DOI: http://dx.doi.org/10.7551/978-0-262-31709-2-ch117

"Artificial spores" - hybrid alginate microcapsules with encapsulated yeast cells

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Abstract

The present work describes the fabrication, structure and functional characterization of composite microcapsules containing encapsulated viable yeast cells, fluorescently labeled liposomes and magnetic nanoparticles embedded in a calcium alginate matrix produced by ink-jet printing. The proliferation of the encapsulated cells under favorable conditions (presence of nutrients, temperature) is used as a biological trigger for the disintegration of the microcapsules and the liberation of the encapsulated sub-micron particles. The principle of "artificial spores", *i.e.* the possibility to repeatedly stop and restart the cell proliferation process has also been demonstrated. Such biologically triggered release from composite microcapsules is novel and offers potentially interesting applications such as autonomous release of bactericides or fungicides only under conditions that are favorable for microbial growth.

Introduction

In biology, a spore is defined as a reproductive structure that is adapted for dispersion and survival for extended periods of time in unfavorable conditions. Once conditions are favorable, spores can develop into new organisms. The activators of such a transformation from spore to cell could be *e.g.* nutrients, temperature, pH, or combination of these parameters. There are several reasons why the spores are formed in the nature:

(i) Spores allow the organisms to survive for many (in some cases, millions of) years under adverse conditions, thus they serve as storage system for genetic information.

(ii) Spores shield cellular components in harsh conditions, and so spores have the protection function.

(iii) A spore must somehow "arrive" at a location and be there at a time favorable for germination and growth. Some spores have flagella or other organelles that serve for the species dispersion to longer distances and new areas. Therefore spores serve as transporters of genetic information.

In present work we adopted the idea of spores and created hybrid alginate microcapsules with embedded yeast cells that are long-term stable and inactive and perform specific target mission only after activation by changing the conditions in their surround. The target aim is to disintegrate and liberate and disperse the encapsulated content in proper time. The principle of such artificial spores is described in Figure 1. Under unfavorable conditions (absence of nutrients) no cell division of embedded yeast occurs and the composite microcapsules are stable in aqueous medium for extended periods of time without disintegration or release of their content. Once the microcapsules encounter favorable conditions (presence of nutrients, here provided by a culture medium), cell division and growth causes a rupture of the alginate capsule and release of the embedded components. Liposomes loaded with fluorescein represent a model "active" particulate substance that is to be liberated from the composite microcapsules. Additionally, iron oxide magnetic nanoparticles were also embedded within the composite microcapsules to facilitate their manipulation and separation by a magnetic field.

The present paper focuses mainly on problem *(i)* described above. Artificial spores were fabricated and their stability and inactivity for long time were investigated. The ability to activate in suitable conditions (in this case nutrients additions) was studied. Artificial spores cultivated in growth medium showed the ability to disintegrate and release embedded object into the surround. This mechanism acts as a biological trigger for controlled opening of the microcapsule.

Further we concentrated on the protection function of spores (task *(ii)* above). Artificial spores were coated by solid silica shell and the viability of encapsulate yeast was tested. Although the coating process does not kill the cells, the cell growth in the microcapsules was not sufficient for the microcapsule disruption. Unfortunately this way of protection shell formation seems to be unsuitable.

Such artificial spores will find applications in biologically triggered controlled delivery *e.g.* of natural fragrances or benign fungicides. Another application of these objects could be as intelligent indicators of storage quality.

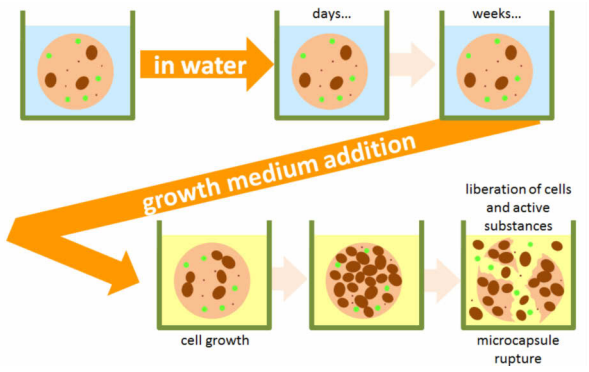

Figure 1: Schematic principle of artificial spore rupture and liberation of an active substance into the environment caused by yeast cell growth in the culture medium.

