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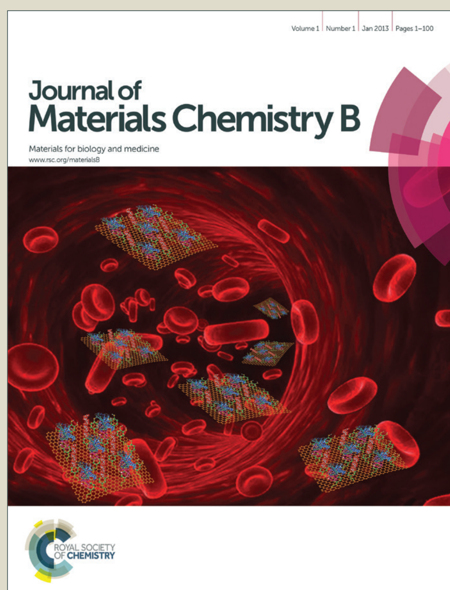

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ARTICLE

Immobilization of urease on magnetic nanoparticles coated by polysiloxane layers bearing thiol- or thiol- and alkyl-functions

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Magnetically retrievable formulations of urease potentially perspective for biomedical and environmental applications were constructed by immobilization of the enzyme on surface of magnetite nanoparticles functionalized by siloxane layers with active thiol or thiol-and-alkyl moieties. The latter were deposited using hydrolytic polycondensation reaction of tetraethoxysilane with either 3-mercaptopyltrimethoxysilane, or with 3-mercaptopyltrimethoxysilane and methyltriethoxysilane, alternatively *n*-propyltriethoxysilane. Immobilization of urease was carried out in different ways for comparison: by adsorption, by entrapment during the hydrolytic polycondensation reaction, or by covalent bonding. For entrapment the enzyme was introduced into solution before functionalization of the magnetite. Entrapment bound high amounts of enzyme (more than 700 mg per g of carrier), but its activity was decreased compared to the native form to between 18 and 10%. In case of covalent binding of urease using Ellman's Reagent, the binding of enzyme was almost as efficient as in case of entrapment but its residual activity was 75%. The residual activity of urease immobilized by adsorption on the surface of thiol-functionalized particles was truly high as compared to native enzyme (97%), but binding was significantly less efficient (46%). Introduction of alkyl functions permitted to increase the amounts of adsorbed enzyme but its activity was somewhat decreased.

Introduction

Magnetite nanoparticles, Fe_3O_4 are magnetic, non-toxic, biocompatible, and easily produced from cheap reagents, iron (II) and iron (III) salts by co-precipitation, which explains the increasing interest to this and related iron oxide materials. Recently, they have received broad application in the study of immunoassays,[1] bioseparation,[2] biosensors,[3] targeted drug delivery,[4] as well as in other domains of biomedical sciences.[5-7] Spherical nanoparticles of these magnetic materials can be easily functionalized, in particular, using alkoxysilanes as precursors. Grafting of functional groups on the surface can broaden the field of their application, for example, in environmental analysis.[8]

Enzymes are universal biocatalysts efficiently catalysing specific chemical reactions in vivo and in vitro.[9] This granted them a broad spectrum of industrial applications covering such domains as fine chemical synthesis, pharmaceutical chemistry, valorisation of food and feed and also in the production processes for biofuels (bioethanol and biodiesel).[10-18] The biological origin of the enzymes sets, however, considerable challenges for their efficient industrial application. Enzymes are commonly highly soluble and are often easily inhibited by substrates, own reaction products and other components in the industrial bioreactor media. They very often display insufficient stability associated with the loss of optimal catalytic functions when applied on non-physiological substrates.[19] Immobilization of enzymes is the simplest approach in addressing the excessive solubility of a protein.[20,21] This approach is often mandatory in order to grant possibility of their repeated use. Immobilization of biocatalyst permits also generally to simplify the construction of a bioreactor and to control effectively its productivity.[22,23] It permits also to tune the conditions of bioreaction, opens possibility to carry it out in continuous regime and helps to avoid the pollution of the reaction products with applied enzymes – a feature highly requested in the food industry. Immobilization of enzymes on solid carriers is a recognized technological approach for improvement of their stability, lifetime and separation from the reaction mixture after completion of the process,[24] which allows also for improved cost efficiency and reuse of the catalyst.

The most common reason for the loss of enzyme activity is the change in conformation of the protein molecule.[25,26] Immobilization helps generally in stabilization of biocatalysts hindering the opening of the molecules and protecting the polypeptide bonds against rupture, providing thus conformational stability in the active sites.[27,28] Efficient stabilization of an enzyme can be achieved through its fixation in the applied matrix through formation of numerous hydrogen bonds with amino acid units in its structure.[29,30] Multicenter fixation of multimeric proteins can even prevent dissociation of their sub-units, decreasing thus even the risk of conformational inactivation of the bound sub-units. This immobilization improves also the thermal stability of enzymes as it enhances the rigidity of the protein molecule creates a protective

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micro environment.[31] Improvement in thermal and chemical stability of enzymes has been broadly demonstrated on their immobilization in gels and sol-gel glasses.[32-35]

Various materials for enzyme immobilization have been described in literature: polymeric membranes,[36] silica,[37,38] chitosan-siloxane hybrid materials,[39] zeolites[40] and other molecular sieves.[41] Compared to those, the magnetic iron oxide nanoparticles are more effective carriers for the immobilization of enzymes,[3,42-45] as they allow for separation of the catalyst from the reaction products using external magnetic field.[46] Additionally, the magnetic nanoparticles possess high active surface area. This helps to decrease the diffusion barriers in the transport of substrate and the reaction products, improving the efficiency of the immobilized enzyme.[35] It has to be mentioned that, on the contrary, immobilization on materials with low active surface area can cause low degree of grafting for the enzyme, and result in its desactivation and desorption in the course of a fermentative reaction.[47]

