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A new precursor for the immobilization of enzymes inside sol–gel-derived hybrid silica nanocomposites containing polysaccharides

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Abstract

Tetrakis(2-hydroxyethyl) orthosilicate (THEOS) introduced by Hoffmann et al. (J. Phys. Chem. B., 106 (2002) 1528) was first used to prepare hybrid nanocomposites containing various polysaccharides and immobilize enzymes in these materials. Two different types of *O*-glycoside hydrolyses (EC3.2.1), 1 → 3-β-D-glucanase L_{IV} from marine mollusk *Spisula sacchalinesis* and α-D-galactosidase from marine bacterium *Pseudoalteromonas* sp. KMM 701, were taken for the immobilization. To reveal whether the polysaccharide inside the hybrid material influences the enzyme entrapment and functioning, negatively charged xanthan, cationic derivative of hydroxyethylcellulose and uncharged locust bean gum were examined. The mechanical properties of these nanocomposites were characterized by a dynamic rheology and their structure by a scanning electron microscopy. It was found that 1 → 3-β-D-glucanase was usually immobilized without the loss of its activity, while the α-D-galactosidase activity in the immobilized state depended on the polysaccharide type of material. An important point is that the amount of immobilized enzymes was small, comparable to their content in the living cells. It was shown by the scanning electron microscopy that the hybrid nanocomposites are sufficiently porous that allows the enzymatic substrates and products to diffuse from an external aqueous solution to the enzymes, whereas protein molecules were immobilized firmly and not easily washed out of the silica matrix. A sharp increase of the enzyme lifetime (more than a hundred times) was observed after the immobilization. As established, the efficient entrapment of enzymes is caused by few advantages of new precursor over the currently used TEOS and TMOS: (i) organic solvents and catalysts are not needed owing to the

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complete solubility of THEOS in water and the catalytic effect of polysaccharides on the sol–gel processes; (ii) the entrapment of enzymes can be performed at any pH which is suitable for their structural integrity and functionality; (iii) a gel can be prepared at reduced concentrations of THEOS (1–2%) in the initial solution that excludes a notable heat release in the course of its hydrolysis.

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1. Introduction

The nanocomposite materials of silica prepared by the sol–gel technique in the aqueous solutions have drawn attention owing to the propensity to entrap enzymes without their covalent bonding to the matrix. It is significant that the immobilized biomolecules retain or even enhance their bioactivity and stability. Furthermore, the porous silica matrix provides enzyme accessibility to external reagents and removal of the reaction products through the pore diffusion. These advantages open up a way for the development of biocatalysts and biosensors for biotechnological and medical uses.

Tetraalkoxysilanes (tetramethoxysilane, TMOS and tetraethoxysilane, TEOS) are common precursors to synthesize silicate. Their hydrolysis in aqueous solutions and further polycondensation reactions cause first a formation of sol particles of which the cross-linking leads to the self-organization into a porous three-dimensional network in the bulk. In view of the fact that the tetraalkoxysilanes are poor-water soluble compounds, methanol or ethanol is generally added to prevent the phase separation [1,2]. If one deals with the enzyme immobilization, the alcohol addition can bring about the denaturation of proteins. This problem has been countered by the use of polyol silicates possessing increased water solubility [3]. The TMOS and TEOS, as shown recently in Ref. [4], can be also exchanged for tetrakis(2-hydroxyethyl) orthosilicate (THEOS). This completely water-soluble precursor forms nanocomposite monolithic material in aqueous solutions in the absence of added alcohol. We have demonstrated that the THEOS is compatible with polysaccharides [5]. The silica synthesized in situ in a biopolymer solution reinforces the gel structure of polysaccharides or transfers it into a gel state, producing a hybrid nanocomposite material.

The main objective of the present article was to apply hybrid silica nanocomposites for immobilization of practically important enzymes (endo-1 \rightarrow 3- β -D-glucanases and α -D-galactosidases). The selected enzymes differ notably not only in the specificity, but also in their molecular weight, stability and conditions for the functioning (optimal pH and ionic strength of aqueous media). Furthermore, their substrates are distinguished by molecular weights as well. It was examined whether these factors and polysaccharide of hybrid material influence the immobilization, activity and lifetime of immobilized enzymes.

The preliminary experimental data considered in the article showed that both the enzymes could be successfully entrapped in the hybrid silica–polysaccharide nanocomposites. They retained their activity in the immobilized state. It was also found to have a significant increase in their stability after the immobilization.

