

Single Living Cell Encapsulation in Nano-organized Polyelectrolyte Shells

Alberto Diaspro,* Daniela Silvano, Silke Krol, Ornella Cavalleri, and
Alessandra Gliozzi

*INFM and Department of Physics, University of Genoa, Via Dodecaneso 33,
16146 Genoa, Italy*

Received February 21, 2002. In Final Form: March 25, 2002

Single living yeast cells have been encapsulated by the alternate adsorption of oppositely charged polyelectrolytes. Exploiting fluorescence techniques, we provide evidence of the shell and cell integrity after the coating procedure. The most relevant result, however, is that, after encapsulation, cells preserve their metabolic activities and they are still able to divide. These hybrid polyelectrolyte cells can provide a cheap model system in a wide range of biophysical and biotechnological applications, thanks to the tunable properties of the polyelectrolyte shell.

The application of nano-organized supramolecular assemblies of surface-modified liposomes, organic and inorganic materials, and ultrathin polymeric films is of significant interest in several fields of applied biophysics. In particular, filled and hollow capsules in the micro- and nanometer size range are of major importance in medical applications, since they can be used as protective shells for cell clusters and tissue encapsulation or as vehicles engineered for local drug delivery. Furthermore, such encapsulation devices constitute physically confined environments for study and control of biochemical events. Because of the different requirements, several encapsulation methods are currently used for the fabrication of micro- and nanosized shells.¹⁻⁷

A comparatively new generation of polymeric shells, known as "nanocapsules", has been recently introduced, on the basis of the layer-by-layer (LbL) technique.^{8,9} The basis of this method is the assembly of composite layers by alternating adsorption of oppositely charged species onto a charged template (core), which is usually removed by chemical action. Hollow capsules exhibit good stability with regard to changes of pH, solvent, and temperature.^{4,8,10} Shell features such as biocompatibility and permeability can be tailored by using specific polyelectrolytes (PEs) as building blocks. In earlier works related to nanocapsule fabrication, biological cells were occasionally used as templates.¹¹ We found the idea of exploiting nanocapsule technology for the design of a new model

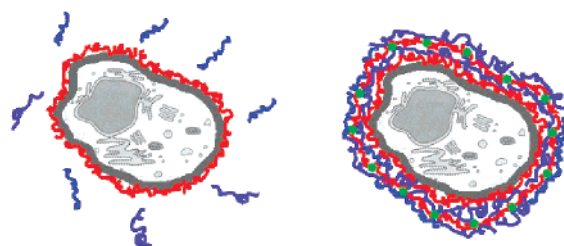


Figure 1. Vignette of the encapsulation process: consecutive adsorption of the polycation PAH (red) and the polyanion PSS (blue) onto a single living cell. To provide evidence of a successful coating, one or two layers are FITC-labeled PAH (green).

system for studying biophysical and biotechnological problems at cell and molecular levels to be extremely attractive.

In this work, we demonstrate the possibility of extending the nanocapsule building scheme to the encapsulation of single living cells, while preserving their metabolic activity and their ability to divide. From this perspective, the core is an intact living cell, not dissolved. The central concern in the encapsulation of living cells resides in the nanocapsule preparation procedures used to obtain the desired features. For example, the stability and tunable properties of the cell coating have to preserve and somehow control the specific cell permeability, since cell viability depends on the diffusion of nutrients through the capsule walls.

As cellular system we used *Saccharomyces cerevisiae* culture stock, the common baker's yeast. *S. cerevisiae* is an interesting class of eukaryotic cells whose genome covers 12 million base pairs and about 6000 genes. Moreover, it was the first eukaryotic organism whose genome was completely sequenced.¹² Mutants are useful for studying eukaryotic DNA replication, transcription, RNA processing, protein sorting, and regulation of cell division.

For encapsulation, poly(styrenesulfonate sodium salt) (PSS, MW 70 000 Da, Aldrich) and poly(allylamine hydrochloride) (PAH, MW 15 000 Da, Aldrich) were dissolved in 0.5 M NaCl at a concentration of 2 mg/mL. Milli-Q-grade water (Millipore GmbH) with a specific resistance of 18.2 M Ω /cm was used for all the preparations.

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* To whom correspondence should be addressed. E-mail: diaspro@fisica.unige.it.

