DEVELOPMENT OF MULTIFUNCTIONAL BIOCATALYST COLLOIDS BY CO-IMMOBILIZATION OF ENZYMES

Szilárd Sáringer

SUPERVISOR:
Dr. István Szilágyi
Assistant Professor

UNIVERSITY OF SZEGED

Faculty of Science and Informatics

Department of Physical Chemistry and Materials Science

MTA-SZTE „Momentum” Biocolloids Research Group

Doctoral School of Chemistry

Szeged

2022

I. Introduction

Enzymes are proteins that act as biological catalysts, which accelerate chemical reactions. In nature, almost all the reactions in cells are catalyzed by cooperation of various enzymes and enzyme catalysis also has a primal role in biochemical industrial processes. Among all the different classes of enzymes, oxidoreductase enzymes are of special importance for the removal of reactive oxygen species (ROS) like superoxide radical anions and hydrogen peroxide. Normal biochemical reactions exposed to environmental effects lead to extended ROS production, which induces oxidative stress giving rise to damage of cellular components and also causes significant loss in the quality of industrial products. In some of the later cases, only peroxidase enzymes are needed, however, when they are used together with superoxide dismutase enzymes, they perform a cascade reaction leading to the complete decomposition of ROS to water and oxygen.

Enzymes, on the other hand, are sensitive biocatalysts that can partially or totally lose their enzymatic activity as a consequence of any major changes occurring in their surrounding environment. This major drawback of their environmental response can be overcome by immobilization on solid supports, which also offers several technical advantages in the industrial applications. Nanostructured materials provide ideal characteristics for the co-immobilization of different enzymes to obtain multi-enzymatic systems. However, one of the main limitations of these type of supports is the particle aggregation in dispersions, which gives rise to inefficient function of the antioxidant enzymes and hence, to failure in ROS decomposition. Since these composite materials are applied in heterogenous systems such as in blood, aqueous environmental samples, or industrial manufacturing processes, the colloidal stability is a critical issue. Experimental conditions such as ionic strength, temperature and pH significantly influence the charging and the aggregation processes and thus, the colloidal stability of these dispersions.

Polyelectrolytes have proved to be efficient aggregation or stabilization agents for nanoparticles dispersed in an aqueous medium. Furthermore, the charging and the aggregation processes are controllable by the adsorption of oppositely charged polyelectrolyte layers on the surface of the particles. Sequential adsorption method with the application of polyelectrolytes is a self-evident way to immobilize multi-enzymatic systems in/on the nanostructures. In this method, the polyelectrolyte multilayers act as a support, and also as a separator between the proteins. On the other hand, they ensure high colloidal stability for the system.

II. Objectives

Our first objective was to study the adsorption mechanism of polyelectrolytes on titanium dioxide nanosheets (TNS) and the enzyme papain (PPN) on polymer latex particles (SL). Our goal was to perform systematic light scattering measurements to determine the surface charge properties and aggregation rates of the bare and functionalized particles in the presence of polyelectrolytes, enzymes, and electrolytes to elucidate the main interparticle forces in aqueous dispersions. By optimizing the polyelectrolyte and enzyme doses, the main goal was to create composite nanostructures with high colloidal stability, which could be ideal carriers for further enzyme immobilization.

This work also aimed to develop bio-nanocomposites that mimic the functions of antioxidant and proteolytic enzymes in stable dispersions. This goal was achieved by immobilizing enzymes on inorganic particle supports functionalized with polyelectrolytes and polymeric particle supports functionalized with enzymes using the sequential adsorption method. The dual enzymatic activity of the hybrid materials was determined in biochemical assays.

III. Experimental

Dynamic light scattering (DLS) technique was used to determine the hydrodynamic size and the aggregation rates of the developed particles in dispersions under different experimental conditions in the presence on polyelectrolytes, enzymes, and electrolytes. The charge of the particle dispersions was measured with electrophoretic light scattering (ELS). The above-mentioned techniques were also used to optimize the dose of the absorbed components on the functionalized particles.

A new method was developed and tested for the determination of the critical coagulation concentration (CCC) or critical coagulation ionic strength (CCIS) data. The procedure is able to estimate the necessary electrolyte concentration for the destabilization of the dispersions from electrophoretic mobility data without direct aggregation rate measurements.

