for this morphology is that the high molecular weight of the LDH enzyme (136 kDa ±2100 Da), could acted as an agglutinant between the nanofibers in order to give place to this morphology. Such type of morphology has been reported in the electrospinning of high molecular enzymes [14], as well as in the electrospinning of concentrated poly(electrolyte) solutions [17, 18]. The change in the morphology was accompanied by an increment in the diameter up to 115 nm ±0.63 (Fig. 1d).

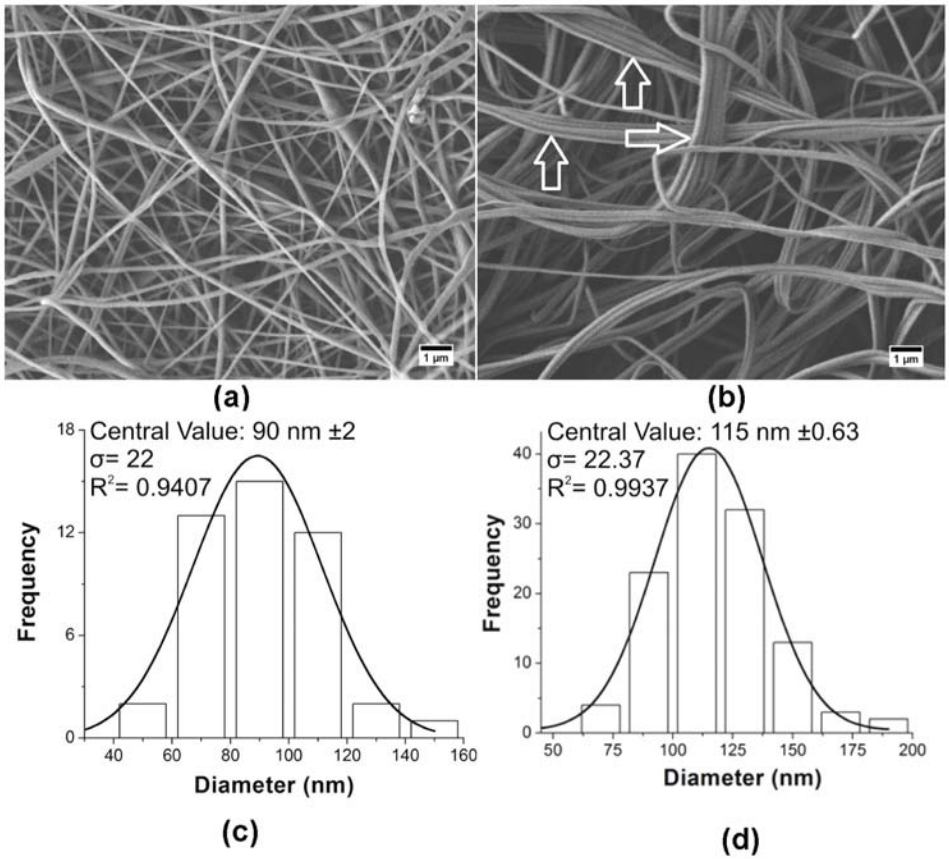

Figure 1. FE-SEM images of electrospun nanofibers showing: (**a**) morphology of PVA nanofibers; (**b**) morphology of ANS-LDH/ PVA nanofibers; (**c**) diameter distribution of PVA nanofibers and (**d**) diameter distribution of ANS-LDH/PVA nanofibers.

