ACS APPLIED MATERIALS & INTERFACES

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Preservation of Cell Viability and Protein Conformation on Immobilization within Nanofibers via Electrospinning Functionalized Yeast

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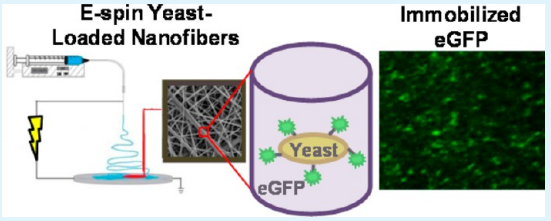
Supporting Information

ABSTRACT: We investigate the immobilization of a model system of functionalized yeast that surface-display enhanced green fluorescent protein (eGFP) within chemically crosslinked polyvinyl alcohol (PVA) nanofibers. Yeast is incorporated into water insoluble nanofibrous materials by direct electrospinning with PVA followed by vapor phase chemical crosslinking of the polymer. Incorporation of yeast into the fibers is confirmed by elemental analysis and the viability is indicated by live/dead staining. Following electrospinning and crosslinking, we confirm that the yeast maintains its viability as well as the ability to express eGFP in the correct conformation. This method of processing functionalized yeast may thus be a powerful tool in the direct immobilization of properly folded, active enzymes within electrospun nanofibers with potential applications in biocatalysis.



KEYWORDS: *electrospinning, nanofiber, protein, biocatalyst immobilization, yeast surface display*

INTRODUCTION

Although enzymes are highly efficient and selective biological catalysts,[1] lack of enzyme stability often limits their practical application.[2−4] For application of biocatalysts, immobilization is often desirable as it improves stability, and eases of recovery of the biocatalysts which facilitates reuse and avoids product contamination.[8−11] One of the foremost challenges in this field is that there is often an apparent decrease in catalytic activity upon immobilization.[8−11] The performance of the immobilized enzyme is significantly affected by the structure (size and shape) of the support material.[12,13] Fibers, especially nanofibers produced by electrospinning, are promising for biocatalyst immobilization due to their high specific surface area.[13−20]

There have been two main approaches using electrospun nanofibers as support materials for biocatalyst immobilization: (1) surface attachment and (2) encapsulation.[14] In surface attachment, biocatalysts, typically enzymes, have been covalently attached to the surface of electrospun nanofibers. However, this approach limits biocatalyst loading and the hydrophobic surfaces employed often require modification to increase their biocompatibility.[14] In contrast, encapsulation within nanofibers can be achieved by direct electrospinning of a biocatalyst and water-soluble and biocompatible polymer, such as polyvinyl alcohol (PVA),[12−14,21−23] and then crosslinking the polymer, which allows for high biocatalyst loading. Coaxial electrospinning as an alternative to direct electrospinning and crosslinking has been reported.[24] However, coaxial electrospinning requires a specialized electrospinning set-up and is complicated by the need to control of multiple feed rates.

The focus of this work is direct electrospinning and crosslinking due to advantages of simplicity and ability to accommodate high biocatalyst loadings. Despite the small length scales of nanofibers, which should minimize mass transfer limitations from the bulk to the biocatalyst, the catalytic activity is often lower upon immobilization.[12,13,21,22] Incorporation of protein into a solid polymer matrix by co-electrospinning may result in conformational changes of the enzyme[24] and crosslinking with glutaraldehyde can affect the conformation of a protein.[20]

Given that cells (bacterial and mammalian) have been successfully incorporated into electrospun nanofibers,[25−29] we propose an alternative to direct immobilization of enzymes that involves processing functionalized yeast that surface-display desired proteins in order to immobilize properly folded, active enzymes. In this approach we are immobilizing yeast cells engineered to express proteins or enzymes of interest via surface display. Yeast surface display is a method to immobilize proteins on the surface of yeast cells in their native conformation with high stability as proteins remain bound through fusion with the Aga2p receptor protein.[30] Although mammalian and other eukaryotic cells are also capable of expressing proteins by surface display, yeast is a stable organism that can withstand harsh conditions, and it is therefore well-suited to immobilization for potential biocatalytic applications.

