Encapsulated Living Cells on Microstructured Surfaces

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The immobilization of cells in defined arrays (cell patterning) is a key step towards cell-based biosensors or other cell-based devices. While cell patterning is usually achieved by modifying the surface on which only the cells should adhere and leaving the cells unmodified, we present here a different approach in which cells are first coated with polyelectrolytes and subsequently immobilized on patterned surfaces. By coating, the cells are protected and their interactions with the substrate are modified such that patterning is simplified. We used microcontact printing of polyelectrolytes to structure surfaces such that regions of opposite charges and the same charge as the cell coating were present and found that we can thus achieve patterning of the coated yeast cells. In accordance with prior work, we find that coating does not kill the cells and coated GFP-expressing cells still function after immobilization, which we checked by fluorescence microscopy.

Introduction

The interaction of living cells with substrates is a vast area of intense research due to its relevance for very different applications: biocompatible surfaces are desirable for the integration of biological materials into artificial systems (cell-based sensors and implants), passivation of surfaces against cell attachment is desired for suppressing negative reactions of the immune system against transplants, and toxic surfaces are sought after as antiseptic coatings, just to mention some examples. Therefore, much effort has been made to understand and control the coupling of living cells with model surfaces and to develop coatings that possess some of the properties mentioned above. One of the most demanding tasks in this context is the creation of patterned surfaces on which both cell-friendly and passivated areas are present. Recently published works of Zheng et al.[1] and Berg et al.[2] show impressively the high impact of the use of microstructured surfaces on the guidance of cell growth. Such patterned surfaces are basic building blocks of single-cell-based biosensors or bioreactors, which are expected to play a major role in future biotechnology. For a broader overview on the potential of single-cell-based biosensors, we refer the reader to the review articles of Bousse.[3] Noteworthy is also the potential use in drug discovery.[4]

The general approach toward creating such surfaces is to functionalize zones of the substrate in different ways, while the cells themselves are completely functional. One main drawback in the use of neat cells on structured surfaces is that an attachment of the cells is only observed in regions with fitting charge or surface properties. Here, we present a novel approach based on polyelectrolyte coating of *both cells and surfaces*. The advantage of cell coating prior to immobilization is that coupling to all kinds of surfaces is simplified due to the fact that the surface properties of the cell coating can be modified as desired. Furthermore, the coating can act as a protective shell for the cells[5] in aggressive environments (paper in preparation, see Supporting Information Figure A). Possible applications for coated cells on microstructured surfaces or charged surfaces could be the recycling of genetically engineered cells used for water clearance purposes or oil pollution in seawater. Furthermore, one can think of the facilitated stepwise deposition of different types of cells on a structured template, for example, for a lab-on-the-chip application.

We chose synthetic polyelectrolyte multilayers as the coating for both cells and substrates in this study, because of the versatility of this approach that should allow extension of the principle to other cell types and substrates more easily. Since its introduction by Decher and coworkers,[6,7] the approach of layer-by-layer (LbL) self-assembly of polyelectrolytes has been successfully applied for a wide range of layer components and surfaces. Polyelectrolyte multilayers can be built up by alternating the deposition of positively and negatively charged polyelectrolytes onto charged surfaces. The thickness of the coating can be controlled on the nanometer scale, and the outermost layer specifies the surface properties. LbL coating is possible with a variety of polyelectrolytes and has only little limitations concerning the substrate, since no specific chemistry is involved.[8] In particular, various biocompatible polyelectrolytes have been used for the buildup of polyelectrolyte multilayers.[9−14] Several groups

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have investigated the interactions of living cells with polyelectrolyte multilayers.[15−21] It was shown that the interactions can be tuned by the composition of the multilayer and that surfaces can be passivated against cell adhesion. However, to the best of our knowledge, so far, surface interactions of coated cells have not been investigated.

Like previously shown, also cells such as other colloids allow for the use of the LbL technique.[22−24] While coating of biological cells was already early demonstrated,[25−27] this was exclusively applied to use fixed, means dead cells as templates for hollow microcapsules. Recently, some of us have demonstrated that living yeast cells can be coated and maintain their metabolic activity after coating.[17,28] In fact, it was also shown that the coating even protects the cells in a lytic environment and the release of large protein molecules through the capsule in the medium is observable (papers in preparation, see the Supporting Information).

