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

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Introduction

Microorganisms most often exist in nature as biofilms

Biofilm formation **protects** microorganisms from various environmental challenges such as pH, salinity, and metal toxicity and confers resistance to antibiotics and microbicides

Researchers presently are attempting to **understand, to create synthetic ones and to exploit** them for biotechnological applications in areas such as environmental remediation, microbial fuel cells and fermentation reactors

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.

The formation of composite microbiological material or biohybrid material containing entire microbial cells as catalytic centers has been pursued for several decades. 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. Thus, they **suffered** from shortcomings such as low viability of microbes, low diffusion through the material, and subsequent loss of biological activity.

Electrospinning has been used to **overcome** these limitations.

These materials, while immobilizing microorganisms, also act as functional materials

Lee and Belcher, Salalha et al.and Gensheimer et al. 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**.

In this research an insoluble fibrous polymeric material was created, these fibers encapsulate industrially relevant bacteria via electrospinning of Pluronic F127 dimethacrylate [FDMA or PEO_{99}-polypropylene oxide $(PPO)_{67}$-PEO_{99} DMA]. FDMA was selected as a model membrane material because of its non-biodegradability and non-toxicity

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. Forexample, *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.

Generation of the biohybrid material

Experimental steps

Electrospun FDMA/PEO fibers containing bacteria

Chemical crosslinking using catalysis in water/glycerol solution

Extraction of PEO and formation of hydrogel

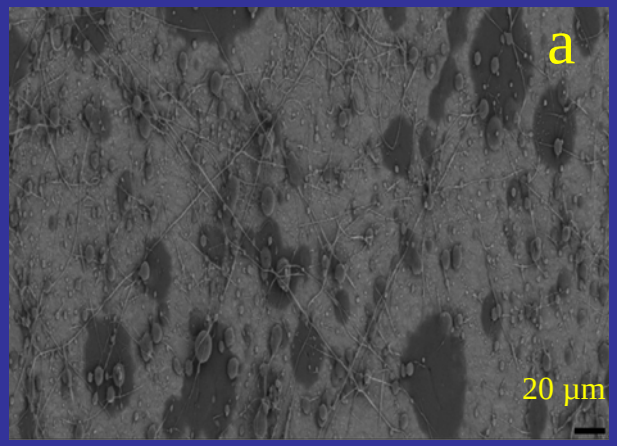
New biohybrid material consisting of insoluble FDMA fibers containing viable bacteria

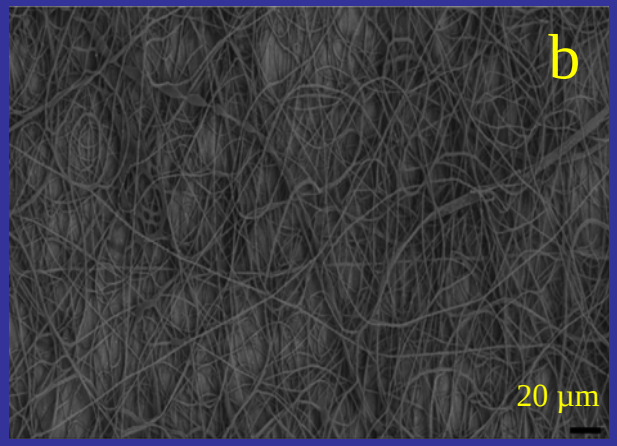
Analysis

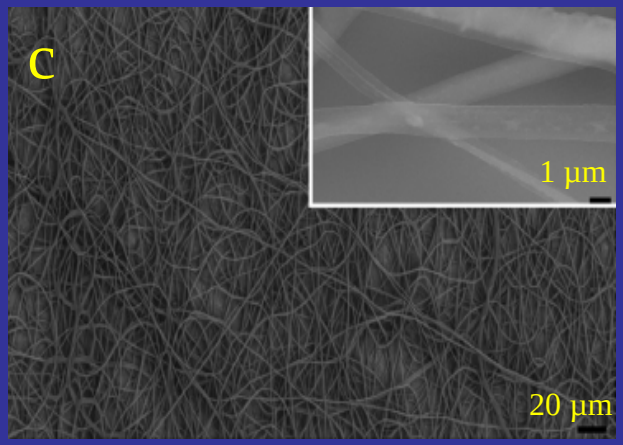
Morphology charecterization and microbal viability assessment under various storage conditions

Morphology charecterization and microbal viability assessment under various storage conditions

Electrospun FDMA/PEO fibers







At an optimized weight ratio of 13:3 (Fig. c), which was used in all further experiments, a uniform fibrous scaffold was obtained.

