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Drug Delivery Systems

Organic–Inorganic Nanospheres with Responsive Molecular Gates for Drug Storage and Release**

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During the last decade, one important objective within the field of biocompatible materials has been the preparation of systems capable of storing and gradually administering active molecules.[1–3] Owing to their versatility, mainly organic systems were employed, for example micelles, liposomes, cyclodextrines, or polymeric nanoparticles.[4,5] Interesting results related to drugs encapsulation have been obtained using liposomal phases.[6] Liposomes are vesicles formed by one or more concentric spheres of lipidic layers separated by water molecules.[7] Owing to their peculiar composition, liposomes are highly effective systems for the encapsulation of active biomolecules (hydrophilic or lypophilic) by interaction with the aqueous or the phospholipidic phase which complement their structure. However, liposomes and, in general, all organic systems used for encapsulation reveal limitations related to their hydrothermal or chemical stability and to the fact that they are quickly attacked and removed by the immunological system.[8–10] Silica nanoparticles were also employed for storage of active molecules, which were incorporated directly during synthesis. In fact, their biocompatibility and stability towards external agents makes them attractive systems.[11,12] These siliceous particles loaded with bioactive molecules were synthesized with a combination of different preparation methods,[13–15] such as hydrolysis and condensation, spray-drying, or emulsion methods, while sol–gel technology was most commonly used.[16–18] This methodology is a very simple technique of inorganic polymerization at room temperature, using neutral silicate precursors as initial agents.[19–24] However, although the methodologies employed to date allow for the precise control of the silica particle size, other problems arise when the internal active molecules are released. Indeed, owing mainly to the porosity of the siliceous matrix and the size of the active molecule, the release is often difficult.

To avoid these problems, hollow silicon nanoparticles with mesoporous external walls and containing active molecules were prepared by using surfactant molecules during the synthesis.[25–28] With this methodology, the diffusion of internal molecules to outside the nanospheres was improved, because the external surface accessibility of these spherical solids was increased.[29] However, even within these systems, problems were observed, as the mesoporous nature of the external walls exhibits a very low hydrothermal stability. Moreover, it was very difficult to control the release of the internal drugs because of the diffusion through the mesopores was a continuous process.[30] To overcome this problem, different studies have been carried out in which the external mesoporous shells were functionalized with other organic groups to reduce the free diameter of the pores, thus controlling the delivery of the bioactive drugs.[31–33] Unfortunately, the results with these methodologies have not to date been satisfactory.[34,35]

Herein, we report the synthesis of a new hybrid organic–inorganic solid with spherical morphology where active compounds are encapsulated. More specifically, these nanospheres are composed of a purely organic internal liposomal phase in which bioactive molecules are encapsulated. Covering this part, we have constructed an external self-assembled organic–inorganic shell comprising covalently connected organic fragments and silica units. This organosilica shell could stabilize the internal liposomal phase and, consequently, isolate and protect the drug molecules. By properly selecting the organic counterpart of the organic–inorganic shell, the spheres, which are designed to be stable in the blood stream and biocompatible, will respond to a particular chemical interaction of the tissues and cells in which the drug is to be delivered. Through this chemical interaction the organic moieties of the external organic–inorganic shell will be broken, thus allowing the drug to be liberated.[36]

We illustrate this general concept by introducing an ester as the organic component of the external organic–inorganic shell. The ester groups are selected in such a way that they are stable at the pH value of blood but they are hydrolyzed by esterase-type enzymes present in the cells. As an interesting pharmaceutical agent active against different tumors, we have used doxorubicin (a drug that acts by intercalating in DNA and is anticarcinogenic), which was trapped and isolated in the internal liposomal phase during the synthesis of the hybrid nanoparticles. The potential of these nanosystems has been confirmed by different in vitro tests, which show how the spheres loaded with doxorubicin enter into human glioma cell cultures and then release the active compound.