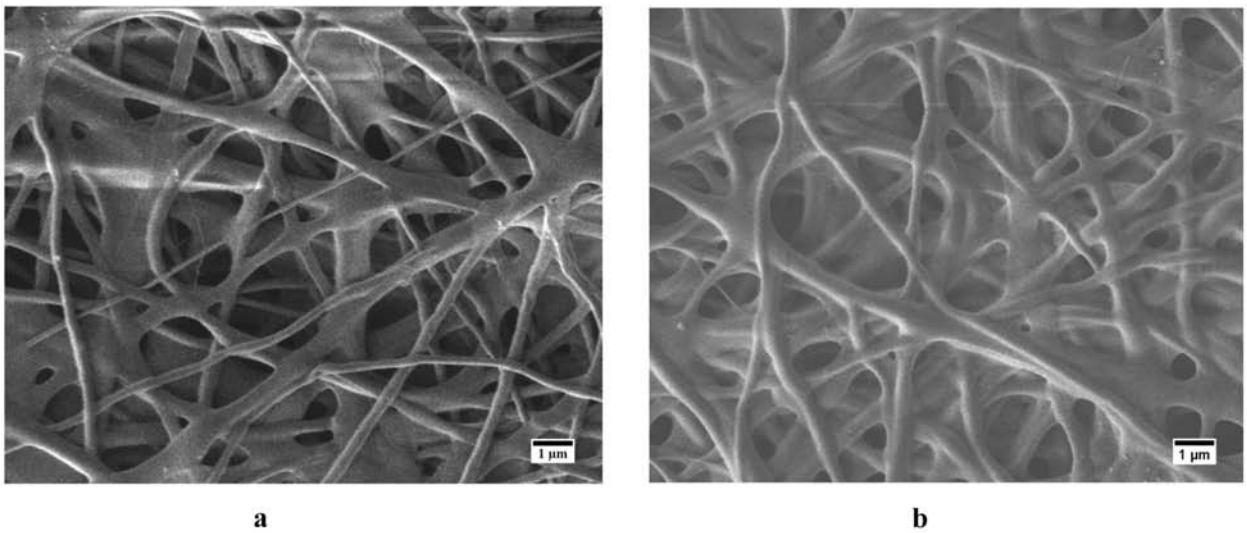

Figure 2. FE-SEM images showing the morphology of ANS-LDH/PVA electrospun nanofibers. **a**) crosslinking with methanol and **b**) already crosslinked nanofibers immersed in water for three days.

Figure 2 shows the nanofibers morphology after crosslinking by immersing the electrospun nanofibers in a methanol bath. From the image, it can be appreciated that the fibrous structure of the nanofibers was conserved even after the crosslinking process (Figure 2a). The nanofibers morphology was preserved even after being immersed in a distilled water bath for three days (Figure 2b). This proved the effectiveness of the crosslinking process due to the formation of nanofiber hydrogel, as has been suggested by others author [19, 20].

The presence of the system LDH-ANS in the electrospun PVA nanofibers was confirmed utilizing LSCM microscopy. PVA nanofibers containing the labeled enzyme were electrospun onto an Indium-Tin-Oxide (ITO) coated glass slide and there-after analyzed with a LSCM microscope. Figure 3 shows the fluorescence images obtained from these samples. The fluorescence appears as a textured mat, where the individual fibers can not be distinguished. However, as can be observed in the image, the whole sample emits, suggesting a uniform distribution of the enzyme in the polymer matrix. A second observation in the same figure indicates that the reflection and fluorescence images obtained by LSCM are coincident, which demonstrates the presence of the ANS labeled enzyme LDH in the electrospun nanofiber. The previous statement is valid since no fluorescence has been reported for poly(vinyl alcohol) (PVA) by itself, in fact, this polymer has been used as inert matrix and fixer in SLCM experiments that involve biologically fluorescent substances [21-24].

Finally, in Figure 4, the obtained data of fluorescence quenching after the ANS-LDH/PVA electrospun nanofibers were exposed to solutions containing different concentrations of lactate,