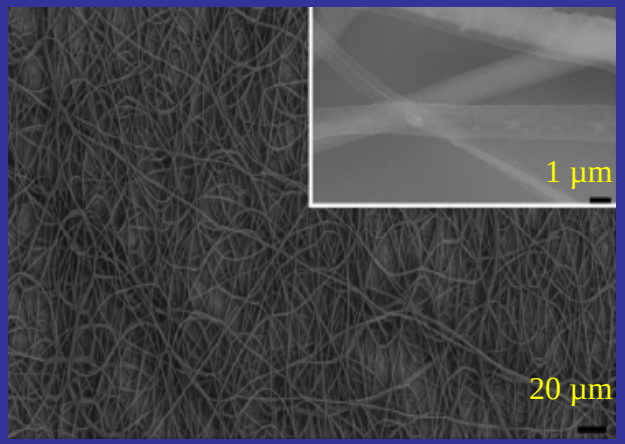
SEM images of the electrospun FDMA/PEO-blend scaffolds with different weight ratios (a) FDMA : PEO = 13 : 1(b) FDMA : PEO = 13:2 and (c) FDMA : PEO = 13:3

Chemical crosslinking using catalysis in water/glycerol solution

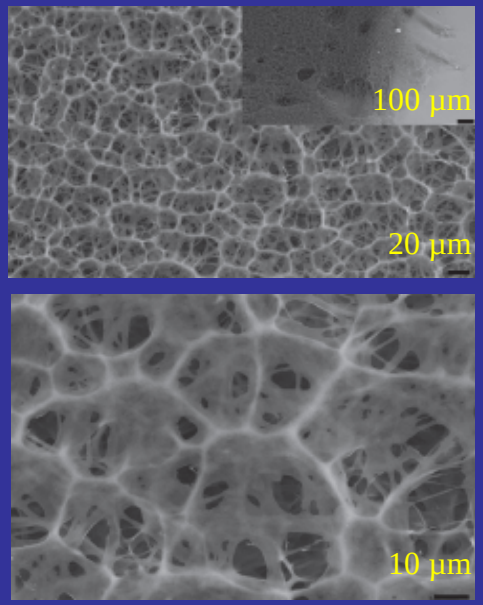
crosslinking

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.

Soaked in deionized water to remove PEO and obtain an FDMA fibrous scaffold



Electrospun fibers
FDMA : PEO = 13:3



Cross-linked scaffold

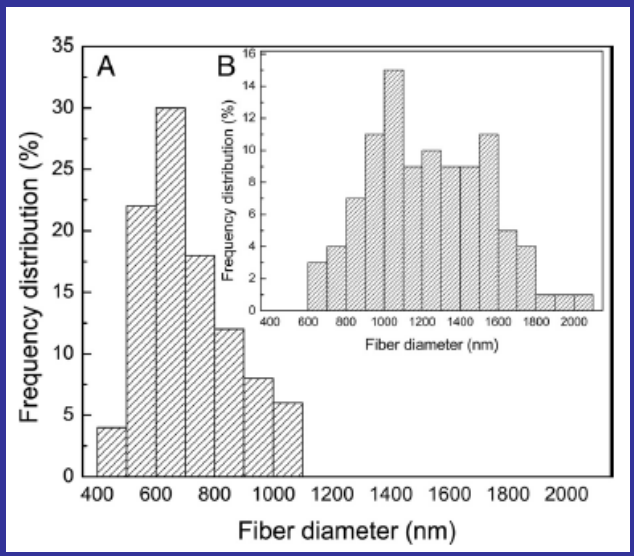
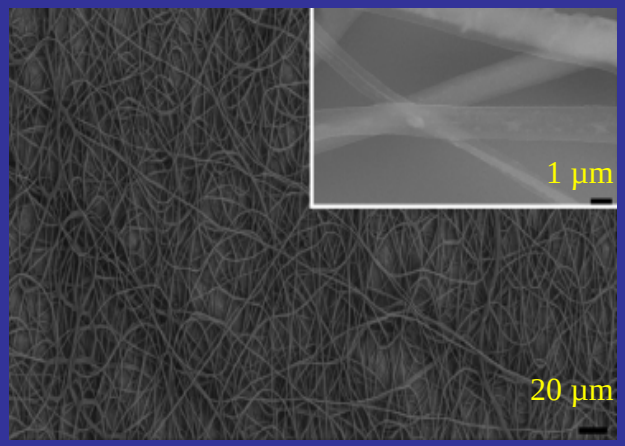
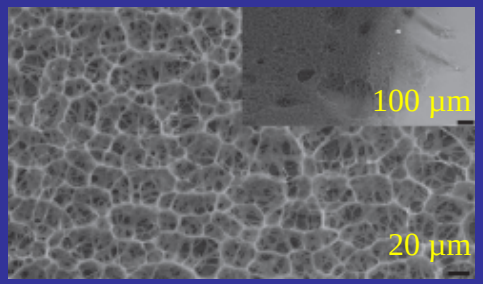