Received: June 11, 2013
Accepted: September 13, 2013
Published: September 13, 2013

   dx.doi.org/10.1021/am4022768 | ACS Appl. Mater. Interfaces 2013, 5, 9349−9354

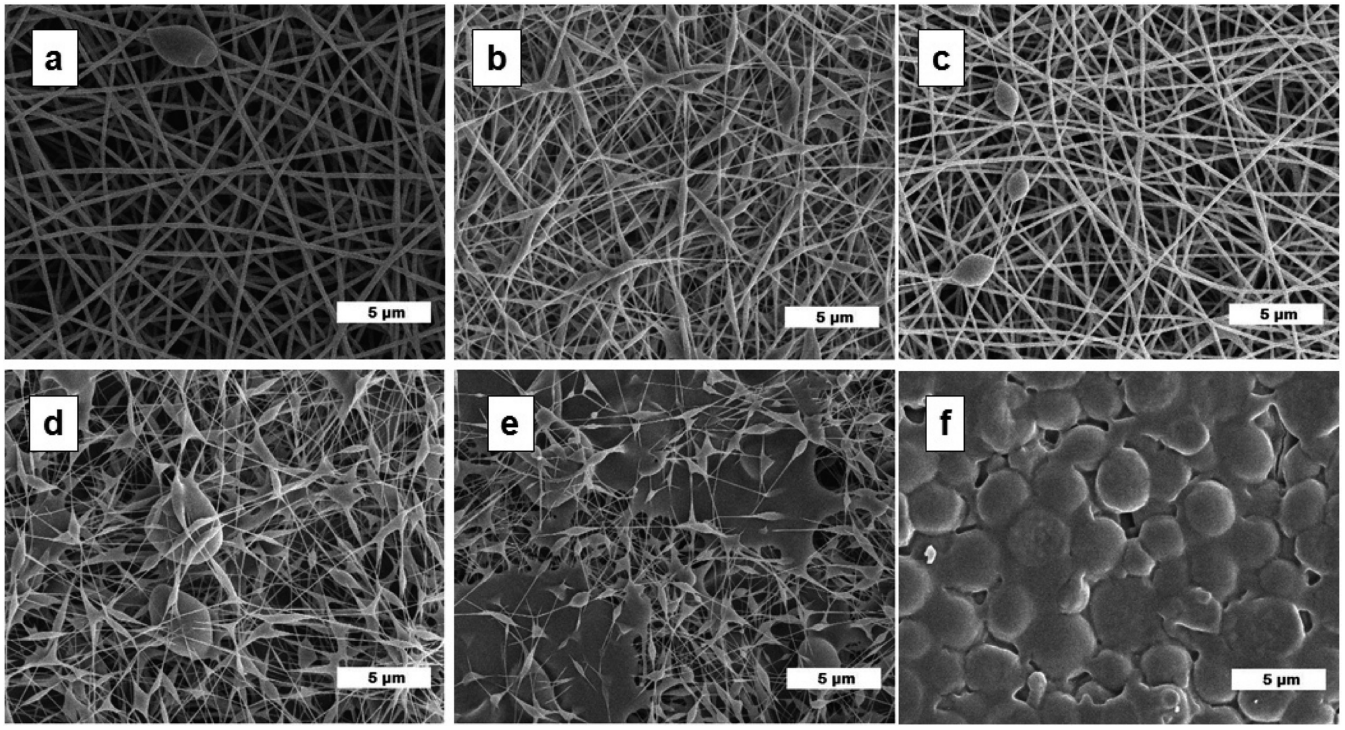


Figure 1. Effect of cell loading on PVA electrospinning (Ratios are given as volume of cell suspension/polymer solution): (a) 1/10 (v/v), (b) 1/8 (v/v), (c) 1/6 (v/v), (d) 1/3 (v/v), (e) 3/5 (v/v), (f) 6/7 (v/v).

We investigate the immobilization of a model system of functionalized yeast that surface-display enhanced green fluorescent protein (eGFP) within chemically crosslinked PVA nanofibers via electrospinning. We examine the effect of cell loading on fiber formation. Incorporation of the yeast cells into the electrospun fibers was confirmed by elemental analysis and the viability of the immobilized functionalized yeast cells was assessed using live/dead staining, and visualization of the displayed eGFP. Remarkably, yeast cells survive the harsh conditions of electrospinning and crosslinking and the surface display of the protein of interest remains intact. Further, we demonstrate that the protein retains its native conformation following direct electrospinning and crosslinking, thereby providing a promising route to immobilization of proteins with maintained structure and function, which has been a formidable challenge.

