J|A|C|S ARTICLES

Published on Web 06/25/2009

Light-Driven Release from Polymeric Microcapsules Functionalized with Bacteriorhodopsin

Svetlana Erokhina,[†] Lucia Benassi,[‡] Paolo Bianchini,[§] Alberto Diaspro,[§] Victor Erokhin,[*,‡,||] and M. P. Fontana[‡,||]

Department of Biochemistry and Molecular Biology, University of Parma, Viale Usberti 23 A, 43100 Parma, Italy, Department of Physics, University of Parma, Viale Usberti 7 A, 43100 Parma, Italy, LAMBS-MicroSCoBiO Research Center, Department of Physics, University of Genoa, Via Dodecaneso 33, 16146, Italy, and CRS SOFT CNR-INFM, Rome 00185, Italy

Received April 17, 2009; E-mail: victor.erokhin@fis.unipr.it

Abstract: Bacteriorhodopsin was incorporated into the shell of polymeric capsules. Light-driven variation of intercapsule volume pH with successive pore opening was demonstrated by scanning electron microscopy. Release of the encapsulated dye molecules was studied by confocal fluorescence microscopy.

1. Introduction

Nanotechnological approaches are intensively used now in different branches of science and technology. In particular, biomedical applications of nanoparticles are widely discussed.[1]

Nanoengineereed polymeric capsules (NPCs)[2−4] have recently become a quite active research subject due to several outstanding and practically unique features they possess. In fact, even if there are several established methods for the encapsulation of materials, NPCs, reported for the first time in 1998,[2] offer several useful functional possibilities.

The method is based on the application of the polyelectrolyte self-assembly technique, known also as layer-by-layer (LbL) assembling,[5,6] for the formation of an organic polymer shell on nonplanar, usually spherical, templates with successive dissolving of the template particle. Thus, a hollow polymeric container with the diameter from several tens of nm to several micrometers and with the shell thickness of some nm can be formed. An important feature of the system is that the shape and sizes of the resultant container are very well determined by the shape and sizes of the templates. Thus, the shape and volume of the microcontainer can be driven not only by the thermodynamically preferential configurations, but can also be controlled, to some extent, by the preparation procedure of the desired particular system.

Furthermore, it is not useful to fabricate a container without the possibility to fill it with desirable substances or molecules. Fortunately, NPCs provide such possibility. The great breakthrough in this field took place when it was shown that it was possible to open and close pores in the shells by varying the pH and/or the composition of the surrounding solution.[7,8] Immediately after this finding, NPCs were considered as very interesting objects for several "smart" activities such as smart drug release. In fact, being encapsulated, the drug can be released only in areas where, for example, there is local pH decrease. Targeting of the capsules can be also realized by several methods. Rough delivery can be performed by the application of external magnetic field, using capsules with magnetic particles in their shell,[7] whereas precise targeting can be realized by the attachment of special receptor molecules to the outer side of the shell.[9]

However, from the practical point of view there are limitations of the NPCs application in medicine. In fact, automatic release can be done only in areas with appropriate pH and/or different composition of the surrounding liquid substance. Very few real diseases provide such possibilities. Thus, it would be useful to modify the capsules in order to have the possibility of pores opening in their shell by some external action which is not hazardous for the patient. Illumination by light is a good candidate for such action. Several attempts were carried out for the modification of capsules with light-sensitive molecules. Very interesting results were obtained when polymer derivatives of azobenzene were incorporated in the capsule shell.[10] Azobenzene is known to exist in cis and trans configurations in dark and light conditions, respectively.[11] It was shown that the illumination of capsules results in the serious variations of the capsule diameter.

[†] Department of Biochemistry and Molecular Biology, University of Parma.
[‡] Department of Physics, University of Parma.
[§] University of Genoa.
[||] CRS SOFT CNR-INFM.