2. Experimental section

2.1. Materials

Tetrakis(2-hydroxyethyl) orthosilicate (THEOS) was synthesized from tetraethoxysilane (ABCR, Germany) as described in details in [6]. Xanthan and locust bean gum were purchased from Fluka. Their structural formulas are shown in Fig. 1. Cationic derivative of hydroxyethylcellulose (cat-HEC) was obtained from Hoechst. It contains glycidyl-trimethylammonium chloride ($-\text{CH}_2\text{-CHOH-CH}_2\text{-N}^+(\text{CH}_3)_3\text{Cl}^-$) as a cationic group (Fig. 1). The molecular weight of cat-HEC was equal to 950 kDa, the molar substitution degree, 0.06 per anhydroglucose unit. The polysaccharides were used as supplied. Sephadex, carboxymethylcellulose, amylose, agarose and *p*-nitrophenyl derivative of α -D-galactopyranoside were purchased from Serva. Laminaran was separated from the brown seaweeds *Laminaria cichorioides* as described in Ref. [7]. $1 \rightarrow 3$ - β -D-Glucanase L_{IV} from marine mollusk *Spisula sacchalinesis* and α -D-galactosidase from marine bacterium *Pseudoalteromonas* sp. KMM 701 were separated and purified in accordance with the procedures developed previously in Refs. [8] and [9], respectively. Distilled water was used for preparing solutions. $1 \rightarrow 3$ - β -D-glucanase L_{IV} was in a solution buffered with 0.05 M of sodium succinate (pH 5.2) and α -D-galactosidase, with 0.05 M of sodium phosphate (pH 7.2).

2.2. Enzyme immobilization

The sol–gel derived silica materials were synthesized as suggested in Ref. [4]. To immobilize an enzyme, 0.1 ml of its buffered solution (1 and 0.2 U for $1 \rightarrow 3$ - β -D-glucanase L_{IV} and α -D-galactosidase, respectively) was added into a pre-jelled aqueous solution (1.9 ml) containing the precursor, one of the three polysaccharides and corresponding buffer. Both the mixed solutions were previously cooled up to 3–5 °C. Once the enzyme was added, the mixture was thoroughly stirred and left in a refrigerator at 3–5 °C.

2.3. Enzyme activity determination

The activity of α -D-galactosidase was determined by using *p*-nitrophenyl- α -D-galactopyranoside as the substrate [10]. The reaction mixture contained 50 μl of the α -D-galactosidase solution (10^{-2} U ml^{-1}) or 50 mg of gel with the immobilized enzyme and 350 μl of *p*-nitrophenyl derivative of α -D-galactopyranoside as the substrate (1 mg ml^{-1}) was dissolved in 0.05 M phosphate buffer (pH 7.2). It was incubated at 37 °C for 2 h. The reaction was stopped by addition of 0.6 ml of 1 M Na_2CO_3 . Then the reaction mixture was centrifuged. The amount of *p*-nitrophenol generated as a product of the enzymatic reaction was determined spectrophotometrically at 410 nm. The enzyme unit (U) corresponded to the enzyme quantity that catalyzed the formation of 1 μM of *p*-nitrophenol for 1 h.

The activity of $1 \rightarrow 3$ - β -D-glucanase L_{IV} was determined by using laminaran as the substrate. The reaction mixture containing 100 μl of the enzyme ($5 \cdot 10^{-2}$ U ml^{-1}) or 100 mg of gel with the immobilized enzyme, 200 μl of laminaran as the substrate (1 mg ml^{-1})