The synthesis of hybrid nanospheres was carried out in two stages: a) the preparation of liposomes with encapsulated doxorubicin by means of emulsion techniques from lecitine in a chloroform/water system, and b) formation of an organic–

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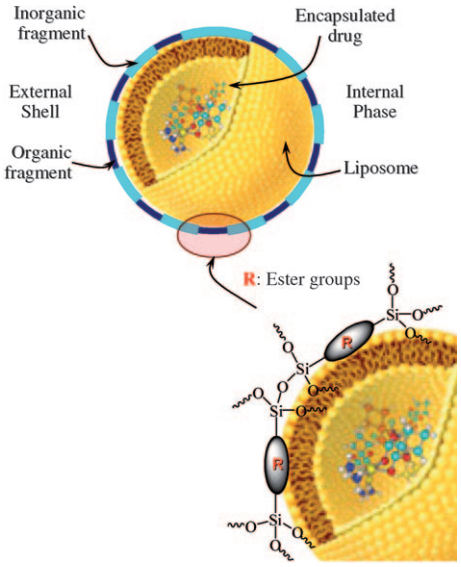
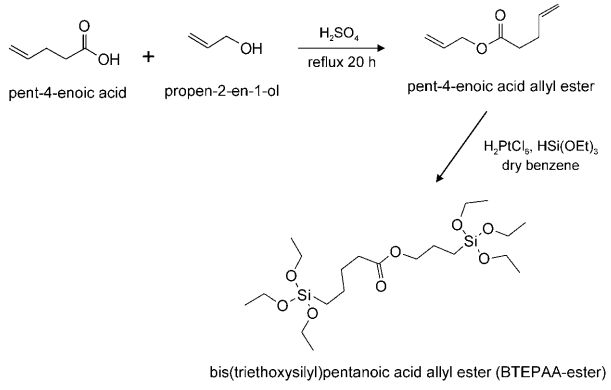


Figure 1. Artistic representation of nanospheres describing their different parts.

inorganic shell around the liposomes, which is formed by ester fragments bonded covalently to silica units. The preparation route is shown in Figure S1 in the Supporting Information. Figure 1 shows an artistic representation of the hybrid nanospheres with a detailed description of the different parts.

An ester-bridged silsesquioxane (BTEPAA, Scheme 1) was employed as the organic–inorganic precursor to synthesize different hybrid nanospheres with ester groups in their external shells. The spherical morphology and topology were observed by transmission electronic microscopy (TEM), and most of the nanospheres were in the range of 80 to 200 nm diameter (Figure S2 in the Supporting Information). In Figure 2, one of these nanospheres is shown in detail, for which the external organic–inorganic shell and the liposomal phase can be observed.

The X-ray diffractograms (not shown) and textural properties indicate that the hybrid systems are amorphous and nonporous. The incorporation of organic ester groups into the external silica framework was monitored by thermogravimetric analysis (TGA; Figure S3 in the Supporting



Scheme 1. Synthesis of the bridged silsesquioxane precursor with ester groups as organic linkers (BTEPAA-ester).

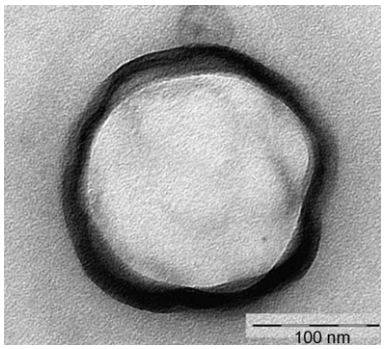


Figure 2. TEM image of one isolated nanosphere showing the internal and external parts.

Information). The presence of internal liposomes forming the internal part of the hybrid nanospheres is clearly corroborated from the TGA of a sample with a purely siliceous external shell (Figure S3a in the Supporting Information). In this case, only one peak is observed at approximately 340 °C, assigned to the loss of the liposomes, which correspond to 33 % of the total weight. When ester groups are incorporated into the external shell by means of the bridged silsesquioxane precursors, the TGA displays a second weight loss at approximately 400 °C (Figure S3b in the Supporting Information). The estimated amount of ester groups in the total weight of the sample is 7 %, consistent with the elemental analysis of the sample. Taking into account the carbon content and the results from TGA, 13 % of silicon atoms are functionalized by ester groups.

