

# Engineering of bio-hybrid materials by electrospinning polymer-microbe fibers

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Although microbes have been used in industrial and niche applications for several decades, successful immobilization of microbes while maintaining their usefulness for any desired application has been elusive. Such a functionally bioactive system has distinct advantages over conventional batch and continuous-flow microbial reactor systems that are used in various biotechnological processes. This article describes the use of polyethylene oxide<sub>99</sub>-polypropylene oxide<sub>67</sub>-polyethylene oxide<sub>99</sub> triblock polymer fibers, created via electrospinning, to encapsulate microbes of 3 industrially relevant genera, namely, *Pseudomonas*, *Zymomonas*, and *Escherichia*. The presence of bacteria inside the fibers was confirmed by fluorescence microscopy and SEM. Although the electrospinning process typically uses harsh organic solvents and extreme conditions that generally are harmful to bacteria, we describe techniques that overcome these limitations. The encapsulated microbes were viable for several months, and their metabolic activity was not affected by immobilization; thus they could be used in various applications. Furthermore, we have engineered a microbe-encapsulated cross-linked fibrous polymeric material that is insoluble. Also, the microbe-encapsulated active matrix permits efficient exchange of nutrients and metabolic products between the microorganism and the environment. The present results demonstrate the potential of the electrospinning technique for the encapsulation and immobilization of bacteria in the form of a synthetic biofilm, while retaining their metabolic activity. This study has wide-ranging implications in the engineering and use of novel bio-hybrid materials or biological thin-film catalysts.

biofilm | cross-linking | immobilization | microbial | *Zymomonas*

Microorganisms most often exist in nature as biofilms, complex and dynamic communities formed by self-encapsulation in a self-developed extracellular polymeric matrix (1). Biofilm formation protects microorganisms from various environmental challenges such as pH, salinity, and metal toxicity (2) and confers resistance to antibiotics and microbicides (3). Biofilms can be viewed as stable and efficient thin-film catalytic systems (4). Researchers presently are attempting to understand the complexity and the unique characteristics of biofilms, to create synthetic ones, and to exploit them for biotechnological applications in areas such as environmental remediation (5, 6), microbial fuel cells (7), particle biofilm reactors (8), and fermentation reactors (4). The shortcomings of traditional batch reactors, such as fermentors, include low cell density and frequent inoculation and start-up. These drawbacks are overcome in a biofilm reactor by virtue of its high cell density and stability. An ideal synthetic biofilm would be used or reused continuously, just as a thin-film catalyst. Further, the development and use of a synthetic biofilm would allow industrial-scale production and reproducibility. This study, which reports the generation of a very thin polymeric fibrous material in which microbes act as functionally active sites, represents a significant step toward achieving that goal.

The formation of composite microbiological material or biohybrid material containing entire microbial cells as catalytic centers has been pursued for several decades (9). In almost all these studies, microorganisms were entrapped in polymeric materials (typically

polyacrylamide or silica) or inorganic spheres that were orders of magnitude larger than thin films (10, 11). Thus, they suffered from shortcomings such as low viability of microbes, low diffusion through the material, and subsequent loss of biological activity (12). We have overcome these shortcomings through the development of thin fibrous materials using the process of electrospinning. These materials, while immobilizing microorganisms, also act as functional materials and thus form a hybrid between immobilization and biofilms.

Previous studies have reported the encapsulation of biological molecules such as enzymes, proteins, or animal cells in polymer fibers by co-electrospinning (13–17). In this process, 2 different solutions are spun simultaneously using a spinneret with 2 coaxial capillaries to produce core/shell fibers. To encapsulate live microbes, however at least 2 problems must be solved: (i) the organic solvent of the outer layer of the fibers must be evaporated completely to decrease the toxicity to the microbes inside the polymer fibers, and (ii) the porosity of the outer layers of the fiber must be increased to facilitate the transfer of material between the microbe and its environment. These problems can be addressed by using polyethylene oxide (PEO)-blend hydrogels, as explained later in this article.

Hydrogels are mechanically soft and flexible in aqueous solution and are capable of hosting enzymes without deteriorating their biological function (18, 19). The ability to make nano- and micro-fibers from water-soluble polymers by electrospinning offers a way to create hydrogels with nano- and microstructures. If these mechanical and biocompatible properties are combined with the high surface-to-volume ratios of fibers, the hydrogel can be used as an efficient support material both for enzymes and for immobilization of microorganisms (20). Lee and Belcher (21), Salalha et al. (22), and Gensheimer et al. (23) had investigated electrospinning as a possible method of encapsulating both bacteria and bacterial viruses. Although these studies are of great value and showed the promise of this emerging field, the electrospun polyvinyl alcohol, PEO, and polyvinyl pyrrolidone materials were water soluble, and thus their use was significantly limited. Furthermore, the microorganisms that were used in those studies, with the exception of *Escherichia coli*, are of minor relevance to the industry. For example *Micrococcus luteus* was selected specifically to survive the dry conditions of their electrospinning process.

