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Silica hybrid biomaterials containing gelatin synthesized by sol-gel method

Research Article

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Abstract: This work reports the sol-gel synthesis of silica hybrids. We determined the effect of the type and quantity of silica precursors and organic compounds on the resulting structure, surface area, nanostructure design and size, and potential applications. The structure of the synthesized hybrids was analyzed using FT-IR, XRD, BET-Analysis, SEM, and AFM. We demonstrate the immovilization of whole living thermophilic bacterial cells with cyanocompound degradation activity in the synthesized silica hybrid biomaterials by entrapment, chemical binding, and adsorption.

Keywords: *Sol-gel synthesis • Silica hybrids • Nanostructures • Bacterial cell immobilization • Biodegradation*

1. Introduction

The families of inorganic-organic hybrid materials have attracted considerable attention due to their valuable properties such as: molecular homogeneity, transparency, flexibility and durability. Hybrid composites can be synthesized by the sol-gel method from various combinations of metal alkoxides and polymers to create a nanoscale mixture of inorganic-oxides and organic polymers [1-9]. The inorganic-organic hybrids consist of an inorganic network and different types of organic components, such as PVA, PEG, PAAG, alginate, agar-agar, and others [10-15]. Recently, silica hybrid materials containing biopolymer gelatin have been synthesized and applied to various applications including: incorporation of Ca^{2+} ions; in vitro deposition of apatite on porous gelatin-siloxane hybrids; porous gelatin-siloxane hybrids for bone tissue engineering; gelatine/silicate interactions from nanoparticles to composite gels; production of highly transparent gelatin/silica coatings; synthesis of microporous silica templated by gelatin [16-21].

Hybrids, composed of inorganic oxides bonded to organic polymers, are of special interest due to the absence of interface imperfections and the biosilicification processes [22,23].

It has been established that silica and silica hybrids are excellent matrices for immobilization of inorganic and organic compounds and various types of biomolecules, including enzymes, whole living cells, antibodies, immune molecules, phospholipids and other proteins. Bioartificial organs are obtained via sol-gel encapsulation of pancreatic, hepatic, or cancer cells. The nanoporous silica matrix may act as a barrier against the host immune system and protect transplants from immune rejection.

The inclusion of cells with nitrilase activity into such matrices could enable the transformation ofhighly toxic, carcinogenic and mutagenic nitriles, containing

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cyano groups (R-C≡N), into more environment-friendly compounds. We have previously demonstrated the use of silica hybrid nanomaterials with different organic components as matrices for the immobilization of living cells.The porous structure of the hybrids is an important parameter for the efficiency of the immobilization process. Immobilization of cells for biochemical conversions has gained increasing interest in recent years owing to its many advantages such as improved catalyst recovery, stability due to the formation of chemical bonds, and reusability which is economically viable and carried out under stabilized conditions and controls over the enzyme reactions [24]. Immobilization of whole cell saves the delicate and expensive separation methods that are required to safely release and isolate the enzymes from the cell, especially for intracellular enzymes that are involved in biochemical conversions [25].

Nitriles are toxic substances, products of the petrochemical and other industries. However,they can be converted to their corresponding acids and ammonia by a single enzyme – nitrilase, without the formation of a stable amide intermediate. Mesophilic bacteria are the primary producers of nitrilases, however only one thermophilic producer, Bacillus pallidus, is currently known [26]. Many of these enzymes have broad substrate specificitieswith a preference for aromatic or heterocyclic substrates such as the nicotinic acid precursors benzonitrile or the cyanopyrydines [27]. The use of microorganisms for the biodegradation of such substrates can be efficiently applied for a bioremediation process. In our previous works we have compared pure silica matrices with organic-inorganic hybrid matrices obtained by using different inorganic precursors (TEOS, TMOS, ETMS, MTES) and organic compounds as: agar-agar, PAAG, carageenan and others [28,29].

In the present work, we present the sol-gel synthesis of nanocomposite hybrids and the influence of the type and quantity of silica precursors and organic components on the resulting structure and potential application.