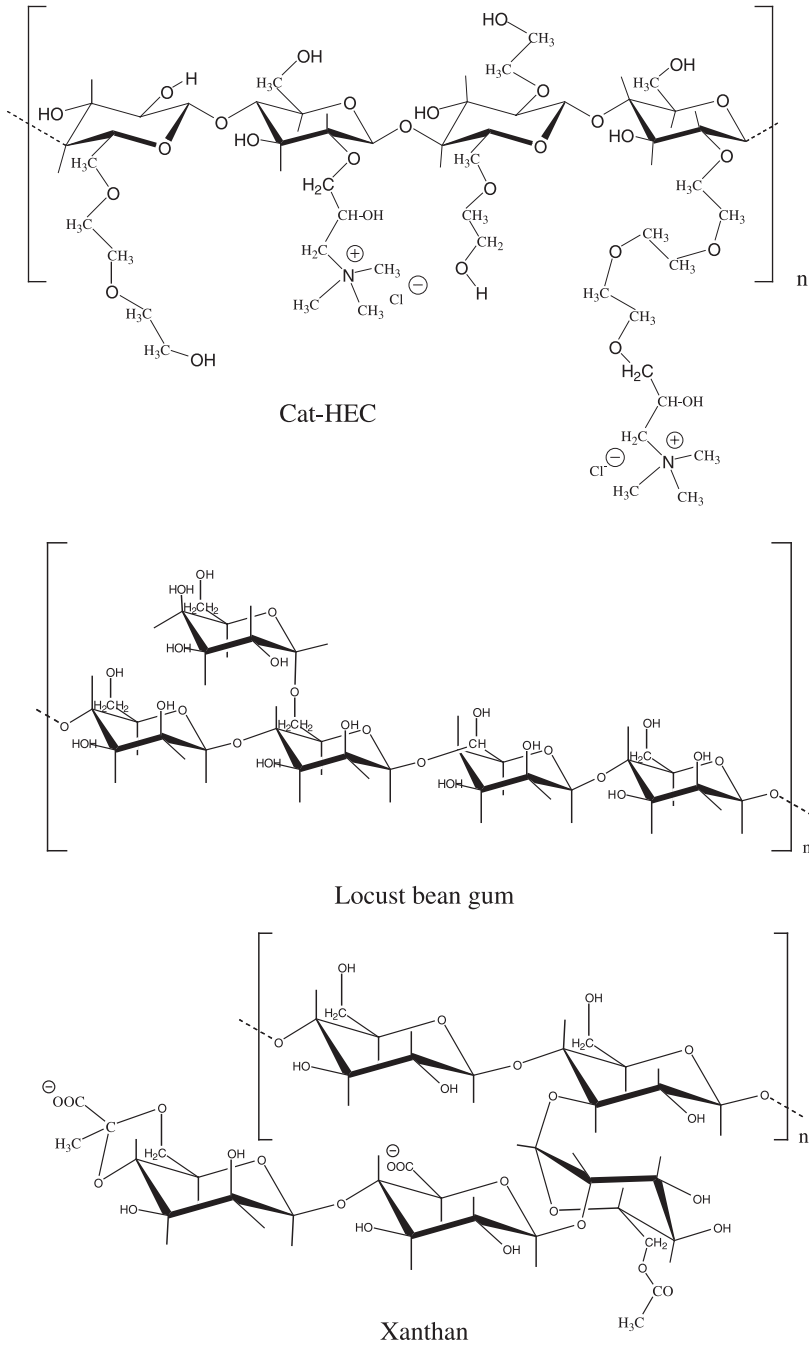


Fig. 1. Structural formula of polysaccharides used for preparing bionanocomposites.

and 200 μl 0.05 M of Na-succinate as the buffer (pH 5.2) was incubated for 1 h at 37 °C. Once the reaction mixture was centrifuged, the amount of reducing sugars released from substrate was determined by Somogyi–Nelson's method [11]. This characterized the hydrolytic activity. The enzyme unit (U) corresponded to the enzyme quantity that catalyzed the formation of 1 μM of glucose for 1 h.

As the control samples were used, enzyme solutions were prepared by the dilution (1:20) of the initial enzyme solutions by the corresponding buffer. They did not contain the precursor and polysaccharides, which were added for the immobilization.

2.4. Rheological measurements

They were performed with a Rotovisco RT 20 (Haake) stress-controlled rheometer. Measuring cells had cone and plate geometry of which the diameter and the angle of the cone were, respectively, 35 mm and 2°. A special chamber was used to decrease the evaporation of water in the course of measurements. The rheometer was run either in an oscillation or creep regime to determine frequency dependencies of the rheological parameters as well as a zero-shear viscosity η_0 and plateau modulus G_0 . The yield stress σ_y , characterizing a critical shear stress at which the gel starts destroying (flowing) under the action of external mechanical force (see, e.g. Ref. [12]), and corresponding critical strain value were determined from stress sweep measurements at the oscillation frequency 1 Hz. The further measuring details are given in Ref. [13]. The temperature during the measurements was 25 ± 0.05 °C.

2.5. Scanning electron microscopy

Samples were prepared by using the cryo-technique. The pictures were made with a JSM-840A (JEOL) scanning electron microscope.

3. Results and discussion

3.1. Characterization of hybrid nanocomposites

The salient physicochemical features of the sol–gel derived hybrid silica–polysaccharide materials synthesized by us are considered here because the results have not been published yet.

It was found that first the precursor and then the silicate generating in situ are compatible with polysaccharides, i.e. with xanthan, locust bean gum and cat-HEC. The organic and inorganic components were mixed with each other without the phase separation, forming a monolithic material. A gel settled within 5–20 min that depended on the precursor concentration, as well as on the polysaccharide amount and type. The enzymes, in their turn, were compatible with polysaccharide–silica materials that enabled us to entrap them into these bionanocomposites.

The initially prepared monolithic biomaterials did not shrink in the course of time. In other words, the syneresis was not observed.

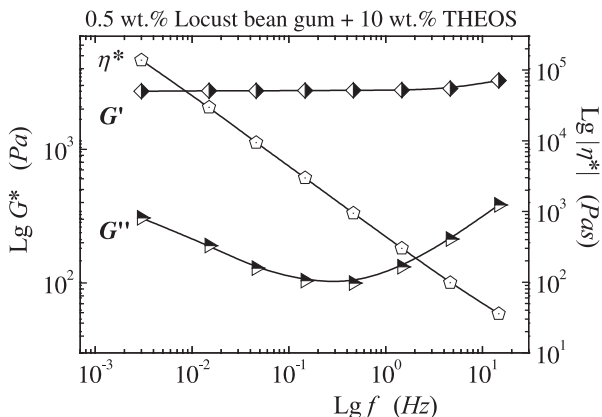


Fig. 2. The complex viscosity $|\eta^*|$, storage modulus G' and loss modulus G'' vs. oscillation frequency f . The biomaterial was prepared by mixing 10 wt.% of THEOS with an aqueous solution containing 0.5 wt.% of locust bean gum.

It should be stressed that the jellification occurred without the addition of a common catalyst, which is applied in a case of TMOS or TEOS. The catalytic effect was provided by the polysaccharide added into a THEOS solution. If it was not introduced, the sol–gel transition was not seen within a few days.