We use the same approach here and combine it with pattering of substrates by microcontact printing (μCP) of polyelectrolytes, the so-called polymer-on-polymer stamping technique that was introduced by the Hammond group.[12,29]

Materials and Methods

Materials. Polyelectrolytes (PEs), poly(styrenesulfonate sodium salt) (PSS, MW 70 kDa), poly(allylamine hydrochloride) (PAH, MW 15 kDa), and branched poly(ethyleneimine) (PEI, MW 25 kDa) were obtained from Aldrich. For visualization of the PE coating, rhodamine-B-isothiocyanate (RBITC; $\lambda_{exc} = 541$ nm; Fluka, Germany) or Alexa (555) Fluor ($\lambda_{exc} = 555$ nm; Molecular Probes, Eugen, OR) was covalently bound to PAH. The procedure was described in detail before.[30,31] Briefly, PAH was solved in a NaH_2CO_3 (2%, pH 9) solution and then 2 mg of the fluorescent dye in 400 mL of dimethyl sulfoxide (DMSO) was added. After 1 h of reaction time at RT, the removal of unbound dyes was performed by dialysis against Milli-Q-grade water. The dialysis by means of a 3.5 kDa cutoff dialysis membrane (Spectrum Laboratory, Rancho Domiguez, CA) lasts for at least 1 week with daily change of water.

The poly(dimethylsiloxane) (PDMS) base silicon elastomer Sylgard 184 and the curing agent were obtained from DowCorning (Midland, MI).

All chemicals were used without further purification except for the PSS, which was dialyzed against water (cutoff of 14 kDa; Millipore, Schwalbach, Germany) and lyophilized prior to use. Milli-Q-grade water (Millipore Inc., Bedford, MA) with a resistance of 18.2 MΩ cm was used for all solutions and cleaning steps.

The yeast strain expressing the green fluorescent protein (GFP) under galactose promotion was a gift from Prof. C. Palleschi from the University "La Sapienza" in Rome, Italy.

Methods. *Encapsulation of Living Saccharomyces cerevisiae Cells.* For the experiments, two different strains of yeast were in use. In most of the experiments, common bakery yeast, *Saccharomyces cerevisiae*, was encapsulated. The coating follows the procedure described in detail in previous papers.[17,28] The following is a short summary of the important steps: First, the cells were stored for 2 h in YPD (10% yeast extract, 20% peptone, and 20% glucose) medium at 28 °C for recovery, and then, the solution was exchanged to 0.5 M saline (NaCl). In accordance with the fact that the surface of uncoated yeast is covered with glycans or other negatively charged molecules, the encapsulation started with the deposition of the polycation.[32] For this, the resuspended cells were incubated in PAH solution (2 mg/mL) containing 0.5 M NaCl, followed by two washing steps with 0.5 M (NaCl) saline solution. Then, the next layer was deposited from 0.5 M saline PSS solution and the self-assembly and the washing were repeated until the desired number of layers was achieved. To achieve capsules with a negative surface charge, we deposited four layers, while, for a positive outermost layer, in one experiment, we encapsulated the cells with seven layers also due to a predicted higher surface charge density.[8]

Because of the fluorescent dye Alexa (555) covalently bound to PAH, we were capable of detecting the capsule homogeneity. The polyelectrolyte deposition gives also information about the cell viability. In case of suffering or dead cells, the labeled polyions enter the cells and stain them red. Only healthy cells keep their membrane integrity and exclude the PE completely. The encapsulation process for the GFP-expressing yeast was equal.

Imaging in fluorescence and phase contrast mode of ordinary yeast cells was performed in 0.5 M NaCl solution. In contrast, the GFP-expressing yeast was incubated in 15% galactose in water for 2 h at a constant temperature of 28 °C to induce the GFP production. Under these conditions, the green protein was kept inside and so colocalization of the capsule and the cell interior was possible.

