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# Functionalized bridged silsesquioxane-based nanostructured microspheres: ultrasound-assisted synthesis and in vitro cytotoxicity characterization

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**Abstract** Different kinds of polymers have been employed in medicine as biomaterials for different purposes. In recent years, considerable attention has been focused on the development of new drug-delivery systems in order to increase bio-availability, sustain, localize and target drug action in the human body. The versatility of the sol–gel processing to synthesize nanostructured materials and the possibility of incorporating organic molecules into the matrix of the final hybrid material, represent a novel and attractive path to the synthesis of new functionalized hybrid biomaterials with advanced properties. In this work, acetylsalicylic acid (ASA)-functionalized hybrid microspheres based on bridged silsesquioxanes synthesized via ultrasound-assisted sol–gel processing, were characterized. An investigation concerning the cytotoxic response of these new microspheres on CHO-K1 cells was accomplished based on ISO 10993-5 standard (Biological Evaluation of Medical Devices). Microspheres incorporating ASA showed a cytotoxic effect when pure extracts of the microspheres were analyzed, however, they strongly diminished their cytotoxicity as the extracts were diluted. When a 10% concentration extract was employed, hybrid microspheres were shown to be non cytotoxic. These results are promising for considering these novel functionalized organic–inorganic microspheres as potential drug-carriers to be employed in drug delivery-related applications.

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## 1 Introduction

In the last years considerable attention has been focused on the development of new drug-delivery systems in order to increase bio-availability, sustain, localize and target drug action in the human body [1, 2]. Resorbable polymer poly (ε-caprolactone), polyanhydrides and lactide/glycolide homo- and copolymers (PLA/GA) have been used in the majority of studies as new biodegradable materials. Polymer microparticles (for instance, monolithic devices in the form of microspheres) have been often employed as supports to deliver drugs, however, with some formulations the amount of drug really absorbed is difficult to control and release kinetics is often quite variable. In this way, microencapsulation of drugs has been proposed in order to solve, at least partially, these problems. Some devices concerning the encapsulation of drugs are largely described in the literature [3–7].

In addition to traditional polymeric materials, well-defined spherical particles of different nature have attracted great attention since they have many applications in advanced technologies, for instance, on biosciences field. If a patterned structure can be generated inside these spheres, some new properties which can not be obtained from simple spheres (for example, stepwise drug-release), are expected. It is well known that block copolymers able to self-assemble can lead to patterned structures in bulk [8], in solution [9] as well as on a surface [10]. However, there are a number of different polymeric materials that offer the possibility of obtaining ordered structures after polymerization. Among these materials, bridged silsesquioxanes are

a family of organic–inorganic hybrid materials which has been recognized to have an enormous potential as building blocks for various advanced nanostructured devices, including those related to biomedical applications [11]. These silica-based bridged hybrids are obtained via hydrolysis and condensation of monomers containing an organic bridging group covalently bonded to terminal trialkoxysilyl or trichlorosilyl groups [12, 13]. Depending on the nature of the bridge, which could exhibit self-assembling properties, different degree of organization at molecular level can be achieved during the hydrolytic condensation of the precursors. In this way, the bridging group can direct the formation of three-dimensional networks [13], supramolecular-assembled films [14], among others. In a previous work [15], we have reported the synthesis of a bridged silsesquioxane monolithic material from a bridged precursor containing a pendant cyclohexyl fragment in the organic bridge. The presence of this fragment led to a hybrid material exhibiting a structure based on elongated organic channels accommodating the pendant group. The presence of functional groups in the organic channels was used to retain acetylsalicylic acid (ASA), employed as anti-inflammatory model drug, inside the generated cavities. This development allows us to plan the desired architecture for a particular material, incorporating organic molecules (for instance, drugs) to synthesize new hybrid devices which could potentially be employed on biomaterials field. On this area diverse techniques have been used to obtain organic–inorganic nano and microparticles for different purposes, among which the oil/water solvent evaporation method [16] and vigorous stirring of the precursors for long periods [17, 18] are two of the most commonly employed ones.

In the last years, the chemical applications of ultrasound have become an exciting new field of research [19], the chemical effects of ultrasound being sorted out into three main areas: homogeneous sonochemistry of liquids, heterogeneous sonochemistry of liquid–liquid or liquid–solid systems, and sonocatalysis. Ultrasonic irradiation differs from traditional energy sources in duration, pressure and energy per molecule because of the cavitation bubble collapse, which means the formation, growth and implosive collapse of bubbles in the liquid. Applications of ultrasound to the synthesis of biomaterials are under rapid development [20]. While the chemical effects of ultrasonic irradiation on aqueous solutions have been studied for many years, the development of aqueous sonochemistry for biomaterials synthesis is very recent. The area of protein microencapsulation has proved to have especial interest. Microencapsulation has important applications; these include diverse uses, among which drug-delivery systems and medical diagnostic agents have been reported [21]. One example is the use of high intensity ultrasound to prepare aqueous suspensions of long-lived proteinaceous microspheres filled with air or water-insoluble liquids for medical applications [22]. However, this technique has not been employed yet in the sol–gel reaction of bridged silsesquioxanes with self-assembling capability, aiming at the synthesis of nanostructured functionalized biomaterials. The versatility of the sol–gel processing to synthesize nanostructured materials and the possibility of incorporating organic molecules into the matrix of the final hybrid material, in combination with the ultrasonic irradiation technique, represent a novel and attractive path to the synthesis of new functionalized organic–inorganic biomaterials with advanced properties.