The jellification caused a sharp rise of the viscosity. It was accompanied by a change in the rheological behavior. An initial Newtonian or non-Newtonian solution, which depended on the type of added polysaccharide, transferred into a state that is characteristic of “soft” or “semisolid materials”. In their case (see, e.g. Ref. [14]), the storage modulus G' , as may be seen in Fig. 2, is higher in magnitude than the loss modulus G'' in the whole frequency range accessible for the measurement.

The introduced polysaccharides influenced all the mechanical properties of hybrid bionanocomposites. This is considered by an example of cat-HEC-containing biomaterials. Fig. 3A and B present dependences, respectively, of the zero-shear viscosity and plateau modulus

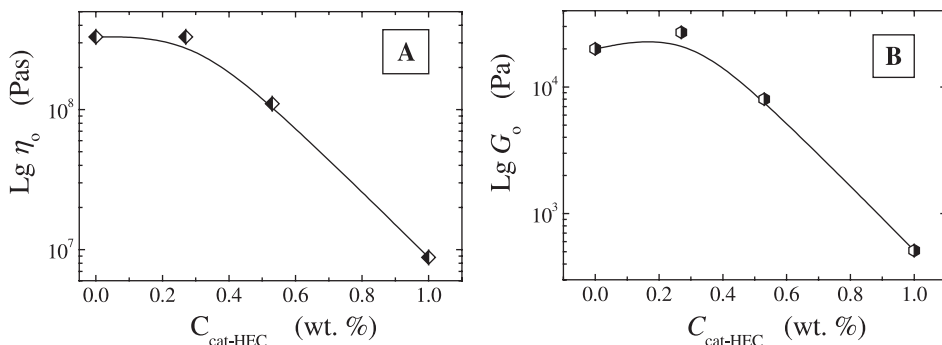


Fig. 3. The zero-shear viscosity (A) and plateau modulus (B) vs. the concentration of cat-HEC. The concentration of THEOS in the initial solution was constant, equal to 10 wt.%.

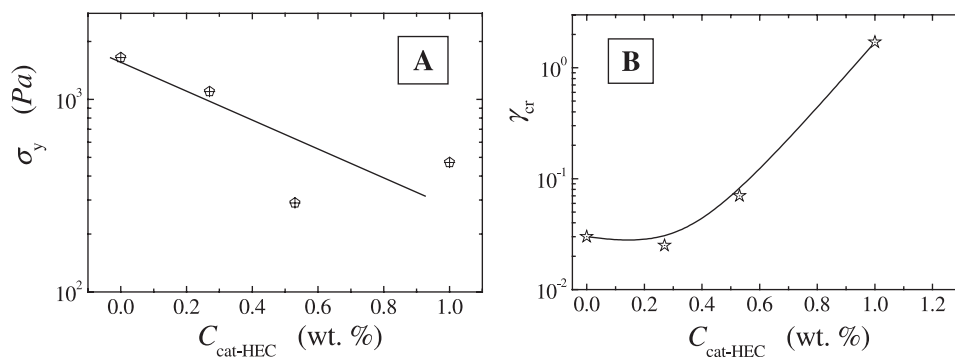


Fig. 4. The yield stress (A) and critical strain (B) vs. the concentration of cat-HEC. The concentration of THEOS in the initial solution was 10 wt.%.

modulus on the polysaccharide concentration C in a bionanocomposite prepared in a solution with 10 wt.% of THEOS in the mixed system. As seen, the polysaccharide induced a decrease in η_0 and G_0 . The effect became obvious at $C > 0.25$ wt.%. The addition of 1 wt.% of cat-HEC decreased the rheological parameters by a factor of about 40 in comparison with those for the unmodified silica gel. Furthermore, the polysaccharide made the gelled material mechanically softer and less strong. This is evidenced by concentration dependencies of yield stress and critical strain given in Fig. 4A and B, respectively. The former is increased, whereas the latter is decreased with increasing the cat-HEC concentration in the hybrid nanocomposite.

A dependence of the rheological parameters of biomaterials on the concentration of precursor in the initial solution is shown in Fig. 5. They were prepared by the addition of 0.2 wt.% of cat-HEC. As one may see from the presented data, a silica content increase resulted in grow of the mechanical strength of hybrid gels. It makes the biomaterial more hard and brittle. This effect is the opposite of that observed for polysaccharides (Figs. 3 and 4). Furthermore, the rheological parameters change much more with increasing the precursor

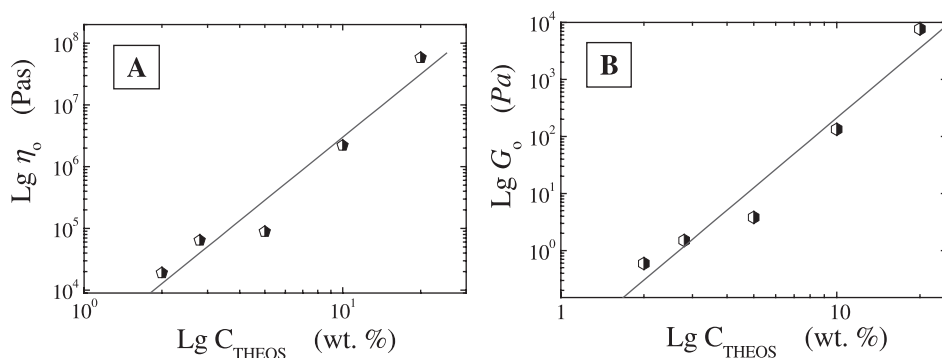


Fig. 5. The zero-shear viscosity (A) and plateau modulus (B) vs. the concentration of THEOS in the initial solution. The concentration of cat-HEC was constant, equal 0.2 wt.%.

concentration than that seen in the case of variation of cat-HEC amount. This counts in favor of the key role of polysilicate in regulating the mechanical properties of synthesized biomaterials.

