O3-4	**Peroxidase encapsulated in chitosan matrix as the basis of optical sensor films**	

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INTRODUCTION AND OBJECTIVES

Horseradish peroxidase is one of the widely used enzymes in analytical biochemistry for determination of xenobiotics, drugs and their metabolites, markers of human diseases and quality of foods. However efficient use of peroxidase in nutritional, clinical and pharmaceutical analysis still has to overcome some limitations, and particularly the low operation stability of the biocatalyst in organic media that is necessary for the determination of analytes in water-insoluble samples.

The objectives of this work were to design of optical sensor films for determination of biologically active phenolic compounds on the basis of peroxidase noncovalent encapsulated in chitosan matrix acting in aqueous-organic media and to demonstrate their analytical possibilities for analysis of biological and pharmaceutical water-insoluble samples.

MATERIALS AND METHODS

Reagents and chemicals

Horseradish peroxidase, low molecular weight (MW) chitosans (5-200 kDa), *o*-dianisidine, hydroquinone, pyrocatechol, quercetin, rutin, esculetin, dimethyl sulfoxide and imidazole were purchased from Sigma and Center "Bioengineering" of Russian Academy of Science, 2-(N-morpholino)ethanesulfonic acid (MES) monohydrate was produced by Fluka, whereas hydrogen peroxide and components of phosphate and phthalate buffer solutions were obtained from Merck. All chemicals were used without any further purification.

Apparatus

Deionized water with specific electroconductivity 18.2 MOhm/cm was prepared employing Millipore Simplicity. The study of the dispersion of particles of peroxidase/chitosan complex in aqueous medium and measuring its hydrodynamic size were fulfilled using dynamic light scattering techniques (Malvern Instruments Zetasizer). All absorption measurements were carried out using Shimadzu UVmini1240 UV–vis spectrophotometer. pH meter was supplied by Mettler Toledo. Atomic force microscopy (AFM) image was obtained using NT-MTD scanning probe microscope.

Design of optical sensor films

Chitosan solution (MW 150 kDa) was prepared by dissolving of chitosan powder in acetic acid (0.5 %, v/v). The viscous chitosan solution was stirred overnight at the room temperature A homogeneous stock solution of a peroxidase/chitosan mixture was prepared from the required amount of 1% (w/v) chitosan solution and 168 U/mL peroxidase solution. The stock solution was pipetted onto a clean glass slide, 14×38 mm (and spread over it), and left to air-dry. The obtained biosensor slides were kept at 4°C before use.

RESULTS AND DISCUSSION

The effect of chitosans on the catalytic activity and stability of peroxidase

The incorporation of horseradish peroxidase into self-assembling complexes with chitosans of different molecular weights (MW 5–200 kDa) yielded highly active and stable enzyme preparations. The catalytic activity of peroxidase in the presence of 0.006 % w/w of chitosan (MW 150 kDa) in 0.05 M phthalate buffer solution (pH 5.8-6.2) is twice higher than the native enzyme.

Particles size distribution of the self-assembling peroxidase/chitosan complex

To study the structure of the peroxidase/chitosan complex in aqueous medium and measure its hydrodynamic size distribution, the light scattering method was applied. It was shown that under conditions mentioned above the particles of the peroxidase/chitosan complex had uniform size with average dynamic radius of 22±3 nm. The formation of nanoparticles with such regular size of the complex is very promising to obtain the homogeneous and reproducible films for design of optical biosensors with excellent analytical performance.

The catalytic activity of self-assembling peroxidase/chitosan complex in aqueous-DMSO media

The choice of DMSO, the polar organic solvent, as a medium to study the catalytic activity of the complex can be explained by the following reasons:
i. it can be mixed with water at all ratios;
ii. it is widely used in medicals and in sample preparations.

It has been shown that the self-assembled peroxidase/chitosan complex is twice more active than the native enzyme in the presence of 30 % v/v of DMSO, acts as the native enzyme in the media containing 60 % v/v of organic solvent, and still keeps the catalytic activity in the presence of 70 % of DMSO. Thus, the mentioned complex is very stable in aggressive media of this organic solvent and is promising for analysis of water-insoluble samples.