The ^{13}C CP/MAS NMR spectrum of the organic–inorganic nanospheres is shown in Figure S4a in the Supporting Information. The peak assigned to the carbon atom directly involved in the ester group in the organic linker appears at 172 ppm (inset, Figure S4 in the Supporting Information), thus confirming that the organic fragment remains intact as in the initial BTEPAA-ester silsesquioxane reagent.[37] It appears that effective incorporation of silicon-bonded carbon species derived from the silicon sources has occurred to form the network of the external shells. The assignment of the other carbon atoms from the starting silsesquioxane is not possible, because the intensity of the peaks is much lower than those of the liposomes, the concentration of which in the nanospheres is higher than that of the ester units. The ^{13}C CP/MAS NMR spectrum of hybrids with a purely siliceous external shell clearly shows the bands assigned directly to liposomes (Figure S4b in the Supporting Information).

While ^{13}C NMR spectroscopy confirmed that the organic fragments preserve their integrity during the synthesis, ^{29}Si MAS NMR spectroscopy was required to confirm that the organic ester groups not only remain intact but also are covalently bonded to silica units in the external shell. The ^{29}Si CP/MAS NMR spectra of hybrid nanospheres obtained with the BTEPAA-ester as the organic–inorganic precursor exhibit characteristic bands around −60 ppm (Figure S5a in the Supporting Information), assigned to T-type silicon species, that is, silicon species having a Si–C bond.[38] More exactly, three bands are detected at −53, −59, and −66 ppm, corresponding to T^1 ($C–Si(OH)_2(OSi)$), T^2 (C–Si(OH)-

$(OSi)_2$), and T^3 ($C–Si(OSi)_3$), respectively. This finding definitively confirms the presence of ester-modified silicon species in these materials. Figure S5a in the Supporting Information also shows one band at −47 ppm arising from not condensed silsesquioxanes. Furthermore, ^{29}Si MAS NMR spectra show three bands at −91, −102, and −111 ppm assigned to Q^2 ($Si(OH)_2(OSi)_2$), Q^3 ($Si(OH)(OSi)_3$), and Q^4 ($Si(OSi)_4$) units, respectively, that originate from the hydrolysis and condensation of tetraethylorthosilicate (TEOS) molecules with the terminal alkoxy groups present in the silsesquioxanes. These results confirm the integrity of the organic ester groups in the nanospheres and their covalent connection to the inorganic silica units to generate the external shells of these loaded systems.

Further confirmation of the complete incorporation of organic linkers into the frameworks of hybrid spheres came directly from ^{29}Si NMR spectra of initial BTEPAA (Figure S5b in the Supporting Information). The pure disilane exhibits only one peak characteristic of silicon atoms centered at approximately −46 ppm. However, when these organic linkers are finally incorporated into the external hybrid framework of nanospheres, the signal corresponding to silicon atoms bonded to carbon units is shifted to approximately −60 ppm (Figure S5a in the Supporting Information), thus confirming the practically complete integration of the disilanes within the material.

To evaluate the amount of doxorubicin encapsulated within the nanosystems and the rate of release of the drug, different experiments were carried out introducing aliquots of the hybrid solid into buffered aqueous solutions with different and controlled pH values from pH 2.0 to 12.0, over 48 h. After these acidic or basic treatments, the solutions were recovered by filtration and analyzed by UV/Vis spectroscopy to determine the concentration of doxorubicin delivered upon acidic hydrolysis or basic saponification of the ester groups. Figure S6 in the Supporting Information shows the percentage of doxorubicin delivered (Figure S6a in the Supporting Information) and the amount of doxorubicin released per gram of sample (Figure S6b in the Supporting Information) as functions of pH value. The results indicate that this type of ester group is very stable at acidic and neutral pH values (such as the physiological pH of 7.5), and only at $pH \geq 10$ does saponification the ester fragments in the external shell occur, thus allowing the complete release of the bioactive drug, as approximately 90% of the initially encapsulated doxorubicin is released after 48 h.

As a control, nanospheres loaded with doxorubicin molecules but with a purely siliceous external shell were synthesized, using only TEOS as silicon precursor and employing the same experimental conditions as described above for bridged silsesquioxanes. Delivery tests at different pH values (from 2.0 to 12.0) showed that the nanosystems were not affected by the pH value and that no release of doxorubicin was detected by UV/Vis spectroscopy. We can then conclude that the presence of predesigned organic linkers that are sensitive to specific external conditions (chemical, photochemical, thermal, etc.) in the external shell of the nanosystems is a decisive parameter to obtain versatile solids for storage and release of bioactive molecules.