Fig. 6A and B show SEM pictures of a gel sample prepared by taking a solution containing, respectively, 1 and 10 wt.% of cat-HEC and THEOS. They were taken at various magnifications. As one can see, the nanocomposite consists of two structural

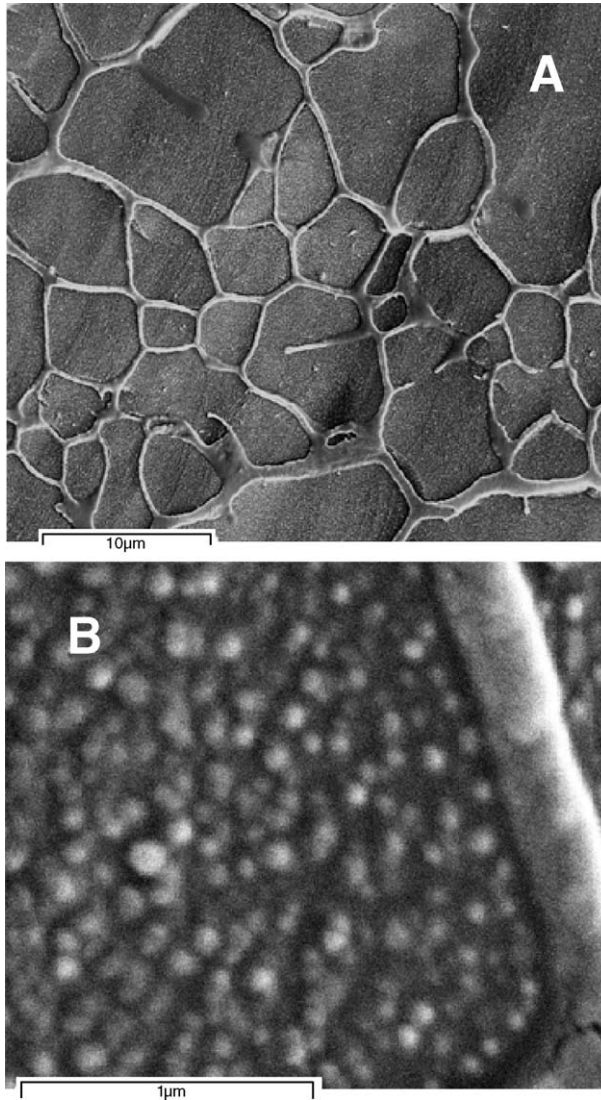


Fig. 6. SEM micrographs of a hydrogel synthesized in an aqueous solution containing 10 wt.% of THEOS and 1 wt.% of cat-HEC.

elements. One is a network formed by crossed (branched) filaments. They represent macromolecules surrounded by a shell made up of silicate. A spacing between the filaments is filled with particles of which shape is close to spherical. They are formed also from silicate. Analogous structural element and hierarchy were found also for bionanocomposites prepared in the presence of xanthan and locust bean gum. This means that the polysaccharides provide the same structural arrangement of silicate when it is generated in situ in their media.

The silica biomaterials prepared by the sol–gel technique are porous that is caused by the peculiarities of processes. The formation of three-dimensional network results from the cross-linking of initially formed sol particles. The porous morphology is typical of silica nanocomposites [1,2] and it is observed also for THEOS-produced materials [4].

The polysaccharides, as followed from the picture in Fig. 6, modified the sol–gel processing in such a manner that the sol particles were left as separated entities, while part of silicate is associated with macromolecules. This signifies that the organic component promotes nucleation and growth of silicon compound, manifesting itself as a template.¹ This result is in line with a current opinion on the role of polysaccharides in the biomineralization in living systems (see, e.g., Ref. [15]). It is believed that the association and polymerization of silica are provided by hydrogen bonding to the organic template.

3.2. Characterization of enzymes immobilized in nanocomposites

1 → 3-β-D-glucanase L_{IV} from marine mollusk *S. sacchalinensis* [8] and α-D-galactosidase from marine bacterium *Pseudoalteromonas* sp. KMM 701 [9] were entrapped into three various gelled matrixes differed by added polysaccharides. This enabled us to examine their effect on the immobilization, activity and stability of entrapped enzymes. The results are summarized in Table 1.

One may see that the enzymes retained their activity after the immobilization in the hybrid silica nanocomposite. There was only an exception for α-D-galactosidase entrapped in the gelled matrix with cat-HEC. In this case, the immobilized enzyme did not demonstrate the enzymatic activity. Meanwhile, 1 → 3-β-D-glucanase L_{IV} was active in the analogous matrix. It is worth of mentioning that the entrapment of both the enzymes into the hybrid silica material containing locust bean gum favored with their functional activity. For 1 → 3-β-D-glucanase L_{IV}, it was about two times greater than that measured for the control sample in the aqueous solution.