This feature explains also the reasons behind growing use of magnetically controlled nanomaterials in biomedicine.[48] Latter have usually a core-shell structure. The shell is commonly composed of silicon dioxide[49] attractive due to its unique chemical and structural characteristics.[50] The shells contain generally certain functional groups, such as hydroxyl, amino, thiol etc., which are introduced via co-polymerization or via chemical modification of the surface.[51] This improves principally the possibility to use these materials for binding to enzymes, antibodies, nucleic acids etc.[52] Another principal advantage in use of enzymes immobilized on magnetic carriers lies in the possibility to create a fluidized bed reactor exploiting rotational-vibrational alternating magnetic field. Magnetic ineractions can be even used in such reactor to prevent formation of a layer of reaction products on the surface of the immobilized enzyme, thus improving the efficiency of the biocatalyst.[53,54]

One of the enzymes most attractive in environmental applications is urease, which belongs to the class of hydrolases. It catalyzes hydrolysis of urea. Urea is the main toxic metabolite in the human body and removing its excess is very important for patients suffering from kidney failure.[55] The most effective way of removing urea from aqueous solution is using immobilized urease. Most common for application in analytical research and biomedicine has been urease immobilized on various polymeric materials.[56,57] However, very little is known about the use of magnetic inorganic nanoparticles for this purpose. Nevertheless, urease immobilized on magnetic nanoparticles as carriers may potentially retain activity at the level of the native enzyme and can be quickly removed from the reaction suspension with external magnetic field. Therefore, the current research is focused on the search for the simple and cost-efficient method for preparation of monodisperse superparamagnetic nanoparticles with immobilized urease that would retain catalytic activity close to that of the native enzyme.

Experimental

Following reagents were used in the present study: tetraethoxysilane, $Si(OC_2H_5)_4$ (TEOS, Aldrich, 98%); 3-mercaptopropyltrimethoxysilane, $(CH_3O)_3Si(CH_2)_3SH$ (MPTMS, Aldrich, 95%); methyltriethoxysilane, $(C_2H_5O)_3SiCH_3$ (MTES, 99%, Aldrich); *n*-propyltriethoxysilane, $(C_2H_5O)_3Si(CH_2)_2CH_3$ (PTES, 97%, Fluka); iron(II) chloride tetrahydrate, $FeCl_2{\cdot}4H_2O$ (Aldrich, 99%), iron(III) chloride hexahydrate, $FeCl_3{\cdot}6H_2O$ (Aldrich, 97%), ammonia (25%); ethanol (96%); acetone (Aldrich, 99.9%); 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's Reagent, Aldrich, 98%); K_2HgI_4, Nessler's reagent (Fluka); NH_4F (Fluka, 98%); 0.1M HCl and 0.1M NaOH (from fixanal); 0.06 M phosphate buffer (pH 7.0), 0.1M EDTA; urea powder (Aldrich). Urease used in this work was derived from soybeans ''Jack beans'' (EC 3.5.1.5, activity 43 Un/mg (pH 7.0), Fluka).

Magnetite was prepared by coprecipitation of iron(II) and iron (III) chlorides with ammonia in a nitrogen atmosphere.[58] Obtained Fe_3O_4 particles were spherical with average diameter about 12 nm, and specific surface area of about 96 m^2/g.[59]

Samples' micrographs were obtained on a JSM 6060LA scanning electron microscope (Jeol, Japan) in the secondary electron mode at an accelerating voltage of 30 kV. The samples were mounted on a specimen stage coated with an adhesive. In order to prevent the buildup of surface charge and to obtain a contrast image, a thin continuous layer of gold was deposited onto the sample surface in vacuo by cathode sputtering.

Content of Si, S and Fe were measured using scanning electron microscopy combined with energy dispersive spectroscopy (SEM-EDS) with Hitachi TM-1000-μDeX microscope (Department of Chemistry, Biocenter, SLU, Uppsala, Sweden).

The elemental analysis of the synthesized samples was carried out in the Analytical Laboratory of the Institute of Organic Chemistry (Kyiv, NAS of Ukraine).

The DRIFT spectra were recorded on the Thermo Nicolet Nexus FT-IR at 8 cm^{-1} resolution using the Spectra Tech collector diffuse reflectance accessory at room temperature. The samples were mixed with KBr (1:30) and were used to fill the DRIFT sample cup before measurements.

Urease enzyme activity was determined by the rate of ammonia formation in the urea hydrolysis reaction at 25°C.[60] In all cases, the average of three parallel experiments (the biggest difference between them <10 %) was used for activity determination. The error of measurement using Student coefficient at rugged probability of 0.95 is less than 10%.

Synthetic procedures.

