Scientific paper

Immobilization of Yeast Cells Within Microchannels of Different Materials

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Dedicated to the memory of the late Prof. Dr. Valentin Koloini

Abstract

Saccharomyces cerevisiae was successfully immobilized on the inner wall surface of channels of submillimeter diameter, which can be further used for the development of a highly productive continuous biotransformation process within a microfluidic device. Covalent bonding by means of 3-aminopropyltriethoxysilane (APTES) and glutaraldehyde was used for immobilization of cells to microchannels made of glass, polystyrene (PS), polytetrafluoroethylene (PTFE), perfluoroalkoxy (PFA) and fluorinated ethylene propylene (FEP). All tested materials were successfully functionalized with H_2SO_4 to promote silanization. The effect of reaction time with acid on immobilization efficiency was studied for polymer materials. This is the first report on cell immobilization onto PTFE, FEP and PFA surface, which enables to develop a microfluidic device with surface bound biocatalyst from low cost and disposable materials.

Keywords: Microchannels, immobilization, yeast, PTFE, FEP, PFA

1. Introduction

Microreactors are miniaturized reaction system devices whose operation depends on precisely controlled design features with characteristic dimensions from submillimeter to submicrometer.[1] They are produced in many different forms, from microfluidic devices consisted of microchannels, to microwells.[2] The main characteristics of microreactors are their small dimensions, high surface to volume ratio, well defined reaction times and conditions, laminar flow, the possibility to automate the process and the concept of numbering-up instead of scale-up.[3,4] In the development of (bio)catalytic processes, small reactor dimensions can be advantageous due to lower running costs and smaller quantities of valuable materials and catalysts needed.[4] High surface to volume ratio is very important for efficient heat and mass transfer and hence, very endo- and exothermic reactions can be run in microreactors. For this kind of reactions, safety can also be considerably improved as we do not have a large amount of dangerous chemicals in a single container.[2] Small dimensions imply that laminar flow is the dominant type of flow and diffusion the main mixing driving force. These simplified conditions allow better reaction control and accurate predictions of the system.[3,5,6] Homogeneous conditions can be easily obtained in microreactors which can result in better product selectivity and better yields. For production purposes, several microreactors can be used in parallel to increase mass flow.[1]

Microreactors are very useful tools for bioprocess development. Strains can be tested and cultivation conditions optimized more quickly and cost effectively than in microtiter plates or bench-scale bioreactors.[7,8] As an important part of this field, biosensors, which particularly take advantage of small sample volumes, shorter analysis time and low cost, have been extensively studied.[9] Furthermore, microreactor technology is applied in genomic and proteomic analyses e.g. improving polymerase chain reaction. Another interesting use of microreactors is the genetic, physiological and biochemical study of individual cells that gives us new perspectives into the life of a cell.[10,11] Namely, microreactors can provide the cell with the environment similar to the *in vivo* state mo-

re easily as it was ever possible, which is important in many areas, including medicine and environmental studies.[12]

Because of the big surface to volume ratio, microreactors are very well suited for surface immobilization.[3] Many enzymes have been immobilized for different purposes, but in some cases, cells are preferable as enzyme extraction and purification can be avoided and cells can have longer lifetime than enzymes.[13] Cell immobilization is essential for some of the applications mentioned before or offers great advantages if used in others, e.g. biotransformations, where immobilization eases product recovery, stabilizes the cells, offers protection against shear forces and may alter physiology of the cell in a favorable manner.[14] Several methods are available to immobilize biocatalysts on supports in conventional reaction apparatus, and these techniques may also be applied to immobilize cells within a microspace. We can distinguish four physical mechanisms that cause immobilization: attachment to a surface, entrapment within a porous matrix, containment behind a barrier and self-aggregation.[15] Cell immobilization techniques used in microfluidic systems include the use of dam structures to hydrodinamicaly trap cells, which is particularly suitable for the development of biosensors, trapping in hydrogels that is used when as much as possible viability has to be retained, or by ultrasound, where a spatially defined distribution of cells can be obtained.[13,16,17] Many organisms were shown to be easily attached to the microreactor surface by non-specific adsorption or biotin-streptavidin system.[13,10] Antibodies were used to specifically attach a class of cells or specific species on the surface.[18] The reports on cell immobilization within microreactors encompass glass, silica, PS, polymethyl methacrylate (PMMA) and polydimethylsiloxane (PDMS).[19]