EXPERIMENTAL METHODS

Materials. PVA (average molecular weight 205 000 g/mol 88% hydrolyzed) and HCl were purchased from Sigma Aldrich. Live/Dead yeast viability kit was purchased from Invitrogen. Glutaraldehyde, 50% aqueous solution was purchased from Alfa Aesar. Yeast extract was purchased from Fisher Scientific, peptone and dextrose were purchased from VWR International LLC.

Cell Cultivation. A 5 mL overnight culture of EBY100 cells was used to inoculate 50 mL YPD media (0.5% (w/v) yeast extract, 1% peptone, and 1% dextrose). Cells were grown with constant shaking at 30°C, 250 rpm for 24 h. After incubation, the culture was centrifuged at 5000 rpm for 5 min and the supernatant was discarded. Cell pellets were kept on ice until used further. We refer to the concentrated cell pellets as cell suspension.

Yeast Cell Surface Display of eGFP. The pEGFP-N1 plasmid harboring the eGFP gene was a kind gift from Dr. Susan Carson (Biotechnology Program, North Carolina State University). The eGFP gene was amplified, ligated into the pCTCON vector, and transformed in Novablue (*E.coli*) cells. Plasmid constructs were subsequently transformed into yeast strain EBY100 for yeast display as described.[31] The yeast cells were cultured and induced for eGFP expression (see the Supporting Information for details). 1×10^7 cells were washed and run through a flow cytometer (FACS Aria, BD Biosciences) to confirm eGFP expression. Uninduced yeast cells were used as a control.

Yeast Cell Immobilization. Immobilization of eGFP within nanofibers required two steps: (1) electrospinning an aqueous solution of PVA and uninduced yeast cells and (2) chemical crosslinking the PVA. To electrospin, PVA and deionized water were stirred at 60°C until homogeneous. Cell suspension (575×10^6/mL)and PVA (8 wt %) solution were combined in various ratios and stirred for 5 min and loaded into a syringe fitted to a stainless steel 22G needle (0.508 mm I.D.) and electrospun. In general, we used 1/6 (v/v) cell suspension/polymer solution concentration for the cell electrospinning and applied 17 kV voltage (Matsusada Precision Inc, NY), 15 cm tip to collector distance and 0.35 mL/h feed rate (New Ear Pump Systems Inc, NY). After electrospinning, we exposed the as-spun yeast-loaded fibers to vapors from glutaraldehyde (50% aq.) and HCl to immobilize the cells within the crosslinked PVA fibers as previously described.[12] The use of glutaraldehyde in the vapor phase minimizes toxicity.[12] Typically, we electrospun for 1 h and crosslinked for 35 min. To determine if the fibers were crosslinked, we exposed the mat to water for 24 h.

Fiber Characterization. Fibers with and without cells, and before and after crosslinking were observed by scanning electron microscopy (SEM) and optical microscopy. For SEM analysis (JEOL JSM-6400F), the nanofiber mat samples were coated with ~10 nm of Au/Pd at 5 kV. The fiber size distributions were determined by measuring the diameter of at least 100 fibers using ImageJ software. Energy Dispersive X-ray Spectroscopy (EDS) analysis at 20 kV in the absence of coating at 1000× and 80 000× using Oxford SiLi detector, Advanced Analysis Technologies amplifier and 4 Pi Pulse processor was performed to confirm incorporation of yeast.

Using fluorescent microscopy (Olympus BX-61) and FITC 31001 filter system with excitation of 430-530 nm and emission of 515-575 nm, we observed the expression of eGFP induced from the immobilized yeast cells within the nanofiber composite structures as