Fig. 4. Distribution of fiber diameters in FDMA/PEO-blend fibrous scaffold electrospun from 13wt% FDMA/PEO aqueous solution with FDMA/PEO weight ratio of 13:3 (*A*) and cross-linked FDMA fibrous scaffold after PEO extraction (*B*).

TG Analysis





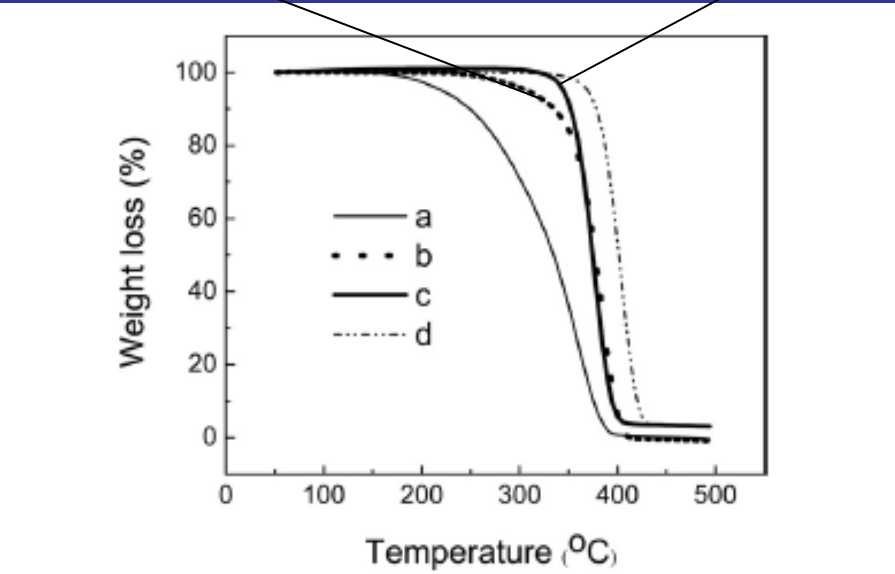


Fig. 5. TG analyses of different samples: (a) F-DMA powder, (b) F-DMA/PEO-blend scaffold, (c) FDMA cross-linked scaffold, and (d) PEO powder.

The increase in the degradation temperature can be attributed to the presence of interchain molecular cross-links.