As evident, several materials have been used for construction of microfluidic devices including glass, PDMS, PMMA and silicon wafers. Although they have many favorable properties, complicated microfabrication methods limit their use for disposable microdevices applications, as well as for their more widespread use as laboratory test systems.[20] An alternative is to use other plastics, which are inexpensive and available in plentiful types of materials and forms.[21] Commercially available PTFE microtubes have already been successfully applied for the development of highly productive continuous lipase-catalyzed isoamyl acetate synthesis within a microfluidic device.[22]

Therefore, our objective was to immobilize cells onto commercially available tubes with inner dimensions corresponding to that of microreactors. According to the literature survey, PTFE, PFA and FEP, which are common materials used in analytics, as optical cables, semiconductors, and in many industrial applications, have not yet been used for this purpose. The unique features such as inertness, excellent heat, chemical and corrosion resistance, long continuous lengths, easy cleaning, good transmission of light and ultraviolet rays, nontoxicity and excellent dielectric strength, make these materials very interesting for the application in microreactor technology.

In this work, *Saccharomyces cerevisiae* immobilization based on covalent bonding was preliminarily studied on glass microchannels by means of 3-aminopropyltriethoxysilane (APTES) and glutaraldehyde treatment. The method developed was further modified for cell immobilization within microchannels from PS, PTFE, PFA and FEP, where different acids and reaction times were tested for polymer material functionalization. Baker's yeast was selected due to its simple manipulation, wide use as expression system and ability to perform different biotransformations. As an example, permeabilized *Saccharomyces cerevisiae* cells were shown to efficiently convert fumaric to L-malic acid without the need for cofactor regeneration, and the biotransformation could be substantially improved by the development of a continuous process within a microreactor.[23]

2. Experimental

2. 1. Chemicals

HNO_3, H_2SO_4, 3-aminopropyltriethoxysilane (APTES) and glutaraldehyde were from Aldrich (St. Louis, MO, USA). Deionized water was used.

2. 2. Microchannels

The majority of channels used throughout the study are commercially available. Glass microchips with Y shaped inlet and outlet channels with dimensions of 33.2 or 66.4 cm in length, 110 or 220 μm width and 100 μm height were purchased from Micronit Microfluidics B.V. (Enschede, The Netherlands). PTFE tubes with internal diameter of 254 μm were from Sigma-Aldrich (St. Louis, MO, USA), while PFA and FEP tubes with i. d. of 254 μm and PFA tubes with i.d. of 500 μm were bought from Vici AG International (Schenkon, Switzerland)

Thermo-shrinkable PS sheets from K & B Innovations (North Lake, WI, USA) were used for PS microchannels manufacturing as described by Chen.[24] The pattern of a microchannel was cut into a PS sheet with a knife. Another sheet was punched through to obtain holes which served as inlet and outlet channels and the third sheet was used to close the microchannel. All three layers were then put together and baked in an oven at 160 °C for 1 min. In the oven, the layers bound together and shrinked resulting in a simple microchannel with submillimeter dimensions.

Chemical structures of polymeric materials used in the form of microchannels are shown in Figure 1.