Preparation of magnetite nanoparticles with monofunctional surface layer (3-mercaptopropyl groups – sample 1). A 100 mg (0.0004 mol) batch of Fe_3O_4 was treated with ultrasound for 10 min in 50 cm^3 of water. Thereafter, pre-hydrolyzed alkoxysilanes were added to the suspension. Their prehydrolysis was carried out on heating (~ 80°C) by mixing 1.0 cm^3 of ethanol, 1.78 cm^3 of TEOS (0.008 mol), and 1.0 cm^3 HCl (0.0024M). The resulting emulsion was stirred until clear solution was formed (~ 20 min). It was cooled to room temperature and then MPTMS, 0.49 cm^3 (0.00266 mol) was added. After 20 minutes of stirring, a clear sol was obtained. This sol was added to a suspension of magnetite in aliquots (0.5 cm^3 every 15 min) for two hours, and then the suspension was stirred for another 30 min. The molar ratio of components was Fe_3O_4/TEOS/MPTMS = 0.0004/0.008/0.0027. The precipitate of modified magnetite was collected by magnet, washed with water (3 x 100 cm^3), ethanol (2 x 50 cm^3), and acetone (2 x 50 cm^3). Further processing of the obtained material depended on the choice of urease immobilization method (either dried at 110°C in an oven or re-dispersed to produce an aqueous suspension). The sulphur content was 0.8 mmol/g according to microanalysis.

Production of magnetite with bifunctional surface layer (3-mercaptopropyl/methyl groups (sample 2) or 3-mercapto-propyl/*n*-propyl groups (sample 3)). Preparation of these samples was the same as of sample **1**, except that 0.49 cm^3 of MPTMS (0.00266 mol) were added together with 0.26 cm^3 of MTES (0.0013 mol) or 0.3 cm^3 (0.0013 mol) PTES. The molar ratio of the

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components was Fe_3O_4/TEOS/MPTMS/MTES (PTES) = 0.0004/0.008/0.0027/0.0013. Sulphur content (mmol/g) was as follows: **2** – 1.8; **3** – 1.2.

Urease immobilization by sorption. Urease sorption was performed by static method for 4 hours at room temperature, applying periodical stirring. A batch of functionalized magnetite (0.05 g) was put into 2 cm^3 of mixed phosphate buffer (pH 7,0) and EDTA solution (volume ratio of 9:1) containing 5 mg urease. The precipitate was then separated from the solution by magnet and washed 5 times with 5 cm^3 phosphate buffer. Urease overall adsorption was determined by the difference between the urease taken for immobilization and its residual content in solution.
To study the kinetics of urease adsorption, a batch of functionalized magnetite (0.05 g) was shaken in a test tube with 2 cm^3 of urease buffer solution with concentration of 2.5 g/l. After 5, 10, 20, 30, and 60 min, and then after 2, 4, 6, and 8 hours, the solution above the precipitate was analysed for the enzyme content. In all cases, the amount of bound enzyme was evaluated by the difference between the urease taken for immobilization and its residual content in solution. The latter was determined in the solution after adsorption and in washing waters from the enzyme activity compared to the specific activity of the enzyme. It was assumed that the specific activity of the enzyme remains the same as that of the native one.
To build urease adsorption isotherms its buffer solutions with concentrations from 1.25 to 7.5 g/l were used. The process was carried out for 4 hours at room temperature with periodical stirring.

Urease entrapment in the polysiloxane shell. 1.78 cm^3 of TEOS (0.008 moles) were mixed with 0.5 cm^3 of ethanol. 1 cm^3 of 0.0024 M HCI solution was added to the mixture that was stirred under heating (70°C) until the clear solution was obtained. After cooling the solution 0.45 cm^3 of MPTMS (0.0027 mol) were added and the mixture was stirred to obtain a transparent sol, the volume of which was adjusted with water to 10 cm^3. Then 0.5 cm^3 of this sol were added in aliquots (0.05 cm^3 every 15 min for 1.5 hours) at constant stirring to the suspension, which consisted of 10 mg of Fe_3O_4 (0.00004 mol) and 5-30 mg of urease in 5 cm^3 of phosphate buffer. After the sol addition was completed, the suspension was stirred for another 30 min. The precipitate of the functionalized magnetite with incorporated urease was separated with magnet, washed with water (3 x 10 cm^3) and re-suspended for further analysis of enzyme activity.

Urease immobilization by covalent bonding. 50 mg of sample **1** was added to an aqueous solution of Ellman's Reagent (0.105 mg per 10 cm^3). The suspension was stirred for 2 hours, and then the precipitate was separated by sedimentation and washed repeatedly with water. Then the sample was mixed with 5 cm^3 of urease (10 mg) buffer solution (pH 7.0) and stirred for 1 hour, at room temperature. The solid phase was then separated, washed with water (3 x 10 cm^3) and re-suspended for further analysis of enzyme activity.

Results and discussion

Hydrolytic polycondensation reaction in two- or three-component systems (considering alkoxysilanes) was carried to create a functional layer on the surface of magnetite nanoparticles (see Fig. 1). It was assumed, that in this case the product of hydrolysis and polycondensation of precursors would create on the surface of spheical Fe_3O_4 nanoparticles a thin layer of polysiloxane network with 3-mercaptopropyl groups (and alkyl radicals), attached by hydrolytically stable ≡Si–C≡ linkages. In order to create homogeneous polysiloxane layer on the surface of Fe_3O_4 nanoparticles, as well as to avoid the formation of separate phases of alkoxysilanes, they were introduced by small aliquots into the reaction medium.

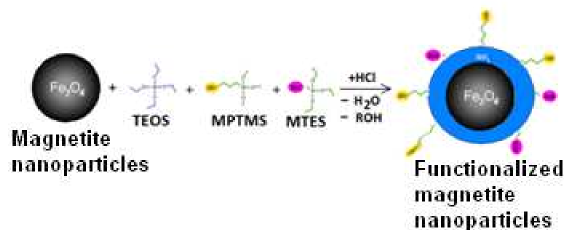

Figure 1. A scheme for producing functionalized magnetite nanoparticles.