Encapsulation of Bacteria in the fiber

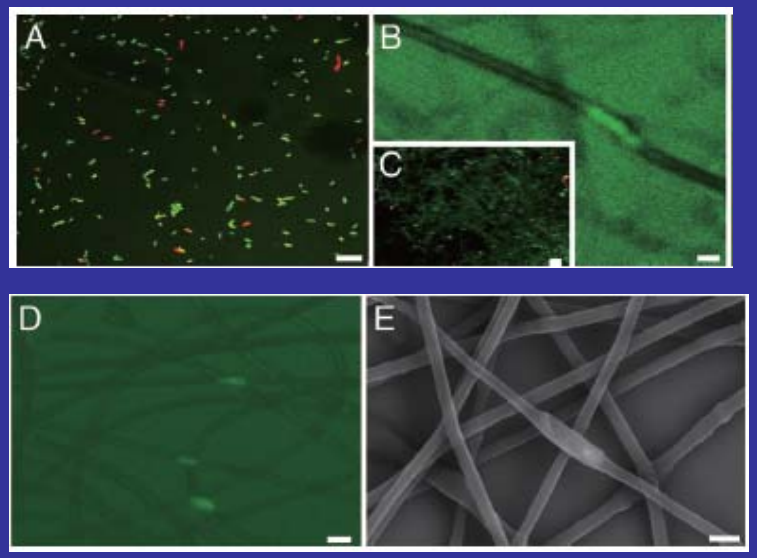


Fig. 6. Confocal images of stained and fluorescent (red, dead cells; green, live cells) cells of (*A*) *P. fluorescens* before electrospinning, (*B* and *C*) *P. fluorescens* inside the dry electrospun FDMA/PEO-blend fibers and, (*D*) *Z. mobilis* in dry electrospun FDMA/PEO-blend fibers. (*E*) SEM image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

The fiber diameters were found to be only slightly larger than theaverage size of the microbes used, thereby encapsulating the bacterium with only a thin layer of the polymeric material.

Electrospinning conditions does not significantly affect the growth of the microorganisms

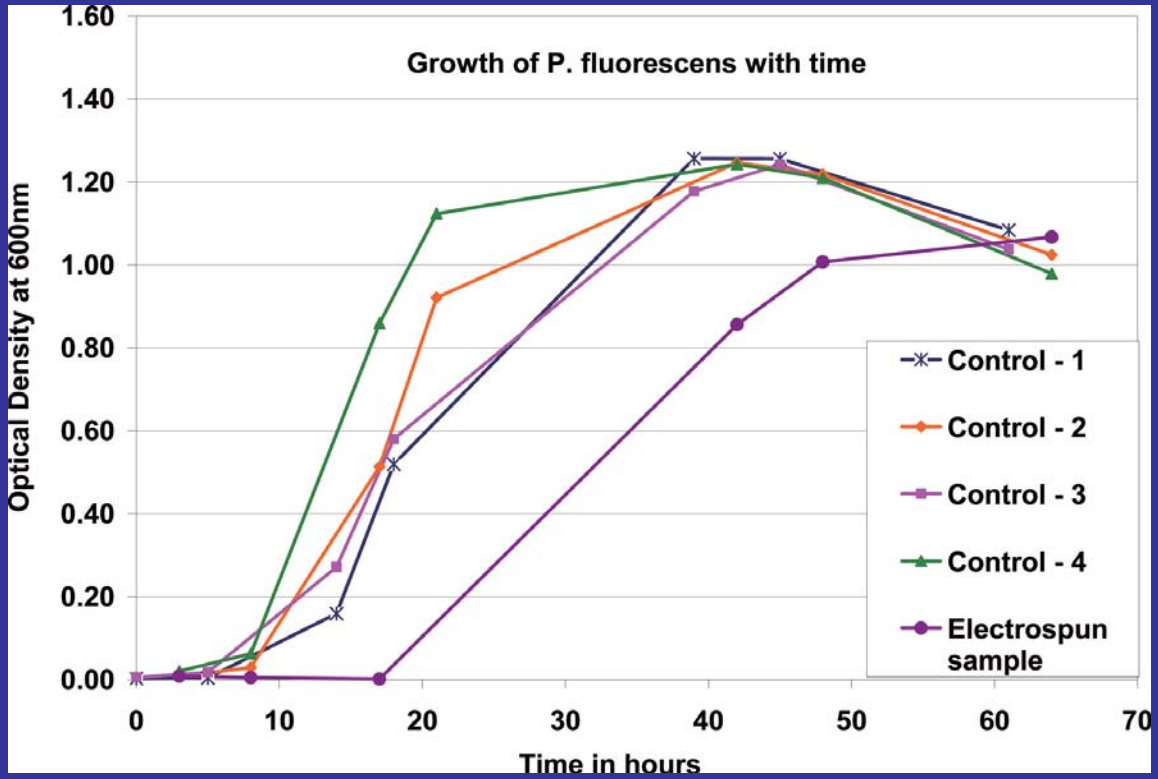
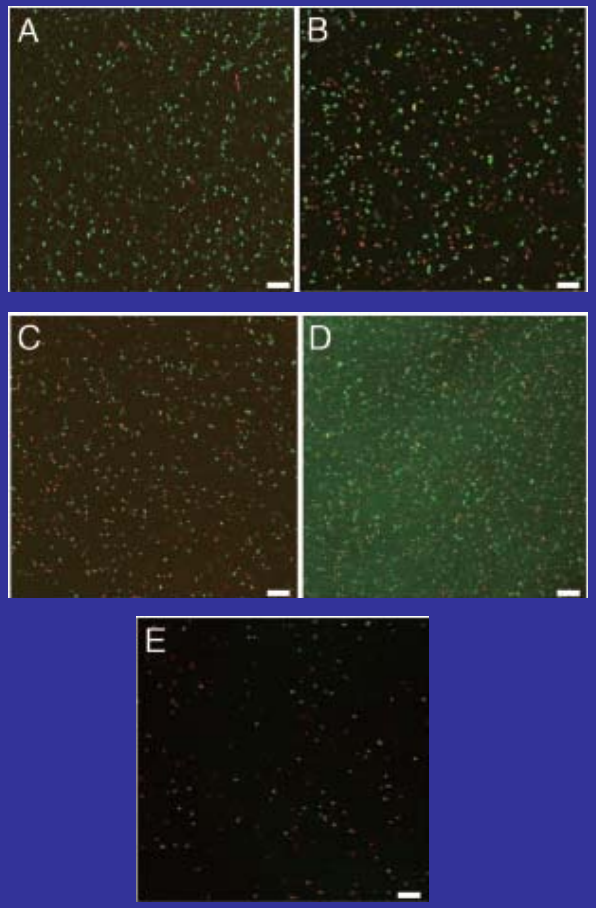


