**Study of Superoxide Dismutase (SOD) Activity Depending on Immobilization Conditions**

Mashkhura Tagirova1, a), Mavjuda Usmonova1, Svetlana Vasina1, Nurali Mukhamadiyev1, Natalia Klyachko2

*1Biochemical Institute of Samarkand State University named after Sharof Rashidov, Samarkand, Uzbekistan  
2Lomonosov Moscow State University, Moscow, Russia*

a)*Corresponding author: mashxurat@mail.ru*

**Abstract.** Oxidative stress is the state of having too many reactive oxygen species (ROS) and not enough antioxidants in the body to protect against them. Superoxide dismutase (SOD) is one of the most important enzymes that helps protect cells. It changes superoxide radicals into oxygen and hydrogen peroxide, which stops cells from getting hurt. In biotechnology, immobilizing SOD is still a big job since it makes the enzyme more stable and opens up new uses for it in medicine, protecting the environment, and making things in factories. The objective of this work is to examine the activity of SOD under various immobilization circumstances.

**Keywords:** oxidative stress, superoxide dismutase (SOD), immobilization conditions, enzymes.

**INTRODUCTION**

Reactive oxygen species (ROS) are natural by-products of cellular metabolism that are present in small concentrations. When their levels rise over normal, however, they begin to damage proteins, lipids, and nucleic acids. A plethora of research have clarified that oxidative damage of this kind is significantly linked to the etiology of numerous diseases, including cancer, diabetes, neurological disorders, atherosclerosis, arterial hypertension, asthma, and acute respiratory distress syndrome [1–3].

Oxidative stress is typically characterized as a state wherein the production of reactive oxygen species (ROS) surpasses the neutralizing ability of the body's antioxidant mechanisms [4–5]. Enzymatic antioxidants are used by living things to inhibit this from happening. Superoxide dismutase (SOD) is one of the most significant ones. This enzyme changes superoxide radicals into hydrogen peroxide and oxygen, which makes them less hazardous to cells.

In the last few years, a lot of individuals have been interested in immobilizing SOD since it makes the enzyme more stable in difficult settings and more useful. Researchers are progressively examining the utilization of immobilized SOD in medical treatments, environmental safeguarding technologies, and industrial procedures. The goal of this study is to find out how SOD works under different immobilization settings in order to find out how useful it is and where it may be used [6–10].

**METHODS**

Nanoparticles Superoxide dismutase (SOD) was obtained using the method described in [9], with modification of the buffer medium conditions. The method is based on the mechanism of spontaneous layer-by-layer self-assembly of oppositely charged polyions, leading to the formation of stoichiometric polymer complexes with a high protein loading (up to 100%).

The reaction mixture was prepared in 60 mM HEPES buffer and 60 mM phosphate buffer (PBS), both with a pH of 6,8. During the synthesis, SOD, which is predominantly negatively charged at physiological pH, was added dropwise to the buffer solution with constant stirring. A protamine solution (Sigma, USA), positively charged at pH 6,8, was then added, and the mixture was incubated for 30 minutes.

The next step was the addition of a negatively charged block copolymer PEG-PG (polyethylene glycol - poly- L - glutamic acid, Alamanda Polymers, USA), followed by incubation for 30 minutes at +4°C. To stabilize the particle structure, a 5% glutaraldehyde solution (Sigma, USA) was added to ensure chemical cross-linking. The reaction mixture was left overnight at +4°C. To restore the Schiff bases after cross-linking, 100 μl of NaBH₄ solution (1 mg/ml) were added. Nanoparticles were purified from by-products and unreacted substances using a filtration system with a molecular cutoff of 300 kDa.

To examine the effect of component ratio on nanoparticle size, synthesis was performed utilizing various reactant charge ratios. This helped us understand what conditions make particles the most stable and spread out. This is important for using them later in systems that spread enzymes around.