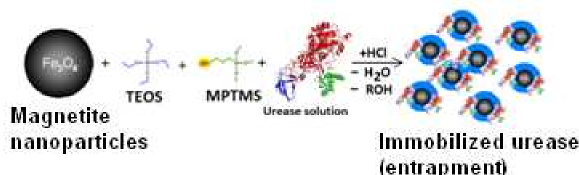

Figure 2. A scheme immobilization of urease by entrapment.

The scheme of urease immobilization by entrapment in the polysiloxane layer with functional groups during the formation of the layer on the surface of nanoparticles is displayed in Fig. 2. It was assumed that in this case, urease is secured on the particle surface not only due to the interaction with the functional groups, but also partly by its mechanical fixation in the polysiloxane network formed during the reaction of hydrolytic polycondensation of alkoxysilanes.
According to SEM images presented in Fig. 3 the particles retain spherical shapes after the formation of functional layers on their surface, but in the applied synthesis conditions their sizes significantly increase compared to the original magnetite particles. Thus, for sample **1** the majority of its particles are 60-70 nm in diameter, for sample **2** about 280 nm, and for sample **3** - 65-80 nm. In other words, the size of the particles bearing a functional layer increases compared with the original one almost five times for samples **1** and **3**, and almost 20 times for sample **2**. Moreover, the size of the formed particles is more influenced by the nature of the functional group additional to the thiol. Thus, under identical conditions of synthesis for samples **2** and **3**, the particle diameter in the sample **2** is more than 3.5 times bigger than in sample **3.**
The EDS analysis of the surface layer of the particles (Fig. 3), and sulfur content according to the elemental analysis indicate that they do contain polysiloxane layer involving sulfur-containing groups. Moreover, higher content of sulfur-containing radicals in the surface layer is observed for sample **3**.
The presence the polysiloxane layer and functional groups on the surface of the magnetite particles was confirmed by IR spectroscopy (Fig. 4). The spectra of all the samples had an intense and broad absorption band at 1020-1120 cm^{-1}, which corresponds to ν_{as}(SiOSi) of the polysiloxane skeleton.[61]

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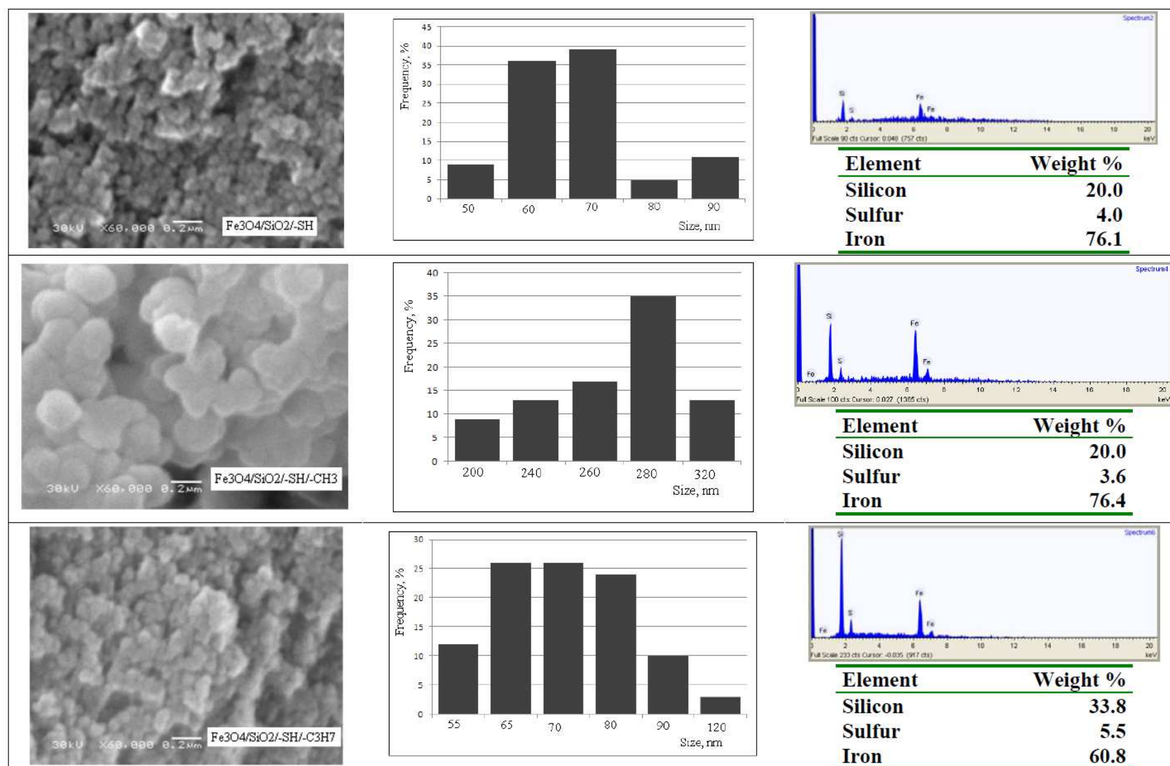

Figure 3. SEM microphotos and EDS characterization of functionalized magnetite samples with 3-mercaptopropyl groups (**1**), 3-mercaptopropyl groups and methyl groups (**2**), 3-mercaptopropyl groups and *n*-propyl groups (**3**).