TNS nanoparticles synthetized by hydrothermal method was used as a support material. Superoxide dismutase (SOD) and horseradish peroxidase (HRP) enzymes were immobilized between and on the PDADMAC and PSS polyelectrolyte layers by the sequential adsorption method to obtain the TNS-PDADMAC-SOD-PSS-HRP and the TNS-HRP-PDADMAC-SOD-PSS composite materials. The polyelectrolyte and the enzyme doses were optimized to ensure high colloidal stability of the system. Transmission electron microscopy (TEM) measurements were carried out to determine the morphology of the bare and the functionalized particles.

On the other hand, sulfate functionalized polystyrene latex spheres were modified with the adsorption of PPN enzyme. It was followed by adsorption of a heparin (HEP) polyelectrolyte layer and a HRP enzyme layer to obtain the SL-PPN-HEP-HRP composite material with remarkable colloidal stability.

The Bradford protein test was applied to determine enzyme concentrations in solutions, while direct stochastic optical reconstruction microscopy (dSTORM) was used to detect labelled enzymes on the particles.

The antioxidant activity of the immobilized and the native HRP and SOD enzymes was determined with the guiaicol and Fridovich assays, respectively. The protease activity of the PPN containing samples was measured with the universal protease activity assay. The color changes in these methods were followed by UV-Vis spectrophotometry.

IV. New scientific results

T1. In the systems containing positively charged TNS, the adsorption of both like-charged PDADMAC and oppositely charged PSS took place and the interparticle forces are in line with the DLVO theory in the presence of electrolytes. For negatively charged TNS, the like-charged PSS did not interact with the particles, while the oppositely charged PDADMAC adsorbed strongly on the surface and possessed the highest colloidal stability in the presence of electrolytes due to the additional non-DLVO repulsive forces.

T1.1. The adsorption of like-charged PDADMAC on the TNS surface was indicated by the slight increase in the electrophoretic mobility values at high polyelectrolyte doses under acidic conditions (pH 4). On the other hand, the strong adsorption of the oppositely charged PSS resulted in charge reversal of the particles. Time resolved DLS measurements revealed that the aggregation processes of the TNS are sensitive to the PSS dose and the major interparticle forces were of DLVO (Derjaguin-Landau-Verwey-Overbeek) origin, however, patch-charge effects (attraction between adsorbed polyelectrolytes (patch) and bare surface of partially covered TNS (charge)) were observed at low surface coverage.

T1.2. For negatively charged TNS, the electrophoretic mobility measurements revealed that negatively charged TNS does not interact with the like-charged PSS, while the oppositely charged PDADMAC adsorbed strongly on the nanosheets. Charge neutralization led to unstable dispersions, while the primary particles were homogeneously distributed at doses below and above the isoelectric point. The results shed light on the fact that PDADMAC adsorbs in a more extended conformation on the TNS and hence, no polyelectrolyte patches were formed on the surface.

T1.3. Results of salt induced aggregation measurements performed at pH 4 yielded the TNS < PDADMAC-TNS < PSS-TNS sequence in the CCC values indicating the adsorption of the like-charged PDADMAC and that the interparticle forces were in line with the DLVO theory. However, additional (non-DLVO) attractive forces were observed with the bare TNS. For negatively charged TNS (pH 7 and 10), the order of the CCC values was TNS ≈ PSS-TNS <<< PDADMAC-TNS, where the different aggregation mechanism and the large increase of the CCC values for the TNS-PDADMAC particles indicate the presence of the additional non-DLVO steric interparticle forces (overlap of adsorbed

polyelectrolyte chains and subsequent rise in osmotic pressure), which was confirmed by model calculations.

T2. Stable dispersions of an antioxidant enzyme cascade involving SOD and HRP was successfully developed by co-immobilization on TNS using the sequential adsorption method with the application of PDADMAC and PSS polyelectrolytes.

T2.1. Polyelectrolyte bilayer was built up on TNS using positively charged PDADMAC and negatively charged PSS with the sequential adsorption method. Optimal polyelectrolyte doses were determined to obtain the TNS-PDADMAC-PSS composite material, which possessed remarkable colloidal stability making it a promising support for further enzyme immobilization.

T2.2. Based on the above results, SOD and HRP enzymes were embedded in the TNS-PDADMAC-PSS structure by the sequential adsorption process. The enzyme doses were optimized to achieve high colloidal stability of the TNS-PDADMAC-SOD-PSS-HRP composite material. The presence of the non-DLVO forces was confirmed upon multilayer formation.