**TABLE 1.** Different charge ratios of the reactants.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| SOD : protamine : PEG-PG | 1:0,5:0,5  1:0,5:1  1:1:1  1:1:2 | 1:2:0,5  1:2:1  1:2:2  1:2:4  1:2:6 | 1:3:0,5  1:3:1  1:3:2  1:3:4  1:3:6 | 1:4:0,5  1:4:1  1:4:2  1:4:4  1:4:6 |

We made SOD nanoparticles using the method given above and a phosphate buffer (PBS) with a concentration of 10 mM at pH levels of 5.0, 5.8, 6.0, 6.5, 6.8, 7.0, 7.2, 7.4, 7.6, and 7.8. The research examined the effect of medium acidity on nanoparticle characteristics to enhance the conditions for producing stable and uniformly sized structures.

The first step was to add a drop of protamine solution to a phosphate buffer solution of SOD (which is mostly negatively charged at physiological pH) and keep shaking it. It was left out for 30 minutes at room temperature.

A solution of PEG-PG (a block copolymer of polyethylene glycol and poly-L-glutamic acid) was then added to the reaction mixture, and the mixture was further incubated at +4°C for 30 minutes. Following this, a 5% solution of glutaraldehyde was added to the resulting intermediate complexes in precisely defined volumes for each sample, depending on the pH, to facilitate subsequent chemical cross-linking. The mixture was left overnight at +4°C.

The next day, 70 μl of sodium borohydride (NaBH₄) solution at a concentration of 1 mg/ml were added to each sample to reduce the Schiff bases formed as a result of the interaction of the aldehyde groups of glutaraldehyde with the amino groups of the SOD protein and protamine.

By-products and unreacted substances were removed by triple centrifugation. The resulting precipitates and supernatants were collected in sterile Eppendorf samples were collected and analyzed. All samples were stored at 4°C until further measurements were taken.

**results and discussion**

### The influence of the buffer medium and composition on the formation of SOD nanoparticles. SOD nanoparticle complex was synthesized in HEPES and PBS buffer solutions with the same pH (6,8). A comparative analysis revealed that the use of HEPES buffer resulted in smaller nanoparticles compared to PBS, possibly due to the lower ionic strength and minimal shielding of intermolecular interactions in the HEPES environment. This is critical for the use of the resulting nanoparticles as a potential enzyme delivery system for the treatment of traumatic brain injury, where high penetrating ability and drug stability are key.

To determine the effect of stoichiometric composition on the size of SOD nanoparticles, complexes were synthesized in various charge ratios of the components: SOD: protamine: PEG-PG. The sizes of the samples were measured before and after cross-linking with glutaraldehyde by dynamic light scattering using a **Zetasizer instrument. Nano ZS** (Malvern Instruments, UK). The table presents the values of Z-average, polydispersity (PdI), autocorrelation intensity (intercept) and numerical size distribution (Number PSD, peak 1).

The results showed (table 2) that at ratios of 1:1:1, 1:2:1, 1:3:0.5, and 1:3:2, it is possible to achieve minimum values of the average diameter of nanoparticles (~88–99 nm) and low PdI values, indicating the homogeneity of the systems. The introduction of an excess of PEG-PG (e.g., 1:2:4, 1:2:6) leads to a sharp increase in particle size (up to 500–550 nm), probably due to over compaction of the structure and the formation of large aggregates. After cross-linking of glutaraldehyde, a slight increase in size (on average by 10–20 nm) was observed, which corresponds to the formation of a dense polymer network without compromising stability.

### The influence of pH on the size of nanoparticles. SOD nanoparticles were studied separately. Synthesis was carried out in 10 mM phosphate buffer (PBS) with pH varied from 5,0 to 7,8. All synthesis steps followed the standard procedure: addition of protamine to the SOD solution, incubation, introduction of PEG-PG, cross-linking with glutaraldehyde, and reduction with sodium borohydride. Nanoparticle sizes were analyzed after completion of all synthesis steps.