The next important conclusion followed from the examination results in Table 1 is that the long-term stability of the immobilized enzyme increased to a great extent. They were functionally active throughout 165 days after the entrapment, whereas the control samples lost the enzymatic activity within 1–2 days. There are some differences between enzymes immobilized in the various matrixes. Their activity was almost unchanged in the silica gels containing xanthan and cat-HEC. A gradual decrease in the activity is seen for 1 → 3-β-D-glucanase L_{IV} entrapped into gelled matrix with locust bean gum.

¹ Further data, which detail a possible mechanism for the mineralization processes, will be presented in a separate article that is under preparation.

Table 1

Relative activity of enzymes in the aqueous solution and immobilized state in silica gel with various polysaccharides

Days elapsed after immobilization	Enzyme activity, % relative to the initial activity of control sample							
	Endo-1 \rightarrow 3- β -D-glucanase L _{IV}				α -D-galactosidase-			
	Control	Locust bean gum	Xanthan	Cat-HEC	Control	Locust bean gum	Xanthan	Cat-HEC
0	100	230	100	120	100	160	52	0
1	40	200	110	90	0	nd	nd	nd
4	0	230	160	160	nd	nd	nd	nd
11	nd ^a	150	145	140	nd	nd	nd	nd
30	nd	160	110	110	nd	120	33	nd
60	nd	70	100	80	nd	nd	nd	nd
90	nd	130 ^a	60	90	nd	nd	nd	nd
165	nd	nd	110 ^a	130 ^a	nd	nd	67 ^b	nd

^a Not determined.

^b The increase in the activity can be caused by a partial drying of samples.

3.3. Discussion of results

The enzymes considered in the article are representatives of two different groups of *O*-glycoside hydrolyses (EC3.2.1). They are presented by endo-1 \rightarrow 3- β -D-glucanase (laminarinase, EC 3.2.1.6) and α -D-galactosidase (EC 3.2.1.22) [16]. The 1 \rightarrow 3- β -D-glucanases are among digestible enzymatic systems of many sea organisms, participating in the reproductive processes and protective reactions, included into PR-proteins of plants, etc. They are promising for biotechnological applications because the glucanases can catalyze processes for the preparation, for example, of oligo- and polysaccharide derivatives possessing immunostimulating, antiviral, anticancer and/or radioprotective activity [17–21].

The α -D-galactosidases play also an important role in plants and animals owing to their ability to separate galactose residues located at nonreducing end of oligo- and polysaccharides, glycosides and various glycolconjugates [16]. A certain of these enzymes possess unique specificity, separating α -galactose residues, for example, from glycoproteins of the blood that serve as a marker causing the blood indispensability to a B(III) group. When this residue has been removed, the blood transfers into O(H) group that is of great interest for preparing the “universal” blood [22].

It is typical of enzymes that they have a short lifetime after the isolation. Their stability decreases sharply with dilution of solutions, whereas it is necessary to have them in the diluted state for using as catalysts. This is one of the main reasons retarding the development of biotechnological processes.

This restriction is valid in full measure for the considered enzymes. Their diluted solutions (control samples) lost the enzymatic activity, as obvious from Table 1, within 1–2 days. Therefore, a sharp increase in the enzyme lifetime after the immobilization comes as a surprise. This is so much great that the immobilization can be considered as a rather simple, convenient method for the conservation of highly purified enzymes.

It is reasonable to mention that endo-1 \rightarrow 3- β -D-glucanase and α -D-galactosidase were entrapped in small amounts inside the hybrid silica nanocomposites. The level is comparable to that in the living cells. The appearance of activity by the enzymes at the small content differs the developed materials from those prepared on the basis of currently used TMOS and TEOS. In their case, much higher amounts of enzymes (up to 20–30 wt.%) are usually immobilized inside silica matrix to provide a reasonable level of activity [23].

The examined enzymes differ notably by the molecular weight and optimum pH. The endo-1 \rightarrow 3- β -D-glucanase L_{IV} is a low-molecular-weight protein (22 kDa) demonstrating the maximum activity at pH 5.2 [24], while α -D-galactosidase is a high-molecular-weight one (200 kDa) for which the optimum pH is 7.2 [22]. Furthermore, the substrates of glucanases are high-molecular-weight polysaccharides, whereas the glucosidases demonstrate activity both in relation to glycoconjugates and low-molecular-weight substrates including oligosaccharides and glycosides. All these factors can be of importance for the enzyme entrapment and functioning in the immobilized state.