(1) Singh, R.; Lillard, J. W. *Exp. Mol. Pathol.* **2009**, *86*, 215.
(2) Donath, E.; Sukhorukov, G. B.; Caruso, F.; Davis, S.; Möhwald, H. *Angew. Chem., Int. Ed.* **1998**, *37*, 2202.
(3) Ibarz, G. L.; Dähne, L.; Donath, E.; Möhwald, H. *Adv. Mater.* **2001**, *13*, 1324.
(4) Antipov, A. A.; Sukhorukov, G. B. *Adv. Colloid Interface Sci.* **2004**, *111*, 49.
(5) Decher, G. *Science* **1997**, *277*, 1232.
(6) Lvov, Y.; Ariga, K.; Kunitake, T. *J. Am. Chem. Soc.* **1995**, *117*, 6117.
(7) Sukhorukov, G. B. In *Novel Methods to Study Interfacial Layers*; Möbius, D., Miller, R.; Eds.; Elsevier Science V.B.: Amsterdam, 2001; p 384.
(8) Déjugnat, C.; Sukhorukov, G. B. *Langmuir* **2004**, *20*, 7265.
(9) Sukhorukov, G. B.; Brumen, M.; Donath, E.; Möhwald, H. *J. Phys. Chem. B* **1999**, *103*, 6434–6440.
(10) Bedard, M.; Skirtach, A. G.; Sukhorukov, G. B. *Macromol. Rapid Commun.* **2007**, *28*, 1517–1521.
(11) Dante, S.; Advincula, R.; Frank, C. W.; Stroeve, P. *Langmuir* **1999**, *15*, 193.

 10.1021/ja903066s CCC: $40.75

Another application of light for the triggered release of encapsulated substances was found with laser irradiation.[12] It was shown that absorbed laser light was transferred into the local heating of the capsules resulting in the release of the encapsulated material. However, in some cases such local heating can be not desirable as it can affect also some internal organs and create some side effects on the patients.

Near IR laser illumination was used for the variation of the shell permeability of membranes containing metal nanoparticles[13,14] and IR dye.[14] However, in both cases local heating can occur.

Recently, the possibility was shown to vary permeability of composite organic−inorganic capsule shells by UV irradiation.[15] However, in many cases UV can be also very undesirable.

Finally, a very interesting application of the light activation of hollow capsules for capture of bacteria with their successive destruction[16] was reported.

Further development of light-sensitive capsules can be the incorporation of the material that can create local pH variation when illuminated into the capsule shell, expecting that light-induced acidity of the internal medium in the capsule will be enough for the pores opening. Bacteriorhodopsin (BR) is a well-known membrane protein working as a proton pump creating a light-driven pH gradient between intracellular and extracellular parts of purple bacteria.[17] Interesting optical and electrical properties together with its extreme stability have resulted in numerous works, both fundamental and applied, on this material.[18,19] Very high photovoltage response was observed in BR layers oriented in an external electric field.[20] For the present study it is important that oriented BR-containing layers could be assembled by the LbL technique.[21] Reported photoresponse measurements have demonstrated the applicability of the method and the existence of a preferential orientation of BR in the film. Thus, the illumination results in the unidirectional proton transfer by the layer. To our knowledge, however, there are no works where BR was incorporated into the shell of NPCs. Thus, the aim of the present work was to realize NPCs with BR layer in the shell and to study their properties in dark and light conditions. The 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt was used as a material for encapsulation and release, and measurements were performed using confocal fluorescence microscopy. Morphology of the capsule surfaces was investigated using scanning electron microscope (SEM).

2. Materials and Methods

Capsules were prepared in the following way. Five μ colloidal $CaCO_3$ particles (PlasmaChem GmbH, Germany) were used as templates for the shell formation, containing alternate layers of polyalylamine hydrochloride (PAH) and polystyrene sulfonate (PSS). Both PAH and PSS were from Sigma-Aldrich. The shell was formed according to a well established procedure.[22] Eight layers were deposited before the BR layer deposition. BR from *Halobacterium halobium* was from Fluka. Befor deposition, BR was dissolved in 50 mM buffer solution (pH 9.4) and sonicated in ultrasonic bath for 5 min. The layer of BR was formed on the PAH last layer. The templates with preformed shell were placed into 1 mg/mL solution of BR (pH 9.4) shaking for 5 min. As the particles were rather heavy, no centrifugation was applied. Deposition of the layer and precipitation of the particles took place in about 20−30 min. Templates were dissolved by adding 0.01 M of HCl.

For this study, 8-Hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (HPTS) (Sigma-Aldrich) was used as a dye. The dye is stable and its absorption peaks are at 403 and 454 nm.[23] Loading with the dye was performed by leaving the formed NPCs in the HPTS solution (0.025 mg/mL, pH 3.0) for 24 h with continuous shaking at 300 rpm/min. Afterward, the capsules were precipitated and the water was substituted in the sample by buffer with pH 9.4 or 7.6.