**TABLE 2.** Hydrodynamic dimensions of SOD nanoparticles before and after cross-linking with glutaraldehyde depending on the charge ratio.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Charge ratio SOD:**  **protomin:PEG-PG** | **SAMPLE DIMENSIONS** | | | | | | | |
| **Before the glutaraldehyde stitching** | | | | **After cross-linking of glutaraldehyde** | | | |
| **Z-average**  **(d,nm)** | **PdI** | **Intercept** | **Number PSD,**  **Peak 1, nm.** | **Z-average**  **(d,nm)** | **PdI** | **Intercept** | **Number PSD,**  **Peak 1, nm** |
| **1:0.5:0.5** | 87,26 | 0,272​ | 0,931 | 37,89 | 104,0 | 0,211 | 0,881 | 53,33 |
| **1:0.5:1** | 300,9 | 0,372 | 0,625 | 4,277 | 374,1 | 0,416 | 0,555 | 193,6 |
| **1:1:1** | 97,32​ | 0,117 | 0,933 | 64,78 | 107,5 | 0,071 | 0,931 | 79,83 |
| **1:1:2** | 272,1 | 0,467 | 0,744 | 4,525 | 254,5 | 0,300 | 0,796 | 67,19 |
| **1:2:0.5** | 94,68 | 0,274 | 0,820 | 46,52 | 110,1 | 0,206 | 0,903 | 57,92 |
| **1:2:1** | 99,03 | 0,268 | 0,902 | 40,66 | 99,31 | 0,199 | 0,934 | 61,09 |
| **1:2:2** | 110,4 | 0,395 | 0,461 | 4,526 | 116,1 | 0,136 | 0,917 | 74,72 |
| **1:2:4** | 498 | 0,469 | 0,461 | 4,526 | 391,2 | 0,404 | 0,735 | 5,250 |
| **1:2:6** | 499,6 | 0,485 | 0,440 | 4,650 | 554,9 | 0,533 | 0,436 | 5,320 |
| **1:3:0.5** | 88,74 | 0,211 | 0,942 | 54,48 | 98,56 | 0,226 | 0,847 | 54,38 |
| **1:3:1** | 120,1 | 0,126 | 0,902 | 83,14 | 349,2 | 0,628 | 0,755 | 114 |
| **1:3:2** | 93,44 | 0,132 | 0,921 | 59,38 | 108,6 | 0,108 | 0,849 | 70,76 |
| **1:3:4** | 109,5 | 0,078 | 0,934 | 80,58 | 126,5 | 0,074 | 0,933 | 97,79 |
| **1:3:6** | 219 | 0,168 | 0,901 | 153,3 | 174,9 | 0,066 | 0,917 | 142,4 |
| **1:4:0.5** | 93,75 | 0,175 | 0,928 | 60,87 | 113,3 | 0,150 | 0,910 | 67,99 |
| **1:4:1** | 82,5 | 0,340 | 0,709 | 44,72 | 122,9 | 0,128 | 0,941 | 80,02 |
| **1:4:2** | 89,81 | 0,102 | 0,944 | 62,08 | 93,81 | 0,135 | 0,935 | 63,47 |
| **1:4:4** | 132,9 | 0,148 | 0,897 | 91,04 | 138,5 | 0,074 | 0,947 | 105,8 |
| **1:4:6** | 159,3 | 0,164 | 0,954 | 103,2 | 193,8 | 0,110 | 0,916 | 150,7 |

The figure 1 shows the dependence of the average hydrodynamic diameter of nanoparticles on the pH of the medium. The obtained data demonstrate a distinct U-shaped dependence: the maximum sizes (~165 nm) are observed at pH 5, which is likely due to the aggregation of nanoparticles due to a decrease in the surface charge of the protein and weakening of electrostatic repulsion. With an increase in pH to 6.8–7.2, the particle sizes decrease to minimum values (~105 nm), indicating high colloidal stability near physiological pH. With a further increase in pH (to 7,6–7,8), the particle size increases again, which may be due to a change in the charge of the components and the ionic strength of the buffer.

**FIGURE 1.** Dependence of sample size from pH buffer

**Conclusion**

The method appears to be effective for diagnosing and monitoring treatment for conditions such as brain injury. Experimental data revealed that the size and activity of the complex depend on the conditions of complex formation : the composition of the buffer solution and the concentrations of the components involved, as well as the ratio of SOD to catalase. The use of nanoparticles as delivery systems appears to be a promising approach in this case, as they can significantly increase the bioavailability of the drug.