The presented results (Table 1) show that both 1 \rightarrow 3- β -D-glucanase and α -D-galactosidase were successfully entrapped in the polysaccharide–silica matrixes. This allows considering THEOS as a convenient precursor for the enzyme immobilization. THEOS efficiency should be associated with the provided experimental opportunities. The precursor makes possible the performance of the sol–gel processes and hence the immobilization at pH and low temperatures which are optimal for an enzyme. The TMOS and TEOS do not usually generate silica gel at these pH (5.2 and 7.2). To carry out the sol–gel processing in their case, the aqueous solution should be acidified or alkalized [1,2]. In addition, TMOS and TEOS produce a non-precipitating gel at a rather large concentration (usually not less than at 25 wt.% [25]). To afford their solubility, it needs to introduce an organic solvent.

There is also another important aspect of the increased precursor concentration. When it is mixed with water, a heat release on the precursor hydrolysis happens. It results in a temperature increase by 10° and more, depending on the precursor amount. This can be critical for the entrapped enzymes. The advantage of THEOS is that its amount can be decreased up to few percent in the solution (Fig. 5) that brings about the temperature change not more than by a 1°.

It is worthy of mentioning that a cross-linking agent is unnecessary to introduce for the efficient immobilization. It is believed [25,26] that the enzymes are entrapped mainly due to the physical interactions including electrostatic ones and hydrogen bond formation. This does not perturb the secondary and tertiary protein structure in such a significant manner as it happens when chemical cross-linkers are used (see, e.g. Ref. [27]). The retention of the activity by 1 \rightarrow 3- β -D-glucanase and α -D-galactosidase after the immobilization means that the reactions proceeding with the silica precursor did not touch the active centers of enzymes.

The second constituent of biomaterials—polysaccharides—plays also a notable role in the enzyme entrapment. This is obvious from a comparison of their enzymatic activity. The influence is mostly pronounced for α -D-galactosidase (Table 1). When positively charged cat-HEC was inside the biomaterial, the entrapped enzyme was inactive. The enzymatic activity was found in a case of hybrid matrix containing xanthan, which bears

negative charges, but its level was nearly half as great as that of the control sample. An activity increase took place when the enzyme was entrapped in the biomaterial with neutral locust bean gum. The same, but more pronounced effect was found for endo-1 \rightarrow 3- β -D-glucanase L_{IV}. On the other hand, there is no notable difference in the activity between the control sample and enzyme inside the hybrid matrixes prepared in the presence of charged polysaccharides. Xanthan and cat-HEC in these cases behaved like neutral additives not influencing on the 1 \rightarrow 3- β -D-glucanase L_{IV} activity in the immobilized state. By this means, the polysaccharides inside the silica gel can produce a modulating effect on the enzymatic activity that depends on the enzyme type. In order to have an insight into the mechanism of their influence, further study is however still necessary on these systems.

The testing of activity of both the enzymes entrapped inside a polysaccharide–silica matrix showed that they are accessible to the low- and high-molecular-weight substrates from an external solution. The accessibility can be associated with the structure of hybrid materials. There is a stiff network consisting of crossed filaments that are formed by mineralized polysaccharide macromolecules (Fig. 6). The mesh size on average is few microns. The gap between filaments is filled with discrete particles of up to 100 nm in diameter. It seems that they are not tightly packed. This structure should not pose severe difficulties for diffused macromolecules. On the other hand, both 1 \rightarrow 3- β -D-glucanase and α -D-galactosidase were immobilized rather strongly and not easily washed out of the porous biomaterials in spite of the significant difference in their molecular weight. To examine it, samples with entrapped enzymes were centrifuged. As found after the 10 min treatment at 4000 \times g, the activity left at the same level as before.

Thus, the forgoing results demonstrated that the new hybrid nanocomposites prepared on the basis of THEOS and polysaccharides can be successfully applied for the immobilization of labile enzymes. This is due to few advantages of the newly introduced precursor. (i) The entrapment can be performed at pH and temperature, which are suitable for the structural integrity and functionality of enzyme. (ii) The organic solvents and catalysts are not needed to solubilize the precursor and promote the sol–gel transition. (iii) A gel can be prepared at reduced concentrations of THEOS that makes the heat release in the course of its hydrolysis as small as possible. (iv) The porous structure of hybrid nanocomposite provides the accessibility of immobilized enzyme by the enzymatic substrate and proper functioning while the protein molecules are sufficiently linked inside of the matrix and not easily washed out of it. It is reasonable to add that the polysaccharide–silica biomaterials hold their integrity after performing the enzymatic processes. This enables one to separate them from the reaction products and be repeatedly used again that is of great importance for biotechnological applications.

The suggested method for the enzyme immobilization is based on the entrapment of protein molecules into pores of hybrid matrix generated in the course of hydrolysis of silica precursor and polymerization into three-dimensional network in bulk solution. Its advantage is that the processes are performed at pH and temperature which are dictated by the immobilized enzyme, but not the sol–gel processing. The most obvious potential applications are in the development of biocatalysts for biotechnology and biosensors.

