# Emulsive Polymerization as a Method of Enzyme Modification Preserving Biological Properties of Nanostructures

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The proposed method of emulsive polymerization provides the possibility of modifying and obtaining insoluble forms of superoxide dismutase (SOD), glutathione reductase, and streptolysin-O preserving nanoobjects (conformationally active centers and antigenic determinants) in their native states. Apart from enzymatic and immunological properties, the samples acquired some new features: resistance to high temperature, resistance to 3 M KCNS solution and buffer solutions with high concentration of hydrogen ions, and resistance to preserving solutions. Magnetic properties provide the possibility of simplifying enzyme-linked immunosorbent and immunofluorescence assays. In addition, sensitivity of these assays was by an order of magnitude higher and the specificity fully preserved. Taking all the facts into account, we prepared agents for long-term and repeated use. Due to preserved enzymatic properties, insoluble forms of SOD and glutathione reductase can be considered as a tool for correction of peroxide-antioxidant balance and associated immunological abnormalities.

**Key Words:** *nanoobjects; polyacrylamide granules; enzymes*

Methods for obtaining in a controllable way and modifying objects with specific properties for using them for diagnostic and therapeutic purposes are a part of biotechnology. They make it possible to preserve conformationally active centers and antigenic determinants in the native states. The method of emulsive polymerization is the most promising and enables immobilization of biopolymers (antigens, immunoglobulins, enzymes, *etc*.) inside or on the surface of polyacrylamide granules. In such a way, nanoobjects are coassembled in totally functional systems of higher level such as immobilized granulated antigenic products (IGAP).

The use of IGAP is necessary in some medical conditions with pronounced autoimmune symptoms, *e.g.* in rheumatoid arthritis, which from the beginning acquires features of autoimmune disease and is maintained without exogenous antigens, which is a dominant factor of its pathogenesis. Production of autoantibodies in this disease was confirmed for many biopolymers including enzymes [7,10].

Here we studied the effects of emulsive polymerization on biological functions of active centers and antigenic determinants of SOD, glutathione reductase (GR), and streptolysin-O (CT-O) in rheumatoid arthritis.

## MATERIALS AND METHODS

Serum samples from patients with rheumatoid arthritis (II-III grade of process activity, II-IV stages of pathological process) as a source of antibodies to the studied molecules were used to compare sensitivity of enzyme immunoassay on polystyrene plates and IGAP with enzymes antigens [2].

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Commercial SOD (EC 1.15.1.1, Sigma, Cat. No. S 9636), glutathione reductase (EC 1.6.4.2, Sigma, Cat. No. G 3664), and streptolysin-O (Streptolysin O leophilized powder, Sigma, Cat. No. S 5265) were used as the antigens. Plasma activities of SOD and glutathione reductase and concentration and activity of streptolysin-O were measured as described previously [5,11,9].

Polyacrylamide granules were prepared using the method of emulsive polymerization in nitrogen flow [3]. The total concentration of polyacrylamide gel (PAAG) was chosen on the basis of published data [6] depending on the molecular weight of the ligand. The concentration of crosslinking agent N,N'-methylenebisacrylamide constituted about 25% PAAG concentration. The pore size in the gel decreased with increasing PAAG concentration, which allowed mechanical fixation of molecules with lower molecular weight and high-molecular-weight biopolymers in its lattice structure. Biopolymers with high molecular weight of 500 kDa and biopolymers with molecular weight about 30 kDa can be firmly fixed in PAAG with the overall concentration of 20%.

The content of antibodies in patients with rheumatoid arthritis was measured by standard enzyme immunoassay and immunofluorescence techniques, and by enzyme immunoassay and immunofluorescent methods with IGAP developed by us [1].

The prepared granules had standard spherical shape and particle size of about 10-100 μ. The active area of the well of polystyrene plates consists of bottom area and wall area ($S=\pi r^2+2\pi rh$, where r=3.17 mm, h=4.18 mm, $S=3.14\times3.17\times3.17+2\times3.14\times3.17\times4.18=114.73$ mm$^2$).

The surface area of a single granule was $S=4\pi r^2=4\times3.14\times625=7850$ μ$^2$, and its volume was $V=4/3\pi r^3=4/3\times3.14\times15{,}625=65{,}416.7$ μ$^3$. In one reaction we used 10 μl of the granules, so their number in this volume would be $10\times10^9$ μ$^3$: 65,416.7 μ$^3$=152,865 units, while the surface area of these granules was S=0.00785 mm$^2\times152{,}865=1199.9$ mm$^2$.