2. Experimental Procedure

The inorganic-organic hybrid materials were prepared by substituting part of the inorganic precursor with an organic component. The inorganic precursors included tetraethylortosilicate (TEOS), tetramethylortosilicate (TMOS), methyltriethoxysilane (MTES), ethyltrimethoxysilane (ETMS) and vynil trimethoxysilane (VTMS). The organic components included: gelatin; gelatin and methylmetacrylate (MMA); gelatin and 2-hydroxyl-ethylmethacrylate (HEMA) in equal quantities. A poly-step sol-gel procedure was carried out at strictly controlled conditions in order to obtain the desired nanostructred materials, permitting the following processes to be performed: hydrolysis of silica precursors leading to the separation of alkoxide groups and producing of silanols; condensation of monomers and formation of nanoparticles; cross-linking of nanoparticles and gelation.

Sol-gel transparent silica hybrid matrix with 5 to 20 wt% of organic compound were synthesized at room temperature. The inorganic-organic hybrid materials were prepared by substituting part of the inorganic precursor with an organic component at 5 wt%. The quantity of sol-gel precursors varied from 19 mL (for the hybrid materials with 5% substitution) to 16 mL (for the hybrids with 20% substitution) depending on the concentration of organic constituent.

Hybrid materials were prepared by mixing the precursor with distilled water and 5 mL of 0.1 N HCl, resulting in a solution with pH~1.5 to enable an increase in the hydrolysis rate. After pre-hydrolysis of the initial mixture, the organic components were dissolved in hot water, with the total amount ranging from 1 to 4 g depending on the desired final concentration.The total volume of organic solution ranged from 20 mL for samples with 5% organic components to 80 mL for samples with 20% organic components. Subsequent homogenization of the solution for 15 minutes was performed in the absence of alcohol co-solvent. In all cases the ratio of precursor to H_2O was kept constant and equal to 1. No phase separation was observed before or after the gelation point, which was confirmed by SEM studies. Phosphate buffer was then added to the homogeneous solution. The gelation time ranged from 2 to 5 min depending on the concentration of organic component. The addition of an organic component resulted in faster polymerization. To keep cell vitality the pH of the final mixture was raised up to pH=7.2±0.02 at 20°C with 15-20 mL of phosphate buffer. The drying procedure was carried out at room temperature overnight. The resulting wet gel was used for cell studies.

The bacterial strain used in the present study was deposited in the NBIMCC-Bulgaria №8021/2001 [30]. Bacillus sp. UG5B with 4-cyanopyridine degrading activity was cultivated in a medium containing 20 mM 4-cyanopyridine (pH 7.5 at 50°C). Cells were separated from the culture medium by centrifugation and resuspended in a phosphate buffer solution (0.06 M, pH 7.2 at 20°C). The resulting cell suspension that was used in the immobilization procedures consisted of 19 mg mL^{-1} dry cells and had an enzyme activity of 1.4 U mL^{-1}. The enzyme activity was measured by the ammonia released due to the action of nitrilase according to the phenol-hypocloride method of Fawcett

and Scott [31].

The following methods were used to study the structure of the synthesized hybrids: X-Ray Diffraction (XRD, PW1730/10 diffracted intensity of Cu Kα radiation λ= 1.54056 Å, 40 kV and 30 mA was measured with scan rate of 0.02° min^{-1} in 2θ range between 4° and 80°), Fourier Transforming-Infrared (FT-IR, IR-MATSON 7000, pellets of ca. 2 mg of hybrid samples were mixed with 200 mg of spectroscopic grade KBr), BET-Analysis (Gemini 2370 V5), Scanning Electron Microscope (SEM) (Philips - 515), and Atomic Force Microscopy (AFM,NanoScope Tapping Mode™). A vertical engage 4842 JV-scanner and Si probes were applied in all experiments. The driving frequency in tapping mode was chosen at the resonant frequency of the free-oscillating cantilever in the immediate vicinity of the sample surface. Height and phase images were recorded simultaneously. The average roughness (R_a) of the hybrids surface was calculated directly from the AFM image.

3. Results and Discussion

Data from the XRD analysis reveals that all the synthesized hybrid biomaterials have an amorphous structure (Fig. 1). The intensity of diffraction peaks of hybrids with ETMS and 5 wt% organic constituent was found to increase in the following order: gelatin < gelatin + HEMA < gelatin + MMA. Additionally, the x-ray patterns indicate that some processes of ordering are carried out in the sample synthesized with addition of gelatin +HEMA and gelatin+ MMA.