We have created an insoluble fibrous polymeric material, the fibers of which encapsulate industrially relevant bacteria via electrospinning of Pluronic F127 dimethacrylate [FDMA or PEO<sub>99</sub>:

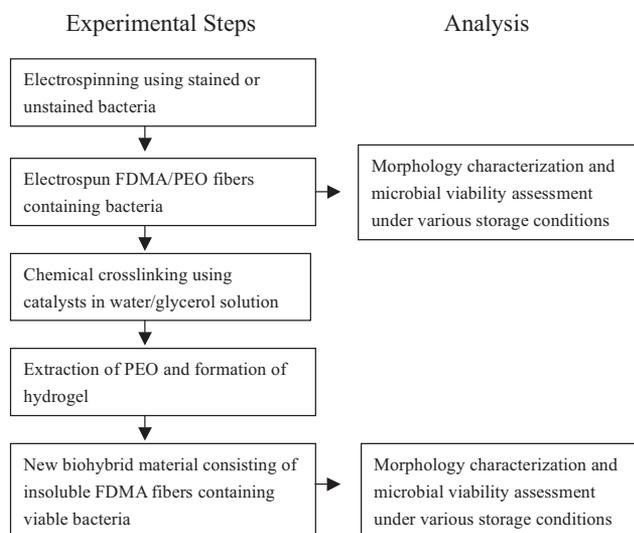
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**Fig. 1.** A schematic describing the different stages of the process that were undertaken to generate the biohybrid material. The chart also shows the various stages at which the fibers were characterized and analyzed.

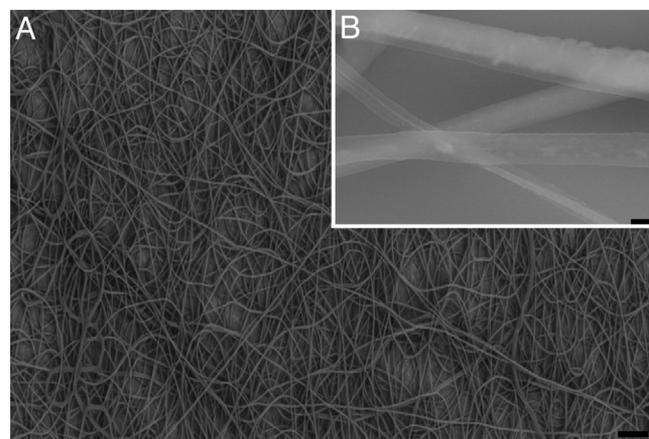
polypropylene oxide (PPO)<sub>67</sub>-PEO<sub>99</sub> DMA]. FDMA was selected as a model membrane material because of its non-biodegradability and non-toxicity. Moreover, it already had demonstrated significant ability to preserve polypeptide bioactivity, enzyme stability, and protein drug delivery (24). During electrospinning, the fibers overlap each other in a completely random manner, giving rise to the open pore structure ideal for use as electrodes or as membranes and in filtration. Our aim was to elucidate the conditions of the electrospinning and cross-linking process that allow the encapsulation of intact bacteria while still maintaining their viability. The microorganisms used in this work were *Pseudomonas fluorescens*, *Zymomonas mobilis*, and *E. coli*. All are rod-shaped bacteria. These species were chosen as examples of industrially relevant genera. For example, *Z. mobilis* is among the most efficient fermentors known to produce ethanol from glucose (25–27). This work opens an avenue for exploring the use of electrospun fibers for more mainstream applications in separation technology as well as in biofilm reactors.

## Results and Discussion

A schematic describing the different stages of the process that were undertaken in this study to generate the biohybrid material is presented in Fig. 1. The various stages at which the fibers were characterized and analyzed also are indicated on the schematic.

The FDMA/PEO-blend solutions with different weight ratios, increasing from 13:1 to 13:3, were electrospun to fabricate fibrous membrane scaffolds. At an FDMA/PEO weight ratio of 13:1, very few electrospun fibers were generated, and these fibers showed a beads-on-string morphology with a high bead density (Fig. S1A). As the FDMA/PEO weight ratio increased from 13:1 to 13:3, the density of beads decreased (Fig. S1B). At an optimized weight ratio of 13:3 (Fig. 2A and B), which was used in all further experiments, a uniform fibrous scaffold was obtained.