Fluorescence imaging was performed on an inverted Leica TCS SP5 AOBS confocal laser scanning microscope (Leica Microsystems CMS, Mannheim, Germany) equipped with a set of lasers covering the 458-, 476-, 488-, 496-, 514-, 543-, and 633-nm lines. Confocal fluorescence imaging was done on these samples at 23 °C. Images were collected using a Leica 63PL APO NA 1.40 oil immersion objective (Leica Microsystems CMS, Mannheim, Germany). Images were obtained using the 488 nm and 543 nm laser lines. Under this imaging configuration, typical confocal resolution is of the order of 150 nm in the lateral and 500 nm in the axial direction.

Investigations were performed on BR-containing hollow capsules (in order to study penetration of the dye molecules) and on capsules already loaded with dye molecules (in order to study the release of the substance).

SEM images were acquired with Zeiss SUPRA 40 instrument at 1 kV and 10 kV accelerating voltage. Measurements were performed on samples prepared by drying of diluted solutions of BR-containing capsules in dark and light conditions.

3. Results and Discussion

Initially, hollow capsules were investigated. NPCs solution was imaged before and after adding to it dye solution. Two capsule solutions were studied. The structure of the capsule shell was identical for both samples. The only difference was that for sample 1 the pH of the solution was equal to 7.6, whereas for sample 2 it was 9.4.

Images of sample 1 measured with confocal fluorescence microscopy before and after the dye solution adding are shown in Figure 1a and b respectively. Image shown in Figure 1b was acquired about 10 s after adding the dye solution.

Difference in the capsule darkness can be connected to the fact that the measurements were performed in the liquid phase which can result in the different relative distance between the microscope objective and individual capsules.

Images of sample 2 before and after adding of the dye solution are shown in Figure 2a and b, respectively. Image shown in Figure 2b was acquired about 10 s after adding the dye solution.

(12) Skirtach, A. G.; Javier, A. M.; Kreft, O.; Kohler, K.; Alberova, A. P.; Möhwald, H.; Parak, W. J.; Sukhorukov, G. B. *Angew. Chem., Int. Ed.* **2006**, *45*, 4612.
(13) Skirtach, A. G.; Karageorgiev, P.; Bédard, M. F.; Sukhorukov, G. B.; Möhwald, H. *J. Am. Chem. Soc.* **2008**, *130*, 11572.
(14) Skirtach, A. G.; Antipov, A. A.; Shchukin, D. G.; Sukhorukov, G. B. *Langmuir* **2004**, *20*, 6988.
(15) Katagiri, K.; Koumoto, K.; Iseya, S.; Sakai, M.; Matsuda, A.; Caruso, F. *Chem. Mater.* **2009**, *21*, 195.
(16) Corbitt, T. S.; Sommer, J. R.; Chemburu, S.; Ogawa, K.; Ista, L. K.; Lopez, G. P.; Whitten, D. G.; Schanze, K. S. *ACS Appl. Mater. Interfaces* **2009**, *1*, 48–52.
(17) Gutman, N.; Nachliel, E. *Annu. Rev. Phys. Chem.* **1997**, *48*, 329–356.
(18) Birge, R. R. *Ann. Rev. Phys. Chem.;* **1990**, *41*, 683–733.
(19) Oesterhelt, D.; Brauchle, C.; Hampp, N. *Q. Rev. Biophys.* **1991**, *24*, 425–478.
(20) Kononenko, A. A.; Maximychev, A. V.; Lukashev, E. P.; Chamorovsky, S. K.; Rubin, A. B.; Chekulaeva, L. N. *Biochim. Biophys. Acta* **1986**, *850*, 162.
(21) Chu, J.; Li, X.; Zhang, J.; Tang, J. *Biochem. Biophys. Res. Commun.* **2003**, *305*, 116–121.
(22) Sukhorukov, G. B.; Donath, E.; Lichtenfeld, H.; Knippel, E.; Budde, A.; Möhwald, H. *Colloids Surf., A* **1998**, *137*, 253–266.
(23) Hakonen, A.; Hulth, S. *Anal. Chim. Acta* **2008**, *606*, 63.

