DEVELOPMENT OF A NANOPARTICLE BASED GLUCOSE BIOSENSOR

Synopsis submitted in fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

By

SHIKHA SHARMA

Department of Biotechnology

JAYPEE INSTITUE OF INFORMATION TECHNOLOGY UNIVERSITY

A-10, SECTOR-62, NOIDA, INDIA

AUGUST 2012

The inimitable properties such as high target specificity and high sensitivity of biosensors, imparted by biological recognition systems, has led to their wider acceptability and commercialization threatening the existing sensors market. Biosensor industry is a global industry having potential application in varied fields, such as medical diagnostics[1], fermentation[2], pharmaceutical[3], drug[4], food and beverage industry[5-7]. Additional areas of biosensor application that still lie dormant include agricultural, environment, mining industry and veterinary investigation. Global market research report by Global Industry Analysts, Inc., proposed the worldwide Biosensors market to reach 12 billion US$ by the year 2015. The intense market analysis indicates that presently United States is globally dominating the biosensor market followed by Europe while Asia-Pacific is expected to emerge as the fastest growing market for biosensors by 2017. It was stated that, "*Glucose Biosensors represents the largest segment cornering a lion's share of the global biosensors market*"[8]. About 90% of the universal biosensors market is seized by glucose biosensor.

Increasing health-related concerns or diabetic population is the crucial driving factor for growth of glucose biosensors in clinical diagnostic sector. However, the stability, response time, quality assurance and high cost are the major factors behind the untapped market of glucose biosensors other than health sector. Low thermal stability of enzymes leads to fast decline in activity of enzyme and hence poor biosensor efficiency, resulting in overall cost escalation leading to a reduced commercial viability. Large response time of currently available membrane or hydrogel based glucose biosensor [9-10] compromises the product quality and reproducibility. Thus the above mentioned two factors - the large response time and instability of biomolecule, are the key reasons limiting the application of current day biosensor in industries such as food, beverage and fermentation where continuous monitoring of glucose is of paramount importance in order to ensure the quality of product. The fusion of current day biosensor technology with nanotechnology is acting as a market propeller in this area. Nano size structures of gold[11], nickel[12], platinum[13], silica[14], carbon[15] etc. have revolutionized the biosensor research by reducing the response time to 2-10 seconds and sensitivities ranging from 5μA mM^{-1} cm^{-2} to as high as 112μA mM^{-1} cm^{-2}[16]. However, increasing sensitivities generally results in reduced linear dynamic range and the reported high sensitivity biosensors suffer from low operational stability (short shelf life). Hence it is imperative to devise alternative nanomaterials and strategies targeting both thermal and operational stability and sensitivity at the same time.

The objective of the presented research is – to increase the sensitivity and linear dynamic range and secondly, to enhance the operational and thermal stability of biosensor. To achieve the targeted goals the fact that nanomaterials of different morphologies exhibit unique physical and chemical properties, have been exploited. Thesis is structured as, Perspectives and Lacunae (chapter 1), Review of Literature (chapter 2), Characterization Techniques (chapter 3) followed by work done presented in the subsequent chapters as Nanostructure Synthesis (chapter 4) and applications of the same for enzyme immobilization and biosensor fabrication (chapter 5-7). Chapter 8 describes a unified approach of nanobiotechnology detailing a novel process to synthesize thermostable enzyme nanoparticles. Design of study is presented in Scheme I.

DESIGN OF STUDY

Chapter 1 presents perspective of the proposed work and identified lacunae of the existing biosensors. This is followed by Review of Literature on glucose biosensors in Chapter 2 discussing the historical perspectives of glucose sensing devices as first-, second- and third- generation biosensors and their limitations. A detailed survey of technological advancements in the field describing characteristics like sensitivity, lower detection limit, response time, linear dynamic range, stability and the material used for immobilization of enzyme has been presented. In addition to this, we have tried to comprehend the vast gap between limited numbers of technology transfers in comparison to large numbers of published papers. The extensive analysis yielded stability of immobilized enzymes as the crucial lacunae behind the slow pace of commercialization.