The aim of this study was to synthesize hybrid microspheres of bridged silsesquioxanes via ultrasound-assisted self-assembly of two different bridged precursors containing pendant groups in the organic bridges. The main objective of using two different pendant organic groups was to obtain hybrid microspheres made up of cavities of different nature for incorporating ASA as anti-inflammatory model drug. In order to employ these new microspheres as potential drug-carriers in advanced biomedical technologies, in vitro cytotoxicity tests were performed on the synthesized materials based on the ISO 10993-5 standard ("*Biological Evaluation of Medical Devices*").

To the best of our knowledge, this is the first report on the ultrasound-assisted preparation and in vitro characterization of bridged silsesquioxane-based microspheres containing functionalized host cavities to be used as potential drug-carriers on biomedical applications.

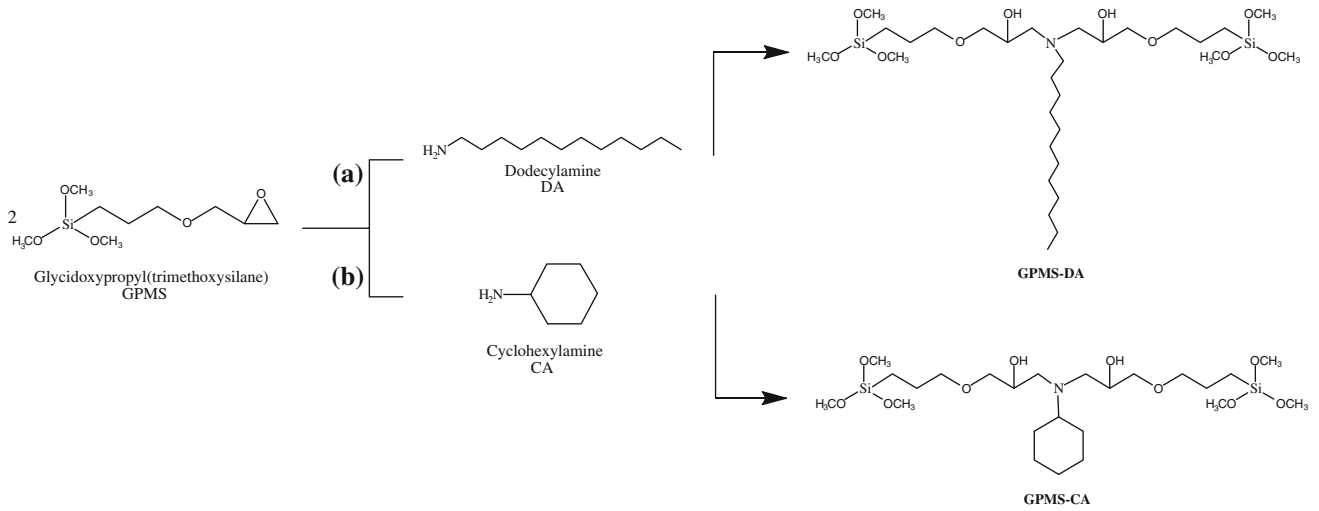
## 2 Materials and methods

### 2.1 Reagents

Glycidoxypropyl(trimethoxysilane) (GPMS, Dow Corning, 99%, density at 20°C = 1.07 g/ml), cyclohexylamine (CA, Merck, 99%, density at 20°C = 0.87 g/ml), dodecylamine (DA, Fluka, 98%, density at 20°C = 0.80 g/ml), formic acid (FA, Cicarelli, 88%, density at 20°C = 1.20 g/ml), acetylsalicylic acid (ASA, Parafarm, 100%), tetrahydrofuran (THF, Cicarelli, 99%, density at 20°C = 0.89 g/ml), and *n*-hexane (*n*-Hex, Sintorgan, 99%, density at 20°C = 0.66 g/ml), were used without further purification.

### 2.2 Synthesis of the bridged precursors

Two different bridged monomers were synthesized from GPMS and two primary aliphatic amines (DA and CA, see Scheme 1). The stoichiometric reaction between GPMS (2 mol) and the employed amines (1 mol) was selected in order to synthesize two precursors containing a long alkyl




**Scheme 1** Bridged monomers (precursors of the hybrid materials) synthesized from: (**a**) GPMS (2 mol) + DA (1 mol), and (**b**) GPMS (2 mol) + CA (1 mol)

chain pendant from the organic bridge, on the one hand; and a cyclohexyl fragment, on the other hand. Details of the syntheses of the bridged precursors were described elsewhere [15, 23].

### 2.3 Synthesis of the functionalized hybrid microspheres

The hydrolytic condensation of the bridged precursors was accomplished in a mixture of THF/*n*-Hex (1:2 volumetric blend), using an aqueous solution of FA as catalyst, in the following molar ratios: FA/Si = 3 and $H_2O$/Si = 1.05. In a typical synthesis, 3.889 g of the prepared monomers (GPMS-DA and GPMS-CA, see Scheme 1) were separately dissolved in 100 ml of reaction solvent mixture in glass vessels (5 cm diameter × 7.5 cm height). After that, 1.77 ml of the 88 wt% FA solution was added dropwise to each solution with continuous application of ultrasonic irradiation. A 6-mm diameter tip Sonic Vibra-Cell (130 W/20 kHz) was employed as irradiation source with 50% power intensity. Solvents and volatile products were continuously evaporated from the open vessels. The processes were ended (at 90 s) when a white suspension appeared in the reaction medium. The obtained solids were filtered and the remaining solvent evaporated at 80°C. The resulting powders were placed in a Petri dish and heated in an oven at 110°C for 3 h. The final products were fine yellow glassy powders. The products were named GDTH (GPMS-DA-THF-*n*Hex) and GCTH (GPMS-CA-THF-*n*Hex), respectively.