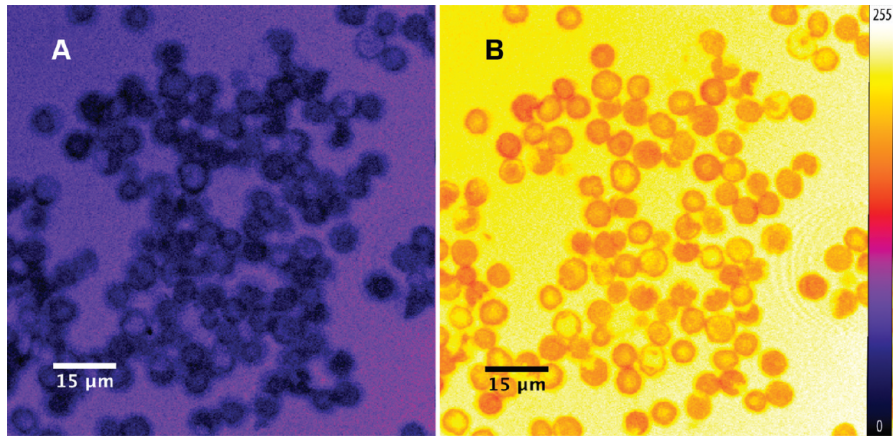


Figure 1. Confocal fluorescence microscopy images of BR-containing capsules solution at pH 7.6 before (a) and after (b) adding the dye solution.

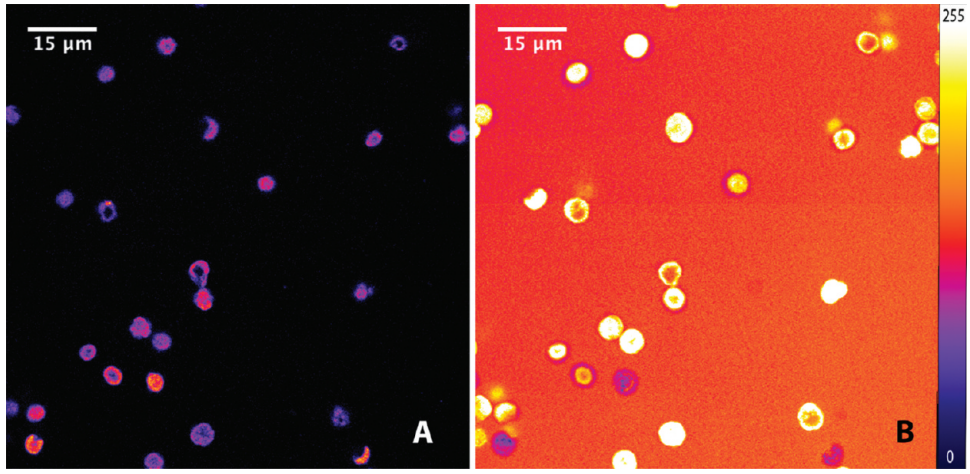


Figure 2. Confocal fluorescence microscopy images of BR-containing capsules solution at pH 9.4 before (a) and after (b) adding to it dye solution.

It is interesting to compare these images. In the first case (Figure 1b) we can see only small penetration of the dye molecules into the capsule's volume, whereas in the second case it is much more pronounced. The capsules in both cases are identical and the only difference is in the pH of the solution. It is to note that if these capsules were constructed without BR in their shells, there must be less pores in the second case (pH is 9.4, whereas it is 7.6 in the first case) and the first capsules should be more permeable to the dye molecules.[8] Instead, experimentally, we have observed the reverse situation. In order to explain this fact we must recall that pH 9.4 is an optimal condition for the BR function. Thus, we can suppose that in both cases BR provides proton pumping through the capsule shell resulting in the local pH variation in the capsule volume. This pH variation is responsible for the pore opening and induced permeability of the shells. In the second case BR activity is higher and the variation of internal pH and increased shell permeability were reached even if the environmental conditions were not preferential for the spontaneous pore opening.

Next, experiments were performed on loaded capsules studying the dye release under the illumination. Two similar capsules were considered. Their images in fluorescent (a) and standard optical (b) microscopies are shown in Figure 3.

Capsule 1 was illuminated locally with green light (543 nm), close to the main absorption peak of BR. Kinetics of the fluorescence intensity variation in time was analyzed in three different areas of the image: inside capsule 1, inside capsule 2,

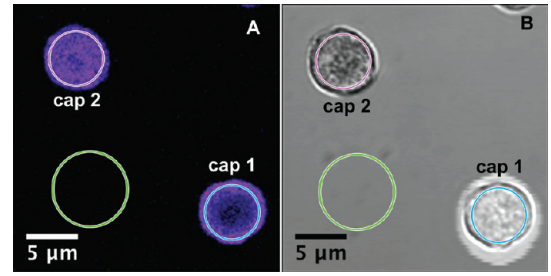


Figure 3. Confocal microscopy images of capsules loaded with HPTS: (a) is fluorescence shown in false color; (b) is the transmission. In the following experiment the capsule 1 was illuminated locally with 543 nm laser line.

and in the area outside both capsules (background). The results of the measurements are reported in Figure 4.

