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Original scientific paper

THE USE OF POLY(ETHYLENE OXIDE) HYDROGELS AS IMMOBILIZATION MATRICES FOR YEAST CELLS

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Hydrogels based on high molecular weight poly(ethylene oxide) (PEO) copolymers of ethylene oxide and propylene oxide, PEO/alginate and PEO/chitosan were synthesized by UV crosslinking of polymer aqueous solutions. These hydrogels were then characterized in terms of their gel fraction yield, degree of equilibrium swelling, shear storage and loss moduli. The physico-mechanical properties of the hydrogels were then correlated to their ability to sustain the viability and the activity of immobilized cells. The production of ethanol by immobilized *Saccharomyces cerevisiae* was used to test the suitability of the PEO based hydrogels as immobilization matrices. The PEO hydrogel with a value of 255 Pa for G` and 11 Pa for G``, showed the best mechanical properties of all the gels tested. Scanning electron microscope (SEM) analysis of the imobilized *S. cerevisiae* showed proliferation of the yeast cells entrapped inside the polymeric matrix.

Key words: hydrogels; poly(ethylene oxide); immobilization; entrapment; *Saccharomyces cerevisiae*

ПРИМЕНА НА ХИДРОГЕЛОВИ НА БАЗА НА ПОЛИ(ЕТИЛЕН ОКСИД) КАКО МАТРИЦА ЗА ИМОБИЛИЗАЦИЈА НА КВАСОЧНИ КЛЕТКИ

Хидрогелови врз база на високомолекуларен поли(етилен оксид) (PEO), кополимери од етилен оксид и пропиленоксид, PEO/алгинат и PEO/хитозан беа добиени со UV вмрежување на синтетичкиот полимер во раствор. Хидрогеловите беа карактеризирани со определување на фракцијата на гелот, рамнотежното бабрење, како и на модулите на еластичност и загубите при деформација на смолк, G` и G``. Физичко-механичките карактеристики на хидрогеловите беа корелирани со нивната способност да ја задржат активноста на имобилизираните клетки. Како модел-реакција за тестирање на погодноста на хидрогеловите за имобилизација беше применета биосинтезата на етанол со имобилизирани клетки на квасецот *Saccharomyces cerevisiae*. Од сите испитани гелови најдобри карактеристики покажаа геловите подготвени од PEO. Размножувањето на квасочните клетки заробени во полимерната матрица беше потврдено и со SEM-анализа.

Клучни зборови: хидрогелови; поли(етилен оксид); имобилизација; заробување; *Saccharomyces cerevisiae*

INTRODUCTION

Immobilized cells, when compared to free suspended cells, permit easier separation from the reaction mixture, provide pure products and can be reused for a number of reaction cycles. Immobi-

lized cell technology also provides the opportunity to co-immobilize different kinds of microorganisms within the same porous matrix, thus allowing the accomplishment of two fermentation steps in one integrated system [1]. Ultimately, all these advantages should lead to lowering the cost of production.

One of the most widely used technique for cell immobilization is cell entrapment, in which the living cells are enclosed in a natural or synthetic polymeric matrix that is porous enough to allow the diffusion of substrates to the cells and of products away from the cells. The choice of the three-dimensional network matrix is very important for the good performance of an immobilized cell system. It is desirable that a cell carrier possesses large surface area, permeability, hydrophilic character, chemical, mechanical and thermal stability, insolubility, suitable shape and particle size resistant to microbial attack [2]. Materials that meet such requirements are hydrogels. Among the materials which have been successfully used for cell entrapment are agar, agarose, alginate, cellulose, kappa-carragennan, collagen, chitosan, polyacrylamide, polyurethane, polyvinylalcohol, etc. [3, 4, 5]. Sodium alginate is commonly used since the hydrogel formation occurs under very mild conditions. Alginate itself is inert and non-toxic to the microorganisms although it is not the best choice for entrapment of the growing cells [6]. Chitosan, another candidate for cell immobilization, is biocompatible and has often been used in drug delivery systems [7]. Chitosan hydrogels formed by the addition of crosslinkers, such as glutharaldehyde, are known to be relatively toxic. Currently, the choice of a safe, biocompatible covalent crosslinkers is quite limited, which is the main drawback of these systems. However, even with a safe biocompatible crosslinker, covalently crosslinked hydrogels would not necessarily be the best choice for whole cell immobilization [8].