Experimental

Materials

Sodium alginate, calcium chloride ($CaCl_2$), (3-aminopropyl) trimethoxysilane (AMPS), tetramethoxysilane (TMOS), *n*-hexane, [7 - diethylaminocoumarin - 3 - carboxylic acid] (DEAC), fluorescein diacetate (FDA), yeast extract and glucose were purchased from Sigma-Aldrich. Instant yeast cells (Labeta a.s., Czech Republic) were suspended in deionized water in various concentrations (1 mg of dry powder corresponds to 3×10^7 cells). Hydrophilic iron oxide nanoparticles were prepared according to a synthesis described in (Tokárová et al., 2012). Fluorescently labeled liposomes (molar ratio of DPPC : cholesterol was 2:1) were synthesized in the same way as described in (Ullrich et al., 2013). Deionized water was produced by a ionex filter (Aqual 25).

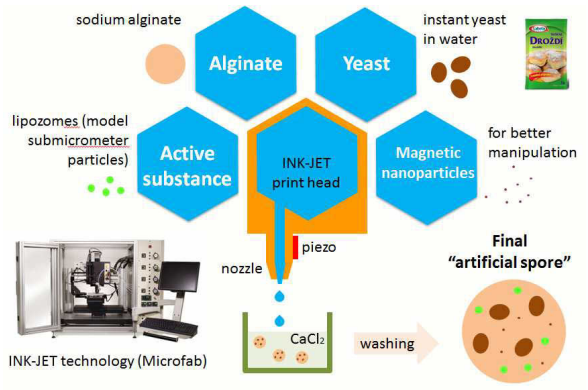

Figure 2: Schema of artificial spore preparation. Solution of sodium alginate, yeast cells, liposomes and magnetic nanoparticles is by means of Ink-Jet printing technology dropped into $CaCl_2$ solution. Precipitated calcium alginate microcapsules are subsequently washed.

Artificial spore preparation

All artificial spores were produced by inkjet printing (Dohnal and Štěpánek, 2010). A piezoelectric drop-on-demand print-head type M5-ABP-01-80-6MX supplied by Microfab, Inc. (Plano, Texas, USA) was used, coupled with a control unit type JetDrive III and a pressure controller type CT-PT-01 also supplied by Microfab, Inc. 2 ml of aqueous solutions of 2% (w/w) sodium alginate and 2 ml of aqueous suspension of yeast cells were mixed and printed into approximately 50 ml of aqueous solution of 2% (w/w) $CaCl_2$ where a rapid ionic cross-linking of the microdroplets occurred. The receiving $CaCl_2$ solution was constantly agitated to avoid microdroplet coalescence after impact. To prepare the magnetic microcapsules, one half of the cell suspension was replaced by citrate-stabilized iron oxide nanoparticle dispersion in water (15 mg/ml). The solution for printing of magnetic capsules containing liposomes was mixed from a sodium alginate solution, the cell suspension, the iron oxide nanoparticle solution and a liposome solution in the volume ratio 4:1:1:2. Cross-linked calcium alginate microcapsules were separated from the $CaCl_2$ solution by using filter or magnet and suspended in deionized water in which they were stored at room temperature until further use. In this state the composite microcapsules were stable for up to 4 months without any significant loss of yeast cell viability or leakage from the liposomes.

Coating of artificial spores by silica shell

The silica shell was formed by a sol-gel process according to our previous procedure (Haufová et al., 2012) derived from the work of Sakai (Sakai et al., 2001): the alginate microcapsules were suspended in *n*-hexane and kept at 4°C in an ice-bath. AMPS and TMOS were then added to *n*-hexane containing the alginate particles. AMPS was added first and stirred for 1 min, followed by TMOS and stirring for another 1 min. The thickness of the silica layer is influenced by the quantity of the silica precursors (AMPS and TMOS). The volume ratios of 10:14:0.8:0.6 for alginate:*n*-hexane:AMPS:TMOS were used in our case. Based on the assumption of complete hydrolysis of the alkoxysilanes and average alginate particle size of 70 µm, the resulting thickness of the deposited silica layer is 0.23 µm. The resulting microparticles were then rinse with 1.0 wt.% $CaCl_2$ solution and then kept in deionized water in a fridge for further use. All the procedures/solutions were kept cold (at 4°C) to enhance the stability of liposomes.