The structure of the synthesized hybrids was analyzed with IR spectroscopy. The FT-IR spectra of the synthesized inorganic-organic materials showed that all samples exhibited bands at 1080 cm^{-1}, 790 cm^{-1} and 480 cm^{-1}, which are assisgned to the v_{as}, v_s and δ of Si-O-Si vibrations.The band at 1080 cm^{-1} can be related to the presence of Si-O-C, C-O-C and Si-C bonds. This peak is split into two separate bands at 1030 and 1130 cm^{-1} in the hybrid structures. This is due to the functionalization of silanols through siloxane bridges by methyl groups. The band at 960 cm^{-1} is due to a stretching Si-OH vibration. The band at 1439 cm^{-1} is assigned to C-O-H vibrations. The characteristic bands at around 3450 cm^{-1} and 1620 cm^{-1} assigned to H-O-H vibration can also be detected. The FT-IR spectra of some hybrids with MTES, ETMS and VTMS show absorption bands at 2975 cm^{-1}, 1255 cm^{-1}, 880 cm^{-1} and 690 cm^{-1}, due to the presence of strong chemical bonds: Si-O-R (CH_3 and C_2H_5) and Si-C (Fig. 2). It could be supposed that gelatin and silica interact by hydrogen bonding between the C=O and N-H groups of gelatin and the silanol hydrogen, in addition to Van der Waals and electrostatic forces. It was established that the spectrum of the sample with ETMS and 5 wt% organic constituent gelatin+HEMA shows the highest transmittance.

Comparing the influence of three silica precursors, ETMS, MTES and VTMS, on the optical properties of synthesized hybrids it is demonstrated that all samples have a highest transmittance when MTES is used.

BET analysis revealed that the surface area of the synthesized samples ranged from 62 to 278 m^2 g^{-1} and decreased with the increase in quantity of the organic component (Fig. 3). On this basis, subsequent experiments concerning the operation stability of biocatalysts were carried out with the samples containing 5 wt% organic component.

In the SEM micrographs of the silica hybrids with 20% gelatin + MMA and VTMS or TEOS, the presence of heterogeneous regions dispersed within relatively homogeneous matrices could be seen (Figs. 4 and 5).

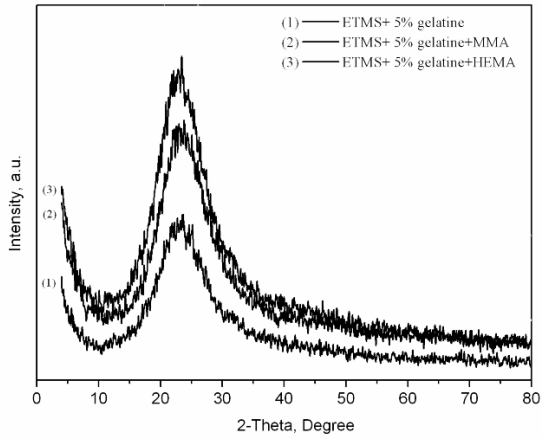


Figure 1. XRD paterns of silica hybrids containing gelatin, gelatin + MMA and gelatin + HEMA

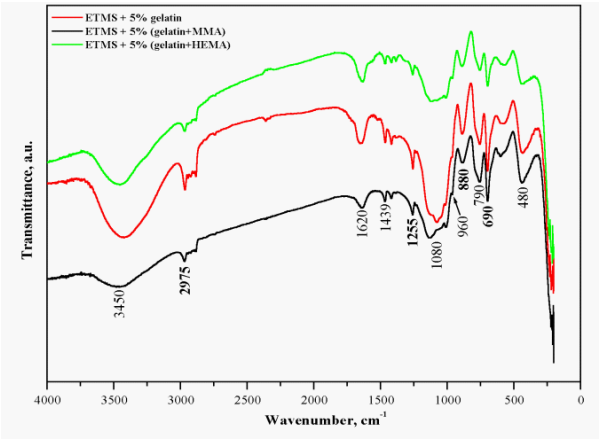


Figure 2. FT-IR spectra of silica hybrids containing gelatin, gelatin + MMA and gelatin + HEMA.