The water-soluble nature of FDMA presents a great challenge, because any contact with water can destroy the fibrous structure immediately, thereby severely limiting the application of this polymer. Cross-linking the fibers after electrospinning creates hydrogel fibers with improved resistance to water. Such cross-linked fibers with encapsulated microorganisms can be used in various applications. However, FDMA cannot be cross-linked using conventional cross-linking approaches such as exposing the fibers to a water-based cross-linking solution (28). Also, the nonvolatile nature of the



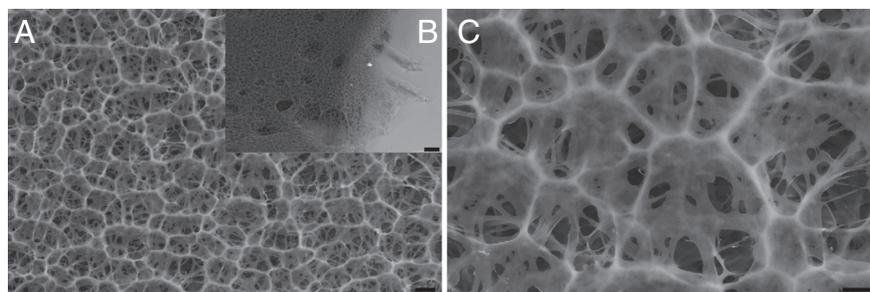
**Fig. 2.** (A) SEM image of an electrospun F127-DMA/PEO-blend scaffold with an FDMA 13wt%: PEO 3wt% (13:3) weight ratio. (Scale bar, 20  $\mu\text{m}$ .) (B) A higher-magnification image of the image in A. (Scale bar, 1  $\mu\text{m}$ .)

cross-linking agent prevents the use of the vapor-phase cross-linking method (29–31). Several studies have described various other methods of creating cross-linked fibers; such as using heat (32–36) or UV radiation (20, 37–39) to initiate the cross-linking reaction during or after the synthesis of the fibers. Heat and UV light, however, have known microbicidal properties and thus are not suitable for use in this study.

It is preferable to cross-link FDMA fibers in an organic solvent to prevent their dissolution. Glycerol was chosen as the organic solvent because of its low toxicity to microorganisms. In fact, glycerol is used for cryogenic preservation of microbes. Although pure glycerol can be used, it does not lead to free-radical polymerization. To initiate free-radical polymerization, the fibers were exposed to a solution of glycerol and water containing a redox system consisting of ammonium persulfate (APS), ascorbic acid (AsA), and ferrous sulfate. As anticipated, the water/glycerol solution did not dissolve the electrospun fibers and allowed the subsequent cross-linking reaction to proceed. Furthermore, the low toxicity of the chosen redox system allowed the microbes to survive.

The as-spun FDMA/PEO-blend scaffold with or without bacteria was cross-linked and subsequently soaked in deionized water to remove PEO and obtain an FDMA fibrous scaffold. The morphological change of FDMA fibrous scaffold (without bacteria) after PEO extraction is shown in Fig. 3. SEM images showed that the cross-linked FDMA scaffold still maintained the 3D porous structure after PEO extraction. This structure is in agreement with the morphology of freeze-dried cross-linked fibers that were obtained via electrospinning of other polymeric materials (28). The porous structure was seen not only on the surface of the electrospun samples (Fig. 3A and C) but through the whole thickness of the sample, as apparent from Fig. 3B (Inset), which shows the cross-section (edge) of the scaffold. However, the presence of a significant amount of water during cross-linking treatment had affected the fiber morphology to some extent: at junctions fibers were fused together, forming bindings. The distribution of fiber sizes before and after PEO extraction, analyzed using the University of Texas Health Science Center at San Antonio (UTHSCSA) Image Tool, is shown in Fig. 4A and B, respectively. Before PEO extraction, more than 85% of fibers were between 500 and 900 nm in diameter. After PEO extraction, the range of fiber diameter became much wider, and more than 74% of fibers were between 1 and 2  $\mu\text{m}$  in diameter.

The thermogravimetric (TG) thermograms of raw FDMA powder, the raw PEO powder, the electrospun FDMA/PEO-blend scaffolds, and cross-linked FDMA fibers are shown in Fig. 5. TG analysis showed that the thermal degradation temperature of



**Fig. 3.** Surface (A and C) and edge (B) images of the FDMA fibrous scaffold obtained by lyophilization after PEO extraction. (Scale bars, 20  $\mu\text{m}$  in A, 100  $\mu\text{m}$  in B, 10  $\mu\text{m}$  in C.)