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References

- [1] Brinker CJ, Scherer GW. Sol–gel science. The Physics and Chemistry of Sol–Gel Processing. Boston: Academic Press; 1990.
- [2] Pierre AC. Introduction to Sol–Gel Processing. Boston: Kluwer; 1998.
- [3] Gill I, Ballesteros A. Encapsulation of biologicals within silicate, siloxane, and hybrid sol–gel polymers: an efficient and generic approach. *J Am Chem Soc* 1998;120:8587–98.
- [4] Meyer M, Fischer A, Hoffmann H. Novel ringing silica gel that do not shrink. *J Phys Chem, B* 2002;106:1528–33.
- [5] Shchipunov YuA, Karpenko TYu. Hybrid silica-polysaccharide materials prepared by the sol–gel processing. In: Maestro P, Roux D, editors. The 2nd Intern. Rhodia Conf. “Physical Chemistry of Polymeric Systems”. Bristol: University of Bristol; 2002. p. 72.
- [6] Mehrotra RC, Narain RP. Reactions of tetramethoxy- and triethoxysilanes with glycols. *Indian J Chem* 1967;5:444–8.
- [7] Zvyagintseva TN, Shevchenko NM, Popivnich IB, Isakov VV, Scobun AS, Sundukova EV, et al. A new procedure for the separation of water-soluble polysaccharides from brown seaweeds. *Carbohydr Res* 1999;322:32–9.
- [8] Sova VV, Elyakova LA. Some aspects of the specificity and action pattern of β -1,3-glucan glucanohydrolase from *Spizula sachalinensis*. *Biochim Biophys Acta* 1972;258:219–27.
- [9] Bakunina LYu, Sova VV, Nedashkovskaya OI, Kuhlmann RA, Likhosherstov LM, Martynova MD, et al. *Biochem (Moscow)* 1998;63:1209–16.
- [10] Zvyagintseva TN, Sundukova EV, Shevchenko NM, Popivnich IB, Stechova SI, Yudakova ZS, et al. The effect of 1,3:1,6-D-glucans on the phytoalexin set and the activity of carbohydrases in the soybean callus cultures and shoots. *Acta Phytopathol Entomol Hung* 1997;32:59–67.
- [11] Nelson TE. A photometric adaptation of the Somogyi method for the determination of glucose. *J Biol Chem* 1944;153:375–81.
- [12] Goodwin JW, Hughes RW. Rheology for Chemists. An Introduction. Cambridge: Royal Society of Chemistry; 2000.
- [13] Shumilina EV, Shchipunov YuA. Chitosan-carrageenan gels. *Colloid J* 2002;64:372–8.
- [14] Larson RG. The Structure and Rheology of Complex Fluids. New York: Oxford Univ. Press; 1999.
- [15] Frausto da Silva JJR, Williams RJP. The biological chemistry of the elements. The Inorganic Chemistry of Life. Oxford: Oxford Univ. Press; 2001.
- [16] Sinnott ML. Catalytic mechanisms of enzymic glycosyl transfer. *Chem Rev* 1990;90:1171–202.
- [17] Solov'eva TF, Elyakova LA, Zvyagintseva TN, Yermak IM. Polysaccharides from the Russian Pacific coast algae and their enzymatic transformation. *J Mar Technol Soc* 1996;30:35–9.
- [18] Zvyagintseva TN, Makareva TN, Ermakova SP, Elyakova LA. The production of *p*-nitrophenyl laminarioligosides through the transglycosylation reaction catalyzed by endo-1,3- β -D-glucanase from a marine mollusk. *Russ J Bioorgan Chem* 1998;24:195–9.
- [19] Shevchenko NM, Zvyagintseva TN, Ivancha LN, Gorbach VI. Preparation and using of antibody to biologically active 1,3:1,6-D-glucan-translam. *Biotechnol* 2000;6:3–10.
- [20] Bulgakov VP, Kusakin MG, Tchernoded GK, Zvyagintseva TN, Zhuravlev YN. Carbohydrase activities of the rolC-gene transformed and non-transformed ginseng tissues. *Fitoterapia* 2002;73:638–43.
- [21] Andreeva AI, Burtseva YuV, Zvyagintseva TN. The effect of soybean mosaic virus on the activity of carbohydrases in leaves of sensitive and resistant soybean plants. *Acta Phytopathol Entomol Hung* 2002; 37:335–45.
- [22] Bakunina LYu, Sova VV, Nedashkovskaya OI, Kuhlmann RA, Likhosherstov LM, Martynova MD, et al.

- α -Galactosidase of the marine bacterium *pseudoalteromonas* sp. KMM 701. *Biochem (Moscow)* 1998;63: 1209–15.
- [23] Lu Z-L, Lindner E, Mayer HA. Applications of sol–gel-processed interphase catalysts. *Chem Rev* 2002; 102:3543–78.
- [24] Yermakova SP, Sova VV, Zvyagintseva TN. Brown seaweed protein as an inhibitor of marine mollusk endo-(1,3)-D-glucanases. *Carbohydr Res* 2002;337:229–37.
- [25] Gill I, Ballesteros A. Bioencapsulation within synthetic polymers (Part 1): sol–gel encapsulated biologicals. *Trends Biotechnol* 2000;18:282–96.
- [26] Dave BC, Dunn B, Valentine JS, Zink JI. Sol–gel encapsulation methods for biosensors. *Anal Chem* 1994; 66:1120A–7A.
- [27] Hennink WE, van Nostrum CF. Novel crosslinking methods to design hydrogels. *Adv Drug Deliv Rev* 2002;54:13–36.