A weaker band at 2557-2570 cm^{-1} in the IR spectra of the samples corresponds to the stretching of the mercapto groups ν(SH).[62] A shoulder at 1260 cm^{-1} in the IR spectrum of the sample **2** synthesized using MTES can be attributed to the $\delta_s(CH_3)$ of methyl group bonded to silicon atom.[62] The IR spectra of the samples (Fig. 4) also reveal absorption bands at 540-560 cm^{-1}, 1625-1635 cm^{-1}, and 2860-2970 cm^{-1}, which can be attributed to ν(FeO), $\delta(H_2O)$, and $\nu_{s,as}(CH)$, respectively. Furthermore, there is a group of low intensity absorption bands in the region of 1300-1500 cm^{-1} associated with vibrations in the alkyl group and/or the propyl chain.

Thus, the synthesized samples feature almost spherical particles containing polysiloxane layer with 3-mercaptopropyl groups on the surface.

To assess protein binding properties of the samples, the kinetics of urease adsorption on them was investigated (see Fig. 5). According to it, the adsorption equilibrium for Sample **1**, which contains only 3-mercaptopropyl groups, is achieved within 1 hour. For the same period of time, samples **2** and **3** sorbed only 70-75% of urease. After 4 hours, the same samples sorbed 80% and 90% of urease, respectively, and the adsorption equilibrium was established only after 8 h of stirring the samples with enzyme solution.

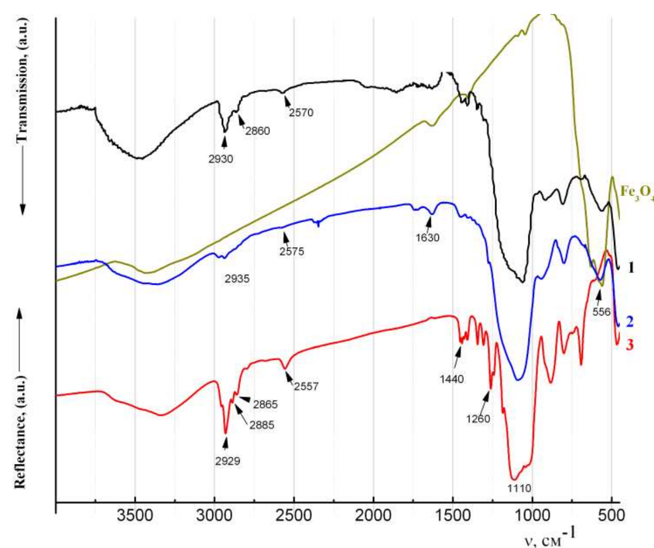

Figure 4. DRIFT spectra of the functionalized magnetite samples with 3-mercaptopropyl groups (**1**), 3-mercaptopropyl groups and methyl groups (**2**), 3-mercaptopropyl groups and *n*-propyl groups (**3**).

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With increasing reaction time, further enzyme binding was not observed. The longer time until establishing equilibrium during urease adsorption by samples **2** and **3** is apparently caused by the presence of alkyl groups.
It should be mentioned, that in the case of magnetite particles functionalized with 3-aminopropyl or 3-aminopropyl/alkyl groups, the sorption equilibrium is reached within 5-10 min.[63] Obviously, this is due, primarily, to the nature of the complexing layer on the particle surface: while 3-mercaptopropyl groups are very weak acids with a low dissociation degree, 3-aminopropyl groups are easily protonated in the presence of excess water, forming alkylammonium cations. It is known, that electrostatic forces of attraction have significant influence on the adsorption interaction of charged macromolecules of proteins with the surface of adsorbent carrying an electric charge.[64,65] Furthermore, it appears that the alkyl groups are more important on the surface of amino than thiol-containing magnetite particles. In the first case, they are likely to participate in the screening of 3-aminopropyl groups from the formation of hydrogen bonds with silanol groups,[63] which is not so important in the presence of thiol groups not causing formation of strong hydrogen bonds.

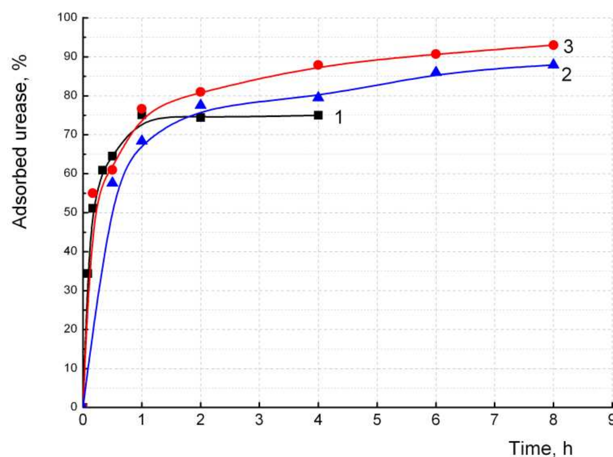

Figure 5. Kinetics of urease adsorption on the magnetite modified with 3-mercaptopropyl groups (**1**), 3- mercaptopropyl groups and methyl groups (**2**), 3-mercaptopropyl groups and *n*-propyl groups (**3**).