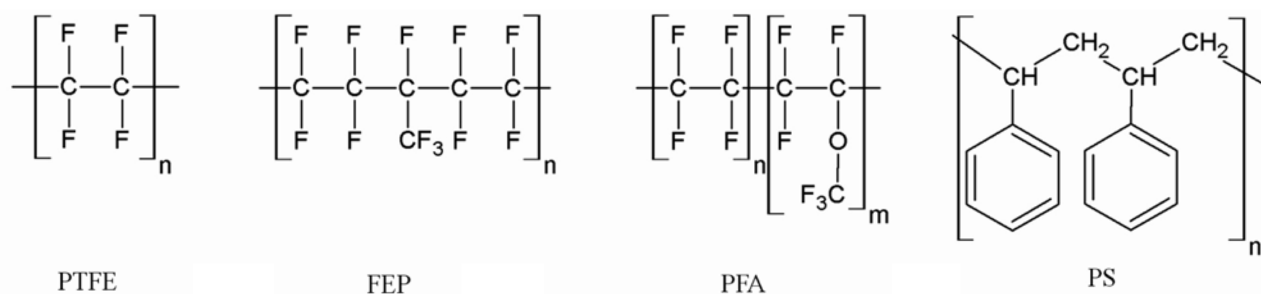

Figure 1: Chemical structures of polymers used in the form of microchannels.

2. 3. Microorganism Cultivation and Preparation

Saccharomyces cerevisiae MZKI K86 (baker's yeast) obtained from Culture collection of National Institute of Chemistry (Ljubljana, Slovenia) was used throughout this study. It was cultured in a medium containing 10 g/l yeast extract, 20 g/l peptone and 20 g/l saccharose (pH 5.5) in 250 ml Erlenmeyer flasks. Cells were grown overnight at 28 °C and 150 rpm in a rotary shaker. After harvesting by centrifugation, the cells were washed twice with water and then resuspended in deionized water in a final concentration of 10^8 cells/ml. This cell suspension was later used for immobilization.

2. 4. Immobilization Procedure

2. 4. 1. Glass Microchips

Glass microchips were first cleaned with 4M NaOH, washed with deionized water and treated with 5M HNO_3 for 1 h at 90 °C. After washing with deionized water, microchannel surface was silanized with 10% APTES aqueous solution for 24 h. Microchannels were washed with deionized water prior to exposure to 5% glutaraldehyde aqueous solution for 2 h. Channels were further washed with deionized water before introducing yeast cells, which were left to attach overnight.

2. 4. 2. Polymer Microchannels

Different polymer tubes of submillimeter diameters and of 3 cm length were first treated either with 67% HNO_3, 97% H_2SO_4, or with both acids in the ratio of 1:1 (v/v), at room temperature for 1, 2, 4 or 24 h. Further procedure (APTES, glutaraldehyde and cells) was the same as used for the immobilization within glass microchips.

Extra care was taken when dealing with concentrated acids or alkali, and adequate safety measures and protective gears were used.

2. 5. Determination of Immobilization Efficiency

After immobilization, the channels were washed with deionized water to remove any unbound cells. Microchannels were then placed under microscope and photographed using a digital camera. Cell surface coverage was evaluated by image analysis of the photographed samples using FIJI software (FIJI, released under GPL) or estimated by visual classification into four different size classes as described in results. All experiments were performed in at least 3 parallels and the results represent the immobilization efficiency of the majority of replicates.

3. Results and Discussion

3. 1. Development of Immobilization Method

A previously described method for bacterial cell immobilization on Siran™ beads reported by Shriver-Lake *et al.*[25] was initially tested for immobilization of *Saccharomyces cerevisiae* cells on glass microchannels. Heated 5M HNO_3 was used for the introduction of hydroxyl groups on the inner surface of the microchannel walls, which enables further silanization.[26] After acid treatment, microchannels were silanized with APTES to introduce amino groups on the surface. Silanization was left to occur overnight at room temperature in order to obtain a thick film of silane. After silanization, the interior of microchannels was exposed to glutaraldehyde to covalently bind amino groups from APTES. At the end, the suspension of yeast cells was introduced into the microchannels and left to attach to free aldehyde group with amino groups present on the cell wall surface.[26] The method is schematically presented in Figure 2. Results of yeast cell immobilization on glass microchips by this method revealed approximately 70% surface coverage with cells, which is close to saturation regarding the inevitable empty spaces between oval cells. Our results are also much better than the ones from the original paper, where 13% surface coverage with *Escherichia coli* cells was reported.[25]