The obtained results demonstrate that the choice of method and carrier for immobilizing superoxide dismutase (SOD) directly depends on its practical application. For medical applications and biosensor development, hydrogels, which ensure high biocompatibility and preserve enzyme activity, are the optimal solution. However, for industrial synthesis and environmental applications, preference should be given to nanomaterials and covalent immobilization methods, which ensure enzyme stability under extreme conditions, including exposure to high temperatures, a wide pH range, and organic solvents. These data allow us to optimize the use of SOD in various biotechnological and applied processes.

**REFERENCES**

# Birben, E., Sahiner, U. M., Sackesen, C., Erzurum, S., & Kalayci, O. (2012). Oxidative stress and antioxidant defense. World Allergy Organization Journal, 5(1), 9–19. <https://doi.org/10.1097/wox.0b013e3182439613>

# Belle, C., Selmeczi, K., Torelli, S., & Pierre, J. (2006). Chemical tools for mechanistic studies related to catechol oxidase activity. Comptes Rendus Chimie, 10(4–5), 271–283. <https://doi.org/10.1016/j.crci.2006.10.007>

# Ghorbani, M., Derakhshankhah, H., Jafari, S., Salatin, S., Dehghanian, M., Falahati, M., & Ansari, A. (2019). Nanozyme antioxidants as emerging alternatives for natural antioxidants: Achievements and challenges in perspective. Nano Today, 29, 100775. <https://doi.org/10.1016/j.nantod.2019.100775>

1. Hepel, M., & Andreescu, S. (2015). Oxidative Stress: Diagnostics, Prevention, and Therapy Volume 2. In ACS symposium series. <https://doi.org/10.1021/bk-2015-1200>
2. Manickam, D. S., Brynskikh, A. M., Kopanic, J. L., Sorgen, P. L., Klyachko, N. L., Batrakova, E. V., Bronich, T. K., & Kabanov, A. V. (2012). Well-defined cross-linked antioxidant nanozymes for treatment of ischemic brain injury. Journal of Controlled Release, 162(3), 636–645. <https://doi.org/10.1016/j.jconrel.2012.07.044>
3. Sahoo, S. K., Dilnawaz, F., & Krishnakumar, S. (2008). Nanotechnology in ocular drug delivery. Drug Discovery Today, 13(3–4), 144–151. <https://doi.org/10.1016/j.drudis.2007.10.021>
4. Kost, O. A., Beznos, O. V., Davydova, N. G., Manickam, D. S., Nikolskaya, I. I., Guller, A. E., Binevski, P. V., Chesnokova, N. B., Shekhter, A. B., Klyachko, N. L., & Kabanov, A. V. (2015). Superoxide Dismutase 1 Nanozyme for Treatment of Eye Inflammation. Oxidative Medicine and Cellular Longevity, 2016(1), 5194239. <https://doi.org/10.1155/2016/5194239>
5. Amatore, C., Arbault, S., Bouton, C., Coffi, K., Drapier, J., Ghandour, H., & Tong, Y. (2006). Monitoring in Real Time with a Microelectrode the Release of Reactive Oxygen and Nitrogen Species by a Single Macrophage Stimulated by its Membrane Mechanical Depolarization. ChemBioChem, 7(4), 653–661. <https://doi.org/10.1002/cbic.200500359>
6. Nukolova, N., Aleksashkin, A., Abakumova, T., Morozova, A., Gubskiy, I., Kirzhanova, Е., Abakumov, M., Chekhonin, V., Klyachko, N., & Kabanov, A. (2017). Multilayer polyion complex nanoformulations of superoxide dismutase 1 for acute spinal cord injury. Journal of Controlled Release, 270, 226–236. <https://doi.org/10.1016/j.jconrel.2017.11.044>
7. Askarov, I., Isaev, Y., Tashtemirova, G., Rustamov, S., Kadirov, M., & Shadmanov, K. (2023). Synthesis of complex compounds of the monoammonium salt of glycyrrhizic acid and thiourea. E3S Web of Conferences, 383, 04023. <https://doi.org/10.1051/e3sconf/202338304023>