Adsorption isotherms of urease from buffer solutions by functionalized magnetite samples are shown in Fig. 6. All isotherms are similar in shape and have a sharp rise at low urease concentrations in the initial solution, which may indicate a strong adsorbent-adsorbate interaction. With a further increase in the concentration of urease, the curves reach a plateau (Fig. 6). It is believed that isotherms of this shape are observed when there is no strong competition of the solvent molecules during the adsorption, or when there is a strong intermolecular interaction between the sorbed molecules.[66] It is possible, that in this case both of the factors should be considered. Urease used for sorption is an enzyme with high molecular weight, which due to the multiple links between the functional groups is in the form of large associated particles in aqueous solutions. Therefore, one would expect a multicenter adsorption, during which competition of water molecules is negligible.
The obtained urease adsorption isotherms (Fig. 6) can be attributed to the Langmuir type.[67] According to the same figure, urease adsorption by samples **2** and **3** containing thiol/alkyl groups in the surface layer is somewhat larger, as compared to sample **1**, containing only 3-mercaptopropyl groups, but only up to a certain concentration of enzyme (approximately 5 g/l, Fig. 6). Above this concentration, urease adsorption by samples **2** and **3** is smaller compared to sample **1**. Apparently, it is due to the presence of alkyl groups, so that urease has lesser opportunities to form more energetically favorable hydrogen bonds and electrostatic bonds with the matrix.[68-72] It is interesting to note that above the limit of the mentioned concentration in solution, the increased length of the alkyl chain can increase enzyme adsorption (compare samples **2** and **3** in Fig. 6). It is worth noting that for the samples with amine groups instead of a thiol,[63] such effect is observed from the first points of sorption series.

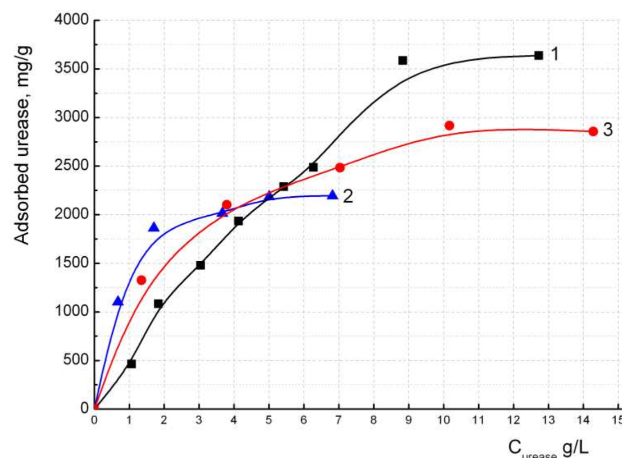

Figure 6. Adsorption isotherms for urease on magnetite modified with 3-mercaptopropyl groups (**1**), 3-mercaptopropyl groups and methyl groups (**2**), 3-mercaptopropyl groups and *n*-propyl groups (**3**).

The nature of the surface layer of functionalized magnetite particles has an impact not only on the quantity of adsorbed urease, but also on its residual activity level. We have shown earlier,[73] that urease adsorption on the not functionalized magnetite is less effective. Introduction of the thiol and thiol/alkyl groups in the surface layer increases enzyme binding in the latter case almost twice when the same amounts of adsorbed urease are used (see Fig. 7).

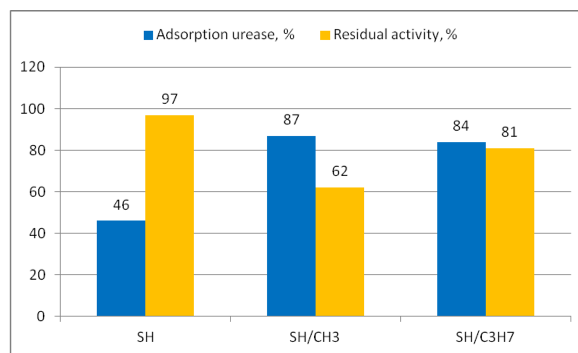

Figure 7. The effect of composition of the surface layer of functionalized magnetite nanoparticles on the quantity of adsorbed urease and its activity (same initial concentration of enzyme, 2.5 g/l, was used for all samples).

However, enzyme activity is partially reduced in the sample with to thiol/methyl groups compared to the one with only thiol

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groups. Entrapment of *n*-propyl groups in the bifunctional layer increases urease activity by ≈20% compared to that of methyl ones (Fig. 7). The latter effect is also observed for urease, adsorbed on the surface of magnetite particles with amine/alkyl groups.[63] Partial reduction of enzyme activity with transition from magnetite particles with 3-mercaptopropyl functionalized groups to the particles with bifunctional layer is obviously due to the influence of the hydrophobic effect. The elongation of alkyl radical would seem likely to reinforce this effect, but on the contrary, an increase, not decrease of urease activity is observed in the experiment (Fig. 7). Consequently, there has to be present other factors. In this case, the enzyme activity may be affected by the distance of the enzyme to the particle surface.

The relationship between the amount of urease, incorporated in the polysiloxane layer of nanoparticles bearing 3-mercaptopropyl groups and its residual activity are displayed in Fig. 8. According to it, all the urease taken for immobilization is incorporated in the matrix. However, the residual activity of incorporated urease is relatively low (approximately 18% of the activity of the native enzyme or less). Moreover, it decreases with increasing amounts of immobilized enzyme (Fig. 8). It appears that this is not only due to the blocking of the incorporated urease, but also because of increased resistance to diffusion of the substrate and its hydrolysis products.

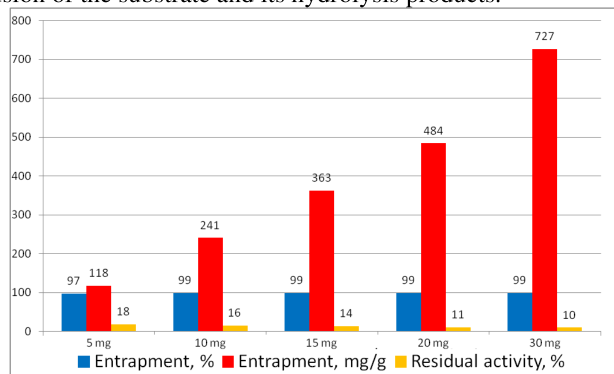

Figure 8. The relation between the amount of urease immobilized by entrapment and its activity for the samples bearing 3-mercaptopropyl groups.