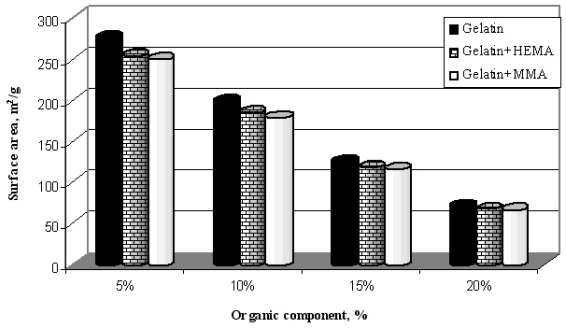


Figure 3. BET analysis of silica hybrids containing gelatin, gelatin + MMA and gelatin + HEMA

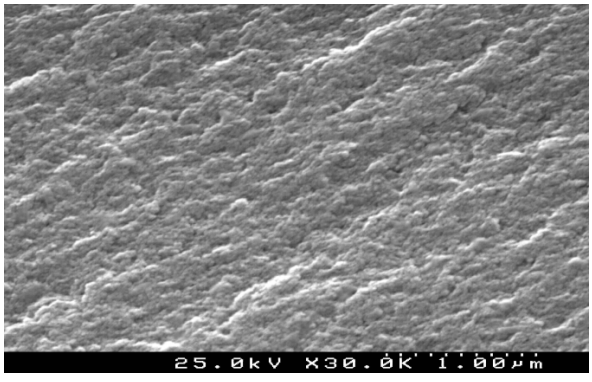


Figure 4. SEM of hybrids with VTMS and 20% gelatin + MMA

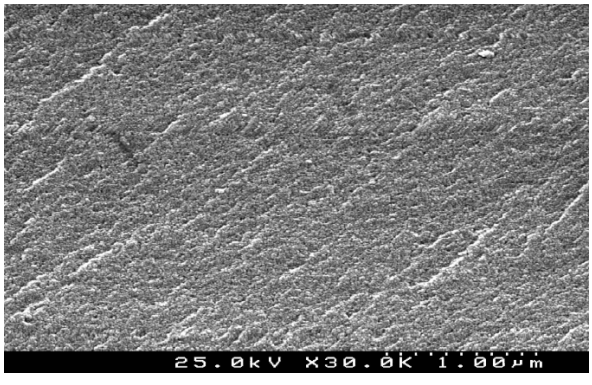


Figure 5. SEM of hybrids with TEOS and 20% gelatin/MMA

The AFM images show that each hybrid material exhibited a different design and morphology on the micro- and nanostructured surface depending on the type of silica precursors utilized and the nature and percentage of the organic component (Figs. 6-8).

Structures with well-defined units and their aggregates were observed by AFM investigations. In the hybrid materials containing 5 and 20% gelatin with silica precursor MTES, the size of particles varied from 5 to 10 nm and the dimensions of their aggregates ranged from about 29 to 45 nm (Fig. 6). For the samples synthesized with MTES and 5% or 20% gelatin +