Performance characteristics of fabricated biosensor are controlled by achieving an understanding and control over the distinctive properties of the materials used. To accomplish the same physical and chemical characterization of materials used in addition to response characteristics of biosensor after each modification step becomes pertinent. Current biosensor technology exploits nanostructures and their varied optical, electronic, magnetic and other properties for enhancing performance and efficiency. Morphological characterization of these nanostructures is of paramount importance since their properties vary drastically with their size and shape. Microscopic techniques like Transmission electron microscopy (TEM), scanning electron microscopy (SEM) and optical microscopy (OM) are exploited for morphological analysis. Moreover, chemical and electrochemical characterization of the nanostructures and biosensors, in the present work, is done through spectroscopic techniques (UV-Visible spectroscopy, energy dispersive X-ray spectroscopy, electrochemical impedance spectroscopy), zeta potential analysis and cyclic voltammetry. Chapter 3 gives a brief description of these characterization techniques.

Chapter 4 describes optimization of synthesis protocols to achieve monodispersed nanostructures. The pioneering method of Turkevich et. al.[17] for synthesis of gold nanoparticles using chloroauric acid as precursor and trisodium citrate as reducing agent has been exploited for exploring effect of various parameters on morphology and size of nanoparticles synthesis. Effect of parameters like concentration of precursor, reducing agent, reducing agent/precursor ratio and rate of addition of reducing agent has been studied. Based on the above studies and analysis thereof, we have proposed probable mechanism for controlled synthesis of charged gold nanochains and/or monodispersed spherical nanoparticles.

The major concern of conventional methods is usage of toxic chemicals. Although most of these conventional methods are simple in implementation, however, high cost and hazardous environmental impact due to usage of organic solvents necessitates the need for alternative eco-friendly route. Spherical and anisotropic gold and silver nanostructures are synthesized via conventional and eco-friendly routes using trisodium citrate and amino acids respectively as reducing agents [18]. Furthermore, we have explored the effect of choice of amino acids, pH of the solution on synthesis of varied shape nanostructures. Finally, probable mechanism controlling the morphology of nanostructures following eco-friendly route has also been proposed.

In addition to optimization of controlled synthesis of gold and silver nanostructures we have also synthesized magnetite nanoparticles following the method of co-precipitation by Zhu et. al.[19] The reason being magnetite nanoparticles are being extensively explored in applications like drug delivery, biological imaging etc and their high biocompatibility behavior. We have included magnetite nanoparticles in our study to compare the biocompatibility and response characteristics of biosensor using these with that of gold nanoparticles.

Biosensing efficiency of these synthesized nanostructures has been evaluated in the next four chapters, i.e., Chapter 5-8. The efficiency of a biosensor, i.e., the sensitivity, stability and response time, is very much subjected to the kind of support material used and the method of immobilization. The most suitable support material and immobilization method vary depending on the enzyme and particular application. Key parameters for selection of suitable material and method are binding capacity of the material, stability, retention of enzyme activity and minimizing leakage of enzyme after immobilization on the material.

Recent advances in material development have led to better understanding and design of proper material surface to immobilize the enzyme. Advances in mesoporous and nanomaterials have brought opportunities for materials scientist to improve the enzyme loading capacities in order to develop an efficient enzyme based sensor [20-22]. Most of the reported literature on immobilization method or matrix is compared with free enzyme response only. However, a comparative study and analysis of different methods, matrices, design and loading capacity of enzyme is rarely reported in a set of experiment or at a laboratory to enable suitable choice of methods and materials for biosensor fabrication.