The reduction in residual activity of incorporated urease is even more pronounced for the samples with a bifunctional surface layer (see Fig. 9).

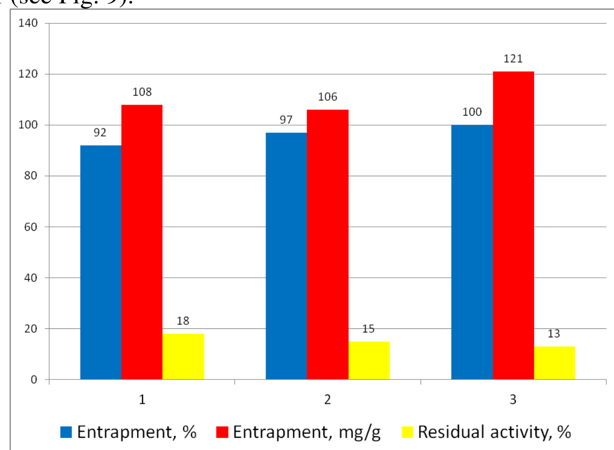

Figure 9. The relation between the amount of incorporated urease and its residual activity for the samples bearing 3-mercaptopropyl groups (1), 3-mercaptopropyl groups and methyl groups (2), 3-mercaptopropyl groups and n-propyl groups (3) (enzyme solution of the same concentration, 2.5 g/l, was used).

Although in this case the binding of the enzyme is almost 100%, its activity remains low. It is further reduced by one third on transition from the sample with 3-mercaptopropyl to the sample with both 3-mercaptopropyl and *n*-propyl groups. The reduction of enzyme activity on transition to the bifunctional surface layer was previously observed.[73] Apparently, the diffusion factor is contributing additionally to the hydrophobic effect of alkyl radicals introduced into the surface of the nanoparticles.

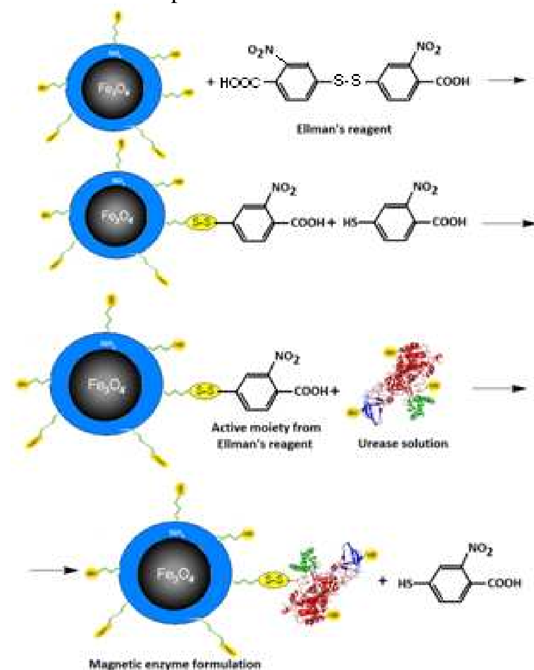

Figure 10. The scheme of urease immobilization on the surface of nanoparticles with 3-mercaptopropyl groups using Ellman's Reagent.

Urease has also been covalently attached to the surface of the sample **1** using Ellman's Reagent (according to the scheme in Fig. 10). The carriers bearing 3-mercaptopropyl groups are very useful for immobilization of urease via disulfide bridges because its molecule is containing itself many mercapto residues. Among the latter the most prone to formation of disulfide links are the peripheral ones, which are not crucial for the enzyme activity.[74] On application of the Ellman reactant, the *5,5'*-dithio-bis(*2*-nitro-benzoic) acid, the reaction proceeds quantitatively resulting in release of the *o*-nitro-*p*-thio-benzoate anion. The latter can easily be followed spectrophotometrically due to a characteristic adsorption band at 412 nm. The amounts of urease attached by this method to the surface of magnetite particles, and the levels of its residual activity are displayed in Fig. 11. This same figure shows similar characteristics for the samples with adsorbed and incorporated urease. From these data, the following conclusions can be drawn: in case of incorporation, there occurs practically complete binding of urease, however, the values of residual activity of the enzyme are the lowest. When the adsorption was used instead, the urease binding is 46% and its residual activity is 97%. Clearly, that during adsorption on nonporous carriers, all urease is located on their surface, wherein there are hardly any obstacles to diffusion of the substrate and the products of enzymatic reaction.

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It is possible that yet another factor contributes to the high residual activity close to that of the native enzyme. It is known that in the reaction of urea hydrolysis, catalyzed by urease, the cysteine group of enzyme located close to the metal center is acting as a proton donor in the formation of ammonia. Upon binding urease with the surface of thiol-containing polysiloxane matrix, a multipoint interaction of a large enzyme molecule with the surface silanol and 3-mercaptopropyl groups should occur.

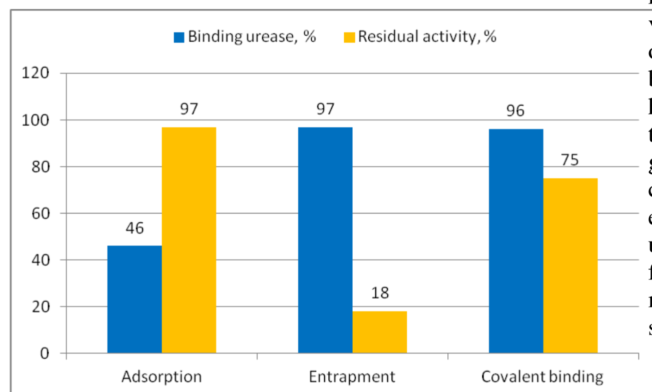

Figure 11. The influence of immobilization method on the amount of incorporated urease and its residual activity (enzyme solution of the same concentration, 2.5 g/l, was used).