ASA was used as a molecular probe to analyze the possibility of incorporating the drug into the microspheres during the hydrolytic condensation of the precursors. Its addition was performed by dissolving it in the THF/*n*-Hex mixture and adding this solution to each precursor in a molar ratio ASA/Si = 0.5 (in a typical synthesis 1.216 g of ASA were employed). The hydrolysis and condensation stages were performed by employing the same procedure used to synthesize the microspheres without drug. The final products were yellow glassy powders. The products were named GDTH-A (GPMS-DA-THF-*n*Hex-ASA) and GCTH-A (GPMS-CA-THF-*n*Hex-ASA), respectively.

### 2.4 Characterization techniques

Morphologies were observed by scanning electron microscopy (SEM), employing a Jeol JXA-8600 microscope after coating the samples with a thin gold layer.

Fourier transformed infrared (FTIR) spectra were recorded with a Genesis II-Mattson device in the absorbance mode, in the range 400–4000 $cm^{-1}$ with a resolution of 2 $cm^{-1}$. Spectra were obtained using pellets of the microspheres with KBr.

1D $^{29}Si$ MAS NMR spectra were measured using a Bruker Avance 500 WB/US spectrometer at MAS frequency $\omega_r/2\pi = 10$ kHz and $B_1(^{13}C)$ field intensity $\omega_1/2\pi = 62.5$ kHz. $B_1(^1H)$ field intensity of TPPM (two-pulse phase-modulated) decoupling corresponds to $\omega_1/2\pi =$ 89.3 kHz. Single-pulse experiments were used with 45° pulse length (2 μs) and 60 s repetition delay. The $^{29}Si$ NMR scale was calibrated by external standard $M_8Q_8$ (−109.8 ppm; the highest field signal).

Small-angle X-ray scattering (SAXS) was performed on the microspheres using a pinhole camera (Molecular Metrology SAXS System) attached to a microfocused X-ray beam generator (Osmic MicroMax 002) operating at 45 kV and 0.66 mA (30 W). The camera was equipped

with a multiwire, gas-filled area detector with an active area diameter of 20 cm (Gabriel design). Two experimental setups were used to cover the $q$ range of 0.007–1.1 $Å^{-1}$ where $q = (4\pi/\lambda)\sin\theta$ ($\lambda$ is the wavelength and $2\theta$ is the scattering angle).

Thermogravimetric analyses (TGA) were performed with a Shimadzu TGA-50 thermal analyzer under argon atmosphere at a heating rate of 10°C/min, from room temperature to 800°C.

### 2.5 In vitro cytotoxicity procedure

Because of cell culture toxicity tests are the international standards for biocompatibility screening, in vitro cell cultures were performed to evaluate the biocompatibility of the hybrid microspheres (with and without ASA). Testing of cytotoxicity was performed by adding extracts of the hybrid microspheres to Chinese hamster ovary cells (CHO-K1) cultured in vitro following stablished protocols [24].

#### *2.5.1 Preparation of microsphere extracts*

0.61 g of each material (GDTH, GCTH, GDTH-A and GCTH-A), previously sterilized by gamma radiation (25 kGy), were mixed with 6.1 ml of culture medium (EMEM with 1% glutamine, penicillin and streptomycin) and incubated for 48 h at 37 ± 1°C in 5% $CO_2$ atmosphere. The supernatant was then filtered through a Millipore membrane (0.22 μm pore size) and three different extract concentrations (100, 25 and 10%) were prepared. Dilutions were performed with sterilized culture medium supplemented with 10% bovine fetal serum. The pH of each solution was registered before and after filtration.

#### *2.5.2 Cytotoxicity testing*

CHO-K1 cells (from ABAC, Argentine Cells Bank) were cultured in appropriate medium under two different conditions: monolayer and freshly suspended cells. When monolayers were used, the culture medium from the subconfluent monolayer was removed and discarded, and an aliquot of the extract (100 or 10%) was added. On the other hand, when suspended cells were employed, the extract (100, 25 or 10%) was immediately added after preparation of the cell suspension. In all cases, the samples were placed in an incubator for 72 h at 37 ± 1°C in 5% $CO_2$ atmosphere. Finally, cultures were examined and photographed by using an inverted Olympus CK-40 microscope.

#### *2.5.3 Determination of cytotoxicity*

Changes in general morphology, vacuolization, detachment, cell lysis and membrane integrity were assessed and compared with a blank (sample not exposed to extracts) on fresh and stained cells. Cytotoxicity was determined according to the following scale: cytotoxicity degree (CD) = 0 (non cytotoxic), 1 (mildly cytotoxic), 2 (moderately cytotoxic) and 3 (severely cytotoxic), based on ISO 10993-5 standard [24].

## 3 Results

### 3.1 Morphology of the hybrid materials

Morphologies and surface features of the organic–inorganic materials were evaluated by scanning electron microscopy observations. Figure 1 shows the SEM images corresponding to the GDTH and GCTH hybrid microspheres.

### 3.2 Conversion to Si–OH and Si–O–Si bonds

$^{29}$Si NMR spectra provide valuable information related to the degree of condensation of the silsesquioxane materials, which is a fundamental parameter to be taken into account when evaluating the possibility of incorporating organic molecules (via hydrogen-bonding recognition strategy) into the final hybrid matrix. Figure 2 depicts the $^{29}$Si NMR spectra obtained for the GDTH and GCTH hybrid microspheres.