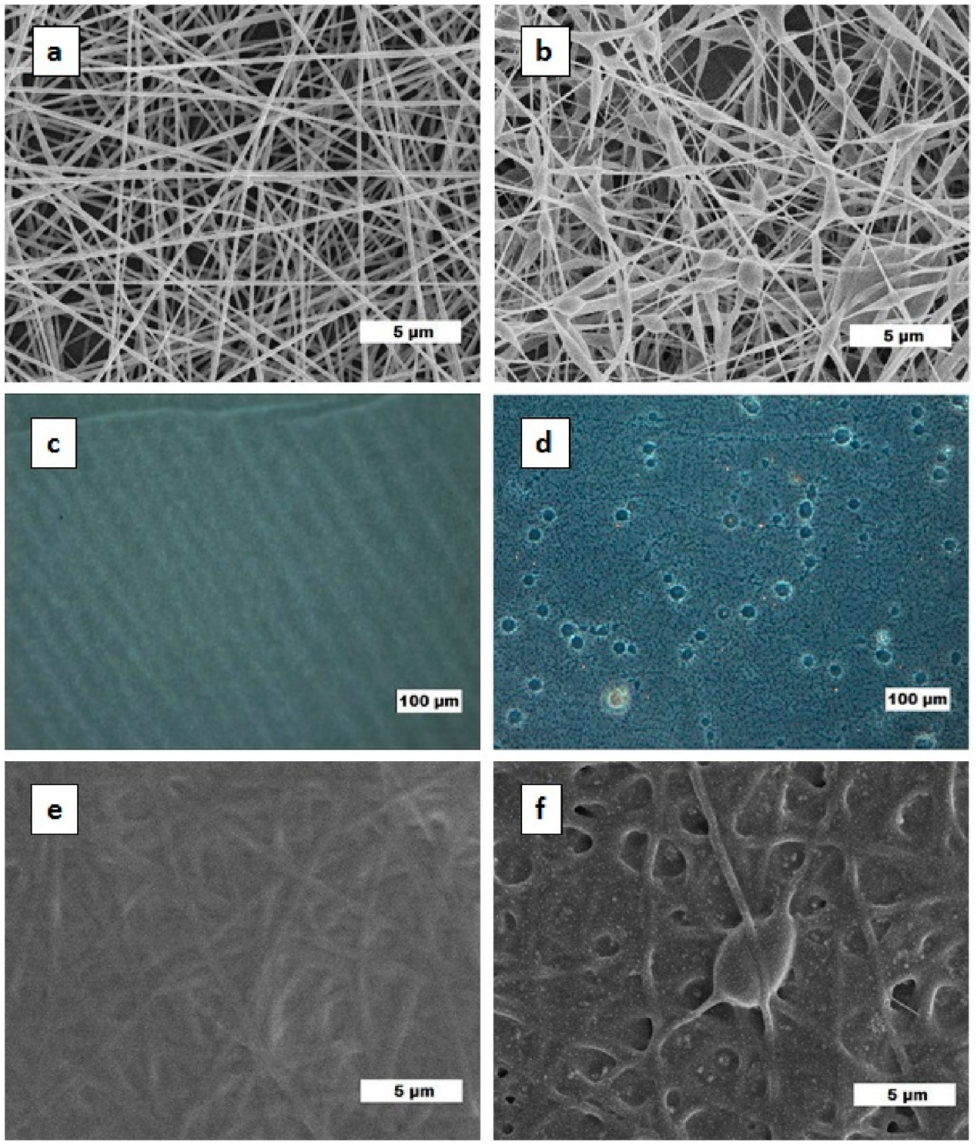


Figure 2. SEM images of (a) 8 wt % PVA nanofibers, (b) 8 wt % PVA dissolved in cell suspension electrospun into PVA nanofibers with yeast cells. Optical microscope images of (c) PVA nanofibers, (d) PVA nanofibers electrospun with yeast cells. SEM images of (e) crosslinked PVA nanofibers after soaking in water for 24 h, (f) crosslinked PVA/yeast nanofibers after soaking in water for 24 h.

well as stained composite structures prepared according to manufacturer's instructions.

RESULTS AND DISCUSSION

Yeast Cell Immobilization. The first step in immobilization was to incorporate yeast cells into electrospun PVA fibers. Initially, we examined the effect of yeast cell loading on electrospinning PVA fibers. The addition of cell suspension (concentrated by centrifugation) to the PVA solution was expected to reduce the viscosity and thus alter the electrospinnability of the system.[32−34] To identify the maximum cell loading, we varied the volume ratio of cell suspension to polymer solution while holding electrospinning process parameters constant (17 kV voltage, 15 cm tip-to-collector distance, and 0.35 mL/h flow rate) and we analyzed the structures produced with SEM (Figure 1). With a 1:6 (v/v) ratio of cell suspension to polymer solution, we achieved good quality fibers. At higher cell concentrations, we observed beaded structures and in some cases fused structures with non-fibrous residues. These results are consistent with the existing literature.[33,35] On the basis of this result, a 1:6 (v/v) ratio of cell suspension to polymer solution was used for all further experiments.