In recent years, synthetic hydrogels have become promising materials for biomedical applications. The high water content of hydrogels contributes to their biocompatibility and thus the hydrogels can be used for controlled drug release systems, as membranes for biosensors, scaffolds in tissue engineering, and for many other applications [9]. Poly(ethylene oxide) hydrogels are non-toxic and biocompatible materials that have been approved by the US Food and Drug Administration for biomedical application, and they meet all of the requirements for strength, absorbency and flexibility [10]. It has been shown that poly(ethylene oxide) films can be successfully crosslinked in aqueous solutions by UV irradiation in the presence of photoinitiators such as benzophenone [11].

The purpose of the present work was to synthesize various hydrogels based on poly(ethylene oxide), determine and compare the physico-mechanical properties of these hydrogels. The hydrogels, including poly(ethylene oxide) and natural polymers alginate and chitosan, were characterized in terms of gel fraction yield, degree of equilibrium swelling, and shear storage and loss moduli. The hydrogels were subsequently used for immobilization of baker's yeast *Saccharomyces cerevisiae,* for batch ethanol fermentation.

EXPERIMENTAL

Materials

Polyethylene oxide (PEO) was purchased from Union Carbide Corp., USA Polyox N12K (Mn = $1 \cdot 10^6$ g/mol), sodium alginate with approximately 70 % G-block content was from Aldrich, and chitosan (medium molecular weight, Mn ~ $4 \cdot 10^5$ g/mol) was from Fluka. The (4-benzoylbenzyl) trimethylammoniumchloride (BBTMAC) purchased from Aldrich was used as a photoinitiator, without further purification. The copolymer of ethylene oxide and propylene oxide (P(EO-co-PO)), (Mn = $1.2 \cdot 10^4$ g/mol), with 20 mol % PO content was synthesized at the Institute of Polymers of the Bulgarian Academy of Sciences. All other chemicals used were purchased from commercial sources and were of analytical grade.

Microorganism and media

Saccharomyces cerevisiae, a commercial grade baker's yeast, with 32 % dry biomass, was used. The nutrient medium for growth and ethanol production had the following composition (per litre): 10 g yeast extract, 2 g KH_2PO_4, 1 g NaCl, 0.2 g $CaCl_2 \cdot 2H_2O$, 1.7 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeCl_3 \cdot 6H_2O$, 2 g NH_4Cl, and 20 g glucose. Initial pH was 5. For the batch production of ethanol, the glucose concentration was increased to 50 g/l. The media were autoclaved at 120°C for 15 min.

Preparation of hydrogels and immobilization of yeast cells

a) UV-crosslinking of aqueous solutions of PEO and P(EO-co-PO)

Aqueous solution of PEO (5 ml, 5 %w/v) or P(EO-co-PO) containing BBTMAC (5 % of the polymer mass) was poured into a teflon Petri dish with a diameter of 5 cm, forming a 2–3 mm thick layer. The polymers were then UV irradiated in a Dimax light curing system, model 5000 Flood, for 2 minutes. For the immobilization of yeast cells, the polymer aqueous solutions were mixed with yeast cells to reach 5 %w/v, followed by the procedure use for the preparation of pure hydrogels.

b) UV-crosslinking of frozen aqueous solutions of PEO/alginate and PEO/chitosan

Aqueous solution of PEO (5 ml, 5 %w/v) with 0.5 %w/v sodium alginate or 0.5 %w/v chitosan containing BBTMAC (5 % of the polymer mass) was poured into a teflon Petri dish with a diameter of 5 cm, forming a 2–3 mm thick layer, which was then placed in a freezer at -40^0C for 2 hours. The frozen solution was then UV irradiated in a temperature-controlled open chamber connected with cryostat apparatus in a Dimax light curing system, model 5000 Flood, for 2 minutes. The yeast was immobilized by adding yeast cells (5 %w/v) to the mixture of polymers, followed by the same crosslinking procedure as already described.