The synthesis of fluorescently labeled silica nanoparticles is specified in (Čejková et al., 2010) with the exception that the fluorescent dye DEAC was used instead of fluorescein isothiocyanate (FITC). For subsequent visualization of the silica layer, the pre-synthesized fluorescently labeled silica nanoparticles (SiO_2-DEAC nano) were added into the silica layer formed by the sol-gel process. 100 mg of SiO_2-DEAC nanoparticles was mixed with 1 ml AMPS for 24 hours prior the further sol-gel procedure. The mean diameter of the SiO_2-DEAC nanoparticles was 150 nm.

Artificial spore characterization

The artificial spores were characterized by means of inverted optical microscope (Olympus CK40) and a laser scanning confocal microscope - LSCM (Olympus Fluoview FV1000). The particle size was evaluated by laser diffraction (Horiba Partica LA 950/V2). The viability of yeast cells was confirmed by using the standard fluorescein diacetate solution method.

Yeast cell division and artificial spore disruption study

For a study of the cell division and disintegration of artificial spores, the composite microcapsules were placed into a Petri dish containing a culture medium (consisting of glucose in a concentration of 10 g/l and yeast extract in a concentration of 5 g/l) and monitored by an optical microscope for 24 hours. The cell growth curves were measured by means of visible spectrophotometer (Specord 205 BU, Analytik Jena, Germany); the wavelength used for the measurement of optical density was 600 nm (OD_{600}).

Results and discussion

Artificial spores characterization

The drop-on-demand inkjet technology was used for the formation of calcium alginate microcapsules with embedded yeast cells by ejecting droplets of a sodium alginate precursor into a pool of calcium chloride solution. The shape of the formed microcapsules was mostly spherical, however, some of them were distorted (flattened) due to droplet deformation upon landing into the $CaCl_2$ solution.

The viability of yeast cells in the composite microcapsules was confirmed by using fluorescein diacetate (FDA). This colorless compound exhibits no fluorescence, however, it is known that it diffuses through cell membrane and living cells are able to hydrolyze it by their enzymatic apparatus and transform FDA into fluorescein (Adam and Duncan, 2001). Typically, 2 ml of microcapsule suspension were incubated with a few droplets of FDA in acetone for 20 minutes, then washed and observed under LSCM. By this test it was proven that the cells are able to retain their viability during the ink-jet printing process. The microcapsules after one month of their fabrication and storage in water that were incubated with FDA show in LSCM images green spots. These spots correspond to living cells, which confirms that the cell viability is preserved for many weeks.

The hybrid microcapsules with embedded yeast cells were stored in water for few weeks and no microcapsule changes and cell division in capsules were observed. Radical changes occurred after incubation with growth medium containing yeast extract and glucose.

To observe the cell division of encapsulated yeast, composite microcapsules were suspended in Petri dish with growth medium and placed under microscope. Images in 1-minutes intervals were grabbed for at least 24 hours. Typical results are shown in Figure 3 for microcapsules containing yeast cells in concentration of 3.75×10^8 cell/ml.

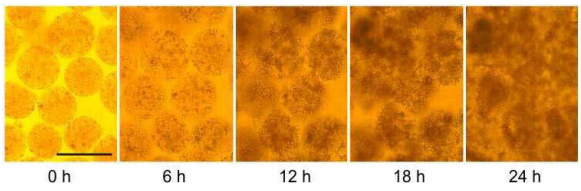

Figure 3: Yeast cell division in alginate microcapsules incubated in a Petri dish with culture medium. Concentration of yeast cells in prepared microcapsules 3.75×10^8 cell/ml. Scale bar represent 100 µm.

Figure 3 shows the changes of microcapsules in various times in static conditions in a Petri dish with cultivation medium. After fabrication the cell concentration in microcapsules corresponded to the cell concentration in alginate matrix that was printed into $CaCl_2$ solution. Time $t = 0$ h corresponds to placing of microcapsules into cultivation medium. Few hours after incubation in growth medium, buds on cells appeared and cells started to divide. Around the time $t = 7$ h, the cell division was highly developed. Around the time $t = 15$ h, alginate microcapsules were almost full of cells and at time $t = 18$ h first ruptures of composite microcapsules started. Due to intensive cell division all microcapsules disintegrate and almost no compact round microcapsules were evident at time $t = 24$ h. In the Petri dish only clusters of yeast cells were present. This experiment confirmed the idea of artificial spore – microcapsules are for long time stable and after external condition changes ruptured and released their content.