In chapter 5, we have presented a comparative study of immobilization techniques and materials for fabrication of glucose biosensor. Conventional matrices (calcium alginate beads, polyacrylamide gel) and membranes (NC and PVDF) have been compared with current-day nano- sized materials (gold and magnetite nanoparticles) for optimum enzyme immobilization. In addition to this, immobilization methods – electrostatic, covalent, physical adsorption and entrapment based methods have also been evaluated for their effect on immobilization efficiency (leakage of enzyme, retention of activity and thermal stability) Comparative analysis demonstrates covalently bound enzyme onto nanomaterials to be the most suitable method and matrix for enzyme immobilization. The characteristic compatible curvature and nanoelectrode behavior of nanomaterials were further exploited for enhancing biosensing capabilities of glucose biosensor. The present comparative analysis may serve as a set of design criteria to help engineers fabricate an efficient biosensor.

As discussed above, nanomaterials were found to be more suitable matrices for enzyme immobilization and biosensor fabrication yielding improved biosensor characteristics as compared to conventional matrices. In Chapter 6, efforts have been directed towards further enhancement of biosensing capabilities. This has been achieved through modulation of the electron transfer properties of gold nanoparticles by inducing coupling amongst the particles. Gold nanoparticles attached in the form of a chain are synthesized using different amino acid, as a reducing and capping agent (as discussed in chapter 4). Usage of the above amino acid facilitates the coupling among the particles (the chain like arrangement of particles). The glucose biosensor developed by immobilization of glucose oxidase enzyme onto amino functionalized chain like coupled gold nanoparticles showed much more enhanced sensitivity and excellent operational stability in comparison to biosensors fabricated using spherical gold nanoparticles. The probable mechanism responsible for enhancement in biosensor characteristics has also been proposed [18].

In chapter 6, we have presented a benign strategy to synthesize gold nanochains by self-assembly of individual gold nanoparticles. However, controlling the chain length was once again major challenge. Template based synthesis is one of the most popular approach for synthesis of controlled length scales of metallic nanostructures [23-24]. Removal of the template in order to obtain pure metallic structures is the major limitation of this approach. Hence it is pertinent to explore more eco-friendly route for synthesis of 1D micro/nanostructures. In Chapter 7 we have exploited unique microbial cell architectures as biotemplates for desired size and morphology. This chapter is dedicated to bio-inspired gold microwires (AuMWs) synthesis, their evaluation as potential microelectrodes for electron transfer between enzyme and electrode surface, in amperometric biosensing applications. To the finest of our knowledge, these bio-inspired gold microwires for the development of amperometric glucose biosensors have not been explored so far. Three different fungal species, having different nutritional characteristics, have been used to understand whether gold microwires synthesis is solely adsorption based or nutritional driven or both. Our work is aimed at understanding the mechanism of synthesis of these functional microstructures of gold and exploring their potential use for efficient biosensor fabrication.

In all the previous chapters, we have exploited nanotechnology for biotechnological application, i.e., biosensor fabrication by immobilization of enzyme on nanostructures. In Chapter 8, we have done hybridization of the two fields and in true sense an example of nanobiotechnology is presented in the form of nanoparticles of enzyme itself [25]. Major driving force behind present work being improving shelf life of enzyme based electrodes due to the major challenge posed by fragile nature of enzymes. Long shelf life of biosensor in particular and any product in general, is of paramount importance for commercialization. Hence, it is imperative to develop methods to enhance the stability of enzymes. Synthesis of nanoparticles of enzymes has been able to achieve the desired aim. We report a novel process to synthesize nanoparticles of enzymes in general and results for two enzymes Glucose oxidase (GOx) and Horse radish peroxidase (HRP) are presented. Optimized synthesis of the enzyme nanoparticles has been achieved by controlling the major governing factors such as, concentrations of desolvating and crosslinking agent and suitable choice of functionalizing agent. The synthesized enzyme nanoparticles exhibit improved thermal stability and biocatalytic activity over a wide range of temperature relative to free enzyme in solution or immobilized over conventional or nanoparticles based matrices.

SUMMARY

The present work can be summarized as:

1) We have proposed probable mechanism for controlled synthesis of monodispersed nanostructures of specific morphologies regulating precursor and reducing agent concentration and rate of addition of reducing agent.