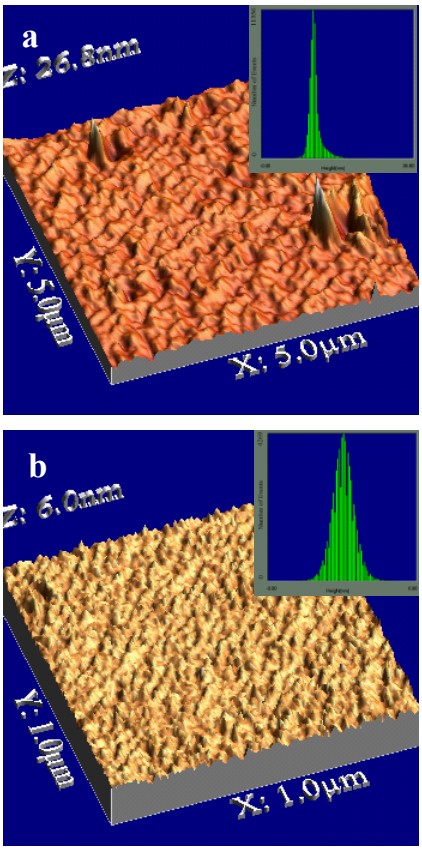


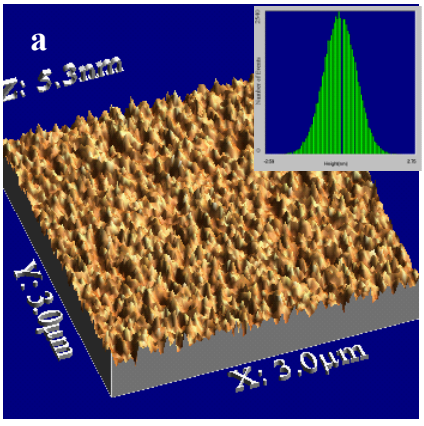
Figure 6. AFM images and height distribution profile of the surface roughness of samples (MTES) with (a) 5 and (b) 20 wt% gelatin.

MMA the size of the resulting particles were 6 to 11 nm and the dimensions of their aggregates were 37 to 52 nm (Fig. 7). From the data presented in Fig. 8 it has been established that the size of particles for samples composed of gelatin + HEMA and MTES are from 7 to 10 nm and their aggregates are about 36-63 nm. In the same figures the height distribution profiles of surfaces roughness are shown. For the hybrid samples with MTES containing 5 and 20 wt% gelatin, gelatin + MMA and gelatin + HEMA, RMS surface roughness varied from 1.02 to 2.43 nm and the average profile peak heights are from 2.29 to 9.13 nm. The histograms of the surface height distribution profiles, obtained from AFM images, showed that all of the inorganic-organic hybrid samples have surfaces with irregularities of relatively small height.

Three methods were used for the immobilization of living cells:

Entrapment - Cells are entrapped in the gel before gelation and 100% of them are included irreversibly in the gel itself. The process is followed by drying at room temperature.

Chemical binding – Surface activation was carried out in order to achieve stronger binding between the cells



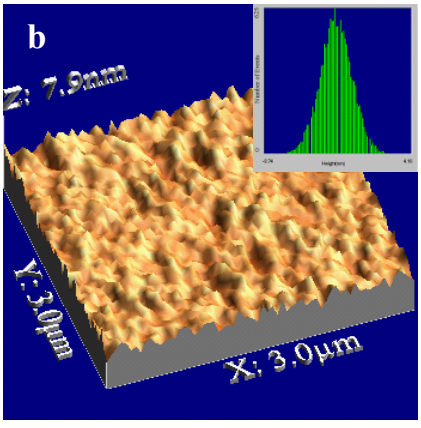
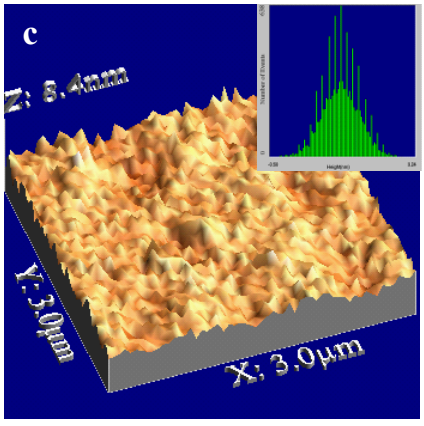


Figure 7. AFM images and height distribution profile of surface roughness of samples (MTES) with (a) 5 and (b) 20 wt% gelatin + HEMA.



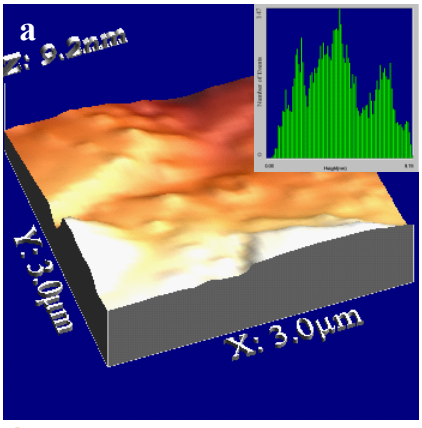


Figure 8. AFM images and height distribution profile of surface roughness of samples (MTES) with (a) 5 and (b) 20 wt% gelatin + MMA.

and the carrier surface. Formaldehyde (10% solution) was used as cross-linker to establish the attachment of cells onto the carrier.