Figure 4 clearly demonstrates the release of the dye from the capsules. First of all, we can see a slow increase of the background intensity due to the increase of the dye concentration in the extracapsular space. Comparison of the kinetics of the intensity variation for illuminated and not illuminated capsules shows that the release in the case of illuminated capsule is more effective. Fitting of the experimental data with exponential decay results in practically the same time constant (25 s) but different coefficients. In the case of the illuminated capsule this coefficient is about twice that of the one not illuminated. The presence of the release in the case of the not illuminated capsule can be

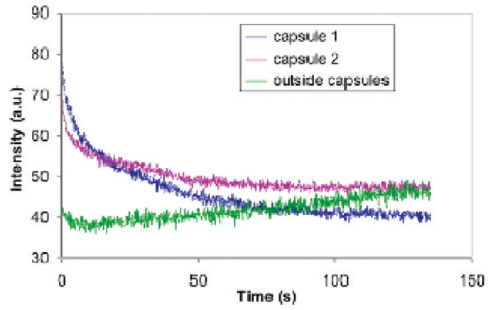


Figure 4. Kinetics of the fluorescence intensity variation measured inside capsule 1 (illuminated with green light), capsule 2 (not illuminated), and outside both capsules. The colored traces reflect the variation of the mean intensity value calculated inside the colored regions in Figure 3.

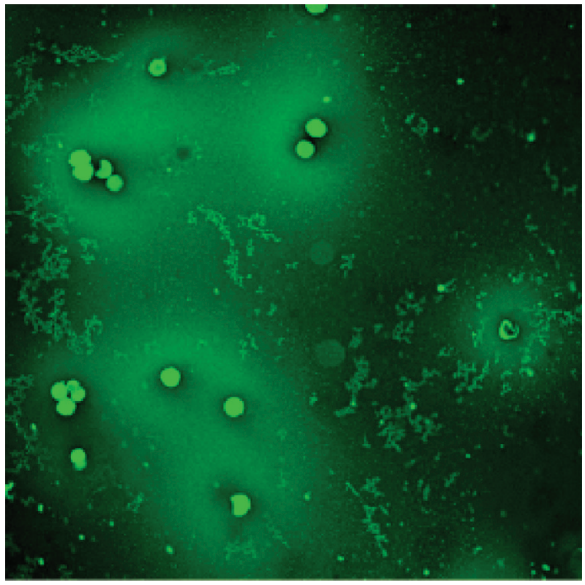


Figure 5. Confocal fluorescence microscopy image of the dye-loaded BR-containing capsule solution dried under the illumination with white light for 3 min.

explained by the scattering of the exciting light and some side effects due to the fluorescence of shell-constituting material (BR, PAH, PSS). The last contribution can be rather significant. In fact, images obtained in the fluorescent mode on the samples never exposed to light reveal the confinement of the fluorescence intensity only in capsule areas and practically zero intensity outside. However, after a couple of minutes of the experiment we have observed an increase of the fluorescence intensity outside the capsules, indicating light-induced functioning of BR. Such behavior cannot be due to the aging of the capsules. Being

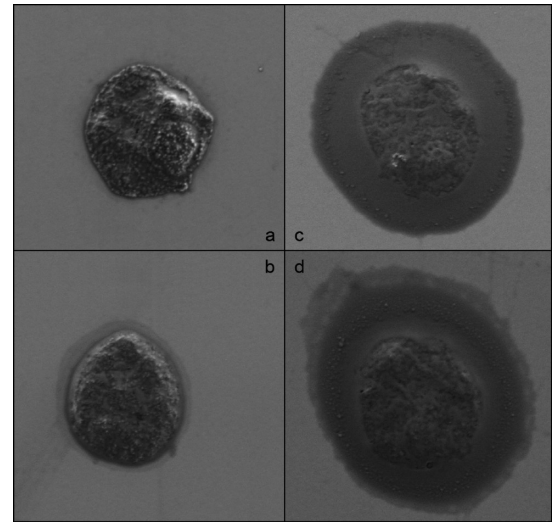


Figure 6. SEM images (1 kV) of dye loaded capsules dried in dark (a, b) and light (c, d) conditions.

stored in the dark, no release of the material was observed for several months.