2) We have presented alternative eco-friendly methods and mechanism for controlled synthesis of monodispersed nanostructures through suitable choice of amino acids, pH of solution and microorganisms as bio-templates.
3) Covalent immobilization onto amino functionalized nanoparticles was found to be the most suitable method and matrix for enzyme immobilization.
4) We have achieved sensitivity of 47.2 μA/mM/cm^2 with linear dynamic range 1μM –5 mM and response time of 4 seconds and more than 85% stability upto two months of storage by modulating the electron transfer properties of coupled gold nanoparticles and suitable functionalization.
5) Gold microwires of few millimeters are synthesized employing microbial architechture as biotemplates. The biosensors fabricated using the same yielded comparatively lower sensitivity of 43.2 μA/mM/cm^2 though there was a substantial increase in linear range from 5 μM – 20 mM while the shelf life was >75% upto two months of storage.
6) We have proposed a novel protocol for synthesis of thermally stable enzyme nanoparticles. The controlled synthesis protocol yielded nanoparticles consisting of 5-6 enzyme molecules. The fabricated biosensor showed high thermal stability, data for the same not presented since the patent application is under process.

IMPLICATIONS AND FUTURE SCOPE

1) The amino functionalized coupled AuNPs could be used for covalent immobilization of enzymes ensuring high catalytic activity for repeated usage and hence ensuring long shelf of the products.
2) Biotemplate based synthesized microwires shall serve as benchmark for fabrication of desired morphology, biocompatible nano-/micro- structures using microbial cell architectures for specific applications.
3) Synthesis of enzyme nanoparticles with enhanced thermal stability could revolutionize the high temperature catalytic applications in biotech and pharma industries.
4) Thermostable enzyme nanoparticles would circumvent low temperature shipping and storage requirements of enzymes, enzymatic diagnostic kits and devices etc., making them more economical.

Table 1 : Comparative Evaluation of all the fabricated biosensors in terms of their performance characteristics

Parameters	***GOx-AuNPs amino spherical electrode* (thesis work)**	***GOx-Fe$_3$O$_4$NPs-electrode* (thesis work)**	***GOx-Au amino NPs-electrode (Nanochains)* (thesis work)**	***GOx-Au citrate NPs-electrode* (thesis work)**	***GOx-AuMWs modified electrode* (thesis work)**	***GOx-electrode* (thesis work)**	***GOx-AgNPs-electrode* (thesis work)**
Dynamic Linear range	1µM to 7mM	5µM to 5mM	1µM to 5 mM	5µM to 5mM	**5 µM-20 mM**	Upto 0.5mM	10µM to 5mM
Sensitivity (µA/ mM/ cm^2)	22.42	14.3	**47.2**	16.4	43.2	1.21	17.36
Detection Limit	**1µM**	5µM	1µM	1µM	5 µM	50 µM	10 µM
Precision (RSD value)	1.43%	1.76 %	0.84%	1.38 %	1.30%	-	1.34%
Interference	No	No	No	No	No	-	No
Stability	73% activity retained after 60 days	56 % activity retained after 60 days	**85% activity retained after 60 days**	64% after 60 days	75% upto 60 Days.	Rapid activity loss	64% after 60 days
K_m	3.24 mM	5.6mM	3.11 mM	4.3 mM	3.6mM	-	4.1mM
k_s	2.62 s^{-1}	1.01 s^{-1}	3.12 s^{-1}	2.4 s^{-1}	2.8 s^{-1}	-	1.16 s^{-1}
Response time	8 sec	11-12 sec	**4 sec**	8 sec	6 sec	15 sec	5-6 sec

REFERENCES

[1] Malhotra B.D., Chaubey A., "*Biosensors for clinical diagnostics industry*", Sensors and Actuators B, vol.91, pp. 117–127, Mar. 2003.

[2] Tothill I.E., Newman J.D., White S.F., Turner A.P.F., "*Monitoring of the glucose concentration during microbial fermentation using a novel mass-producible biosensor suitable for on-line use*", Enzyme and microbial technology, vol. 20, pp. 590–596, Jun. 1997.