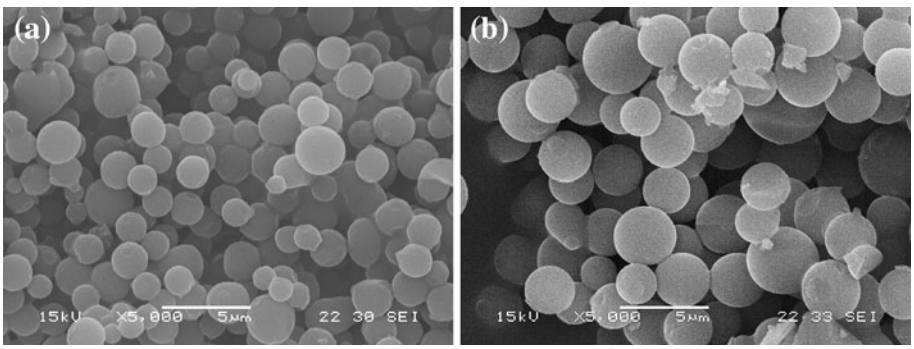



**Fig. 1** SEM images of the hybrid microspheres: (**a**) GDTH, and (**b**) GCTH

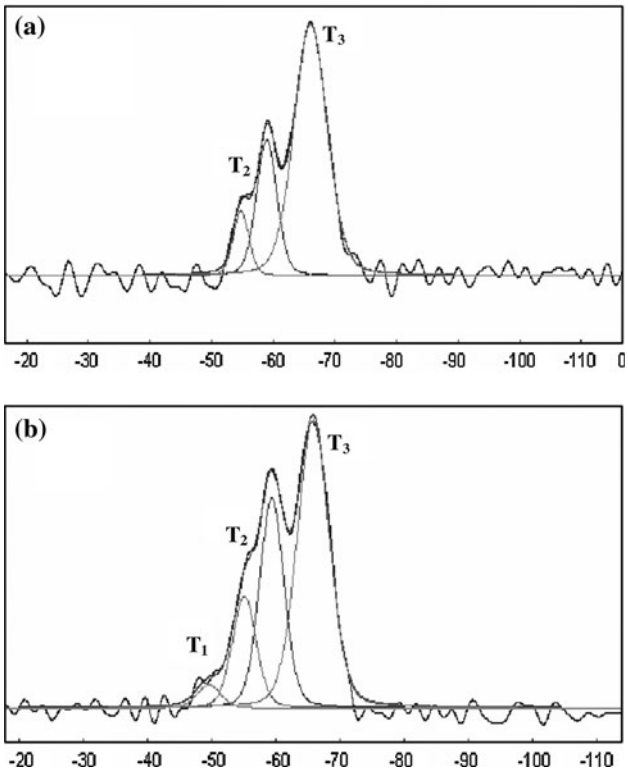



**Fig. 2** $^{29}$Si NMR spectra corresponding to synthesized hybrid microspheres: (**a**) GDTH, and (**b**) GCTH

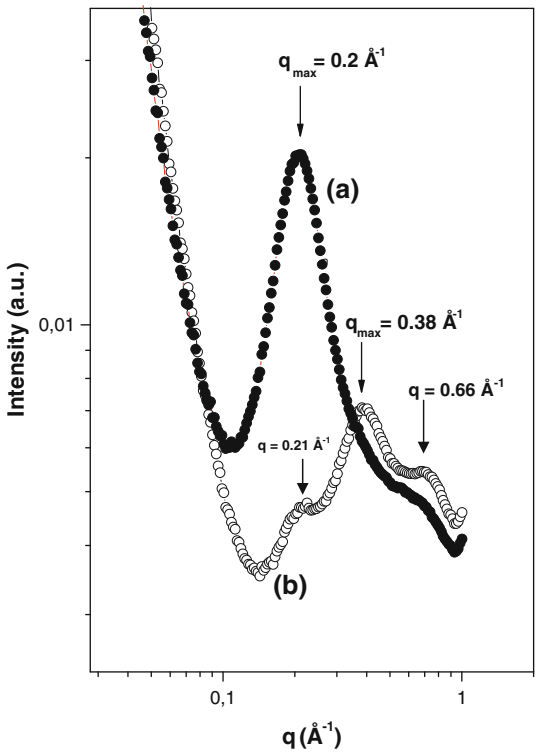



**Fig. 3** Small angle X-ray scattering (SAXS) spectra corresponding to: (**a**) GDTH, and (**b**) GCTH

### 3.3 Nanostructuring of the microspheres

In order to evaluate the existence of some degree of organization at molecular level into the developed hybrid materials, SAXS measurements were performed on the synthesized microspheres. Figure 3 depicts the obtained SAXS spectra corresponding to the GDTH and GCTH microdevices.

### 3.4 Incorporation of acetylsalicylic acid as anti-inflammatory model drug

The main purpose of employing bridged silsesquioxane precursors to synthesize the hybrid microspheres is related to the possibility of obtaining different architectures at molecular level to incorporate organic molecules (for instance, drugs) into the final hybrid matrices, to make use of the organic–inorganic microspheres as potential drug-carriers and delivery devices. The presence of acetylsalicylic acid (ASA) into each nanostructured hybrid material was evaluated by FTIR. Figure 4 shows the FTIR spectra corresponding to the GDTH and GDTH-A microspheres.