Microstructuring of the Surface. The process of polymer-on-polymer stamping we used for the structuring of coverslips was described in detail before.[33] The coverslips (Menzel-Glaser, Braunschweig, Germany) were cleaned by the RCA method[34] prior to coating. For the coating, the slides were put in a solution of 0.5 mg/mL PEI without added salt for 20 min, rinsed carefully, and immersed into a 1 mg/mL solution of PSS containing 0.5 M NaCl. PDMS silicon elastomer and the curing agent were mixed in a 10:1 ratio, respectively. The masters (GeSim, Dresden, Germany) were covered with the prepolymer, degassed, and cured for 12 h at 60 °C. The stamps were cut out, cleaned in $CHCl_3$, dried at 60 °C for 1 h, and hydrophilized in an air plasma (PDC-32G-2, Harrick, U.S.A.; pressure 2×10^{-1} mbar; 60 s).[35] The stamp was inked with a 1 mg/mL solution of RBITC−PAH with an ionic strength of 0.5 M (NaCl). After 15 min, the stamps were briefly rinsed with water and excess solution was removed in a nitrogen stream. The inked stamps were brought into contact with dried, coated coverslips for 20 min and then removed. The

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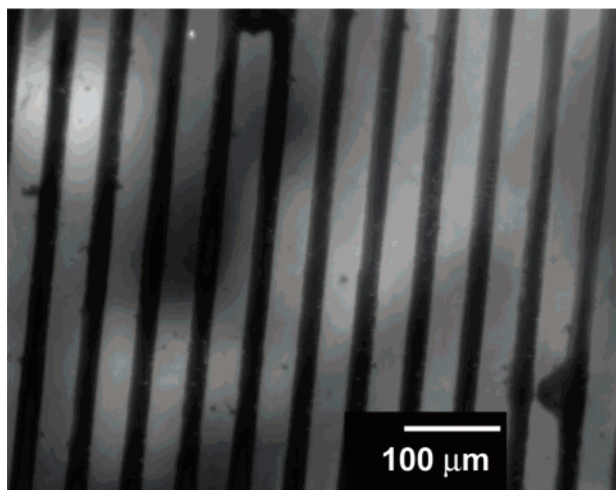
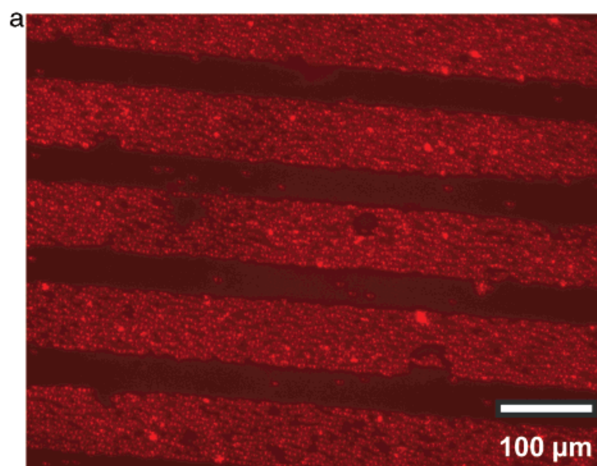


Figure 1. Fluorescence image of 40 μm wide stripes transferred by microcontact printing of RBITC−PAH onto a PEI/PSS-covered glass slide.



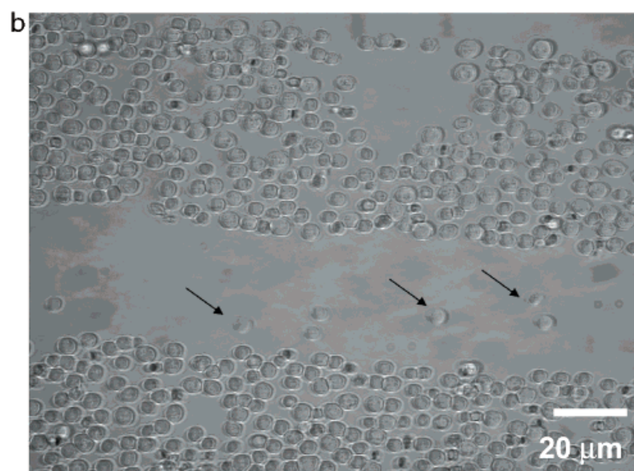


Figure 2. (a) Fluorescence micrograph showing the selectivity of coated (PAH/PSS/Alexa (555)−PAH/PSS) cell adsorption on a patterned (PEI/PSS/RBITC−PAH) substrate over a large area. (b) Phase contrast image of encapsulated yeast cells attached by electrostatic self-assembly to a 100 μm stripe structure. Unattached cells in the like-charged region of the glass are blurred due to movement (arrows).

structured surfaces were carefully rinsed with water, characterized using fluorescence microscopy, and stored under water until use.