[3] Tombelli S., Mascini M., "*Aptamers biosensors for pharmaceutical compounds*", Combinatorial Chemistry & High Throughput Screening, vol. 13, pp. 641-649, Aug. 2010.

[4] Yu D., Blankert B., Vire J.C., Kauffmann J.M., "*Biosensors in Drug Discovery and Drug Analysis*", Analytical Letters, vol. 38, pp. 1687-1701. Aug 2005.

[5] Terry L.A., White S.F., Tigwell L.J., "*The Application of Biosensors to Fresh Produce and the Wider Food Industry*", Journal of Agricultural and Food Chemistry, vol. 53, pp. 1309-1316, Feb. 2005.

[6] Baeumner A.J. "*Biosensors for Environmental Pollutants and Food Contaminants*", Analytical and Bioanalytical Chemistry, vol. 377, pp. 434-445, Aug. 2003.

[7] Monosik R., Stredansky M., Tkac J., Sturdik E., "*Application of Enzyme Biosensors in Analysis of Food and Beverages*" Food Analytical Methods, vol. 5, pp. 40-53, Mar. 2012.

[8] Biosensors in Medical Diagnostics: A Global Strategies Business Report "Report by Global Industry Analysts, Inc." Mar. 2012

[9] Doretti L., Ferrara D., Barison G., Lora S., "*Glucose sensors based on enzyme immobilization onto biocompatible membranes obtained by radiation-induced polymerization*", Applied Biochemistry and Biotechnology, vol. 49, pp. 191-202, Dec 1994.

[10] Zhu J., Tian C., Wu W., Wu J., Zhang H., Lu D., Zhang G., "*Fabrication and characterization of glucose sensors based on a microarray H_2O_2 electrode*", Biosensors and Bioelectronics, vol. 9, pp. 295-300, Jan. 1994.

[11] Si P., Kannan P., Guo L., Son H., Kim, D.H., "*Highly stable and sensitive glucose biosensor based on covalently assembled high density Au nanostructures*", Biosensors and Bioelectronics, vol. 26, pp. 3845-3851, May. 2011.

[12] Wang X., Zhang Y., Banks C.E., Chen Q., Ji X., "*Non-enzymatic amperometric glucose biosensor based on nickel hexacyanoferrate nanoparticle film modified electrodes*" , Colloids and Surfaces, B: Biointerfaces, vol. 78, pp. 363-366, Jul. 2010.

[13] Baby T.T., Ramaprabhu S., "*SiO2 coated Fe3O4 magnetic nanoparticle dispersed multiwalled carbon nanotubes based amperometric glucose biosensor*", Talanta, vol. 80, pp. 2016–2022, Mar. 2010.

[14] Zhang F.F., Wan Q., Wang X.L., Sun Z.D., Zhu Z.Q., Xian Y.Z., Jin L.T., Yamamoto K., "*Amperometric sensor based on ferrocene-doped silica nanoparticles as an electron transfer mediator for the determination of glucose in rat brain coupled to in vivo microdialysis*", Journal of Electroanalytical Chemistry, vol. 571, pp. 133-138, Oct. 2004.

[15] Lin Y., Lu F., Tu Y., Ren Z., "*Glucose Biosensors Based on Carbon Nanotube Nanoelectrode Ensembles*", Nano Letters, vol. 4, pp. 191-195, Feb. 2004.

[16] Chen K.J., Lee C.F., Rick J., Wang S.H., Liu C.C., Hwang B.J., " Fabrication and application of amperometric glucose biosensor based on a novel Pt Pd bimetallic nanoparticle decorated multi-walled carbon nanotube catalyst ", Biosensors and Bioelectronics, vol. 33, pp. 75-81, Mar. 2012.

[17] Turkevich J., Stevenson P.C., Hillier J., "*"A study of the nucleation and growth processes in the synthesis of colloidal gold*", Discussions of the Faraday Society, vol. 11, pp. 55–75, May. 1951.