From the studies of gels of different concentrations it is clear that biopolymers with high molecular weight of 500 kDa and biopolymers with a molecular weight about 30 kDa can be optimally fixed in PAAG with a concentration of 20%. The studied antigens had following molecular weights: SOD 30-35 kDa, glutathione reductase 50-55 kDa, and streptolysin-O 60-61 kDa. They could be retained in granules with epy used gel concentration.

Diffusion of labeled tropomyosin ($H^+$-TM) was studied on a Beta-2 apparatus for radiobiochemical studies.

Processing of the results was performed using the Statgraphics 3.0 and Statistica 6.0 software.

## RESULTS

For evaluation of the fixation strength, we studied diffusion of the immobilized ligand through porous gel structure with $H^+$-TM from human skeletal muscle with a molecular weight of 34 kDa close to the molecular weight of the studied enzymes.

During granule preparation, the percentage of inclusion of various concentrations of tropomyosin was approximately the same (~90%).

Analysis of $H^+$-TM diffusion showed that maximum removal of the protein was observed on days 2-3 and then the process sharply slowed down.

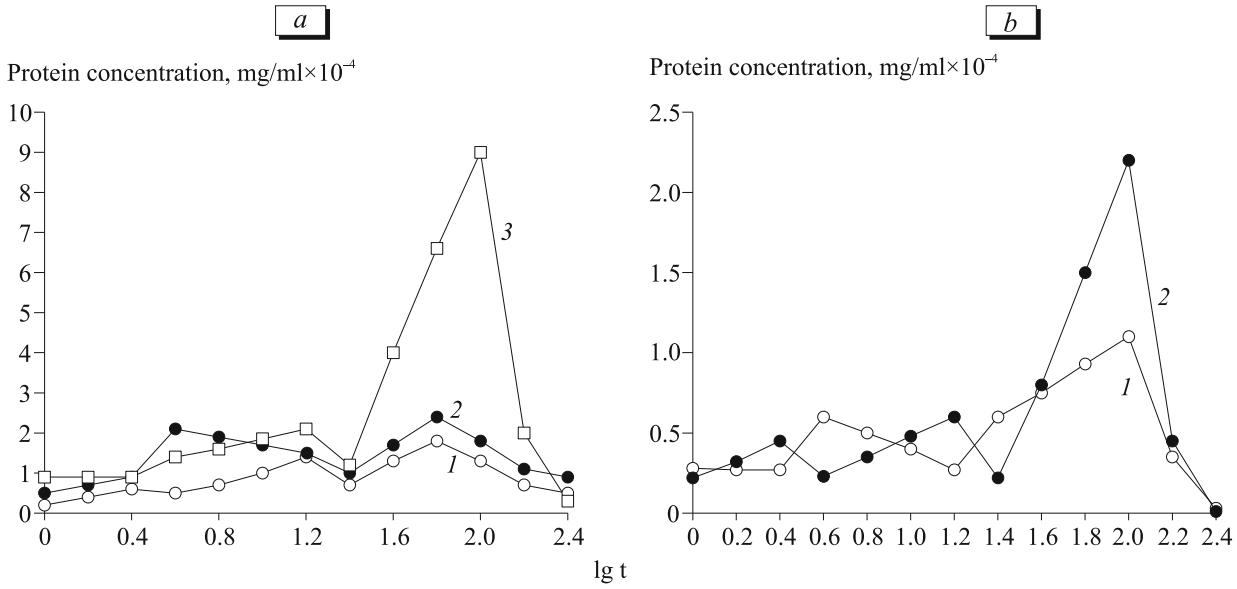


**Fig. 1.** Dynamics $^3$H-TM diffusion from polyacrylamide granules at different temperaturs and time (*a*) and exposure to dissociating agents and time (*b*). *a*: *1*) 4°C; *2*) 20°C; *3*) 37°C. *b*: *1*) potassium rhodanide; *2*) glycine--HCl-buffer.

Protein release from the granules at temperatures 4, 20, and 37°C was negligible. The overall loss of the protein at the maximum temperature was 0.093%. The protein losses were negligible during treatment of the immobilized labeled tropomyosin specimens with 3 M potassium rhodanide and glycine-HC1 buffer pH 2.2 (0.09 and 0.087%, respectively). The method of radioactive tracers showed that individual selection of ligand concentration is required (Fig. 1).

Addition of 10% of magnetic substance in the immobilized systems considerably improved them and reduced the time of immunoassay to 18 hours.