Microscopy. Fluorescence microscopy measurements were carried out on a Zeiss Axiovert 200 instrument (Zeiss, Germany).

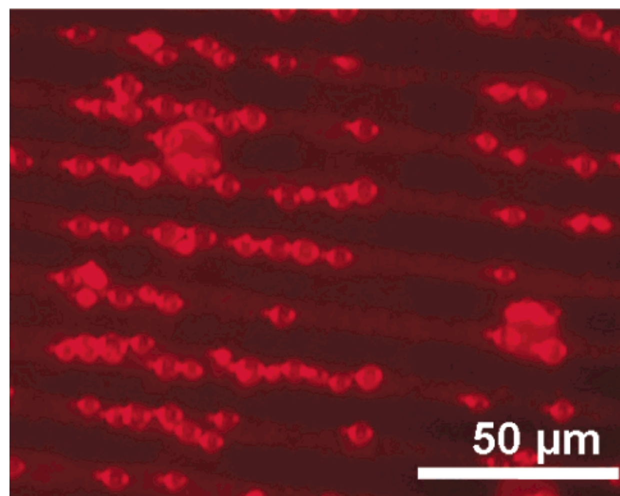


Figure 3. Fluorescence image of 5 μm wide stripes transferred by microcontact printing of RBITC−PAH onto a PEI/PSS-covered glass slide. The attached yeast cells were coated with $(PAH/PSS)_2$ with the third layer labeled by Alexa (555). It is obvious that they adsorb preferentially to the small lines. The micrograph was taken with 20× magnification, and both red dyes were excited at $\lambda_{max} = 514$ nm.

An LDA-Plan 20× objective was used. The light source was a Hg lamp. The excitation and emission wavelengths were filtered between 450−490 and 515−565 nm, respectively. A Zeiss AxiocamHR high-resolution monochromatic camera was used for collecting the images.

The confocal micrographs of the coated yeast cells were obtained by a Nikon C1 (Nikon Instruments, Florence, Italy) confocal laser scanning microscope (CLSM). The C1 scanning head was mounted on a Nikon inverted optical microscope. An Ar-ion laser (488 nm, 514 nm) and a He−Ne laser (543 nm) source, enclosed in a common multilaser module, provided the excitation beams, and a Nikon Plan Apochromat 40× and a 100×/1.4 NA oil immersion objective were used for imaging.

Results and Discussion

To develop new biosensors based on the use of whole living cells, it is crucial to immobilize the cells in a way that does not alter their interaction with the environment and specify the lateral position as well as the cell density. In the present study, we focused on the interaction between microprinted surfaces and encapsulated living cells. The microstructure on the glass slides was induced by stamping with a polyelectrolyte-covered PDMS master on a cleaned glass surface. Several different stamps were used to vary the stripe width or the structure form. The printed structure was mostly fluorescent labeled with RBITC bound to PAH to allow a visualization of the transferred structure. Living yeast cells were coated with the same polyelectrolytes (PAH and PSS) used for the patterned substrates. The only exception was that in this case the PAH was labeled with Alexa (555) which is brighter red than RBITC and permits one to distinguish between underlying layer and coated cells.

First, we visualized by means of CLSM or conventional fluorescence microscopy the glass slides to control the structure transfer from the stamp pad to the template, which was printed with RBITC−PAH. A pattern with a stripe width of around 40 μm is shown in Figure 1. The bright red fluorescence indicates a good transfer of the polycation ink to the PEI/PSS-coated glass slide. The thickness of the transferred polyelectrolyte layer is ~2.5 nm, as determined earlier by atomic force microscopy (AFM) measurements for patterns of the same kind with comparable fluorescence intensities.[33] Thus, it is clear that