Fig. The growth of electrospun-immobilized and free *P. fluorescens* in an Erlenmeyer flask containing 50 mL of sterile growth medium as monitored by absorption at 600 nm against a culture medium blank. Controls 1, 2, and 3 are from inoculation of 50, 100, and 250 µ L of a fresh culture. Control 4 and the electrospun sample show the growth observed when the sample was inoculated with 100 µ L of the polymer solution containing the bacteria just before and immediately after electrospinning.

Viability of cells over time



After 1, 3, and 7 days, bacteria-encapsulatingFDMA/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: 62%, 47%, and 23% were found to be viable after 1 day (Fig. 7C), 3 days (Fig. 7D), and 7 days (Fig. 7E), respectively.

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 μm.)

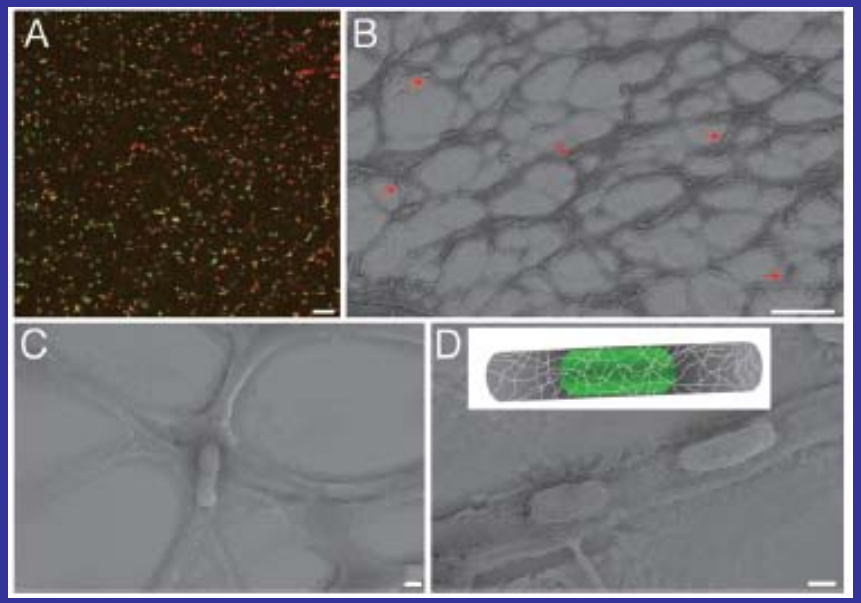
Electrospinning process does not adversely affect the metabolic pathway of the microbes.