Further the growth curves of cells in alginate microcapsules were measured by using visible spectrophotometry as a function of optical density at the wavelength 600 nm. First few hours no changes were evident, because yeast cells were still in lag phase of their growth, they adapted themselves to growth conditions and rarely cells divided. About at the time $t = 7$ h, the cells entered into the exponential phase of their growth which corresponded with rapid increase in measured absorbance. Approximately until the time $t = 24$ h the increase of absorbance is evident, which shows the cell division. After the time $t = 24$ h, the absorbance did not increase, because cells entered into the stationary phase of their growth due to the lack of nutrients.

As was shown above, composite microcapsules are stable in water, whereas after cultivation in growth medium they are able to disintegrate and release their content. To confirm this fact, the liberation of fluorescently labeled liposomes was observed by laser scanning confocal microscope. Directly after their fabrication, the fluorescence signal is obtained only in microcapsules and this confirms the successful encapsulation of liposomes into microcapsules. The same microcapsules imaged one day after fabrication and storage in pure water show the fluorescence signal again only in microcapsules, no liberation of liposomes from microcapsules occurred. On the other hand, after cultivation of microcapsules in growth medium, the cells divide and cause the rupture of capsules, the liberation of all encapsulated substances and their release into the surround. After one day of incubation in culture medium no compact microcapsules were present, only the clusters of cells were evident and the fluorescence signal was detectable from whole medium because the liposomes were released during the microcapsule disintegration.

Temperature effects on artificial spore germination

In biology, a spore is defined as a reproductive structure that is adapted for dispersion and survival for extended periods of time in unfavorable conditions. The interesting property of the transformation of spores into cellular entities is as follows: once the conditions seem to be suitable for germination, the spores enter a lag phase and activate specific genes that trigger certain signal pathways leading to swelling and cell emergence. Once a spore has swollen, germination becomes irreversible, but during the lag phase activated spores can return to dormancy (Van Dijken and Van Haastert, 2001).

To cover the idea of “artificial spores”, additional experiments with switching favorable/unfavorable conditions were performed. In previous section the experiments with nutrient additions were described. It was shown, that the growth medium can start the process of cell division with consequent microcapsule rupture. We were interested if it is

possible to stop the division process anyway and then to trigger the growth again. Therefore following experiments with temperature changes were done.

The experiment mimicking spore response to conditions changes was performed in spectrophotometer equipped with heating/cooling facility. The growth curves were measured and with temperature changes compared. The experiment started at unfavorable conditions, where microcapsules were stored in water (without nutrients). The first change consisted in placing of capsules in the culture medium at temperature 30°C (corresponding to time t = 0 h in the growth curves in Figure 4). Although they had enough of nutrients and the temperature was acceptable, the first few hours the yeast embedded in microcapsule were still dormant, because they were in the lag phase. When they entered the exponentially phase of their growth, we have decreased the temperature rapidly to 8°C. Such a temperature shock stopped the cell growth resulting in a return into dormancy of microcapsules. During this time no change of optical density was observed corresponding to no division of cells. This horizontal line in the growth curve confirmed the possibility to paralyze artificial spores with temperature decrease that represents coming back of unfavorable conditions. To provide the better conditions again, the temperature was increased back to 30°C. Such a sudden suitable condition establishment lead to rapid cell growth again. The next temperature decrease caused the next interruption of cell division. The example of three repetitions heating/cooling represents Figure 4(B) shows the possibility to "freeze" the cell growth in microcapsule for almost two days.

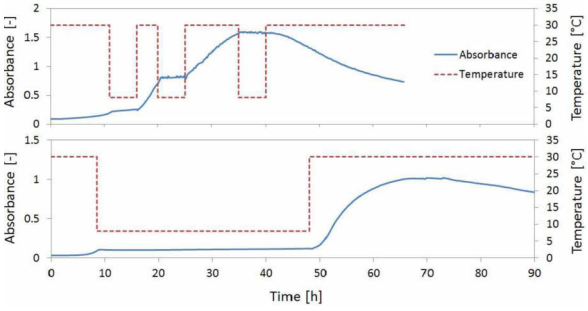

Figure 4: Interruption of yeast growth by temperature decrease and resumption of growth by increase of temperature. (A) Three interruption of cell growth by three temperature decreases (at time 11-16 h, 20-25 h, 35-40 h). (B) Long cell growth interruption for almost two days (8-48 h).