Characterization of the hydrogels

For complete removal of the sol fraction, the hydrogels were kept in distilled water that was changed frequently and then dried to constant mass under vacuum and weighed. The gel fraction (GF) was calculated as:

$$\text{Gel fraction} = \frac{\text{mass of the dried sample after extraction}}{\text{initial mass}} \cdot 100\,(\%) \quad (1)$$

The equilibrium degree of swelling (ES) was determined at room temperature. Disks of dried hydrogel (ϕ 14 mm) were equilibrated in distilled water or chloroform for at least 72 h, removed from the solvent, blotted with filter paper and weighed. They were then dried to constant mass under vacuum and weighed again. The ES was calculated as grams of swollen gel sample per gram of dried gel sample:

$$\text{Equilibrium swelling} = \frac{\text{mass of equilibrated swollen sample}}{\text{mass of dry sample}}. \quad (2)$$

Samples for rheological measurements were prepared as disks with thickness of cca 4 mm. Shear storage modulus, G` and loss modulus G`` were measured with ThermoHaake rheostress RS600, cone-plate system (2^o) at 25 ^{o}C. They values were recorded in a frequency range of 0.1 to 10 Hz. The values presented in Table 1 are recorded at 1 Hz.

Activity test of the immobilized yeast cells

Immobilized cells of *Saccharomyces cerevisiae* in the hydrogel matrices were incubated in 50 ml nutrient medium in 250 ml Erlenmeyer flasks on a rotary shaker (150 rpm) at 28^{o}C. After 20 hours, the immobilizates were washed several times in sterile distilled water and transferred into the fermentation medium for ethanol production. The conditions for ethanol production were the same as those for growth of the cells.

Scanning electron microscopy

The hydrogels, with and without yeast cells, were characterized using scanning electron microscopy (SEM) (Jeol JSM–5510). The composite hydrogels were first quenched in liquid nitrogen, freeze dried, and coated with gold in a Jeol JFC–1200 fine coater.

Analytical methods

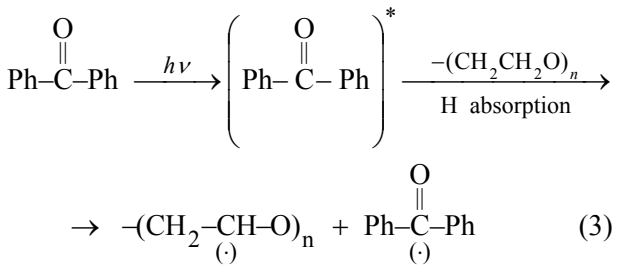
Samples were taken periodically and centrifuged. Cell concentration was estimated turbidometrically at 620 nm, after diluting the samples within the range of 0.05 to 0.5 units. One optical density unit corresponded to 0.3 mg dry cell mass/ml. Ethanol was analyzed by gas chromatography using a Varian CP 3800 with a capillary column WCOT fused silica (30 m × 0.32 mm), CP WAX 52 CB, d_f = 0.25 μm and a manual injector type 1709. The injector and detector temperatures were 250 ^{o}C, and the column temperature was 200 ^{o}C. Nitrogen was used as a carrier gas with a flow rate of 30 ml/min. Isopropanol was used as an

internal standard. Glucose concentration was determined by a dinitrosalycilic acid procedure of Miller [12]. To determine the rate and the degree of cell leakage from the matrices after the immobilization procedure, the immobilized cells were placed in 250 ml Erlenmeyer flasks filled with 50 ml distilled water and shaken at 150 rpm and 28°C.

RESULTS AND DISCUSSION

Characterization of PEO based hydrogels

UV crosslinking of poly(ethylene oxide) occurs through a recombined reaction of two macroradicals (Eq. 3) producing carbon-carbon bonds between the main chains [11].



(3)

Different hydrogels were prepared by covalent crosslinking of PEO or P(EO-co-PO) and as semi-interpenetrating networks of PEO/alginate and PEO/chitosan. In order to determine the efficiency of the UV-crosslinking, the prepared gels were subjected to physico-chemical characterization. The properties of the hydrogels are presented in Table 1.