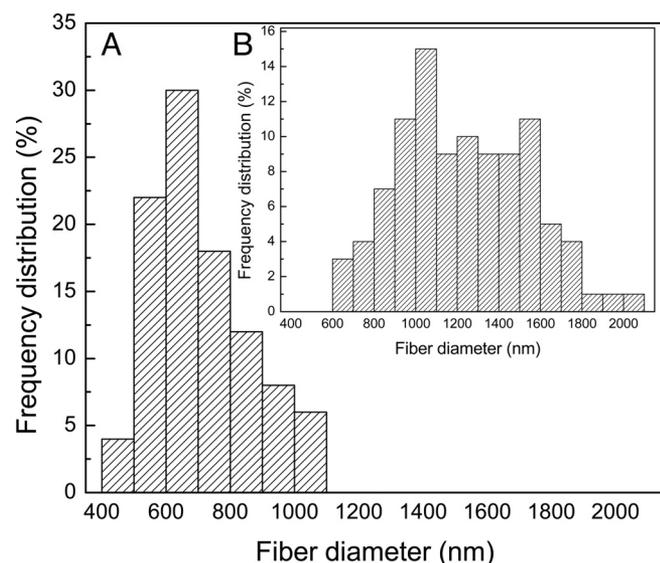
FDMA/PEO scaffolds was  $\approx 350^\circ\text{C}$ , indicating its thermal stability in the temperature range where microorganisms are used (usually lower than  $45^\circ\text{C}$ ). Also, the thermal degradation temperature of the FDMA/PEO blend was between the thermal degradation temperature of pure PEO powder and that of FDMA powder, suggesting the mixture of PEO and FDMA in the electrospun-blend scaffold. On the other hand, TG analysis showed that the thermal degradation temperature of cross-linked FDMA scaffolds increased from  $200^\circ\text{C}$  to  $350^\circ\text{C}$ . The increase in the degradation temperature can be attributed to the presence of interchain molecular cross-links.

We then used the method described earlier in this article to encapsulate rod-shaped bacteria in a polymer matrix, which forms a composite fiber during electrospinning. The bacteria initially were suspended in the FDMA/PEO aqueous solution, in which they were found to be randomly oriented (Fig. 6A). After electrospinning, the rod-like bacteria were found to be oriented mainly along the direction of the fibers (Fig. 6B and C). A lower-magnification image (Fig. 6C) showed that the microbes were distributed over the entire area of the electrospun fibers. With higher magnification using confocal microscopy, individual bacteria could be discerned within these fibers. Fig. 6D shows a representative cell of *Z. mobilis* bacterium inside the electrospun FDMA/PEO fibers. The microorganisms were found to be fully encapsulated by the fibers and oriented in the longitudinal direction of the fiber. To obtain higher magnification images and to confirm the cellular integrity of bacteria within the single fiber, electron microscopy was required. To this extent, uranyl acetate was used as a contrast agent to

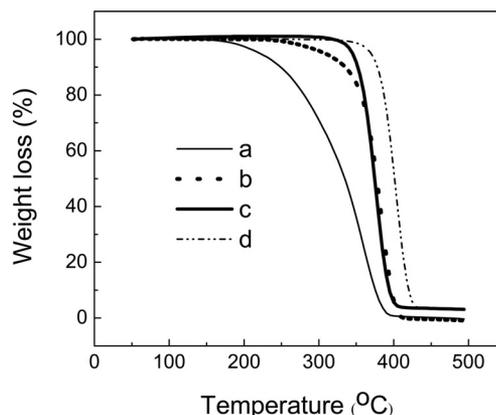
differentiate the microbe from the polymer. Fig. 6E presents the SEM image of an uranyl acetate-stained *P. fluorescens* encapsulated in the fiber. Fig. 6E clearly shows that the polymeric matrix has fully encapsulated the bacterial cell, causing a local widening of the fiber. In all these circumstances, the fiber diameters were found to be only slightly larger than the average size of the microbes used, thereby encapsulating the bacterium with only a thin layer of the polymeric material.

We found that exposure to FDMA and PEO had little or no effect on the viability of the bacteria, even when the bacteria were maintained in this solution for up to a week before assaying them. As described earlier, electrospinning is an efficient method for encapsulating bacteria in the polymer fiber. However, in the electrospinning process, the removal of water by rapid evaporation is anticipated to cause drastic changes in the osmotic environment of the organism (23). Furthermore, an electric field, which may be harmful to the bacteria, is generated by applying a high voltage between the metal capillary and the collector. To assess this effect, non-cross-linked fibers containing *P. fluorescens* were dissolved in culture medium, thereby releasing the bacteria from the fibers. The growth of the bacteria was then monitored by measuring the absorbance at 600 nm (Figs. S2 and S3). Although the electrospun microorganisms had a slightly longer lag-phase of growth, their growth was barely affected by electrospinning.