According to the literature, a 24 h incubation of glass microchannel with APTES at room temperature resulted in the formation of approximately 15 nm thick silane layer with roughened surface.[27] It was assumed that silane covers the entire surface of the microchannel and that surface properties after silanization are thus governed only by silane layer and not by the surface properties of the underlying material. By providing adequate glutaraldehyde treatment, efficient immobilization can be expected on

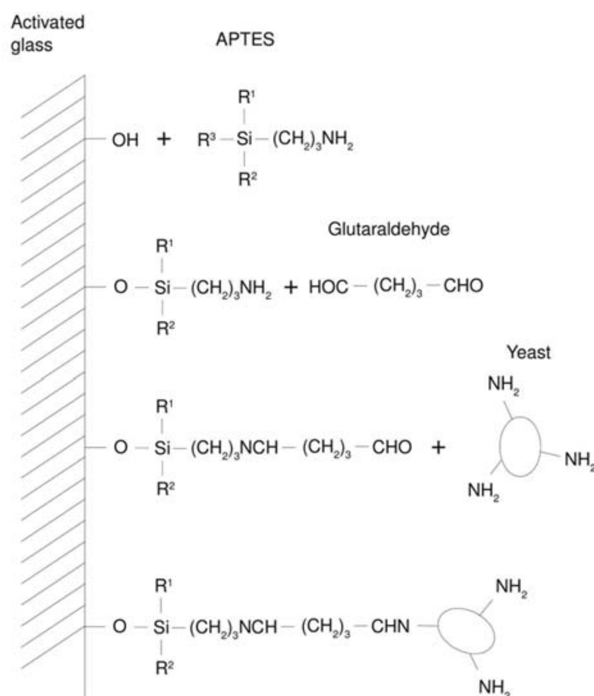

Figure 2: Procedure and the proposed mechanism for immobilization of yeast on the glass microchannel surface.

all surfaces that can be silanized and cell immobilization efficiency directly correlates to silanization success.

The method used for glass was therefore adapted for the use with tested polymer materials. Polymer microchannels were treated with concentrated HNO_3, H_2SO_4 or a combination of both acids at room temperature. Treatment of PS with HNO_3 or this acid together with H_2SO_4 is known to introduce nitro functional groups on the surface, which promotes silanization.[28, 29] It was assumed that acid treatment of PTFE, FEP and PFA would also activate the surface of polymers to allow further silanization.

Depending on immobilization conditions, different immobilization efficiency in the microchannels was observed. An arbitrary four level scale was used to describe the estimated surface coverage, namely: low, average, good and excellent immobilization. Examples of all four levels of immobilization efficiency are shown in Figure 3 and the corresponding estimated surface coverages are shown in Table 1.

Table 1: Explanation of terms used for the description of immobilization efficiency.

Descriptive term used	Approximate surface coverage with cells [%]
Poor	5
Average	15
Good	30
Excellent	70