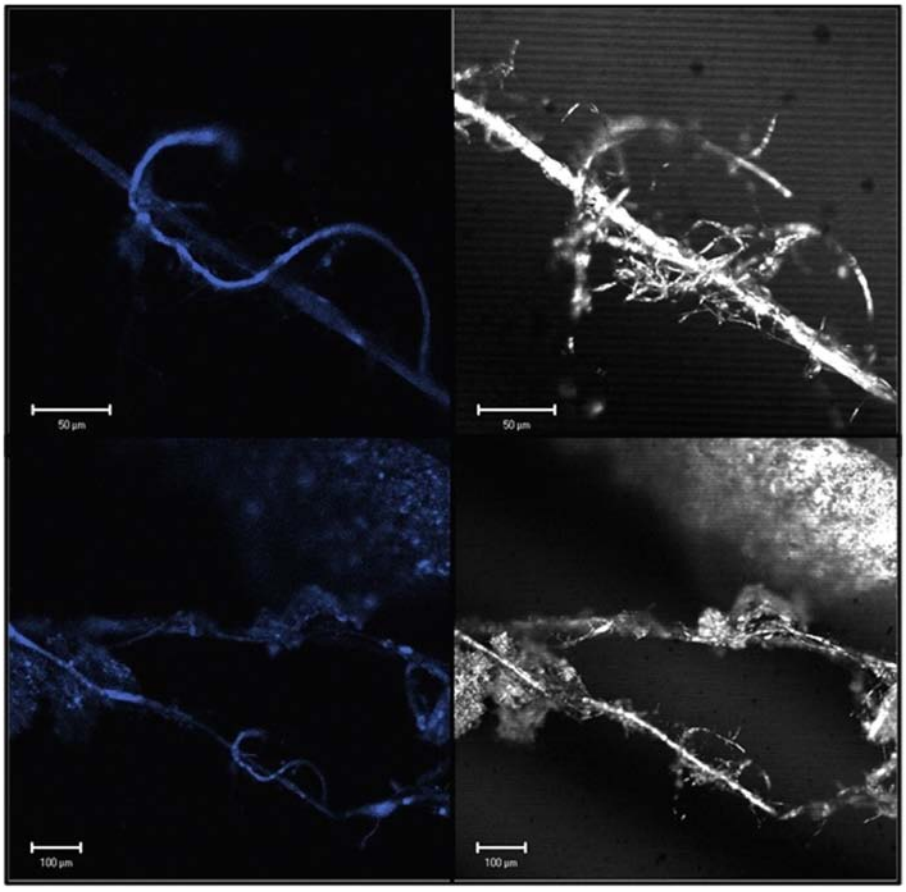

Figure 3. LSCM images of PVA nanofibers containing the immobilized ANS-labeled enzyme lactate dehydrogenase.

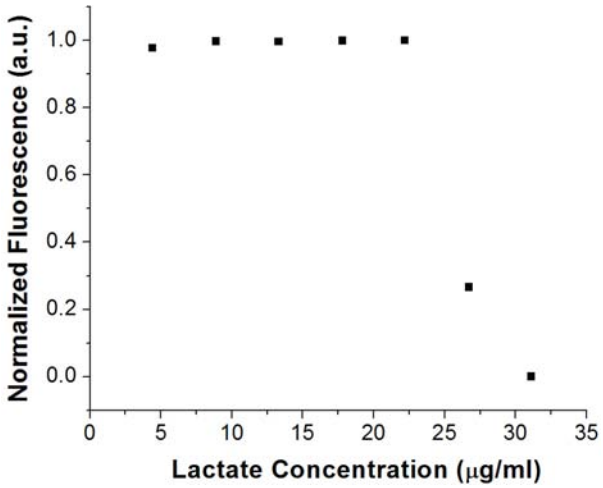

Figure 4. Fluorescence quenching in the presence of different concentrations of lactate using LDH-ANS system immobilized in PVA nanofibers.

are shown. This represents a preliminary assay for the detection of lactate in solutions. There was no quenching in the fluorescence of the system in the first five concentrations (4-22 μg/ml). The system did not show any response until a 27 mg/ml concentration was reached, at this point, the system underwent a drastic decrease in its fluorescence. This decrement in fluorescence of the system continued until a minimum was reached at a concentration of 31 μg/ml. This behavior can be attributed to the diffusion path that the analyte molecules have to follow through the porous structure of the nanofibers until they reach the immobilized enzyme molecules. In a similar way, this could be explained by the fact that the enzyme molecules in solid state and bound to solids had much more limited access to the substrate than the free ones. In addition, it is not expected that all the enzyme molecules of lactate dehydrogenase incorporated in the polymer matrix resided on the surface, or were exposed to the substrate, in order to participate in the enzymatic reaction [11].

CONCLUSIONS

The immobilization of the ANS labelled lactate dehydrogenase (LDH) enzyme in poly(vinyl alcohol) (PVA) nanofibers was demonstrated in the present work. The nanofibers showed interesting morphologies that were attributed to the high molecular enzyme acting as an agglutinant between the fibers. The presence of the LDH labelled with ANS in the nanofibers of PVA matrix was confirmed by LSCM microscopy; where the membrane nanofibers mat showed a strong fluorescence in the blue region. The immobilized LDH-ANS system in PVA nanofibers was used to detect lactate in solutions with concentrations as low as 22 μg/ml. This preliminary result could represent advancement for the development of robust and more versatile lactate sensing systems, based on polymer nanofibers.

ACKNOWLEDGMENTS

Ivan Moreno thanks CONACYT for financial support through 185172 scholarship during the time involved in preparation of this article. The authors also thank Gabriela Padron for the technical support.

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