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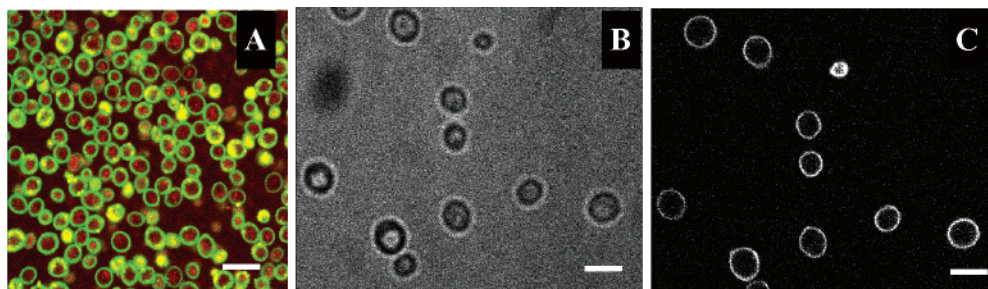


Figure 2. Confocal images of freshly coated cells: (A) overlapping of reflection (red) and fluorescence (green); (B) transmission non-confocal; and (C) fluorescence images. The high percentage of coated cells is evident. Scale bar: 10 μm (A); 5 μm (B and C).

Each polyelectrolyte adsorption step was performed by yeast cells incubation in the proper polyion solution (5 min for PAH, 10 min for PSS) followed by centrifugation and twice repeated washing in 0.5 M NaCl.

The number of layers, influencing the shell thickness (~ 1.5 nm/layer), is defined by the number of assembly steps carried out by adsorption at supersaturating bulk PE concentration.⁸ Four to six PE layers were typically adsorbed. As sketched in Figure 1, the inner layer was built by the cationic polyion, because of the negative surface charge of the outer cell membrane. Evidence for PE adsorption was obtained by using fluorescein isothiocyanate (FITC; MW = 389 Da, Aldrich)-labeled PAH (prepared following the standard procedure for protein labeling¹³). All steps of the coating process, as well as the first investigations, were performed in the absence of culture medium, so as to keep the cells in a stationary phase and avoid duplication activity. On the other hand, to check the duplication activity, coated yeast cells were incubated at 28 °C in YPD medium (yeast extract 1%, Peptone 1–2%, and Dextran 2%) overnight.

Image acquisition was performed using a two-photon excitation (TPE) microscope built around a conventional confocal laser-scanning microscope, Nikon PCM2000, where conventional excitation was preserved.¹⁴ A Nikon Planachromat 100 \times , 1.3 oil immersion objective was used. TPE fluorescence of the dyes was achieved using a 720 nm Ti:sapphire ultrafast laser source (Tsunami, Spectra Physics, CA) at approximately 6 mW of average power in the focal plane, 150 fs of pulse width, and 80 MHz of repetition rate. Confocal imaging was performed, both in fluorescence and reflection modes, using 488/543 nm beamlines from Ar ion and He–Ne coupled lasers. Occasionally, transmission (non-confocal) images were collected using a conventional lamp and the descanned optical pathway of the PCM2000 scanning head. Special barrier filters were used for collecting the TPE fluorescence signal to block possible high-peak power IR reflections.

The following fluorescent dyes were used: FITC-labeled PAH (conventional fluorescence excitation 488 nm, TPE 720 nm, emission 515 nm) for shell staining; 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI; MW = 350 Da) (TPE 720 nm, emission 461 nm (DNA-bound DAPI)) for DNA staining; DASPMI (TPE 720 nm, emission 605 nm) for the metabolic activity test. For all experiments visualizing the metabolic activity, DAPI and DASPMI were added at different temporal intervals.

Flow cytometry measurements were performed by using a EPICS XL flow cytometer (Beckman-Coulter, Hialeah, FL) with an Ar ion laser operating at 488 nm. Forward and side scatter signals were collected in linear mode,

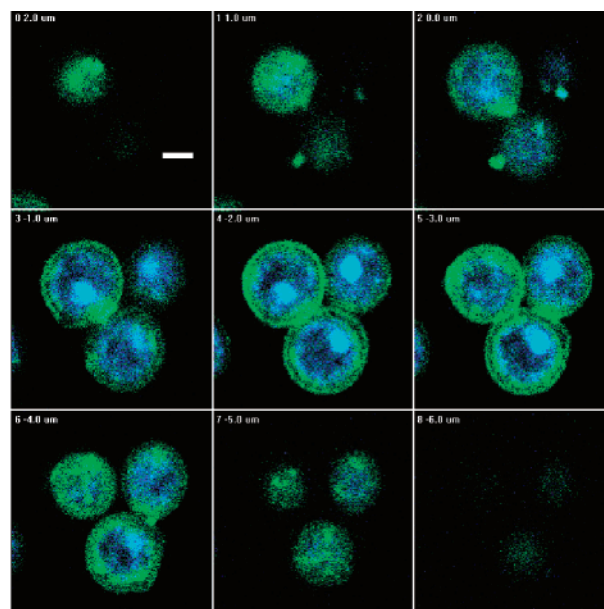


Figure 3. 3D optical sections of encapsulated cells after incubation in DAPI. The homogeneous FITC–PAH green fluorescence is indicative of a uniform cell coating while the DAPI blue fluorescence, staining the mitochondrial and nuclear DNA, is indicative of the integrity of the main cell biostructures. Scale bar: 2 μm .