Table 1

Efficiency of UV-crosslinking in PEO based hydrogels

System	Gel comp. (%w/v)	Gel fraction (%)	Equilibrium swelling in H_2O	Equilibrium swelling in $CHCl_3$	Shear storage modulus G′ (Pa)	Loss modulus G″ (Pa)
PEO	5	85	44	65	255	11
P(EO-co-PO)	5	62	36	36	157	54
PEO/alginate	5/0.5	75	33	14	725	102
PEO/chitosan	5/0.5	53	19	11	562	134.5

The hydrogels with shear storage modulus, G` >200 Pa are considered to posses acceptable mechanical properties. It is obvious, from the results, that the copolymer P(EO-co-PO) does not fulfill the basic requirements for mechanical strength. The results for the PEO/alginate and PEO/chitosan hydrogels with rather high values of G` lead to the conclusion that their mechanical properties are satisfactory. However, the high loss moduli and lower values of gel fractions show the necessity of crosslinking of the natural polymer. The high G` values of the PEO/alginate and PEO/chitosan systems (Table 1) could be explained by the crosslinked PEO structure and also by the presence of natural polymers. As Kong *et al*. [13] stated, the stiff alginate chains contribute to high shear storage modulus. Thus, the PEO hydrogel with a value of 255 Pa for G` and 11 Pa for G``, and the highest value of gel fraction, exhibited the best properties of the four gels examined. Despite these considerations, in the further experiments involving cell immobilization, all hydrogels were investigated for their possible application.

Cultivation of Saccharomyces cerevisiae entrapped in the poly(ethylene oxide) hydrogels for ethanol production

The biosynthesis of ethanol by immobilized *Saccharomyces cerevisiae*, a baker's yeast, was used as a model reaction for testing the suitability of the PEO based hydrogels as immobilization matrices. The PEO, P(EO-co-O), PEO/alginate and PEO/chitosan hydrogels were examined with respect to their biocompatibility and diffusion properties by using the immobilized *S. cerevisiae* for ethanol production. In order to be used as immobilization matrices, these hydrogels have to be permeable to the substrate (glucose), to the other nutrients, and to the product formed (ethanol). The fermentation profiles of immobilized *S. cerevisiae* on the gels are illustrated in Figs. 1 and 2.

In the fermentation with the P(EO-co-PO) and PEO/chitosan immobilized yeast, ethanol was not detected in the media before 24 hours, while with PEO and PEO/alginate immobilized cells, ethanol was detected as early as the 3rd hour. With the PEO hydrogel, the concentration of ethanol increased till the 24th hour, and with the PEO/alginate, the ethanol peaked at the 9th hour and the highest ethanol concentration recorded was 1.86 g/l. The highest ethanol production coincided with the fastest rate of glucose consumption (Fig. 3). The concomitant increase in biomass with a decrease in ethanol concentration indicated that this yeast strain efficiently utilized ethanol as carbon source in

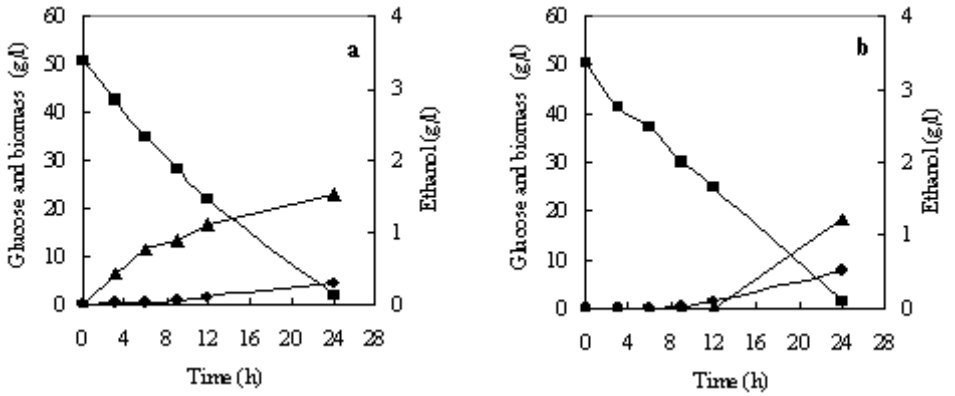


Fig. 1. Glucose (■), ethanol (▲), and biomass (●) concentration during batch cultures of immobilized *S. cerevisiae* in synthetic hydrogels (a) PEO and (b) P(EO-co-PO)