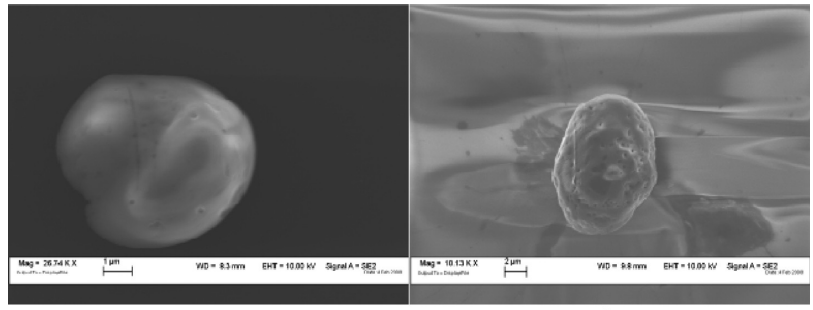
The profile of the distribution of the released dye was studied on the capsules, dried under illumination conditions. In the liquid, the dye during the release propagates in all three dimensions, which makes difficult to quantify this process by fluorescence microscopy. Thus, the sample was prepared in the following way. Small volume (about 5 μL) of capsule-containing solution was dropped on the cover glass support and dried under the illumination with white light for 3 min. The resultant image is shown in Figure 5.

The image demonstrates the effective release of the dye and its propagation to distances of about 3−5 times more than the capsule diameter (about 10 μm).

Comparison of the dye release in capsules dried in the dark and light conditions was also performed using a scanning electron microsocope. Typical images of the particles, loaded with the dye and dried in dark conditions are shown in Figure 6a and b. Typical images of the capsules dried under day light illumination are shown in Figure 6c and d.

Most of the capsules dried in dark conditions have revealed no dye leakage after drying (Figure, 6a). In a few cases, a small insignificant leakage was observed (Figure 6b). Instead, in the case of the capsules dried under the illumination, the effective release was observed for all studied capsules.

Finally, the possibility of the light induced pore opening was studied by electron microscopy. SEM images (10 kV) of BR-



a b

Figure 7. SEM images of BR-containing capsules dried in dark (a) and illuminated (b) conditions.

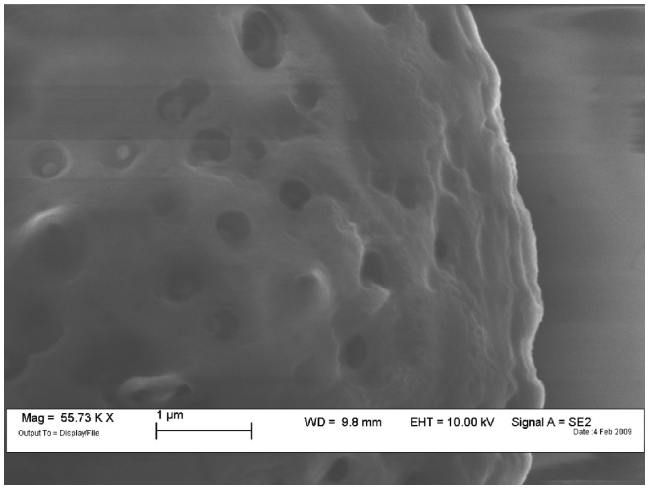


Figure 8. SEM image of a part of the capsule dried in light conditions.

containing capsules dried in dark and illuminated conditions are shown in Figure 7 a and b respectively.

As it is clear from the images, the illumination has really led to the opening of significant amount of pores in the capsule shell and deformation of the capsule shape. Therefore, it is possible to conclude that significant local pH gradient was realized by the light-induced proton pumping, as the surrounding solution pH was 9.4, while it is necessary to reach pH less than 6.0 for the pores opening.

Average diameter of pores is an important parameter that can be useful for the prediction of the release rate. A higher resolution SEM image (10 kV) of the capsule, dried in light conditions is shown in Figure 8.

Analysis of the image shown in Figure 8 allows us to estimate an average pore diameter of about 400 nm.

4. Conclusions

This work is the first attempt to incorporate BR molecules into the NPCs shell and to use its property of light-driven proton pumping for the creation of the local pH variation, necessary for the pore opening and the modification of the shell permeability. It was shown that induced shell permeability and encapsulated material release can occur even under the illumination with low light intensities (daylight illumination). The obtained results can be useful for the design of smart drug releasing systems, where even low intensity of the excitation light will result in the triggered release without any local heating of the body which could be a cause of undesirable side effects.

Acknowledgment. The work was supported by regional project SITEIA of Emilia Romagna (Italy).

JA903066S