T2.3. The catalytic activity of the immobilized enzymes was determined in biochemical assays. It was found that the co-immobilization of the enzymes did not cause significant loss in the enzymatic activities, i.e., the proteins kept their structural integrity upon immobilization. The use of different sequence of the enzymes (TNS-HRP-PDADMAC-SOD-PSS instead of TNS-PDADMAC-SOD-PSS-HRP) resulted in even higher antioxidant efficiency.

T3. The surface charge and aggregation properties of SL particles were tuned with the adsorption of PPN enzyme. The protease activity of the immobilized enzyme was preserved upon the immobilization.

T3.1. Electrophoretic mobility and time resolved DLS measurements revealed that the PPN enzyme strongly adsorbed on the oppositely charged SL particle. Such a polyelectrolyte-type adsorption was not reported earlier for enzyme-particle systems. The charging and aggregation mechanism was highly affected by the dose of the enzyme and the formation of a saturated enzyme layer on the surface was confirmed and it resulted in a stable SL-PPN dispersion.

T3.2. Results of electrolyte induced aggregation studies shed light on that the major interparticle forces could be described within the DLVO theory, however, steric interactions between adsorbed enzyme chains and subsequent stabilization of the particles occurred at high papain coverage, where formation of tails and loops of the adsorbed enzyme molecules took place.

T3.3. The protease activity of the SL-PPN composite was determined by a biochemical assay and it was found that the enzymatic activity of the immobilized papain is 67% lower than the native enzyme in solution. Nevertheless, the obtained latex-PPN composite possessed the advantages of a heterogeneous catalyst such as easier separation from the reaction mixture.

T4. PPN and HRP enzymes were successfully co-immobilized on SL support by the sequential adsorption method with the application of HEP polyelectrolyte as a separator. The obtained SL-PPN-HEP-HRP composite material possessed high colloidal stability and dual oxidative-hydrolytic function.

T4.1. A bilayer containing PPN enzyme and HEP polyelectrolyte was successfully built-up on the SL nanoparticle with the sequential adsorption method to obtain the SL-PPN-HEP composite material. It was found that a saturated HEP layer provided excellent colloidal stability for the system, i.e., the particles could not be aggregated even at elevated ionic strengths.

T4.2. HRP enzyme was attached to the above composite to obtain the SL-PPN-HEP-HRP material. The dose of the HRP was optimized to maintain the colloidal stability of the system. The irreversible immobilization of the PPN and the HRP was proved by Bradford protein test and dSTORM method, respectively.

T4.3. The time dependent antioxidant and protease activities of the immobilized enzymes were determined, and it was revealed that such a dual activity is maintained at least for five days. However, in the mixed solution of the native enzymes, the HRP lost its activity after three days due to hydrolysis by the PPN enzyme.

V. Applications

The method developed for the calculation of the CCC values allows other researchers to determine CCCs solely from electrophoretic mobility data. This tool can be of special importance in systems, in which the direct measurement of the CCC is not possible due to non-ideal sample conditions for aggregation rate measurements and in dispersions containing electrolyte mixtures, as in many industrial and environmental processes. The TNS based antioxidant materials are promising candidates in biomedical treatments to reduce the ROS level, including simultaneous decomposition of O_2^- and H_2O_2 in liquid medium, where colloidal stability is an important parameter. A typical example for such an application is the antioxidant treatment of inflammatory bowel diseases. Furthermore, such a broad-spectrum ROS scavenging ability can be utilized in industrial manufacturing processes like formulation of cosmetics or protecting textiles. The combined application of PPN and HRP enzymes is desirable in certain industrial areas such as food and pharmaceutical industry, on the other hand, the successful application requires separation of the enzymes to protect the HRP from the proteolytic activity of PPN. With the use of the developed immobilized multi-enzymatic SL-PPN-HEP-HRP composite material, one can overstep such a limitation. Apart from these examples, sustainable and processable multi-enzymatic systems are needed in many areas both in fundamental research and more applied disciplines. Therefore, the knowledge developed during the doctoral work should attract considerable interest in the scientific and technological communities dealing with biocatalytic procedures.

VI. Scientific publications

MTMT ID: 10062649

Papers related to the dissertation

1. **Szilárd Sáringer**, Tamás Valtner, Árpád Varga, József Maléth, István Szilágyi

   Development of polymer-based multifunctional composite particles of protease and peroxidase activity

   JOURNAL OF MATERIALS CHEMISTRY B 10 (2022) 2523-2533

   DOI: 10.1039/D1TB01861B SJR indicator: Q1
   Independent citations: 0 IF_{2020}: 6.331

2. **Szilárd Sáringer**, Paul Rouster, István Szilágyi

   Co-immobilization of antioxidant enzymes on titania nanosheets for reduction of oxidative stress in colloid systems

   JOURNAL OF COLLOID AND INTERFACE SCIENCE 590 (2021) 28-37.