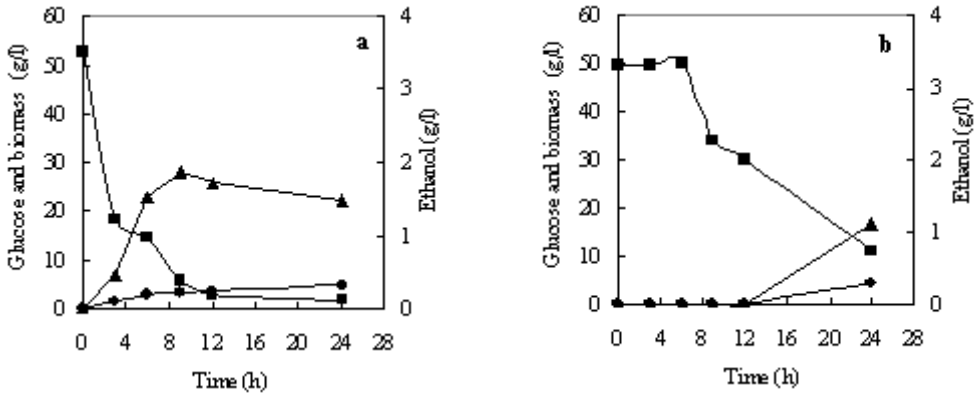


Fig. 2. Glucose (■), ethanol (▲), and biomass (●) concentration during batch cultures of immobilized *S. cerevisiae* in synthetic and biopolymer networks of (a) PEO/alginate and (b) PEO/chitosan.

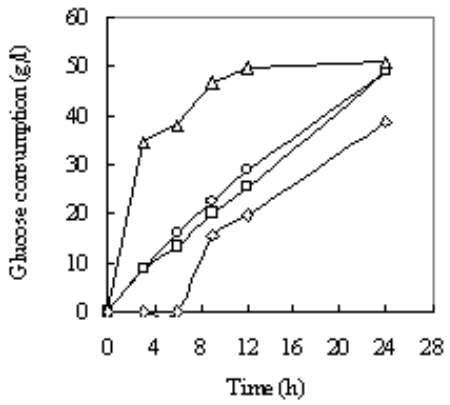


Fig. 3. Dynamics of glucose consumption in fermentation experiments with immobilized *S. cerevisiae* in PEO (○), P(EO-co-PO) (□), PEO/alginate (△), and PEO/chitosan (◇) hydrogels

the absence of glucose. Such behaviour has also been found with other yeasts, free or immobilized [14–16].

After 24 hours of cultivation, in the immobilized systems with PEO, P(EO-co-PO), PEO/alginate, the substrate consumed in each case was about 97 %. This was in contrast to the immobilization system of PEO/chitosan where only 77 % of substrate was consumed. Such high glucose consumption should have resulted in a much higher ethanol production. However, the low ethanol concentration obtained could probably be due to fact that some of the glucose was used for biomass production and the less than optimum fermentation conditions employed rather than diffusion limitations in the hydrogels. Furthermore, the fact that the glucose was consumed very fast is evidence that there had been no diffusional limitations in the hydrogels. In all cases the biomass concentration increased to about 4.5 g/l except in the system with P(EO-co-PO) immobilizates where the concentration was 7.7 g/l (Figs. 1 and 2) which could be due to leakage of some of the cells from the gels in to the media.

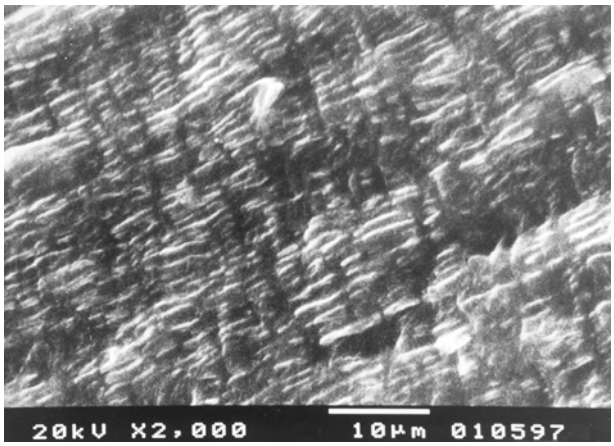
Concurrently with the fermentation experiments, the loss of yeast cells from the gels was studied in water and nutrient medium. The immobilized cells were placed in distilled water and the nutrient medium and the biomass released measured. The cell leakage varied from 0.02 g/l for P(EO-co-PO) to 0.75 g/l for PEO hydrogels (Table 2). The P(EO-co-PO) system exhibited the highest and lowest cell losses in the nutrition medium, and water respectively. Apparently, the cells were not immobilized into the matrix and in the nutrient medium they leaked out as freely suspended cells. This observation with the copolymer system could be attributed to its weak mechanical properties as shown by the value of its G' modulus, 157 Pa (Table 1). The high cell leakage in pure water out of the PEO matrix can be due to its degrees of equilibrium swelling, 44 in water and 65 in chloroform, which are the highest among the gels examined.