Adsorption – When adsorption takes place the cells are attached to the matrix surface as a result of hydrogen bonding, van der Waals forces and electrostatic interactions. The enzyme-substrate reaction is achieved without mass transfer limitations.

The thermophilic bacterial cells with cyanocompound degradation activity were immobilized by entrapment in the obtained biocatalysts. They were applied in the process of enzyme degradation of the toxic and carcinogenic environmental pollutant 4-cyanopyridine (Fig. 9).

The toxic organo-cyanide compound was subjected to degradation using the three types of matrices and the operational stability was followed for 12 reaction cycles. The enzyme activity was highest for the biocatalyst, composed ofTEOS and gelatin with acell suspension of 5 mL - 2.2 U mL^{-1}. The residual enzyme activity was 48% after 12 cycles of operation. The addition of another organic constituent in the hybrid matrix did not lead to increased enzyme activity and stability where the residual activity was higher for HEMA as an organic additive-39%.

When the operation stability was determined by chemical binding the residual activity of about 50% was achievedafter the tenth cycle for gelatin-containing hybrids and after the eighth cycle for the hybrids with other organic components. For the adsorption method these data are much smaller.

Comparing the activity of immobilized cells in hybrids with different organic components it was established that the organic components increase the activity of immobilized cells in the following order: PAAG<Ca alginate<Agar-Agar<Gelatin.

The surface structure of the hybrid matrix and the immobilized bacterial cells on it have been observed by SEM. It is seen that bacteria are randomly dispersed, the vitality of cells is kept, and the matrices are well preserved.

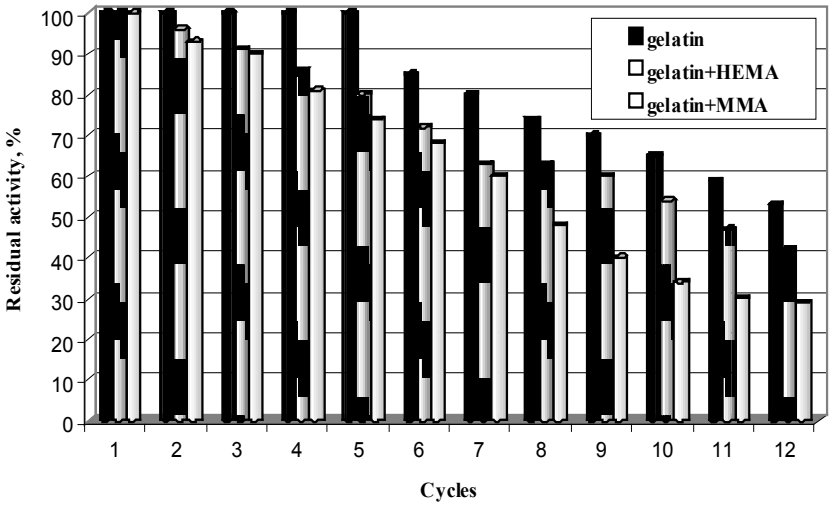


Figure 9. Operational stability of the biocatalysts

4. Conclusions

The silica hybrid biomaterials, containing gelatin, gelatin and MMA, and gelatin and HEMA have been synthesized via the sol-gel route. From the AFM images of hybrid samples the size of nanoparticles and their aggregates has been determined and the surface roughness of most samples shows irregularity of relatively small height.

With the help of IR studies the inorganic and organic components were found to interact by van der Waals forces, hydrogen bonding or electrostatic forces in the synthesized hybrids with TEOS and TMOS. In the synthesized hybrids with ETMS, MTES and VTMS, FT-IR revealed strong chemical bonds between the organic and inorganic components.

The influence of the nature and quantity of precursors and organic components on their structure development and application potential as a carrier for immobilization of cells with nitrilase activity has been established.

It has also been determined that the enzyme activity of immobilized cells depends on the chemical composition and the structure of synthesized hybrid nanomaterials, as well as on the type of immobilization procedures utilized.

All the experiments have collectively shown that the synthesized hybrid nanomaterials were successfully applied for prokaryotic cell immobilization, since these carriers keep their vitality and did not cause damage to the enzyme systems involved in nitrilase synthesis.

Acknowledgements

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