   DOI: 10.1016/j.jcis.2021.01.012 SJR indicator: D1
   Independent citations: 8 IF_{2020}: 8.128

3. Marco Galli, **Szilárd Sáringer**, István Szilágyi, Gregor Trefalt (shared first authorship)

   A Simple Method to Determine Critical Coagulation Concentration from Electrophoretic Mobility

   COLLOIDS AND INTERFACES 4 (2020) 20.

   DOI: 10.3390/colloids4020020 SJR indicator: –
   Independent citations: 14 IF_{2020}: –

4. **Szilárd Sáringer**, Rita Achieng, Adél Szerlauth, István Szilágyi

   Papain Adsorption on Latex Particles: Charging, Aggregation, and Enzymatic Activity

   THE JOURNAL OF PHYSICAL CHEMISTRY B 123 (2019) 9984-9991.

   DOI: 10.1021/acs.jpcb.9b08799 SJR indicator: Q1
   Independent citations: 8 IF_{2019}: 2.857

5. **Szilárd Sáringer**, Paul Rouster, István Szilágyi

   Regulation of the Stability of Titania Nanosheet Dispersions with Oppositely and Like-Charged Polyelectrolytes

   LANGMUIR 35 (2019) 4986-4994.

   DOI: 10.1021/acs.langmuir.9b00242 SJR indicator: Q1
   Independent citations: 11 IF_{2019}: 3.557

Papers not related to the dissertation

1. Nizar B. Alsharif, Gergely F. Samu, **Szilárd Sáringer**, Adél Szerlauth, Dóra Takács, Viktória Hornok, Imre Dékány, Istvan Szilagyi

   Antioxidant colloids via heteroaggregation of cerium oxide nanoparticles and latex beads

   COLLOIDS AND SURFACES B: BIOINTERFACES 216, (2022) 112531.

   DOI: 10.1016/j.colsurfb.2022.112531 SJR indicator: Q1
   Independent citations: 0 IF_{2022}: 5.268

2. Adél Szerlauth, Lilla Szalma, Szabolcs Muráth, **Szilárd Sáringer**, Gábor Varga, Li Li, István Szilágyi

   Nanoclay-based sensor composites for the facile detection of molecular antioxidants

   ANALYST 147 (2022) 1367-1374.

   DOI: 10.1039/D1AN02352G SJR indicator: Q1
   Independent citations: 0 IF_{2022}: 4.616

3. Adél Szerlauth, Edina Balogh, Dóra Takács, **Szilárd Sáringer,** Gábor Varga, Gábor Schuszter, István Szilágyi

   Self-assembly of delaminated layered double hydroxide nanosheets for the recovery of lamellar structure

   COLLOID AND INTERFACE SCIENCE COMMONICATIONS 46 (2022) 100564.

   DOI: 10.1016/j.colcom.2021.100564 SJR indicator: Q1
   Independent citations: 0 IF_{2022}: 4.914

4. Bojana Katana, Dóra Takács, Adél Szerlauth, **Szilárd Sáringer**, Gábor Varga, Andrej Jamnik, Felix D Bobbink, Paul J Dyson, Istvan Szilagyi

   Aggregation of halloysite nanotubes in the presence of multivalent ions and ionic liquids

   LANGMUIR 37 (2021) 11869-11879

   DOI: 10.1021/acs.langmuir.1c01949 SJR indicator: Q1
   Independent citations: 1 IF_{2021}: 3.882

5. Lívia Vásárhelyi, Tímea Hegedűs, **Szilárd Sáringer**, Gergő Ballai, István Szilágyi, Zoltán Kónya

   Stability of Boron Nitride Nanosphere Dispersions in the Presence of Polyelectrolytes

   LANGMUIR 37 (2021) 5399-5407.

   DOI: 10.1021/acs.langmuir.1c00656 SJR indicator: Q1
   Independent citations: 0 IF_{2020}: 3.882

6. Nizar B. Alsharif, Katalin Bere, **Szilárd Sáringer**, Gergely F. Samu, Dóra Takács, Viktória Hornok, Istvan Szilagyi

   Design of hybrid biocatalysts by controlled heteroaggregation of manganese oxide and sulfate latex particles to combat reactive oxygen species

   JOURNAL OF MATERIALS CHEMISTRY B 9 (2021) 4929-4940.