Figure 5 depicts the TGA diagrams corresponding to GDTH and GDTH-A materials. From the obtained profiles it was possible to determine the total amount of ASA incorporated during the ultrasound-assisted sol–gel method. This amount was calculated to be 14.5 wt%. As to the GCTH-A material (not shown), the total amount of ASA incorporated into the microspheres was calculated to be 19 wt%.

### 3.5 Cytotoxicity results

It is well known that the cytotoxicity of nano and microparticles frequently differs from that of the bulk materials that they are derived from. In most cases the reason of such a toxicity is unknown, however some results have demonstrated that specific characteristics such as the high surface area to volume ratio of the particles or their surface chemistry could be the main reasons for such a cytotoxic effect. In order to employ the synthesized hybrid microspheres as biomaterials (for instance, as drug-delivery devices), cytotoxicity tests were performed on the four synthesized organic–inorganic materials (with and without ASA) following stablished protocols.

Figure 6 shows microphotographs corresponding to CHO-K1 cells exposed to the extract of the GDTH microspheres. The two first lines correspond to fresh cells observed under two different magnifications (4× and 40×). The third line corresponds to cells fixed and stained with hematoxiline (observed at 20× magnification). Figure 7 summarizes the cytotoxicity results obtained for all tested microsphere samples.

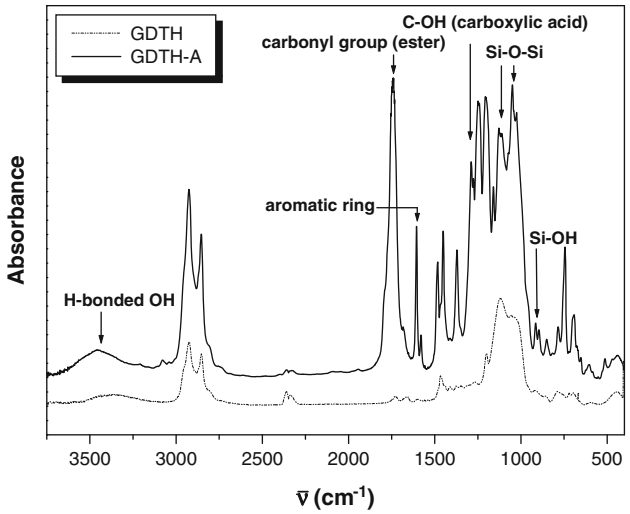



**Fig. 4** FTIR spectra of the microspheres synthesized with and without ASA

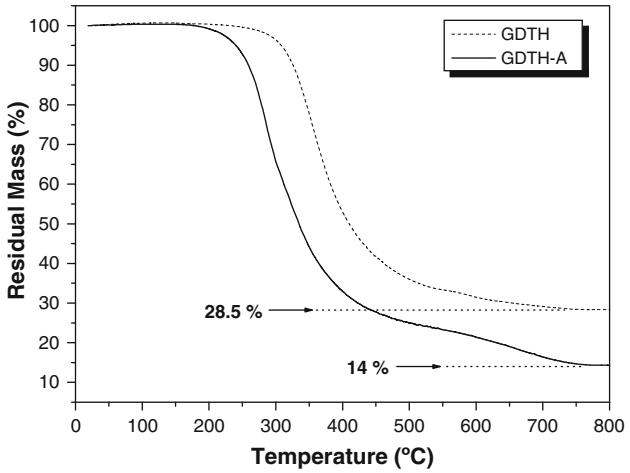



**Fig. 5** TGA profiles corresponding to GDTH and GDTH-A microspheres

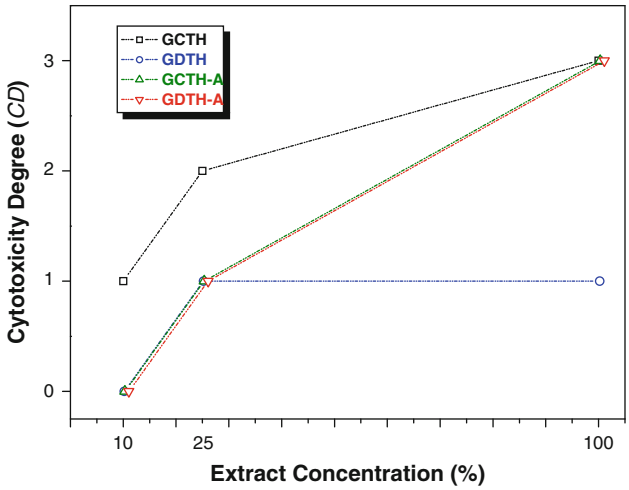



**Fig. 7** Cytotoxicity degree as a function of extract concentration corresponding to all tested samples (dotted lines were drawn for clarity)

## 4 Discussion

Considering the SEM images (Fig. 1) obtained from the GDTH and GCTH hybrid materials, in both cases individual microspheres with a very low polydispersity were observed. A mean particle size of 2.4 ± 0.1 μm corresponding to GDTH and 3.4 ± 0.2 μm corresponding to GCTH, was obtained by image analysis. The microspheres (in both formulations) showed a smooth surface without any apparent microporosity. The microspheres synthesized in the presence of ASA showed a similar size and morphology as those obtained in the absence of drug.