[18] Sharma S., Gupta N., Srivastava S., "*Modulating electron transfer properties of gold nanoparticles for efficient biosensing*", Biosensors and Bioelectronics, vol. 37, pp. 30-37, Aug. 2012.

[19] Zhu Y., Wu Q., "*Synthesis of Magnetite Nanoparticles by Precipitation with Forced Mixing*" Journal of Nanoparticle Research, vol. 1, pp. 393-396, Apr. 1999.

[20] Trewyn B.G., Giri S., Slowing, I., Lin SY.V., "*Mesoporous silica nanoparticle based controlled release, drug delivery, and biosensor systems*", Chemical communications, vol. 31 pp. 3236-3245, Mar 2007.

[21] Lu X., Xiao Y., Lei Z., Chen J., Zhang H., Ni Y., Zhang Q., "*A promising electrochemical biosensing platform based on graphitized ordered mesoporous carbon*", Journal of Material Chemistry, vol. 19, pp. 4707-4714, May 2009.

[22] Wang J., "*Nanomaterial-based electrochemical biosensors*", Analyst, vol. 130, pp. 421-426, Jan. 2005.

[23] Liang H.W., Liu S., Yu S.H., "*Controlled Synthesis of One-Dimensional Inorganic Nanostructures Using Pre-Existing One-Dimensional Nanostructures as Templates*", Advanced Materials, vol. 22, pp. 3925-3937, Sep. 2010.

[24] Yan Y, Yang H, Zhang F, Tu B, Zhao D., "*Surfactant-templated synthesis of 1D single-crystalline polymer nanostructures*", Small, vol. 2, pp. 517-521, Apr. 2006.

[25] Indian Patent for process to make thermostable enzyme nanoparticles, Sudha Srivastava and Shikha Sharma (2010) "Novel process to enhance thermal stability of enzyme nanoparticles" Indian Patent Application No 2782/DEL/2010.

LIST OF PUBLICATIONS DURING Ph.D THESIS WORK

PATENT(S)

- Filed Indian Patent for process to make thermostable enzyme nanoparticles, Sudha Srivastava and Shikha Sharma (2010) "Novel process to enhance thermal stability of enzyme nanoparticles" Indian Patent Application No 2782/DEL/2010.

PUBLICATIONS

International Journals

- Shikha Sharma, Nidhi Gupta, and Sudha Srivastava, "Modulating Electron Transfer Properties of Gold Nanoparticles for Efficient Biosensing" in Biosensors and Bioelectronics, 2012, 37(1), 30-37.
- Sharma S., Goswami N., Gupta N., Srivastava S., "*Amino Coated Gold Nanorods Based Amperometric Glucose Detection*" International Journal of Advancements in Technology, vol. 3, pp.195-202, Jul. 2012.
- Sharma S. and Srivastava S., "Synthesis of Branched Gold Nanostrcutures with Improved Biocompatibility" Nano Trends (In press : to be published in Volume 13, Issue 1, 2012)

International Conference

- Shikha Sharma, Aditi Shrivastav, Nidhi Gupta, and Sudha Srivastava, "*Amperometric biosensor: increased sensitivity using enzyme nanoparticles*" Proceedings of international conference on nanobiotechniology and biosensors, Hong Kong, China, pp 24-27, Dec 2010.

ACADEMIC HONORS

- Selected for Travel Support from University of Luxembourg(2011-2012) for attending Cycle 7 of “European Ph.D. School on Nanoanalysis using finely focused ion and electron beams” Belvaux, Luxembourg.
- Best Poster Award, at “ ALIGARH NANO – I ,Workshop on Nanoscience and Nanotechnology”, Aligarh Muslim University.(2011)
- Selected to attend “International conference on Nanobiotechnology and biosensors”. Travel Support from Department of biotechnology (DBT), Government of India.(2011)
- Second Runner-Up, at National conference on "Potentials of Biotechnology and Microbiology in India" Institute of applied Medicines and Research (IAMR) poster presentation.(2009).