   DOI: 10.1039/D1TB00505G SJR indicator: Q1
   Independent citations: 1 IF_{2021}: 6.331

7. Nizar B. Alsharif, Gergely F.Samu, **Szilárd Sáringer**, Szabolcs Muráth, Istvan Szilagyi

   A colloid approach to decorate latex particles with Prussian blue nanozymes

   JOURNAL OF MOLECULAR LIQUIDS 309 (2020) 113066.

   DOI: 10.1016/j.molliq.2020.113066 SJR indicator: Q1
   Independent citations: 8 IF_{2020}: 6.165

8. Szabolcs Muráth, Nizar B. Alsharif, **Szilárd Sáringer**, Bojana Katana, Zoltán Somosi, Istvan Szilagyi

   Antioxidant Materials Based on 2D Nanostructures: A Review on Recent Progresses

   CRYSTALS, 10(3) (2020) 148.

   DOI: 10.3390/cryst10030148 SJR indicator: Q2
   Independent citations: 12 IF_{2020}: 2.589

9. Szabolcs Muráth, **Szilárd Sáringer**, Zoltán Somosi, István Szilágyi

   Effect of Ionic Compounds of Different Valences on the Stability of Titanium Oxide Colloids

   COLLOIDS AND INTERFACES 2 (2018) 32.

   DOI: 10.3390/colloids2030032 SJR indicator: –
   Independent citations: 17 IF_{2018}: –

10. Paul Rouster, Marko Pavlovic, **Szilárd Sáringer**, Istvan Szilagyi

    Functionalized Titania Nanosheet Dispersions of Peroxidase Activity

    THE JOURNAL OF PHYSICAL CHEMISTRY C 122 (2018) 11455-11463.

    DOI: 10.1021/acs.jpcc.8b03271 SJR indicator: D1
    Independent citations: 7 IF_{2018}: 4.309

11. Ildikó Y. Tóth, Márta Szekeres, Rodica Turcu, **Szilárd Sáringer**, Erzsébet Illés, Dániel Nesztor, Etelka Tombácz

    Mechanism of in Situ Surface Polymerization of Gallic Acid in an Environmental-Inspired Preparation of Carboxylated Core–Shell Magnetite Nanoparticles

    LANGMUIR 30 (2014) 15451–15461.

    DOI: 10.1021/la5038102 SJR indicator: D1
    Independent citations: 43 IF_{2014}: 4.457

Scientometric Data

Sum of peer reviewed publications:	**16**	In relation to the theses:	**5**
Cumulative impact factor:	**62.670**	In relation to the theses:	**20.873**
Sum of independent citations:	**130**	In relation to the theses:	**41**

Oral presentations and posters related to the dissertation

1. **Szilárd Sáringer**, Tamás Valtner, István Szilágyi (oral presentation)

   Multifunctional composite polymeric particles with dual protease and peroxidase activities

   33rd Australian Colloid and Surface Science Student Conference, 31 January-2 February 2022, Mawson Lakes, Australia

2. **Szilárd Sáringer**, Tamás Valtner, István Szilágyi (oral presentation)

   Co-immobilization of papain and horseradish peroxidase on latex nanoparticles

   35th Conference of the European Colloid & Interface Society, 5-10 September 2021, Athens, Greece (online)

3. **Szilárd Sáringer**, István Szilagyi (poster)

   Immobilization of an antioxidant enzyme cascade on titania nanosheets

   Geneva Colloids 2021, 8-9 April 2021, Geneva, Switzerland (online)

4. **Szilárd Sáringer**, István Szilágyi (oral presentation)

   Immobilization of an antioxidant enzyme cascade on titania nanosheets

   XLIII. Kémiai Előadói Napok, 27-29 October 2020, Szeged, Hungary (online)

5. **Szilárd Sáringer**, Paul Rouster, István Szilágyi (oral presentation)

   Regulation of the stability of titania nanosheet dispersions with oppositely and like-charged polyelectrolytes

   17th European Student Colloids Conference, 18-22 June 2019, Varna, Bulgaria

6. **Szilárd Sáringer**, Paul Rouster, István Szilágyi (poster)

   Colloidal stability of polyelectrolyte functionalized titania nanosheets (poster)

   5th International Conference on Solution Chemistry, 26-30 August 2018, Szeged, Hungary