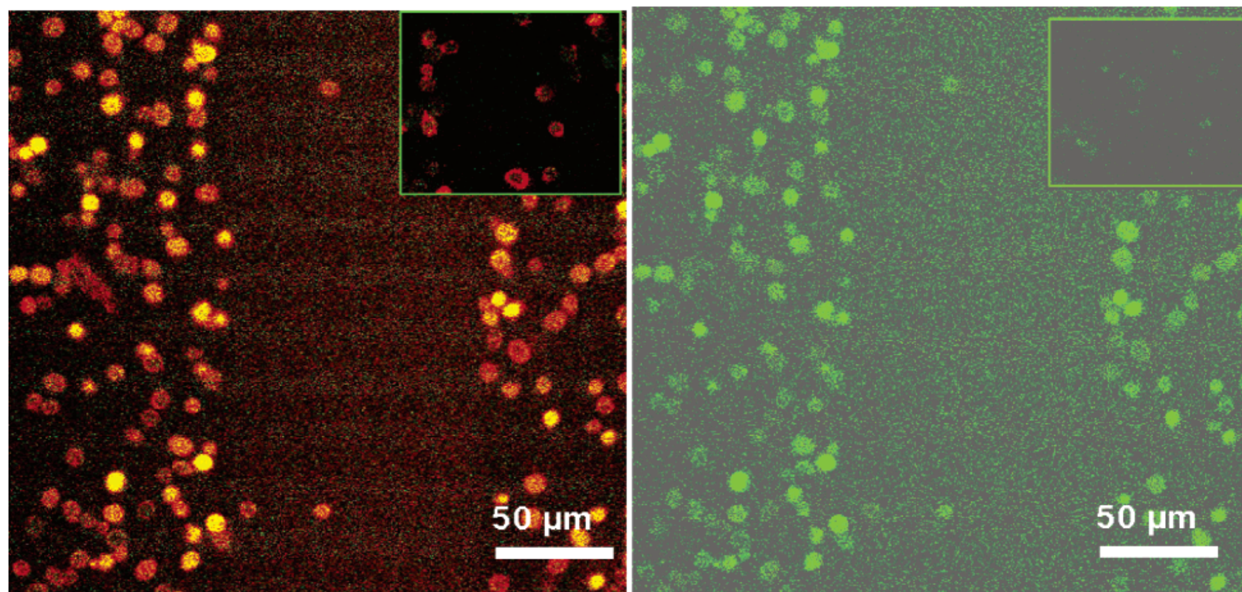


Figure 4. CLSM micrograph of GFP-filled, coated yeast self-assembled to a stripe structure induced by microcontact printing. The printed polyelectrolyte was RBITC−PAH onto PEI/PSS coated glass, while the shell on the cells consisted of $(PAH/PSS)_2$, with the third layer labeled with Alexa (555). Fluorescence was excited with two lasers, GFP (green) with λ_{max} = 488 nm and the red dyes at λ_{max} = 514 nm. The yellow appearance of the cells in the left image is due to colocalization of the green and red emission. The right image shows only the green channel of the same image. Both insets show the cells before incubation.

the imprinted pattern topography has no effect on the ordering of the cells.

Next, we investigated the self-assembly of coated cells onto the μCP glass. In the case of the coated cells having a surface charge opposite to that of the assigned regions of the glass slide, a clear ordered arrangement of the cells over large areas was observable after carefully rinsing with water (Figure 2a). The low magnification for the image was chosen to show that large areas are covered with coated cells. These appear as small bright red spots, but the fact that they bind only to printed regions of the template is clearly visible. In the phase contrast image (Figure 2b), the cells are fixed along the stripes of opposite charge. Here, it is noteworthy that cells that are in the likely charged region of the glass are still in movement and due to this appear blurred (arrows).

In a time series performed after rinsing the template and attached cells with water, we were able to see that the free cells carried on drifting around until they reached an area of opposite charge (data not shown). Next, we investigated the influence of the stripe size, which were in the now presented set of experiments of the same size like the cells. For yeast, the dimension is 5 μm, and also in this case, self-assembly along the stripes is observable (Figure 3). Due to the higher magnification of the images with respect to the former ones, one can clearly see the red polyelectrolyte capsule of four layers, labeled with Alexa (555).

These observations support the idea that the attachment is clearly electrostatically driven. Furthermore, the present findings are in accordance with the results found in our previous work concerning the selective deposition of hollow capsules.[33,36] The coupling of the cells is strong.[37] The structure is still recognizable after rinsing and even after the substrate was removed from water and dried out, which is accompanied by large capillary forces acting on the cells (data not shown). The strong adhesion for capsules of the same size supports the experimental findings.