Table

Sample ID	Storage temperature in Celsius	Days in storage	Ethanol concentration (vol %)
Sterile medium 0.00			
Control – ZM			0.92
Electrospun samples			
Electrospun ZM	Added to media immediately 0.69		
Electrospun ZM	4	1	0.58
Electrospun ZM	4	3	0.62
Electrospun ZM	4	7	0.79
Electrospun ZM	-70	31	0.82
Electrospun ZM	-70	60	0.52

Amount of ethanol produced by *Z. mobilis* after inoculation of the sterile medium (composition provided in *Methods*) with various microbial samples beforeand after electrospinning. The amount of ethanol produced by the electrospun samples (immediately after electrospinning and after storage) is slightly lower than that produced by the control (free culture, before electrospinning). This difference could be explained by the longer lag-phase of growth experienced by the electrospun samples, thereby providing more aeration and leading to lower ethanol production. ZM, *Z. mobilis*.

Cross-linked fibers



Microbes were found to be encapsulated both at the junctions where fibers fused together (Fig C) and in single fibers (Fig *D*); the cellular integrity of the microorganism seemed to be well preserved,regardless of the location.

FigImages of *Z. mobilis* within the cross-linkedFDMAfibers. (*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 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 μ m in *B*, and 1 μ m in *C* and *D*.) Sketch (*Inset*, *D*) illustrates a bacterium encapsulated in the cross-linked FDMA fibers.

Do cells proliferate inside the scaffold?

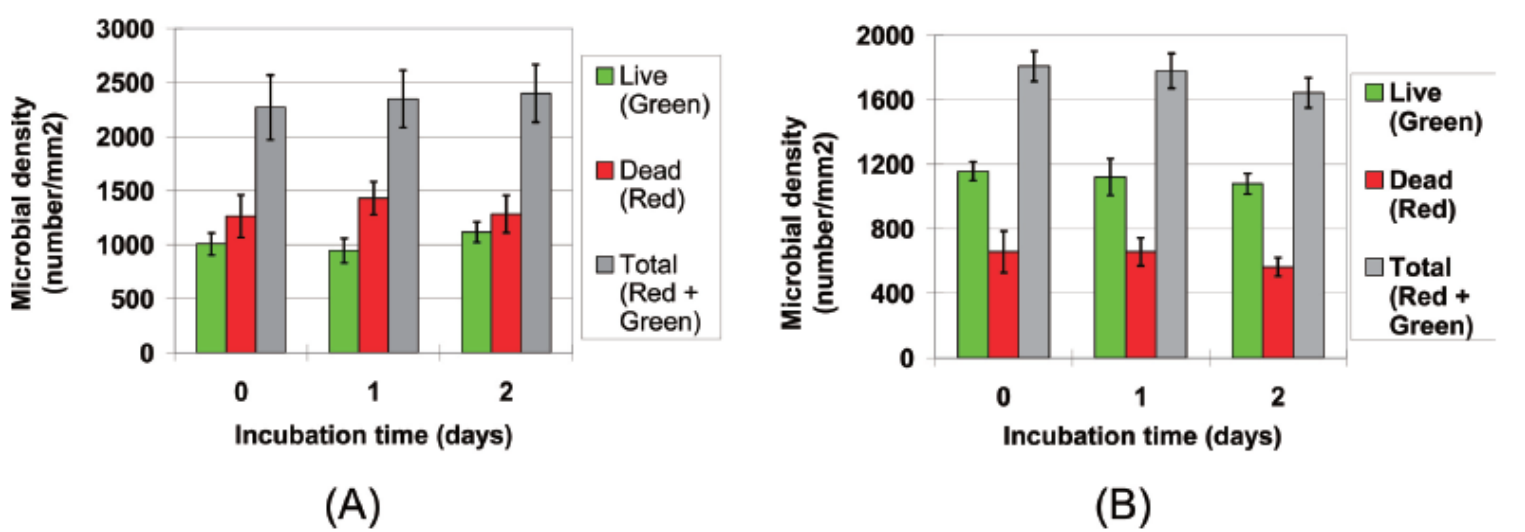


Fig. Microbial cell counts in cross-linked FDMA fibers (*Left*) and bulk hydrogels (*Right*), performed using LIVE/DEAD BacLightTM bacterial viability kits, at different times: day 0 (just after cross-linking); day 1 (after cross-linking and culturing in the growth medium for 24 h); and day 2 (after cross-linking and culturing in the growth medium for 36 h). Microbes with intact cell membranes fluoresce green, and microbes with damaged cell membranes fluoresce red.