while fluorescence signals (through a band-pass filter at 425 ± 5 nm) were collected in log mode. More than 30,000 events were acquired per sample. Analysis was performed using the resident software of the cytometer. No gates were employed to preselect the analyzed population. The percentage of cells expressing a significant fluorescent signal and the mean fluorescence channels under different experimental conditions were calculated.¹⁵

Confocal and two-photon fluorescence microscopy have been mainly used for this study, allowing three-dimensional imaging at low perturbation levels.^{16–18}

As a first step, using flow cytometry, we checked the efficiency of cell encapsulation. Analysis of scattering and fluorescence signals from freshly coated cells indicated that approximately 98.5% of the cellular population was coated. Microscopic observations comparing fluorescence¹⁹ and optical images also confirmed this result (Figure 2).

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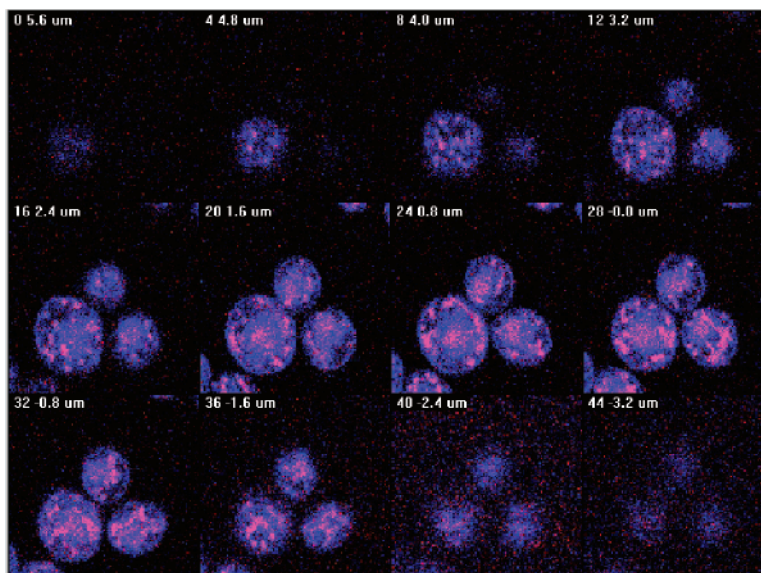


Figure 4. 3D optical sections of encapsulated cells loaded with DAPI and DASPMI. Bright violet areas are due to red DASPMI fluorescence colocalized with the blue DAPI signal. The accumulation of DASPMI in the mitochondrial matrix is indicative of cell oxygenation activity. The image is acquired utilizing the two available channels for DNA and DASPMI fluorescence. Green fluorescence of the capsule wall is checked but not shown. Moreover, the DAPI fluorescence signal is acquired in saturation to provide a morphological reference based on DNA distribution. Scale bar: 5 μm .

Evidence for a complete cell encapsulation was achieved by means of two-photon excitation imaging. In point of fact, three-dimensional analysis of the fluorescence distribution allows a check for capsule and cell integrity. Figure 3 provides a view of some coated cells, showing costaining of both the shell structure and the cell biostructures. The set of images shows the green fluorescence of the FITC-labeled PE shell in every optical section, indicating a complete coating of the cell. To get information on cell integrity, we routinely incubated the encapsulated yeast in a DAPI solution. DAPI is a low-molecular-weight fluorescent dye, which penetrates easily both the capsule and the cell wall, and binds to nuclear and mitochondrial DNA. This procedure can be considered as a check for cell structure preservation: intact cells exhibit well-defined bright fluorescent regions, while corrupted ones have a diffuse blue emission. Confocal microscopy was occasionally used for visualizing the shell walls alone (data not shown). By inspecting confocal and two-photon fluorescence microscopy images, we can conclude that encapsulated cells preserved their mitochondrial and nuclear DNA distribution.

Another relevant aspect to be investigated is whether the cells maintain their normal functions, such as the mitochondrial activity related to aerobic energy metabolism. DASPMI, a fluorescent lipophilic cationic dye, was used for this purpose. DASPMI accumulates exclusively in the mitochondrial matrix as a result of the membrane potential.¹⁹ It is known that a higher oxygenation activity leads to an increasing number of mitochondria, which results in a more intense DASPMI signal.²⁰ In Figure 4, one can observe bright violet areas due to red DASPMI fluorescence, colocalized with the blue DAPI signal, which in this case is used as a cell landmark. These experiments allow us to conclude that the polyelectrolyte coating does not influence the metabolism in terms of cell respiration.