Table 2

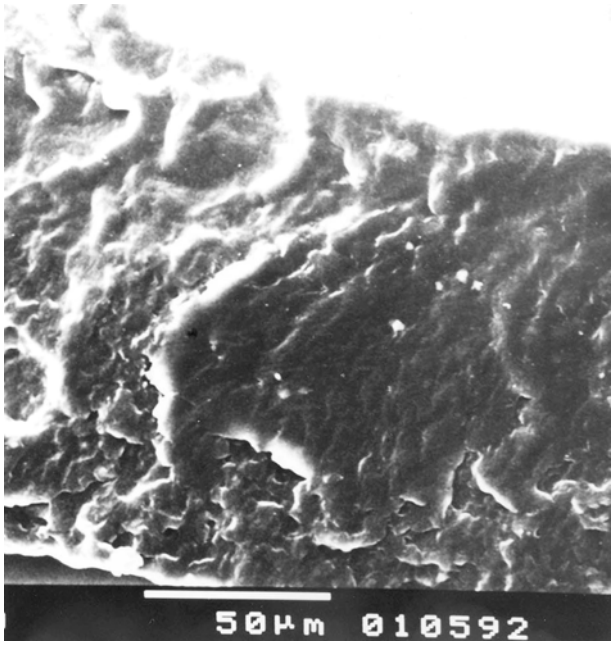
Cell leakage from hydrogel immobilizates after 24 hours

System	Cell leakage (g/l) in	
	distilled water	fermentation medium
PEO	0.75	4.35
P(EO-co-PO)	0.02	7.67
PEO/alginate	0.01	4.81
PEO/chitosan	0.03	4.43

The fact that the substrate was almost completely consumed with very little ethanol produced, implied that the substrate could have been utilized by the yeast cells entrapped in the hydrogels. However, immobilization is known to cause changes not only in the activity but also in the total metabolism of the microorganisms [17, 18]. To determine whether the yeast had grown inside the polymeric matrix, the PEO hydrogel, with and without yeast cells, was cut and the morphology of the gel was examined by scanning electron microscope (SEM). Figure 4 shows a series of SEM images representing the surface and the cross section of the PEO gel without cells, and PEO gel loaded with cells. The image in Fig. 4c shows the proliferation of the yeast cells entrapped inside the polymeric matrix after 24 hours of batch cultivation. The SEM photographs support our assumption that glucose was utilized for biomass production by the immobilized *S. cerevisiae* cells.



(a)



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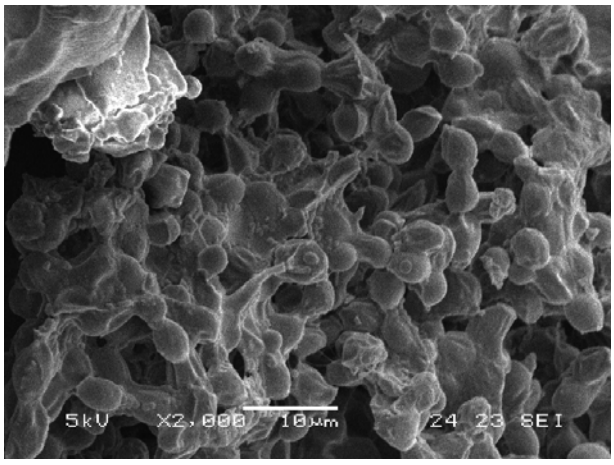

(c)

Fig. 4. Scanning electron micrographs of the (a) surface and (b) cross-section of PEO hydrogel, and (c) cross-secction of the PEO hydrogel loaded with *S. cerevisiae* cells

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