Aqueous solutions of the enzymes (SOD, glutathione reductase) and streptolysin-O exhibiting hemolytic activity were used for studies of the properties of modified nanoobjects incorporated into immobilized systems. The concentrations of the antigens incorporated into the gel lattice were selected experimentally and specifically for each antigen. Immobilized forms of SOD and glutathione reductase were prepared for detection of antibodies for the enzymes from patients with rheumatoid arthritis. The concentrations of the proteins in solutions were 0.5-2.0 mg/ml. The optimal concentrations for SOD and glutathione reductase were 1.4 mg/ml and 1.6 mg/ml, respectively (Fig. 2).

The effects of immobilization on the function of bioactive substances were studied using native and immobilized preparations of SOD, glutathione reductase, and streptolysin-O. Immobilization of these substances did not change their properties. Hemolytic activity of streptolysin-O after immobilization remained virtually unchanged: the number of red blood cells in the Goryaev chamber with soluble and immobilized forms decreased from $4.9\times10^{12}$ to $0.3\times10^{12}$. Activities of immobilized and soluble SOD were 37.30±0.19 and 37.50±0.08, respectively ($p$=0.343), and immobilized and soluble glutathione reductase 128.10±0.11 and 128.40±0.14, respectively ($p$=0.109).

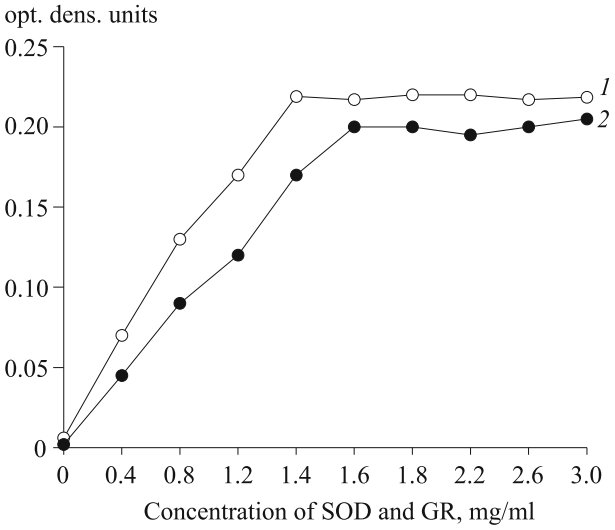


**Fig. 2.** Dynamics of extinction depending on SOD (*1*) and GR (*2*) concentration in IGAP during study of antibody content in pooled serum from patients with rheumatoid arthritis.

These findings suggest that immobilization does not significantly affect biological activity of these insoluble products.

Immobilization increased the stability of bioactive substances to high temperatures. Autoclaving at 170°C for 1 h reduced activity of soluble enzymes to

**TABLE 1.** Sensitivity of Two ELISA Versions Employing Pooled Sera from Patients with Rheumatoid Arthritis ($M\pm m$)

| Antigens | Maximum extinction in working dilution of pooled sera ($n$=10) | | Minimum titer of pooled sera with positive extinction values | |
|---|---|---|---|---|
| | standard ELISA | ELISA with IGAP | standard ELISA | ELISA with IGAP |
| SOD | 0.173±0.007 | 0.216±0.010 | 1:400 | 1:1600 |
| GR | 0.142±0.008 | 0.205±0.012 | 1:400 | 1:1600 |

**TABLE 2.** Sensitivity of Immunofluorescent Method on IGAP (in mV) with Pooled Sera from Patients with Rheumatoid Arthritis ($M\pm m$)

| Antigens | Dilutions of pooled sera from patients with rheumatoid arthritis | | | | | |
|---|---|---|---|---|---|---|
| | 1:4 | 1:10 | 1:20 | 1:50 | 1:100 | 1:200 |
| SOD | 32.70±2.34 | 25.30±1.63 | 8.46±1.05 | 4.52±0.39 | 0 | 0 |
| GR | 31.90±2.26 | 23.60±1.88 | 7.71±0.91 | 5.00±0.37 | 0 | 0 |

**TABLE 3.** Specificity of IGAP (*M*±*m*)

| IGAP on the basis of | IGAP used for controlling specificity of removed antigens | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | collagen | | | IgG | A | TM | SOD | GR |
| | type I | type II | type III | | | | | |
| SOD | 0.198±0.030 | 0.188±0.030 | 0.189±0.030 | 0.192±0.030 | 0.194±0.020 | 0.199±0.020 | 0.190±0.020 | 0.198±0.030 |
| GR | 0.204±0.020 | 0.185±0.020 | 0.195±0.030 | 0.197±0.030 | 0.193±0.020 | 0.197±0.020 | | |

**Note.** A: actin.

zero, whereas activity of immobilized enzymes only slightly decreased. Activity of immobilized form of SOD before and after autoclaving was 37.30±0.49 and 36.50±0.68 ($p$=0.352). Activity of immobilized form of glutathione reductase before and after autoclaving was 128.10±0.71 and 127.40±0.94 ($p$=0.559). The effect of temperature on the immobilized form of streptolysin-O and its commercial form was studied by heating of the specimens to 56°C, because streptolysin-O is thermolabile and can be completely inactivated after 30 min at 56°C [4]. In contrast to its soluble form, functional activity of heated immobilized granulated streptolysin-O was confirmed by similar dynamics of hemoglobin content and erythrocyte count.