The degree of condensation of the silsesquioxane materials was evaluated by $^{29}$Si NMR measurements. In the spectrum corresponding to the GDTH sample (Fig. 2a) three signals were obtained from the deconvolution process. The main peak located at −66 ppm is characteristic of $T_3$ species: Si–(O–Si)$_3$ [25], while the two peaks

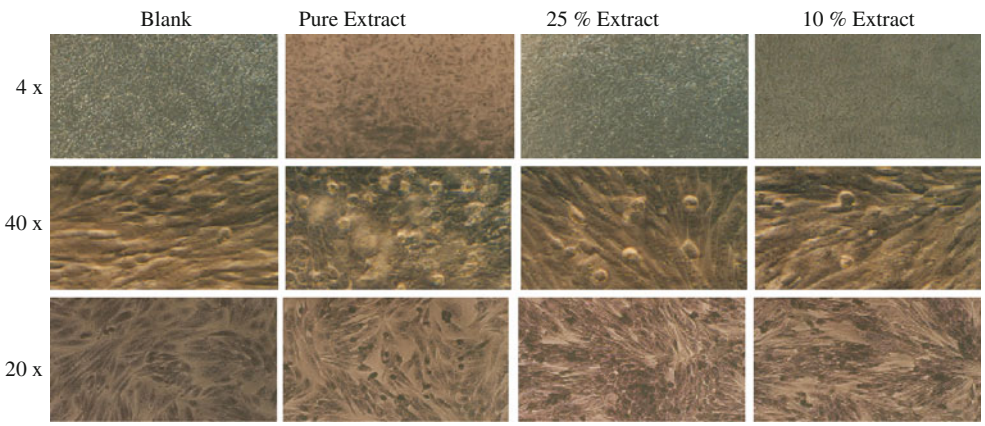



**Fig. 6** CHO-K1 cells exposed and not exposed (blank sample) to extracts of GDTH microspheres

observed at −54.8 and −58 ppm were assigned to $T_2$ structures: Si–(O–Si)$_2$(OH) [26]. No peaks corresponding to $T_1$ and $T_0$ structures were detected. The area under the $T_3$ peak represented 68.5% of the total area, whereas the contribution of the two $T_2$ peaks was 31.5%, which led to a total conversion to siloxane bonds (Si–O–Si) of 89.5% after the hydrolytic condensation of the bridged monomer (GPMS-DA). On the other hand, in the spectrum corresponding to the GCTH microspheres (Fig. 2b), in addition to the three mentioned peaks, a small peak located at −49.6 ppm was attributed to inorganic $T_1$ species: Si–(O–Si)(OH)$_2$ [27]. The area of this peak was calculated to be 3.2% of the total area. Taking into account the area corresponding to the four signals detected for this sample, a conversion to Si–O–Si bonds of 83.1% was calculated.

In summary, both kinds of microspheres led to an incomplete condensation after the hydrolysis and polymerization processes, which means that a number of silanol groups (Si–OH) remained unreacted. It has been already demonstrated [28] that the presence of Si–OH groups on the surface of glasses and bioglasses makes these materials to behave as bioactives when immersed in physiological fluids (or simulated body fluids, SBF). These Si–OH groups act as nucleation sites promoting the precipitation of carbonated apatite similar to that of bone tissue. In addition, considering the case of bioglasses, these basic materials provoke a local pH increase of SBF leading consequently to a decrease of hydroxyapatite (HA) solubility. This promotes a very large acceleration of apatite nucleation which is reinforced by the release of calcium ions from the bioglass [29]. The formed thin apatite layer plays a key role in generating a chemical bond between these biomaterials and bone tissue. Once this apatite layer is formed, it rapidly grows from calcium and phosphate ions present in the physiological medium. Considering the obtained results (an incomplete condensation in the hybrid microspheres), the presence of unreacted Si–OH groups could potentially be useful to promote a bioactive response from these new hybrid materials in physiological fluids.

The degree of organization at molecular level was studied by performing SAXS measurements on the synthesized hybrid samples (Fig. 3). Due to the contrast of electronic densities between the organic and inorganic phases in a hybrid material, when a correlation length is found in SAXS spectra it is associated to a characteristic distance separating inorganic domains (inorganic domains are the electron-richest phases). In the case of self-assembled bridged silsesquioxanes, this characteristic distance is frequently found to be equal to the length of the organic bridges separating silica domains [30]. In the spectrum corresponding to the GDTH sample (Fig. 3a), a strong correlation peak at $q = 0.2$ Å$^{-1}$ was observed. This position represents a characteristic distance given by $2\pi/q_{max} = 31.4$ Å. In this case, this distance cannot be attributed to the length of the organic bridge, which was determined to be 19 Å (as calculated using the software ACD Labs/3D Viewer). As previously reported [23], this distance corresponds in a good agreement to the length of two dodecylamine chains in a tail-to-tail association. This conformation leads to a hybrid material made up of cavities (bilayer structure composed of a tail-to-tail association of alkyl chains with all trans conformations) which could be employed as reservoir-like device for incorporating organic molecules. As to the GCTH spectrum (Fig. 3b), three scattered patterns were observed at 0.21, 0.38 and 0.66 Å$^{-1}$. The relative positions of these patterns correspond to a progression characteristic of a two-dimensional hexagonal structure consisting of cylindrical self-assemblies crystallized in a hexagonal lattice [31]. In summary, in both materials (GDTH and GCTH), the structuring obtained at molecular level led to the formation of organized cavities which could be functionalized with drugs or other organic molecules. This possibility of synthesizing microspheres structured at molecular level allows us to employ this architecture to incorporate organic molecules during the sol–gel processing, obtaining rapidly (the synthesis takes only 90 s) microdevices functionalized with active agents (e.g. antiinflammatory drugs, antibiotics, analgesics, among others). The incorporation of ASA into the synthesized microspheres was evaluated by the FTIR technique. The FTIR spectrum of the GDTH microspheres (Fig. 4) shows the characteristic bands corresponding to Si–O–Si (1040 cm$^{-1}$, 1120–1130 cm$^{-1}$) and unreacted Si–OH (910 cm$^{-1}$) groups [32]. The broad band located at 3400 cm$^{-1}$ was attributed to C–OH groups present in the organic bridges of the hybrid materials, including the unreacted fraction of Si–OH groups. In the spectrum corresponding to the GDTH-A microspheres (Fig. 4), characteristic peaks of ASA are present at 1300 cm$^{-1}$ (H-bonded C–OH of the carboxylic acid), 1600 cm$^{-1}$ (aromatic ring) and 1740 cm$^{-1}$ (carbonyl group of the ester group) [32]. The absence of bands in the region 2500–2700 cm$^{-1}$ indicates that there is no dimerization of carboxylic acids, meaning that ASA is dispersed in the material forming hydrogen-bonds with affine groups of the organic bridge (tertiary amine, OH and ether groups) [32]. Analogous qualitative results were obtained for the GCTH and GCTH-A microspheres. As a consequence, the ultrasound-assisted sol–gel reaction of bridged monomers represents a very useful method to synthesize nanostructured microspheres able to incorporate ASA via hydrogen-bonds with the organic groups present in the organic bridges of the precursors. In this way, this hydrogen-bonding recognition strategy can be successfully employed to retain