As a result, the urease molecule may have such location on the surface that some of the HS-groups would be near the catalytical center of urease and act as proton donors, resulting in the observed increased speed and completeness of the enzymatic process. It is possible that "free" 3-mercaptopropyl groups binding inhibitory substances, such as heavy metal ions, make potentially also certain contribution to the observed increase in the enzyme activity, as compared to its native form.

Interesting results were obtained for covalent binding of urease. In this case, almost complete binding of enzyme (96%, see Fig. 11) is observed, which is approximately twice more than on it's adsorption. However, the residual activity decreases slightly from 97% to 75%. The matter is that the formation of covalent bonds may to some extent influence the conformation of the protein molecule, which causes the observed decrease in the activity of urease.

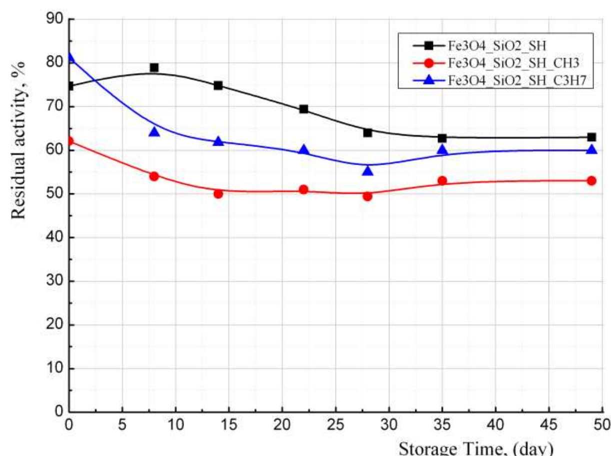

Figure 12. Storage stability of immobilized urease on magnetite functionalized by 3-mercaptopropyl groups, 3-mercaptopropyl groups and methyl groups, and 3-mercaptopropyl groups and *n*-propyl groups.

Finally, the changes in activity of the immobilized urease were also studied on it's storage. The residual activities of the enzyme immobilized on the carriers bearing 3-mercaptopropyl groups (black line), 3-mercaptopropyl and methyl groups (red line) and 3-mercaptopropyl and *n*-propyl groups (blue line) are displayed for comparison in Fig. 12. It is well-known that immobilization leads generally to preservation of the enzymatic activity.[75] This can be explained by stabilization of the native conformation of the ferment via inclusion into a matrix mimicking its natural environment in the cell and supporting it via hydrogen bonding. It is interesting to note that the activity of immobilized urease is initially decreased (with 10-15% loss), but is subsequently stabilized (see Fig. 12). It can be hypothesized that during the initial storage phase the aging of the polysiloxane matrix (additional condensation of silanol groups etc.) can cause some changes in the enzyme conformation. In continuation, however, the environment of the enzyme molecule is stabilized and the activity remains unchanged at the level of 50-60% of the initial activity in the following 50 days. The immobilized enzyme can easily be magnetically retracted from the medium even after this long storage period(Fig. 13).

Figure 13. Demonstration of the magnetic retraction of immobilized enzyme from solution.

Conclusions

Comparison of the binding efficiency and the activity of the immobilized enzyme permits to identify the covalent bonding as the most perspective approach for creation of a magnetically retrievable formulation (96% binding together with 75% in residual activity), especially if biomedical applications such as dialysis are the principal aim. All formulation components are biocompatible, making both biomedical and environmental applications of produced materials feasible.

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Notes and references

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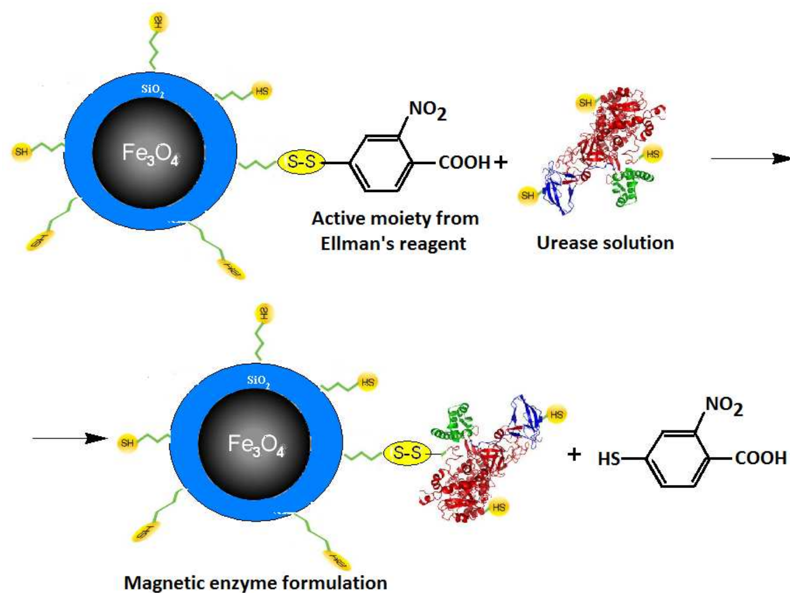
Optimized strategy for production of highly active magnetic formulation of urease has been elaborated via systematic studies of the enzyme immobilization on magnetite nanoparticles coated by functional siloxane layers.



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