For evaluation of antigen–antibody complex dissociation and effects of sterilization and long-term storage of immobilized products, solutions with high ionic strength (3 M KCNS), high concentration of $H^+$ ions (0.1 M glycine-HCl-buffer, pH 2.2), 70% ethyl alcohol used for sterilization, and thiomersal solution (1:10,000) were used. The same immobilized enzymes were used as the markers. Granules containing the studied markers were put into solutions of 3 M KCNS, 0.1 M glycine-HCl-buffer (pH 2.2), 70% ethyl alcohol or thiomersal (1:10 000), mixed thoroughly for 1 h, and then settled. The volume of the granules in tubes with the buffer, thiomersal, and alcohol was almost unchanged, but in tubes containing KCNS it decreased by 0.2 ml. Biological activity of the immobilized granules was virtually stable (SOD activity before and after treatment was 37.10±0.17 and 36.90±0.28, respectively, $p$=0.549). The dynamics of erythrocyte count coincided with the dynamics of hemoglobin content.

Solutions with high ionic strength and high $H^+$ ion concentration, 70% ethyl alcohol, and solution of thiomersal 1:10,000 had no significant effect on variation in functional activity of IGAP with SOD, glutathione reductase, and streptolysin-O.

The use of immobilized granulated enzymes (SOD and glutathione reductase) 4-fold increased sensitivity of ELISA in our version compared to standard assay (Table 1) due to increase in antigen concentration (by 20-100 times), surface of antigen–antibody interaction, and the frequency of interaction between immobilized antigens with magnetic properties and the corresponding immunoglobulins when magnetic stirrer was used.

Conversion of soluble biopolymers into insoluble forms with preservation of biological properties enables their use in different immunological methods of diagnostics. Insoluble forms of antigens made it possible to use indirect immunofluorescence method with commercial luminescent serums for detection of the corresponding antibodies (Table 2).

Thus, the IGAP usage in the immunofluorescence method makes it possible not only to detect the pres-

ence of antibodies, but also to evaluate the amount of antibodies to traditionally dissolved antigens showing regularities which can be obtained using standard ELISA. The sensitivity of the immunofluorescent method is about 20 times lower.

Specificities of the specimens of immobilized antigens were tested in immunoenzyme method by blocking or inhibition of antibodies from the blood serum with specific antigens. In the set of experiments ($n$=10) we used samples of the serum from patients with rheumatoid arthritis with maximum extinction in ELISA for different antigens frequently used in rheumatoid arthritis studies (collagens 1, 2, and 3; IgG, actin, tropomyosin, SOD, and glutathione reductase). The immobilized form of the examined antigen was used as the adsorbent in each experiment. Granule suspension (10 %, 1 ml) with collagens 1, 2, 3, IgG, actin, tropomyosin, and SOD or glutathione reductase was mixed with 1 ml serum and stirred with magnetic stirrer for 1 h at 37°C. After that the granules were removed and the presence of antibodies to the specific antigen was tested in each serum sample. We demonstrate here the results reflecting removal of specific antibodies by each tested IGAP used in ELISA (Table 3).

The nearly zero values suggested that antibodies were completely removed from the serum samples by specific immobilized antigens. These experiments confirm preservation of immunological specificity for our IGAP.

The proposed method of emulsive polymerization provided the possibility of modifying and obtaining insoluble forms of superoxide dismutase (SOD), glutathione reductase, streptolysin-O preserving nanoobjects (conformationally active centers and antigenic determinants) in its native states. Apart from enzymatic and immunological activities of these samples, they acquired new properties: resistance to high temperature, 3 M KCNS and to buffers with high concentration of hydrogen ions, and preserving solutions. Additional magnetic properties gave the possibility of simplifying immunoenzyme and immunofluorescence assays. Sensitivity of these assays was an order of magnitude higher with full preservation of specificity.

Taking into account all these facts, we prepared specimens of long-term and repeated use. Preserved enzymatic properties of insoluble forms of SOD and glutathione reductase allow considering these products as a tool for correction of peroxide-antioxidant balance and associated immunological impairments.

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