drugs during the hydrolytic condensation of the bridged monomers as the proposed ultrasound-assisted sol–gel synthesis takes place.

Figure 6 depicts the microphotographs corresponding to the CHO-K1 cells, exposed and not exposed to the extracts of the GDTH sample, showing the biological response of this material when assessed in vitro. Blank cells (not exposed to the extract) showed both normal phenotype and homogeneous growth, after 72 h of incubation time. When cells were exposed to pure extract (not diluted), a monolayer with some changes was observed. Although the attached cells showed a similar phenotype as that of the control ones, there were few free spherical cells suspended in the culture medium. This was correlated with a CD (cytotoxicity degree) = 1. When cells were exposed to 25% extract, about 70% of the total cells maintained their characteristic aspect, keeping their adhesion properties and leading consequently to the formation of a monolayer. A few suspended spherical free cells were also observed in this sample. In accordance to these results, a CD = 1 was stated. Finally, when extracts were diluted to 10%, a similar image as that of the blank sample was observed. A well-constituted monolayer with few free areas was observed, which was related to a CD = 0.

Figure 7 summarizes the cytotoxicity results obtained for all tested microsphere samples. According to these results, GDTH microspheres evidenced a minimum cytotoxicity degree, no matter the concentration of the evaluated extracts. On the other hand, the GCTH sample showed a severe cytotoxic effect when the pure extract was tested. Microspheres incorporating ASA (GDTH-A and GCTH-A samples) behaved in a similar way as GCTH microspheres when pure extracts were assessed, however the ASA-containing microspheres strongly diminished their cytotoxicity as the extracts were diluted. When a 10%-concentration extract was employed, GDTH-A and GCTH-A microspheres showed a non cytotoxic behavior. These results are promising for considering these novel nanostructured functionalized microspheres as potential drug-carriers in drug delivery-related applications.

In vitro ASA-release tests from the functionalized hybrid microspheres (in simulated physiological conditions) are currently in progress.

## 5 Conclusions

The versatility of the sol–gel method in combination with the ultrasonic irradiation technique allows synthesizing nanostructured hybrid microspheres via self-assembling of bridged monomers containing organic pendant groups in the organic bridges. This novel method leads to microspheres with very low polydispersity in a very short reaction time (90 s). The architecture reached at molecular level can be employed to incorporate acetylsalicylic acid (ASA) as anti-inflammatory model drug. Although ASA-containing hybrid microspheres showed a cytotoxic effect on CHO-K1 cells when pure extracts of the microspheres were assessed, this cytotoxicity considerably diminished as dilutions of the extracts were performed. ASA-containing microspheres were demonstrated to be non cytotoxic when 10%-concentration extracts were tested based on the ISO 10993-5 standard.

These results are promising for considering these novel nanostructured functionalized microspheres as potential drug-carriers on biomedical fields. In vitro ASA-release tests from the functionalized hybrid microspheres, in simulated physiological conditions, are currently in progress.