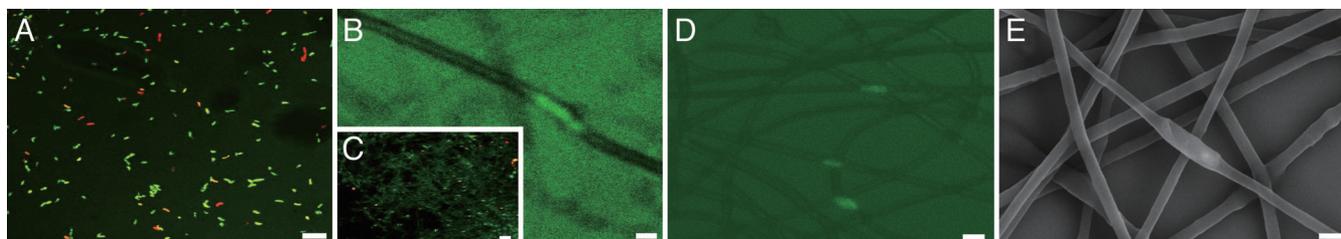
*Z. mobilis* suspended in a polymeric solution were electrospun, stained with bacterial viability kits, and examined immediately after electrospinning. The images obtained before and after electrospinning are shown in Fig. 7A–E. Fig. 7B shows that most (about 93%) of the bacteria were viable (shown as green in the images) immediately after the electrospinning process, before the cross-linking and PEO extraction steps were conducted. The effect of storage on the viability of encapsulated bacteria is an important issue for their potential deployment in industrial-scale processes, because the application of these novel bioactive materials requires the bio-hybrid system to be intact and functional (i.e., that microorganism



**Fig. 4.** Distribution of fiber diameters in FDMA/PEO-blend fibrous scaffold electrospun from 13wt% FDMA/PEO aqueous solution with FDMA/PEO weight ratio of 13:3 (A) and cross-linked FDMA fibrous scaffold after PEO extraction (B).



**Fig. 5.** TG analyses of different samples: (a) F-DMA powder, (b) F-DMA/PEO-blend scaffold, (c) FDMA cross-linked scaffold, and (d) PEO powder.



**Fig. 6.** Confocal images of stained and fluorescent (red, dead cells; green, live cells) cells of (A) *P. fluorescens* before electrospinning, (B and C) *P. fluorescens* inside the dry electrospun FDMA/PEO-blend fibers and, (D) *Z. mobilis* in dry electrospun FDMA/PEO-blend fibers. (E) SEM image of uranyl acetate-stained *P. fluorescens* cells after electrospinning. (Scale bars, 10  $\mu\text{m}$  in A, 1  $\mu\text{m}$  in B, 20  $\mu\text{m}$  in C, 2  $\mu\text{m}$  in D, and 1  $\mu\text{m}$  in E).

are viable) at the time of use at a desired site (22). To test the viability of the microbes in the fiber over time, the bacteria-containing scaffolds were maintained under saturated humidity conditions and under exclusion of light at 4 °C, for up to 7 days. After 1, 3, and 7 days, bacteria-encapsulating FDMA/PEO fiber was dissolved in deionized water to liberate the microorganisms. Although the viability of bacteria decreased over time, a significant percentage of the bacteria remained viable:  $\approx 62\%$ , 47%, and 23% were found to be viable after 1 day (Fig. 7C), 3 days (Fig. 7D), and 7 days (Fig. 7E), respectively. Further, to test whether the functionality (metabolic pathway) of the microbe was affected, the non-cross-linked fibers containing encapsulated microbes were assayed in growth media, and the metabolic products were assessed. *Z. mobilis* is well known for its ethanogenic activity, and thus the amount ethanol produced (vol %) was assessed. The results showed that the metabolic activity of the microorganism was not affected by the electrospinning process, and the amount of ethanol produced by the encapsulated microbes was in good agreement with the amount produced by un-encapsulated (free culture) control (Table S1), after 1 week of storage at  $-4^\circ\text{C}$  and after up to 2 months of storage at  $-70^\circ\text{C}$ . It thus is apparent that the electrospinning process does not adversely affect the metabolic pathway of the microbes.

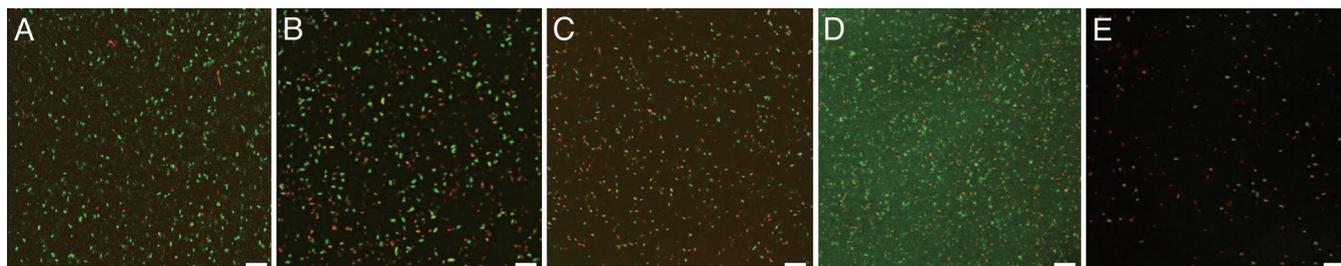
Although the cross-linking treatment improved the water resistance and thermal properties of the electrospun FDMA fibrous membranes, such treatment eventually could be cytotoxic to bacteria encapsulated in the fiber. The cytocompatibility of the cross-linking step is critical to the ultimate success of this study. Although polymerization of monomers with carbon-carbon double bonds has been investigated extensively in the past using photopolymerization/photo cross-linking (40–42), this method cannot be used for the preparation of cross-linked FDMA fibers encapsulated with bacteria, both because UV light has known microbicidal properties and because photopolymerization cannot be carried out uniformly in a large or thick system. Furthermore, the penetration depth of UV light is quite limited, and the distribution of light is inhomogeneous (43). Chemical cross-linking seems to be a more suitable method for the purpose of this study, although it could be toxic to the bacteria. The most commonly used free radical