The results indicate that the total number of live and dead cells of *Z. mobilis* remained almost constant (A), and there was no proliferation.

Similar results also were obtained with bacteria encapsulated in the cross-linked bulk hydrogel - without undergoing electrospinning (B), indicating that some aspect other than the confinement in the fibers is responsible for hindering their proliferation.

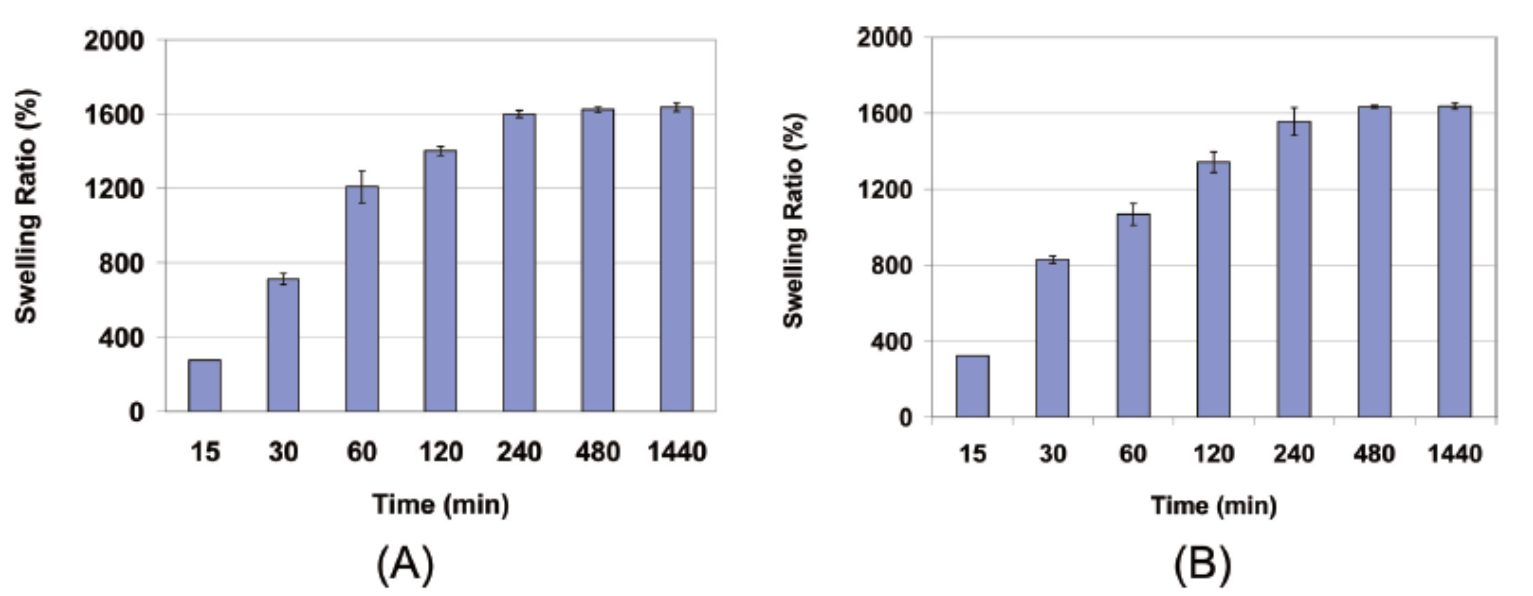
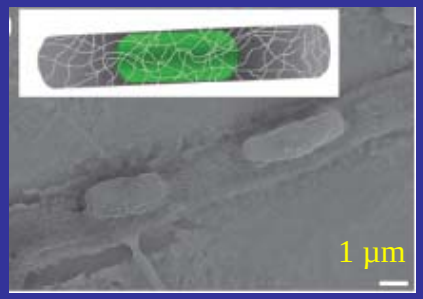


Fig. Swelling ratio of cross-linked FDMA fibers (*Left*) and bulk hydrogel (*Right*) in deionized water at room temperature.

$$\text{Swelling \%} = (Ws - Wd) \times 100$$



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.

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 °C and for more than 2 months at -70 °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.