Next, we looked more closely at the nanofibers formed. PVA nanofibers with and without cells are shown in Figure 2. In the case of the PVA without cells (8 wt %), we observe uniform fibers with average fiber diameter of 269 ± 57 nm (Figure 2a). For samples electrospun with cells, we observe beaded portions that we believe encapsulate cells because the average size of a beaded structure is 3 μm (Figure 2b), as has been previously reported.[26,27,36] Optical microscopy images of fibers with and without yeast cells (Figure 2c, d, respectively) augment the results with SEM. The larger structures (on the order of 10 μm) observed under optical microscope indicate the presence of multiple cells. Inspection with SEM indicates that the sample is comprised of individual cell-encapsulating fibers rather than aggregates of cells. Elemental analysis of the PVA/yeast fibers using EDS indicated the presence of phosphorous, sulfur,

dx.doi.org/10.1021/am4022768 | ACS Appl. Mater. Interfaces 2013, 5, 9349−9354

potassium, and silicon (Table 1) that were not present in a PVA-only sample, indicating the presence of yeast cells.[37] We note that the presence of silicon is likely in the form of silicon dioxide, a commonly used anticaking agent.[38]

Table 1. Elemental Analysis Results of Pure PVA and Yeast-Cell-Incorporated PVA Nanofibers

element	PVA	PVA/yeast
C	12 968	12 622
O	1554	1665
P	N/A	390
S	N/A	177
K	N/A	346
Si	N/A	385

We next chemically crosslinked the fibers and examined the fibers after crosslinking and soaking in water for 24 h. SEM micrographs (Figure 2e, f) show that crosslinked material remains after soaking in water, although the fibers appear to swell and fuse upon soaking. Importantly, the beaded structures remain after soaking, which suggests that the yeast cells may be successfully immobilized within the crosslinked material.

Functionality of Immobilized Yeast Cells. Finally, we assessed the viability of the immobilized yeast cells after electrospinning and after crosslinking. The fibers containing yeast stained using the Live/Dead kit (Invitrogen) were imaged using optical microscopy (Figure 3). Results showing the stained Live cells appear red in Figure 3, indicating that yeast cells remain viable after electrospinning (Figure 3a) and after crosslinking (Figure 3b). We next confirmed the preservation of the yeast surface display system by inducing protein expression and observing the fluorescence of eGFP. Using fluorescence microscopy, stable eGFP expression was observed following electrospinning and crosslinking (Figure 3c, d, respectively). On the basis of these results, we confirmed the immobilized engineered yeast remain viable and capable of surface display of proteins in the proper conformation, which cannot be obtained by processing GFP directly.[24] Therefore, this may be a promising method to immobilize protein and whole cell biocatalysts within polymer nanofibers via direct electrospinning.

Further quantification of eGFP fluorescence was obtained using flow cytometry. For control purposes eGFP expression of yeast cells before electrospinning was examined and 85.8% of the cells showed eGFP fluorescence, confirming the proper folding (Figure 4a). After electrospinning, the PVA/yeast fibers were dissolved in SGCAA media, but only 2.4% of cells showed positive eGFP fluorescence (Figure 4b). When these cells (electrospun and dissolved in SGCAA media) were then cultivated for 48 h, we obtained 63.8% positive eGFP fluorescence indicating that the cells remain viable and multiply (confirmed by cell counting, see the Supporting Information). The relatively small fraction of yeast cells showing positive eGFP fluorescence after electrospinning is consistent with previous reports of significant losses in cell viability following electrospinning.[24,39−41] Importantly, despite the low percentage of cells showing positive eGFP fluorescence, the fibers show fluorescence (Figures 3) indicating that the immobilized engineered yeast maintain the ability to stably express significant amounts of eGFP after electrospinning. We note that cross-linked samples could not be redissolved and analyzed by flow cytometry.