In experiments with positively terminated coating, we could observe no ordering on patterned surfaces. The behavior changed from a patterned adhesion observed for negatively terminated coatings to a random distribution. This effect does not depend on the number of layers that were assembled on the cells. The number of layers was varied between four and seven, which rules out the idea that there were defects in the coating.[8] Rather, preliminary experiments give evidence that the living cells actively are able to change the surface charge of the attached polyelectrolyte layers. A detailed quantitative analysis of the surface charge density of the coated cells as a function of the outermost layer and its evolution with time is on the way but goes beyond the scope of the present paper.

The viability as well as the addressability of the attached cells was investigated. Encapsulated yeast was assembled on the pattern in a 0.5 M NaCl solution, which was exchanged to 15% galactose in water. The template was then stored for 2 h at 28 °C in an incubator. Under the fluorescence microscope, the GFP level was visualized as a detector for induced protein production and was therefore considered as a viability indicator. In the left image of Figure 4, a CLSM micrograph is depicted that shows the coated and GFP-filled cells as small yellow spots due to colocalization of the green GFP and red Alexa (555) fluorescence. The right image of Figure 4shows only the green channel of the same picture to visualize the production of GFP more clearly. Both insets show the lack of GFP fluorescence before incubation with galactose.

Also, in these experiments, a preferential deposition on the unlike-charged printed structure is evident. Higher magnification of the cells reveals in the focal plane a small red fluorescent rim, while the interior is completely occupied by GFP, which emits green fluorescence (Figure 5).

Our presented results give evidence that living cells can be immobilized in an easy and elegant way on glass or other charged surfaces structured by microcontact

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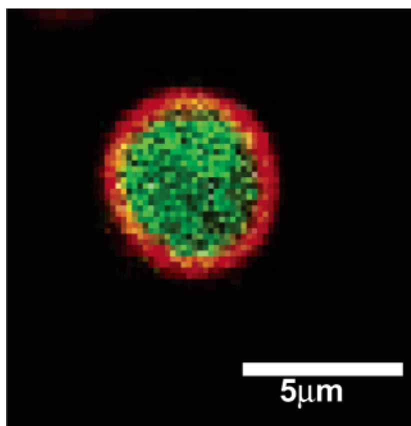

Figure 5. CLSM image of the focal plane of a single GFP-expressing yeast cell with a polyelectrolyte capsule ((PAH/PSS)$_2$, third Alexa (555)−PAH) and green GFP signal inside. Excitation at λ_{max} = 488 and 514 nm at 100× magnification. The cells were imaged after 18 h of storage in 15% galactose and incubation at 28 °C.

printing. With the capsule on the cell surface, the cells are more protected against shear stress or aggressive environments. Furthermore, the encapsulation procedure did not alter the viability of the coated organisms, which is in accordance with former experiments.[17,28] Beyond that, in the current study, it was shown that the shell allows also for inducing a signal after coating. Moreover, for capsules on the permeable core prepared under similar conditions, a cutoff between 20 and 40 kDa was stated, which is good enough for larger molecules but as mentioned in the Introduction variations and fine-tuning of the cutoff is possible (see Supporting Information Figures A and B).

Due to the extraordinary features inherent to the applied polyelectrolyte capsule, which allow for an opening and closing of the wall as well as tuning of the permeability, the coating can serve as a regulation of the flux.[38,39] The capsule walls are mechanically robust and can protect cells against external forces[40] and the attachment of hollow capsules is strong, allowing for high shear rates without detachment, which is an important feature for applications.[33,36,37]

An interesting perspective of the principle demonstrated here is that it can be expanded to other nonelectrostatic interactions: specific interactions can be incorporated like those demonstrated by Huang.[41] Indicated by the numerous studies dealing with biosensors based on whole cells or the interaction of biological components with polyelectrolytes, the combination of immobilized coated cells and a pattern induced by microcontact printing is an exploitable new tool.

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Supporting Information Available: Figures showing protection of *Saccharomyces cerevisiae* encapsulated with different ionic strengths and the cutoff of an encapsulated amorphous $CaCO_3$ core. This material is available free of charge via the Internet at http://pubs.acs.org.

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