To check the real usefulness of the hybrid nanospheres synthesized loaded with doxorubicin in biomedical applications, they were introduced into human glioma cells. The presence of esterases in the cells should favor the breaking of external ester groups of the outer shells, allowing the release of the internal drug molecules. For this experiment, human glioma cell cultures were treated with free doxorubicin and with hybrid spheres loaded with doxorubicin (3 wt%) with the aim of testing in vitro chemotherapeutic effects of the drug. After treatment, culture viability was studied using different approaches: flow cytometry, fluorescence inverted microscopy, and MTT (3,4,5-dimethylthiazol-2,5-diphenyltetrazolium bromide) cytotoxicity assay.

The results obtained from flow cytometry show that the cell density values for nanospheres with doxorubicin decrease with increasing drug concentration, reaching mortality rates of 44% with respect to 90% achieved using free drug (see Table S1 in the Supporting Information). The dose response curves confirm that the hybrid materials are capable of causing cell death. The lower mortality rates for hybrid materials compared with the free drug could be attributed to diffusion problems of nanospheres into the cell cultures owing to the formation of small aggregates. Nevertheless, the mortality values achieved with the nanospheres can be considered very good, taking into account literature reports for other types of drug delivery systems based on solids.[39–44]

The study of absorbance measurements from MTT assays (Figure S7 in the Supporting Information) obtained for glioma cultures shows significant differences ($p > 0.05$) between controls, free drug samples, and organic–inorganic spheres (7 μM). However, insignificant differences were observed between free drug samples and hybrid nanospheres. These results are in agreement with dose response curves and corroborate the cytotoxicity of hybrid materials with stored doxorubicin.

Finally, fluorescence images definitively demonstrate that nanospheres enter into the cells, and doxorubicin molecules are released as a consequence of the breaking of external ester groups by esterase enzymes in the cells. Figure 3b shows an image with red cell silhouettes produced as a result of doxorubicin intercalation into nuclear and mitochondrial DNA.[45] Furthermore, the merging of phase-contrast and fluorescence images (Figure 3c) reveals the location of hybrid nanospheres and the liberation of drug molecules into the human cells, thus confirming the effectiveness of the delivery nanosystem presented herein.

Novel organic–inorganic nanospheres formed by a hybrid organosilica shell and an internal liposomal core containing bioactive molecules have been successfully synthesized. The external shell including labile silsesquioxanes linkers containing ester functional groups provides stability to the system at the typical pH value of the blood stream (pH 7.5), but the ester groups are broken within the glioma cells, thereby liberating the drug. We have shown that the nanoparticles, stable at physiological pH values, can be used to store and release bioactive molecules, such as doxorubicin, which is effective in the clinical treatment of cancer cells. With in vitro experiments, it has been observed that the presence of esterase enzymes in the cells leads to the generation of open

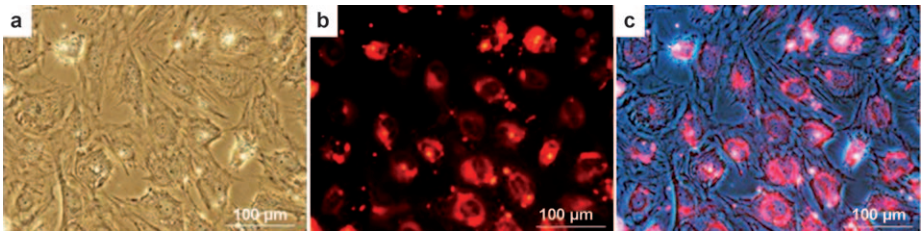


Figure 3. Images obtained from fluorescence-inverted microscopy of 42-MGBA human flioma cell culture in the presence of hybrid nanospheres (7 μM) loaded with doxorubicin (3 wt%): a) phase-contrast image, b) fluorescence image, and c) merged phase-contrast and fluorescence images.

gates on the external shell by saponification, thus favoring the release of the encapsulated drug to the outside and counteracting the carcinogenic effect. By combining the nanoparticle delivery system described herein with organic linkers responsive to different pH values and to thermal, photochemical, or chemical stimuli, a large variety of delivery systems can be prepared. These properties, combined with the fact that target-directing molecules can be easily attached to the external silica surface of the material, can increase the specificity of this system for drug delivery.

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