initiator consists of N,N,N',N'-tetramethylethylenediamine (TEMED) and peroxydisulfate (potassium or ammonium salt) (15, 43–47) and works by polymerization of the methacrylate groups (48). However, TEMED is toxic to microbes and was not considered in this work (49).

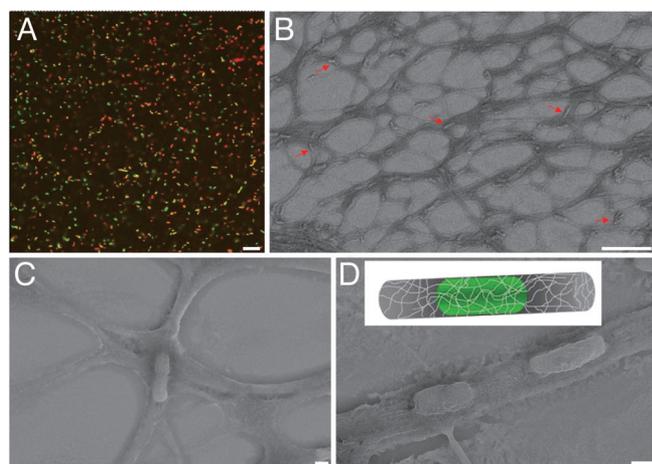
Previous studies also have reported that FDMA could be cross-linked by free radical polymerization at 37 °C using a redox system that included APS and sodium metabisulfite (50, 51). However, sodium metabisulfite releases  $\text{SO}_2$  when exposed to water. To prevent any interference from gas evolution and possible changes in the pH caused by  $\text{SO}_2$  during the cross-linking reaction, we used ferrous sulfate and AsA instead of sodium metabisulfite. Ferrous sulfate is a component of several bacterial growth media and, unlike metabisulfate, does not degrade in water. The concentration of the APS, ferrous sulfate, and AsA was adjusted to be as low as possible, by iterative experimentation, to minimize the oxidation of microbes by APS.

The FDMA cross-linked fibers encapsulating *Z. mobilis* were washed with deionized water 3 times and stained with LIVE/DEAD BacLight™ bacterial viability kits (Molecular Probes) to visualize the bacteria under the confocal microscope. The confocal microscopy image shown in Fig. 8A revealed that about 40% of the bacteria were still alive after the electrospinning and cross-linking process. To verify encapsulation of *Z. mobilis* cells by the FDMA fiber, freeze-dried cross-linked FDMA fibers encapsulating the bacteria were examined. Normally, the cross-linked FDMA fibers are multilayered. A single layer of FDMA fibers encapsulating *Z. mobilis* cells is shown here, because it provides better contrast between the microorganism and fiber. Fig. 8B shows a homogeneous distribution of bacteria in the cross-linked material. Microbes were found to be encapsulated both at the junctions where fibers fused together (Fig. 8C) and in single fibers (Fig. 8D); the cellular integrity of the microorganism seemed to be well preserved, regardless of the location.

To determine whether the bacteria proliferate within the fibers, we monitored the total number of live and dead *Z. mobilis* cells in cross-linked FDMA fibers for 2 days (Fig. S4A). The results indicate that the total number of live and dead cells of *Z. mobilis* remained almost constant, and there was no proliferation. Similar results also



**Fig. 7.** Confocal images of *Z. mobilis* cells (A) before electrospinning; (B) immediately after electrospinning; and after storage at 4 °C under saturated humidity, with the exclusion of light for (C) 1 day; (D) 3 days; and (E) 7 days. (Scale bars, 20  $\mu\text{m}$ .)



**Fig. 8.** Images of *Z. mobilis* within the cross-linked FDMA fibers. (A) Confocal microscopic image of *Z. mobilis* within the cross-linked FDMA fibers shows that about 40% of the bacteria were still alive after the electrospinning and cross-linking process. (Scale bar, 10  $\mu\text{m}$ .) (B–D) SEM images of *Z. mobilis* in the cross-linked FDMA fibers. Arrows in B indicate the locations of a few bacterial cells. Microbes were found to be encapsulated both at the junctions where fibers fused together (C) as well as in single fibers (D). (Scale bars, 20  $\mu\text{m}$  in B, and 1  $\mu\text{m}$  in C and D.) Sketch (inset, D) illustrates a bacterium encapsulated in the cross-linked FDMA fibers.

were obtained when we monitored the bacteria encapsulated in the cross-linked bulk hydrogel (Fig. S4B), without undergoing electrospinning, indicating that some aspect other than the confinement in the fibers is responsible for hindering their proliferation.