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## References

1. Parejo C, Gallardo A, San Román J. HEMA-based methacrylic carriers incorporating ketoprofen: chain flexibility and swelling behaviour. J Biomater Sci Polym Ed. 2000;11:1429–41.
2. Parejo C, Gallardo A, San Román J. Controlled release of NSAIDs bound to polyacrylic carrier systems. J Mater Sci Mater Med. 1998;9:803–9.
3. Alfabrouchard C, Doelker E. Méthode de préparation des microparticules biodégradables chargées en principes actifs hydrosolubles. STP Pharma Sci. 1992;2:365–80.
4. Jameela SR, Suma N, Jayakrishnan A. Protein release from poly(ε-caprolactone) microspheres prepared by melt encapsulation and solvent evaporation techniques: a comparative study. J Biomater Sci Polym Ed. 1997;8:457–66.
5. Guzman M, Molpeceres J, Garcia F, Aberturas MR. Preparation, characterization and in vitro drug release of poly-ε-caprolactone and hydroxypropyl methylcellulose phthalate ketoprophen loaded microspheres. J Microencapsul. 1996;13:25–39.
6. Arshady A. Preparation of biodegradable microspheres and microcapsules: 2. Polyactides and related polyesters. J Control Release. 1991;17:1–22.
7. Elvira C, Fanovich A, Fernández M, Fraile J, San Román J, Domingo C. Evaluation of drug delivery characteristics of microspheres of PMMA-PCL-cholesterol obtained by supercritical-$CO_2$ impregnation and by dissolution-evaporation techniques. J Control Release. 2004;99:231–40.
8. Abetz V, Simon PFW. Phase behaviour and morphologies of block copolymers. Adv Polym Sci. 2005;189:125–212.
9. Gohy JF. Block copolymer micelles. Adv Polym Sci. 2005;190:65–136.
10. Segalman RA. Pattering with block copolymer thin films. Mater Sci Eng R. 2005;48:191–226.
11. Mori H, Lanzendörfer MG, Müller AHE. Silsesquioxane-based nanoparticles formed via hydrolytic condensation of organotriethoxysilane containing hydroxy groups. Macromolecules. 2004;37:5228–38.

12. Shea KJ, Loy DA. Bridged polysilsesquioxanes: molecular engineering of hybrid organic-inorganic materials. MRS Bull. 2001;26:368–76.
13. Shea KJ, Loy DA. Bridged polysilsesquioxanes. Molecular-engineered hybrid organic-inorganic materials. Chem Mater. 2001;13:3306–19.
14. Moreau JJE, Vellutini L, Wong Chi Man M, Bied C, Dieudonné P, Bantignies JL, Sauvajol JL. Lamellar bridged silsesquioxanes: self-assembly through a combination of hydrogen bonding and hydrophobic interactions. Chem Eur J. 2005;11:1527–37.
15. Romeo HE, Fanovich MA, Williams RJJ, Matejka L, Plestil J, Brus J. Bridged silsesquioxanes with organic domains self-assembled as functionalized molecular channels. Macromol Chem Phys. 2007;208:1202–9.
16. Jeong JC, Lee J, Cho K. Effects of crystalline structure on drug release behavior of poly(ε-caprolactone) microspheres. J Control Release. 2003;92:249–58.
17. Liu S, Lang X, Ye H, Zhang S, Zhao J. Preparation and characterization of copolymerized aminopropyl/phenylsilsesquioxane microparticles. Eur Polym J. 2005;41:996–1001.
18. Moreau JJE, Pichon BP, Bied C, Wong Chi Man M. Structuring of bridged silsesquioxanes via cooperative weak interactions: H-bonding of urea groups and hydrophobic interactions of long alkylene chains. J Mater Chem. 2005;15:3929–36.
19. Suslick KS. Sonochemistry. Science. 1990;247:1439–45.
20. Suslick KS, Didenko Y, Fang MF, Hyeon T, Kolbeck KJ, McNamara WB, Mdleleni MM, Wong M. Acoustic cavitation and its chemical consequences. Phil Trans R Soc Lond A. 1999;357:335–53.
21. Dai C, Wang B, Zhao H. Microencapsulation peptide and protein drugs delivery system. Colloids Surf B Biointerfaces. 2005;41:117–20.
22. Suslick KS, Grinstaff MW, Kolbeck KJ, Wong M. Characterization of sonochemically prepared proteinaceous microspheres. Ultrason Sonochem. 1994;1:565–8.
23. Romeo HE, Fanovich MA, Williams RJJ, Matejka L, Plestil J, Brus J. Self-assembly of a bridged silsequioxane containing a pendant hydrophobic chain in the organic bridge. Macromoleculaes. 2007;40:1435–43.
24. International standard: biological evaluation of medical devices – Part 5: tests for cytotoxicity: in vitro methods. ISO 10993-5, 1992.
25. Rikowski E, Marsmann HC. Cage-rearrangement of silsesquioxanes. Polyhedron. 1997;16:3357–61.
26. Feher FJ, Soulivong D, Nguyen F. Practical methods for synthesizing four incompletely condensed silsesquioxanes from a single $R_8Si_8O_{12}$ framework. Chem Commun 1998;1279–80.
27. Matejka L, Dukh O, Brus J, Simonsick WJ Jr, Meissner B. Cage-like structure formation during sol-gel polymerization of glycidyloxypropyltrimethoxysilane. J Non Cryst Solids. 2000;270:34–47.
28. Hench LL, Wilson J. An introduction to bioceramics, Advanced series in ceramics, vol. 1. Singapore: World Scientific Publishing Co. Pte. Ltd.; 1993. Editors in-chief: McLaren and Niesz.
29. Ramila A, Vallet-Regi M. Static and dynamic in vitro study of a sol-gel glass bioactivity. Biomaterials. 2001;22:2301–6.
30. Fasce DP, Williams RJJ, Matejka L, Plestil J, Brus J, Serrano B, Cabanelas JC, Baselga J. Photoluminescence of bridged silsesquioxanes containing urea or urethane groups with nanostructures generated by the competition between the rates of self-assembly of organic domains and the inorganic polycondensation. Macromolecules. 2006;39:3794–801.
31. Romeo HE, Fanovich MA, Williams RJJ, Matejka L, Plestil J, Brus J. Fast synthesis of nanostructured microspheres of a bridged silsesquioxane via ultrasound-assisted sol-gel processing. Macromol Chem Phys. 2009;210:172–8.
32. Colthup NB, Daly LH, Wiberley SE. Introduction to infrared and Raman spectroscopy. New York: Academic Press; 1975.