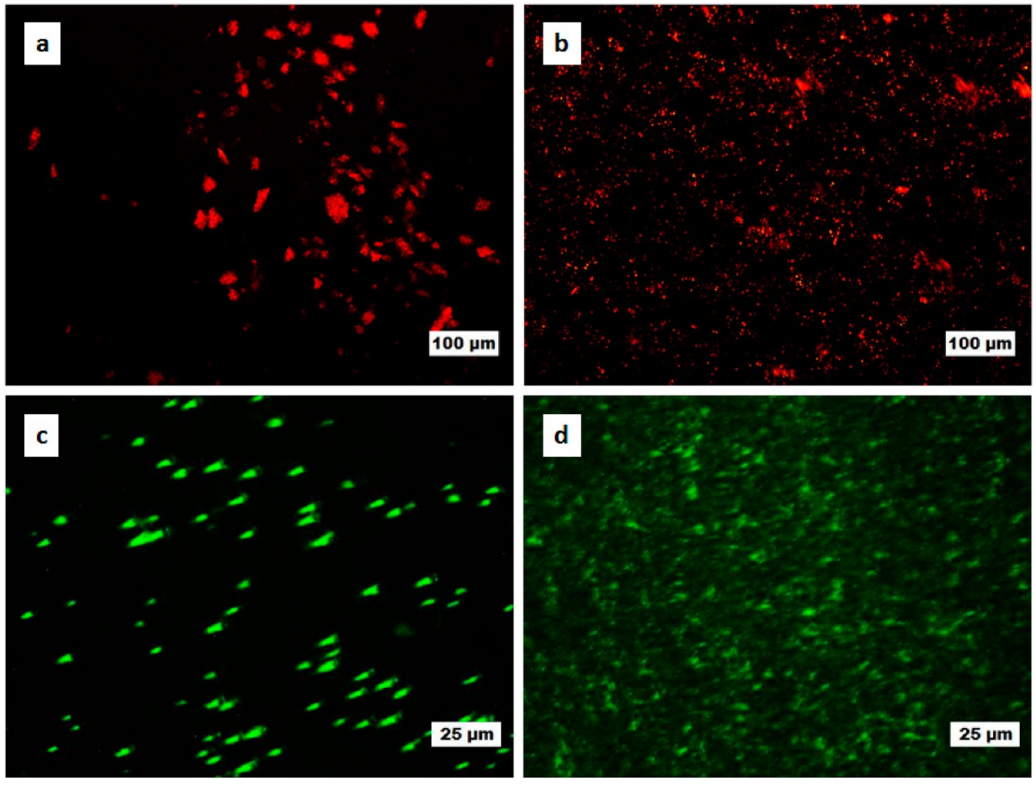


Figure 3. Live-dead staining results where red indicates live cells for (a) yeast cells electrospun with PVA, (b) yeast cells electrospun with PVA after crosslinking. and fluorescence microscopy images for eGFP expression induced (c) after electrospinning and (d) after crosslinking.

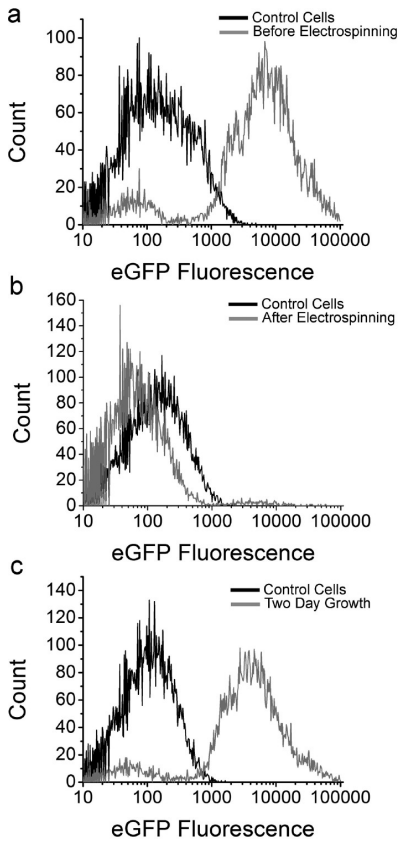


Figure 4. Fluorescence of yeast surface-displayed eGFP: (a) eGFP fluorescence before electrospinning (85.8% showed fluorescence), (b) eGFP fluorescence immediately after electrospinning (2.4% cells showed fluorescence), (c) eGFP fluorescence after electrospinning and grown for 2 days (63.8% cells show fluorescence), where uninduced cells were used as a control (shown in black).

CONCLUSIONS

We immobilized engineered yeast designed to express eGFP via surface display within electrospun and chemically crosslinked PVA nanofibers. Notably, the yeast cells survive the electrospinning and crosslinking process and thus are able to express eGFP in the correct conformation. This method of processing functionalized yeast may thus serve as a powerful tool in the direct immobilization of properly folded, and thus presumably active enzymes within electrospun nanofibers with potential applications in biocatalysis.

ASSOCIATED CONTENT

Supporting Information

Details of the cell viability tests and analysis and eGFP yeast surface display. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We acknowledge the Nonwovens Cooperative Research Center (NCRC) and Defense Threat Reduction Agency (DTRA, Grant HDTRA1-10-1-0024) for partial support of this work. M.F.C. thanks the Turkish Government for supporting his graduate studies.

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