The next crucial point to be understood is whether cells entrapped in a polyelectrolyte matrix with tunable mechanical properties²¹ are still able to divide. To this end,

we incubated coated yeast cells at 28 °C in YPD medium overnight. In this case, we observed both CO₂ production, indicative of metabolic activity, and an increase of turbidity due to cell growth, as confirmed by spectrophotometric measurements (data not shown).

Moreover, by means of microscopic techniques, three cell species were identified in the cell population. The comparison of transmission and fluorescence images revealed a small number of cells with a fluorescent-labeled shell, cells without fluorescence, and very few fluorescent mother cells with mainly nonfluorescent buds. An encapsulated mother cell with its forming bud is shown in Figure 5.

The heterogeneity in the cell population reflects the fact that cellular divisions occurred, demonstrating that cells were still able to divide even if entrapped in the polyelectrolyte matrix. We further confirmed the cell growth by using flow cytometry measurements. After incubation (18 h), we found around 2% fluorescent-labeled cells versus the 98.5% measured before incubation. On the assumption of cell duplication every 2 h, the growth of only the uncoated organisms (1.5%) would result in a ratio of fluorescent to nonfluorescent cells of 11:100. On the other hand, a division of all cells would result in 0.2% fluorescent cells after incubation. The measured value (2%) confirms the microscopic observation that the coated cells are still able to divide.

The discrepancy between the data obtained by flow cytometry and the value expected under the hypothesis of duplication of the complete cell population is not surprising and can be easily explained by the fact that the PE shell can hamper the first division of the coated cells. In any case, the value found by flow cytometry supports the evidence that the shell does not prevent cell duplication. Moreover, encapsulated cells maintain their viability up to at least one-week under standard storage conditions.

Considered together, the above experimental observations allow us to conclude that coated cells are alive and in good vital condition. This fact suggests that the capsules are biocompatible and noncytotoxic with respect to cell activity. A further intriguing step would be the extension of this technique to the coating of mammalian cells.

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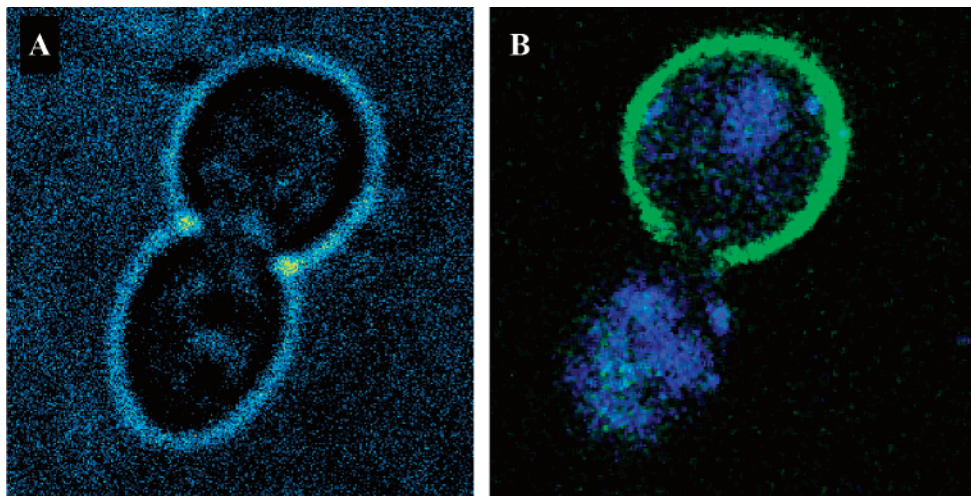


Figure 5. Transmission image (A) of a duplicating cell. This is confirmed by the fluorescence image (B) where the lack of green fluorescence in the mother cell coating reveals where the bud has formed. The DAPI-labeling of the DNA distribution allows the imaging of both mother and uncoated daughter cell.

Encapsulation of living cells opens interesting perspectives for biophysical studies. They provide a model system to investigate the mechanical properties of the cell in relation to the presence of the coating. For instance, one can exploit the tunable mechanical properties of the shell to study the forces involved in cell division, as well as to protect the cells from shear stress. Because of the simplicity of the coating procedure, this hybrid PE–cell system is also a very promising tool for biotechnological applications. Encapsulation, which protects cells from an immunological response, can be combined with shell biofunctionalization. This could allow the in situ targeting

of cells, while preserving at least part of their functions, such as protein expression or division capability.

Acknowledgment. We would like to thank Paola Ramoino, Paola Ballario, Prisca Ornaghi, Teresa Rinaldi, and Claudio Palleschi for their help and comments. We are indebted to Gianni Melioli, Advanced Biotechnology Center, Genoa, Italy, for flow cytometry measurements. S.K. acknowledges the EU for support in the form of a research fellowship (EU Project HPRN-CT-2000-00159). LA025646E