Silica shell of artificial spores

To develop the concept of artificial spore it is necessary to develop the strategy to coat the particle with any hard thin shell that could protect the encapsulated content. For this reason we decided to cover the hybrid alginate microcapsule by silica shell because of our previous experience with this process (Haufová et al., 2010).

Figure 5 represents image of microcapsules covered by thin fluorescently labeled silica layer (it is visualized by blue color). Green spots correspond to viable encapsulated yeast cells visualized by using FDA as described in previous sections. It was proven that the sol-gel coating process in harsh conditions (temperature 4°C, in *n*-hexane solution) does not kill all the cells and some cells are still viable. It can be concluded that at least some of the yeast cells were able to survive both the inkjet printing and the silica coating processes, although there seems to be also a fraction of dead (non-fluorescent) cells. However the coating lead to microcapsule shrinkage (*ca* to 30 µm compared to 80 µm original diameter).

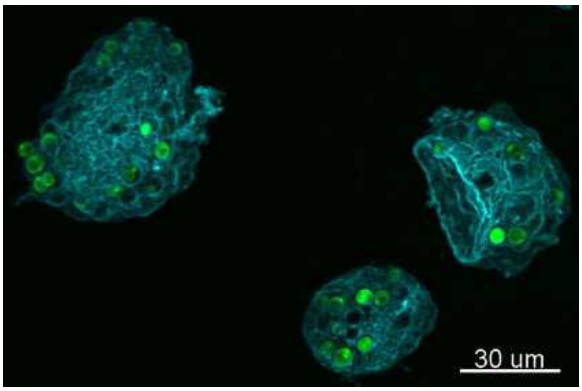

Figure 5: Laser scanning confocal microscopic images of alginate microcapsules with encapsulated yeast cells (viable cells visualized by green color) and covered by fluorescently labeled silica shell (blue color).

Further we focused on cell division in silica-coated microcapsules. Figure 6 summarized the results of silica-coated artificial spores' cultivation in growth medium. It is evident, that yeast cells entrapped on the surface of microcapsules are able to divide and growth freely in surrounding medium, however the growth of cells inside the microcapsule is not sufficient to microcapsule disruption and following rupture. Unfortunately these results exclude the sol-gel silica coating method as a suitable process for protecting surface layer formation. Our future work will focus on other cover techniques, such as layer-by-layer method.

Dry artificial spores

All experiments described above were performed in aqueous conditions. Artificial spores were fabricated and stored in aqueous solutions and never during their studies were dried. Following test focused on the properties changes after particle drying.

The artificial spores were air dried and Figure 7 represents microscopic images of their rehydration. In (A) dry shrunken microcapsules are displayed. Figure (B) shows the result of microcapsule incubation in water for 1 hour. Dry alginate is not able to swell in pure water and microcapsule retain in the shrunken state. On the other hand, growth medium containing various ions is able to facilitate the microcapsule swelling (Figure C). Unfortunately, cell division in rehydrated artificial spores was not sufficient for microcapsule rupture. This observation recommends the artificial cells applications in hydrated state without any drying step.

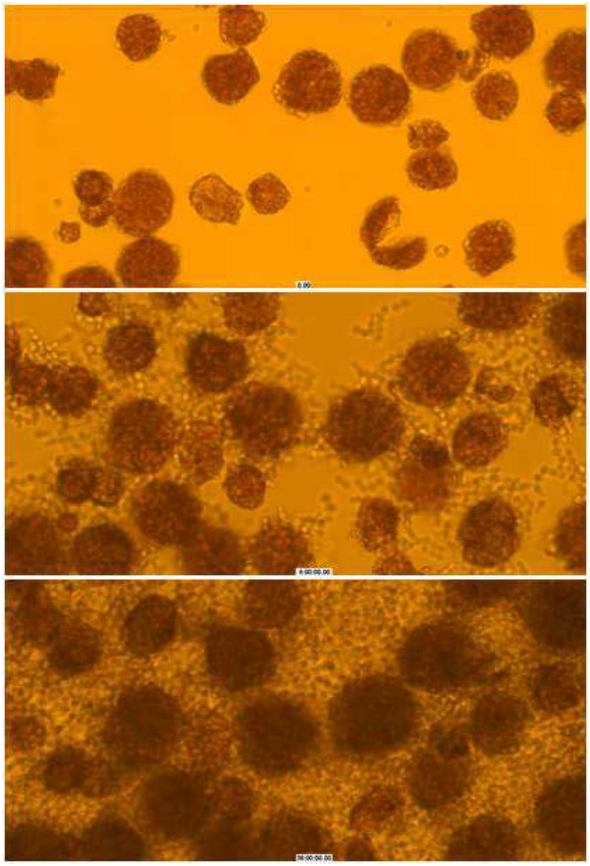

Figure 6: Cultivation of silica-coated artificial spores in growth medium. Optical microscope images from times t = 0, 6 and 36 hours after placing into the medium.