Formation of optically transparent self-assembling peroxidase/chitosan films

Chitosan is an excellent film-forming agent and its films are transparent and almost do not absorb light. Therefore UV-vis spectroscopy in absorbance mode was chosen to measure the response of the biosensor. Transparent glass slides were selected as the support for a sensitive biolayer. A simple technology described above in the materials and methods was developed to obtain uniform and transparent films of peroxidase/chitosan complex on a glass slide**.**

The transparency (T, %) of the self-assembling films on the surface of glass slides after aging into aqueous buffer solution and aqueous-organic medium in the presence of 30% v/v DMSO was compared. It was shown that films generated from aqueous-DMSO solution were characterized by higher transparency than films from aqueous medium. The values of transparency equaled to (96±2) % and (81±4) % respectively (n=5).

The morphology and topography of the films obtained from different media were studied by AFM method. It was shown that the peroxidase/chitosan films formed in the presence of 30% v/v DMSO are considerably more uniform and even than the films generated from aqueous medium in the absence of organic solvent (the fluctuations in height within 1 square micron pattern are 20-30 and 140-150 nm correspondingly). The film was also studied using optical microscopy and, as a result, its thickness was estimated to be about 5 microns.

Analytical performance of the optical biosensor

Hydroquinone-containing pharmaceutical creams and gels for skin bleaching are typical and popular real samples for the evaluation of performance of biosensors for hydroquinone determination. For these reasons, commercially available cream was chosen for the demonstration of the applicability of the proposed biosensor for the analysis of water-insoluble pharmaceutical preparations. The performance of the biosensor is based on the following conjugated reactions:

i. enzymatic oxidation of a phenolic compound into a quinonic product;

ii. interaction of the formed quinonic product with amino groups of chitosan yielding a strongly light absorbing adduct. The mechanism of chitosan-quinone adduct formation has been previously much speculated by Payne G. (1996) and Michael's addition of quinone to chitosan amino groups was proposed to be the most probable mechanism of this reaction.

Under the chosen conditions (0.05 M phosphate buffer solution, pH 6.5, reaction mixture volume 5 mL, hydrogen peroxide concentration 1 mM), hydroquinone can be determined with the following analytical characteristics: linear range corresponds to 20 – 200 μM, calibration curve equation is $A=(6.8\pm0.2)\times10^3\times c+(0.09\pm0.02)$ (P = 0.95, n = 20), RSD (at hydroquinone concentration (c) 20 μM, n=4) equals to 8%, the r^2 0.9949, the detection limit (estimated using 3 σ of noise value) is 3 μM.

The analytical concentration obtained using the biosensor and the standard addition technique, was in accordance with the result of HPLC determination of hydroquinone in the same sample: (1.9±0.1)% (n=4, P=0.95). The value reported by the producer 'Alen Mak' (Bulgaria) was 1.9%. The matrix of the sample did not interfere the determination of hydroquinone using the developed biosensor.

The fabricated biosensor shows excellent stability (it was investigated using 100 μM of hydroquinone) and thus can be stored for at least a month before use (the biosensor, stored for 30 days at 4°C, provides 98±8% (n=4, P=0.95) of the response provided by a new biosensor).

Another notable feature of the developed biosensor is that the analytical signal is measured as the absorbance of the biosensor slide itself. As a result, emulsions and solutions, which are not transparent, can be analyzed without preliminary treatment. In case if any particles of the sample (e.g., ointment) are left on the surface of the slide, the film can be carefully washed with water. No risk of the analyte leaching from the film exists since it is bound covalently to chitosan.

The biosensor on the basis of peroxidase noncovalent encapsulated in chitosan films was tested in the analysis of some water-insoluble pharmaceuticals, organic extracts from herbal materials and dietary supplements for determination of hydroquinone, pyrocatechol, quercetin, esculetin et al.

CONCLUSIONS

Novel optical biosensor on the basis of highly active and stable peroxidase noncovalent encapsulated in chitosan films operating in aqueous-organic media were developed. Analytical properties of the optical biosensors for determination of biologically active phenolic compounds were demonstrated in the analysis of water-insoluble samples.

REFERENCES

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