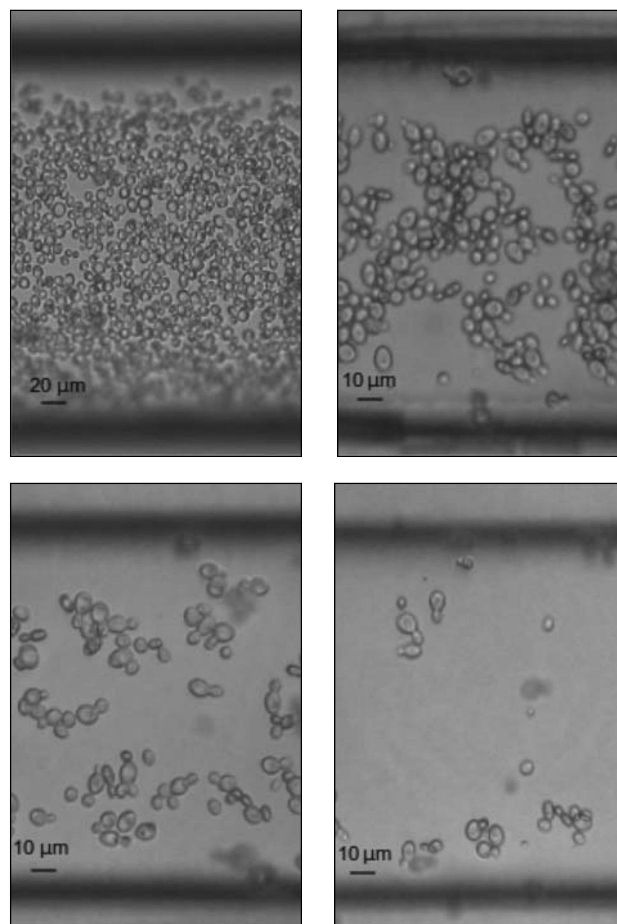

Figure 3: Different levels of yeast cell immobilization in glass microchannels of various dimensions (from left to right: excellent, good, average and poor).

3. 2. The Influence of Acid Treatment on Immobilization Efficiency

Different acids and various reaction times were tested for their effect on the immobilization efficiency on polymer materials.

As evident from Figure 4, the best immobilization efficiency on all polymeric materials was observed after sulphuric acid treatment. On all materials, except for FEP, it enabled excellent cell immobilization with 70% surface coverage, which was also achieved on glass microchannel surface. The best results were obtained already after 1h treatment (Figure 4a), while in the case of nitric acid and nitric/sulphuric, acid treatment had to be longer to obtain the same results. 24h treatment with combined nitric and sulphuric acid was needed for PTFE, FEP and PFA to achieve excellent immobilization. However, nitric acid was only suitable for FEP treatment, while PTFE and PFA microchannels could not be modified as required for excellent cell immobilization with this acid. Therefore, sulphuric acid treatment was further used throughout this study.

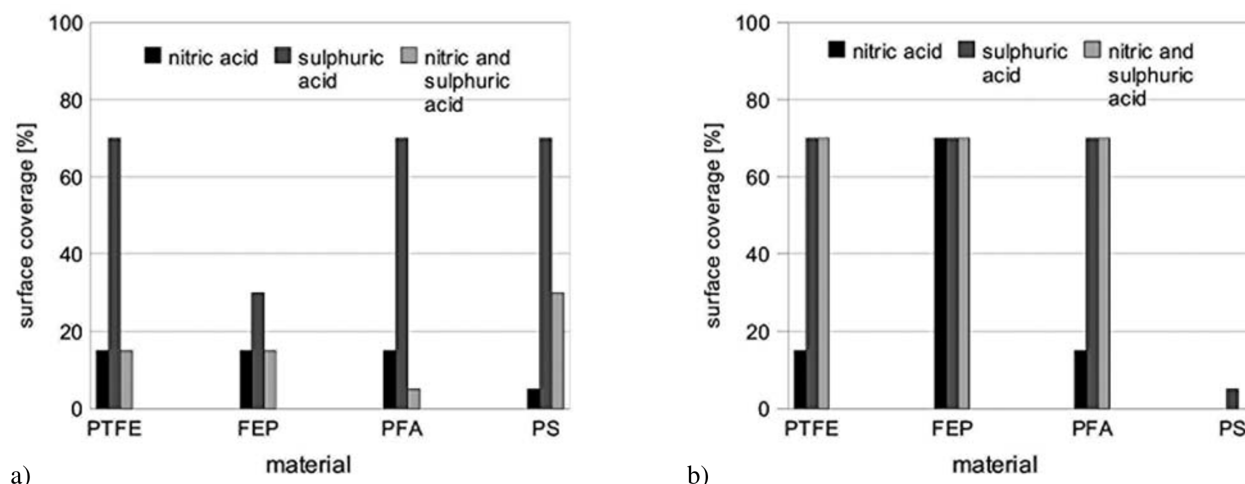

Figure 4: The effect of acid type on immobilization efficiency on different materials after (a) 1 and (b) 24 h treatment.