Conclusions

We fabricated a new type of microcapsules with embedded microorganisms that can locally liberate sub-micrometer objects, and act as a biological trigger for controlled opening of the microcapsule. Such a hybrid microparticle cover the idea of artificial spore, that is inactive for long time without any changes and start to rupture after external condition changes (here nutrient addition). Artificial spores prepared here were able to liberate encapsulate model substance in form of liposomes. It was shown, that silica coating by means of so-gel process does not allow the microcapsule rupture as in the case without any shell presence. Further it was observed, that also dried artificial spores are not able to rupture after rehydration in cultivation medium.

Our future work will focus on microcapsule covering to form a shell that will protect the alginate and encapsulated objects and subsequently will not prohibit the particle rupture. Because the rupture of capsules is also impossible for particles that were dried, we will concentrate to find applications for artificial spores in aqueous solution, such as in biologically triggered controlled delivery, *e.g.* of natural fragrances or benign fungicides or as quality indicators.

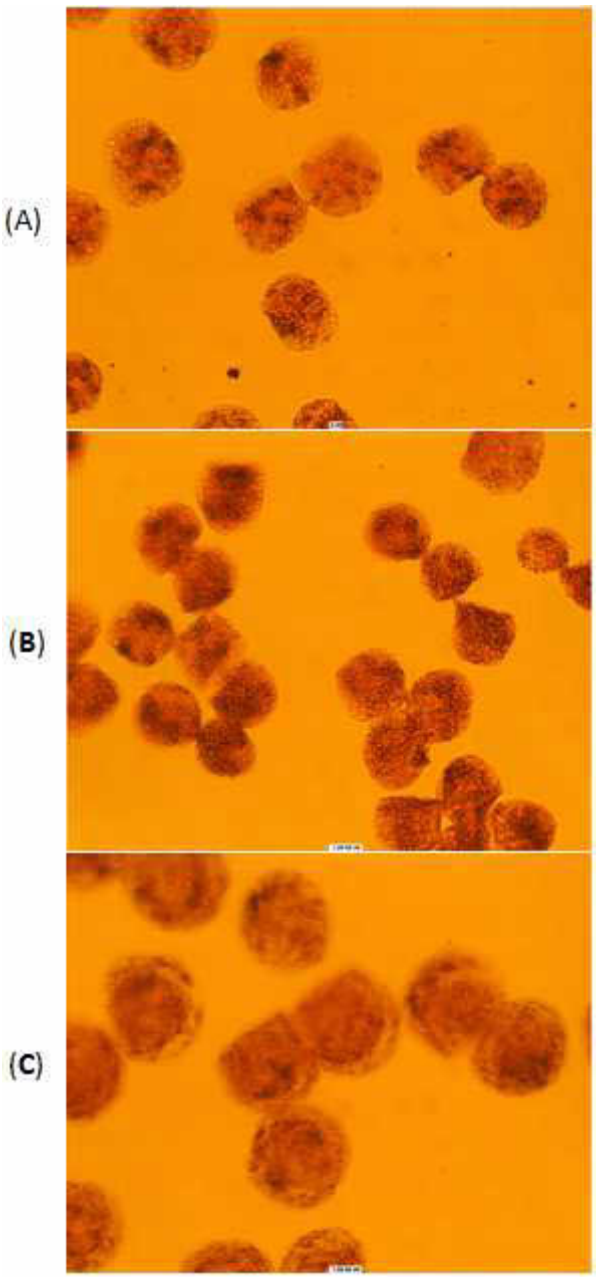

Figure 7: Microscopic images of rehydration of dry artificial spores (A) in water (B) and in growth medium (C) after 1 hour.

Acknowledgments. Thanks Jaroslav Hanuš for fluorescently labeled liposomes. Financial support from the European Research Council (grant number 200580-Chobotix) is gratefully acknowledged.

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