The sketch shown as an inset in Fig. 8D illustrates the conditions around a bacterium cell inside a cross-linked FDMA fiber. In contrast to the fibers reported previously (13–17), which are composed of a water-insoluble polymer shell around a hollow core formed by dissolution of a hydrophilic component, the fibers discussed here are formed around the bacterium from a uniform hydrophilic polymer solution. When immersed in water, the cross-links prevent the fibers from dissolving and lead to the formation of a mesh-like network of the swollen polymer within a short time (Fig. S5). When the gel fibers are fully cross-linked, we can estimate that the greatest distance between cross-links is the stretched end-to-end distance of the polymer, which is less than 100 nm. This distance is large enough to allow the exchange of nutrients and microbial metabolic products between the microbe and the environment, but it is much smaller than the size of the bacteria, thereby immobilizing the microbes inside the open mesh-like enclosure. As an indication of the rate of nutrient exchange, it was observed that complete exchange of glucose at a concentration of 80 g/L occurred in  $\approx 2$  h (Fig. S6).

GFP *E. coli* also were encapsulated using this process and were examined by confocal microscopy. The innate fluorescence of GFP *E. coli* was used so image the bacteria could be seen easily in confocal microscopy images (Fig. S7). With higher magnification, single bacteria within the cross-linked fibers could be observed to be oriented in the longitudinal direction of the fiber, with no morphological changes caused by the cross-linking reaction being observed. Encapsulation of *E. coli* shows the broad applicability of this process.

**Summary.** This study describes the development and formation, via electrospinning, of an FDMA fibrous hydrogel material with encapsulated microbes. The microbes in the material were found to be viable for more than 1 week in the dry FDMA/PEO-blend scaffold at 4  $^{\circ}\text{C}$  and for more than 2 months at  $-70$   $^{\circ}\text{C}$ . The FDMA fibers were cross-linked using a water/glycerol solvent mixture and the APS, ferrous sulfate, and AsA catalytic system. The occurrence of

the cross-linking reaction was demonstrated by TG analysis. The integrity and the viability of the bacteria were maintained through the cross-linking process. The mesh-like network of the polymer effectively immobilized the microbe while allowing the exchange of nutrients and metabolic products between the microorganism and the environment. This study has significant implications for application of immobilized microorganisms and/or synthetic biofilm-based bioreactors.

## Materials and Methods

**Synthesis of FDMA.** The synthesis and characterization of FDMA was described in an earlier article, and the chemical reaction is shown in Fig. S8 (50). The weight average ( $M_w$ ) and number average molecular weight ( $M_n$ ) of FDMA were determined using calibrated gel-permeation chromatography to be  $M_w = 21,900$  Da and  $M_n = 12,600$  Da ( $M_w/M_n = 1.3$ ).

**Bacterial Cultures.** *P. fluorescens* (ATCC 55241) were cultured in a medium containing citric acid, 2.0 g/L;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 2.0 g/L;  $\text{NH}_4\text{Cl}$ , 1.0 g/L;  $\text{KH}_2\text{PO}_4$ , 1.0 g/L;  $\text{K}_2\text{HPO}_4$ , 1.0 g/L; and NaCl, pH 6.1, 5.0 g/L, adjusted using NaOH. *Z. mobilis* (ATCC 31821) were cultured in a medium containing glucose, 20 g/L; yeast extract, 10 g/L;  $\text{KH}_2\text{PO}_4$ , 2 g/L, pH adjusted to 6.0 using NaOH. Functionality was assessed by growing immobilized *Z. mobilis* and pure culture (control) in a fermentation medium containing 20 g glucose, 10 g yeast extract, and 2 g  $\text{KH}_2\text{PO}_4$  per L  $\text{H}_2\text{O}$ , pH 6.0. Recombinant *E. coli* bacteria expressing GFP were grown in LB medium. The cultures were grown in Erlenmeyer flasks in an incubator at  $27 \pm 1$   $^{\circ}\text{C}$ . Typically, cultures at the end of the log phase of growth were used for electrospinning.

**Fabrication of FDMA/PEO Blend Fiber.** An aqueous solution of FDMA is not optimal for electrospinning into fibers, even at high concentrations. Therefore, PEO ( $M_w = 900$  kDa; Sigma-Aldrich Inc.) was blended with FDMA to facilitate fiber formation during the electrospinning process. Electrospinning solution was prepared by dissolving PEO powder in deionized water at various concentrations, followed by the addition of FDMA powder at a concentration of 13wt%. The mixture was kept at 4  $^{\circ}\text{C}$  until the solution became clear. For the encapsulation experiment, a predetermined amount of the bacteria, as required, was dispersed homogeneously in the FDMA/PEO solution before electrospinning.