The effect of sulphuric acid treatment time of different materials on immobilization efficiency is presented in Figure 5. It can be seen that PTFE and PFA immobilization efficiency does not depend on acid treatment time, as excellent immobilization was observed at all times tested. On the other hand, FEP should be treated for at least 2 h to obtain the best results. Interestingly, PS immobilization efficiency decreased with prolonged acid treatment, as the best result was observed after 1 h treatment. Microscope observations of sulphuric acid treated PS microchannels showed intense yellow pigmentation and cracked surface, indicating material decomposition.

4. Conclusions

We have proven that glass, PS, PTFE, FEP and PFA can all be functionalized by means of strong acids, APTES and glutaraldehyde as to promote *Saccharomyces cerevisiae* immobilization. Using appropriate conditions, the highest possible cell surface coverage (70%) was obtained on all tested materials. To our knowledge, PTFE, FEP and PFA have not been previously used for cell immobilization. We believe that these materials can have important applications in microreactor technology as they are readily commercially available, relatively cheap, and available in different dimensions and, as we showed, susceptible to modify. We conclude that H_2SO_4 is the most universal reagent for surface activation before silanization with APTES among acids tested. In summary, we have demonstrated a simple but highly effective approach to develop a microfluidic device with immobilized biocatalyst from low cost and disposable materials.

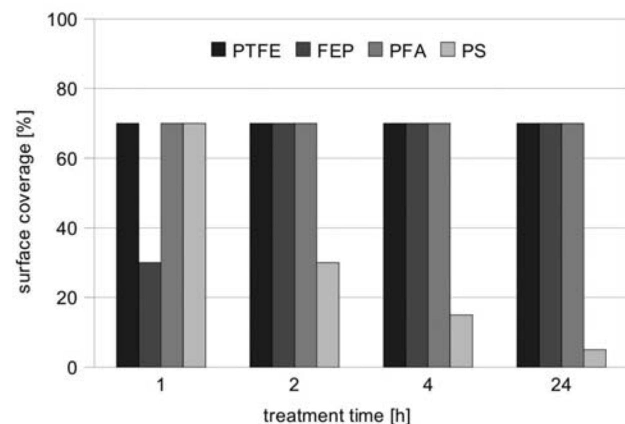

Figure 5: Effect of acid treatment time on cell immobilization efficiency on different materials using sulphuric acid.

Nomenclature

APTES	3-aminopropyltriethoxysilane
CD	cell density
FEP	fluorinated ethylene propylene
PDMS	polydimethylsiloxane
PMMA	polymethyl methacrylate
PFA	perfluoroalkoxy
PS	polystyrene
PTFE	polytetrafluoroethylene

5. Acknowledgement

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Povzetek

Celice *Saccharomyces cerevisiae* smo uspešno imobilizirali na notranje stene mikrokanalov, kar nam v nadaljevanju omogoča razvoj visoko produktivnega kontinuirnega procesa biotransformacije v mikroreaktorskem sistemu. Uporabili smo mikrokanale iz stekla, polistirena (PS), politetrafluoroetilena (PTFE), perfluoro-alkoksija (PFA) in fluoriranega etilen-propilena (FEP). Celice smo pritrdili s kovalentno vezavo na silanski sloj z glutaraldehidom, silanizacijo sten mikrokanalov pa smo predhodno izvedli s 3-aminopropiltrietoksisilanom (APTES). Pred obdelavo z APTES smo površine mikrokanalov obdelali z različnimi kislinami in ugotovili, da najboljše rezultate na izbranih materialih daje obdelava s H_2SO_4. Proučili smo vpliv časa obdelave posameznih polimernih materialov s kislinami na uspešnost imobilizacije. To je prvi opis pritrditve celic na površino PTFE, FEP in PFA, ki jih v obliki komercialno dosegljivih in nizkocenovnih cevk submilimetrskih dimenzij lahko uporabimo za razvoj mikroreaktorjev s površinsko vezanimi biokatalizatorji.