The experimental set-up of the electrospinning stage was as described elsewhere (52). The fibers were electrospun and collected on a sterile silicon wafer for about 30 min to form a 3D structure.

**Cross-Linking of the Electrospun FDMA Matrix.** The catalytic system consists of AsA (Aldrich), ferrous sulfate (Aldrich), and APS (Aldrich). The principle of this reaction is similar to the Fenton reaction, as reported earlier (53). APS is the free radical initiator; ferrous sulfate and AsA are used to catalyze the breakdown of the APS and, therefore, to accelerate the cross-linking reaction. This catalytic system is effective at room temperature even if the concentration of the initiator is very low. However, for the system to be highly efficient, the ratio of the APS, AsA, and ferrous sulfate had to be optimized.

AsA, APS, and ferrous sulfate solution were prepared freshly in deionized water, and predetermined amounts of those solutions then were added to the glycerol/deionized water solvent at differing glycerol:water ratios. The electrospun fibers (along with the silicon wafer support) were placed into a glass vial containing 2 mL of the cross-linking solution and were allowed stand overnight at room temperature. The cross-linked membrane then was washed 3 times with deionized water to remove unreacted monomers and catalyst. Finally, the membrane was soaked in deionized water for 24 h to ensure complete extraction of the PEO and full swelling of the scaffold.

**Characterization of Electrospun FDMA Fiber Mats.** The surface morphology of the electrospun FDMA/PEO-blend fibers and FDMA fibers (with and without bacteria) were characterized using SEM (LEO 1550). The swollen FDMA fibers were freeze dried (Consol 1.5, Virtis Inc.) at  $-40$   $^{\circ}\text{C}$ , followed by lyophilization. Samples were sputter-coated with gold for 15 seconds twice before SEM imaging. The fiber diameter distributions of the FDMA/PEO-blend scaffolds and cross-linked FDMA scaffolds were calculated by analyzing the SEM images using the UTHSCSA Image Tool in a manner similar to that described by Boland et al. (54).

TG measurements were conducted to analyze the thermal behavior of the electrospun FDMA/PEO-blend fibers before and following cross-linking. TG measurements were conducted in nitrogen gas at a heating rate of 5  $^{\circ}\text{C}/\text{min}$  in the temperature range between 50  $^{\circ}\text{C}$  and 500  $^{\circ}\text{C}$  using a Mettler Toledo TGA/SDTA 851 thermal analyzer. Samples with a weight of  $\approx 15$  mg were loaded in a  $\text{SiO}_2$  crucible under dry conditions.

**Characterization of Microbes.** The viability of the microbes was assessed using the LIVE/DEAD BacLight bacterial viability kits (Molecular Probes). Live microbes (intact cell membranes) stain fluorescent green, whereas dead microbes (damaged cell membranes) stain fluorescent red. Live and dead bacteria later were viewed simultaneously by Leica TCS SP2 laser-scanning confocal microscopy (LSCM) (Leica Microsystems Inc.).

The morphologies of the bacteria inside the FDMA/PEO fibers were characterized both by LSCM and SEM. For LSCM, bacteria were spun down from culture media, stained with bacterial viability kits, and then mixed with the FDMA/PEO solution before electrospinning. For GFP *E. coli*, GFP was excited at 488 nm with an argon ion laser source without any staining. For SEM studies, the bacteria were rinsed with deionized water twice, stained with 2% (wt/vol) uranyl acetate for 2 min at room temperature, spun down, and mixed with the FDMA/PEO electrospinning solution. To image the bacteria inside the cross-linked FDMA fiber by SEM, the swollen fibers were freeze dried and coated with gold as described earlier in this article.

**Cytotoxicity and Storage Evaluation.** The viability of the bacteria, before and after electrospinning, was evaluated after various durations of electrospinning. Bacteria-containing fibers were stored under the exclusion of light at 4 °C for up to 7 days and at -70 °C for up to 2 months. We used 2 methods to analyze the bacterial survival rate. The encapsulated microbes were stained with LIVE/DEAD

BacLight bacterial viability kits (Molecular Probes) immediately after they were liberated from the FDMA/PEO-blend fibers and then were observed under LSCM. Photomicrographs of the stained bacteria were obtained. The number of live bacteria was averaged over several views of the same condition. Microbial counts at each time point was performed in triplicate. In the case of *Z. mobilis*, the uncross-linked fibers were dissolved in sterile bacteria culture medium and/or fermentation medium described earlier, so that the immobilized microorganisms were released from the fibers. As controls, free culture and microorganisms mixed with polymeric material (before electrospinning) were used as inocula. After incubation, the metabolic activity of the *Z. mobilis* was tested by analyzing the spent media for residual glucose and ethanol concentration using HPLC. The cytotoxicity of the chemicals and the cross-linking process to the bacteria also was evaluated using the LIVE/DEAD staining procedure.

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