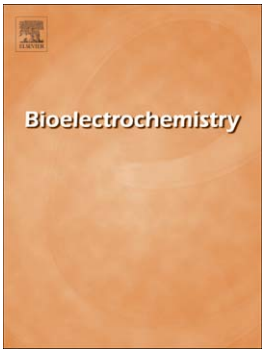


Effective immobilization of glucose oxidase on chitosan submicron particles from gladius of *Todarodes pacificus* for glucose sensing

J.R. Anusha, Albin T. Fleming, Hee-Je Kim, Byung Chul Kim, Kook-Hyun Yu, C. Justin Raj

PII: S1567-5394(15)00022-5
DOI: doi: 10.1016/j.bioelechem.2015.02.004
Reference: BIOJEC 6815

To appear in: *Bioelectrochemistry*

Received date: 2 June 2014
Revised date: 10 February 2015
Accepted date: 15 February 2015

Please cite this article as: J.R. Anusha, Albin T. Fleming, Hee-Je Kim, Byung Chul Kim, Kook-Hyun Yu, C. Justin Raj, Effective immobilization of glucose oxidase on chitosan submicron particles from gladius of *Todarodes pacificus* for glucose sensing, *Bioelectrochemistry* (2015), doi: 10.1016/j.bioelechem.2015.02.004

Effective immobilization of glucose oxidase on chitosan submicron particles from gladius of *Todarodes pacificus* for glucose sensing

J.R. Anusha[a], Albin T. Fleming[a], Hee-Je Kim[b], Byung Chul Kim[c], Kook-Hyun Yu[c], C. Justin Raj[c,*]

[a] *Department of Advanced Zoology and Biotechnology, Loyola College, Chennai-600034, India*

[b] *Laser and Sensor Lab, Department of Electrical Engineering, Pusan National University, Gumjeong-Ku, Busan-609735, South Korea*

[c] *Department of Chemistry, Dongguk University-Seoul, Seoul-100-715, Republic of Korea*

* Corresponding author: Tel.: +82 2 2260 3709; Fax: +82 2 2268 8204

E-mail: cjustinraj@gmail.com (C. Justin Raj)

ABSTRACT

An effective enzymatic glucose biosensor was developed by immobilizing glucose oxidase on chitosan submicron particles synthesized from the gladius of *Todarodes pacificus* (GCSPs). The chemically synthesized chitosan from gladius were pulverized to submicron particles by ball milling technique, which was further characterized and compared with the standard chitosan (SCS). The degree of deacetaylation of GCSPs was determined using FTIR spectroscopy which was comparable to the value of standard chitosan. The glucose oxidase (GOx) was immobilized over GCSPs on porous zinc oxide/platinum nanoparticles (ZnO/Pt) based electrode. The morphological and structural properties of the electrodes were analyzed using scanning electron microscopy and X-ray diffraction analysis. The glucose sensing behaviour of electrode was estimated using electrochemical analysis and showed an excellent analytical performance. The electrode ZnO/Pt/GCSPs conjugated with GOx displayed high sensitivity (88.76 μA $mM^{-1}cm^{-2}$) with low detection limit in short response time. In addition, the very low value of Michaelis-Menten constant for GCSPs based electrode contributes a better affinity of the electrode surface towards glucose oxidase.

Keywords: Biopolymers; Chitosan; Submicron particles; Glucose oxidase; Biosensors

1. Introduction

Biopolymers are now being used in diversified applications due to the variety of beneficial properties such as low toxicity, biodegradability, antitumor efficacy, enhanced immunogenicity, biocompatibility and high mechanical strength [1]. Chitosan (CS) is an attractive natural linear polysaccharide consists of copolymer, glucosamine and N-acetyl glucosamine which can be obtained by the partial decacetylation of either α, β or γ-chitin from crustaceans, gladius of squid and microorganisms respectively [2-4]. Major source for commercially available chitosan are shells of crabs, shrimps and other arthropods. Usually, the β-chitin rich gladius of squid is treated as refuse from food processing industries. Due to the presence of less impurities and absence of coloured compounds in gladius, the β-chitin and chitosan extraction made so easy by preventing acid hydrolysis and thus reduce processing cost [5] . Furthermore, the β-chitin produced from gladius shows more open structure, better quality, reactivity, solubility and swelling than α-chitin due to its low molecular hydrogen bonding [6]. Chitosan, deacetylated from the β-chitin of gladius, has been developed into membranes and scaffolds to assess their various biomedical applications [7-9].

In recent decades, chitosan in the form of submicron or nano sized particles were attracted due to their unique physicochemical and biological properties [10, 11]. Moreover, reducing the particle size improves the property of material such as physicochemical, optical, catalytic and other reactive properties [12, 13]. Despite the above mentioned applications, its excellent film forming ability with high sensitivity, stability and low cost holds them suitable for enzyme based biosensor for detection mechanisms [14, 15]. In biosensors, enzymes have been immobilized onto biopolymer by means of covalent bonding, precipitation, emulsion

cross linking and ionic gelation technique [16]. Recent studies on enzymatic biosensors indicates enzyme immobilized over chitosan particles shows better performance than that of bare electrode [17]. Although, chitosan improves the catalytic activity with controlled release and ameliorates the stability of enzyme from variation of pH, temperature and denaturing compounds.

Among various glucose biosensors, electrochemical biosensors have been employed successfully for the determination of glucose because of its low complexity, robustness and high range of detection [18, 19]. Glucose oxidase (GOx) is widely used enzyme in glucose sensor due to its stability, high catalytic properties, real time detection and less expensive [20]. To enhance the direct electron transfer from GOx enzyme to sensitive layer, a functional material is required to attain their excellent performance. Zinc oxide (ZnO) is one among such versatile nanomaterial with low dimensionality and inadequate properties like biocompatibility, non-toxicity, electric conductivity, high electron transfer etc., which provides an incentive to the research and development especially in biomedical and sensor applications [21, 22]. Moreover, metallic nanoparticles have been considered much in the field of catalysis and sensor due to its better catalytic and sensing properties than non-metallic components. Generally, platinum nanoparticles can serve as an effective material for improving the performance of biosensor due to their potential properties such as low overvoltage for H_2O_2 redox reactions, efficient electron transfer, high surface energy and surface to volume ratio[23, 24]. Since, organic polymer chitosan over inorganic nano composite have been effectively used for the fabrication of potential biosensor to utilize the benefits of both polymer and nanoparticles [25-28]. The main focus of this research is to

develop valuable alternative chitosan material from endoskeleton of squid by converting waste into economically and environmentally valuable biomaterial for sensing application.

In this study, we report a new strategy for fabricating glucose biosensor using simple technique on FTO substrate using ZnO/Pt nanoparticles with a layer of chitosan submicron particles from the gladius of squid, *Todarodes pacificus*. The comparative analysis of as prepared chitosan from gladius with commercially available chitosan were performed and discussed in detail. Furthermore, the electrocatalytic activities of electrodes in context with glucose were also discussed in detail using electrochemical analysis like cyclic voltammetry (CV) and amperometric measurements. As per our knowledge, this is the first report to fabricate a biosensor based on chitosan submicron particles from gladius of squid.

2. Experimental

2.1. Materials

The chemicals such as sodium hydroxide and hydrochloric acid (Sigma-Aldrich) were used for the extraction of chitosan (CS) from gladius of squid. The other chemicals used for the fabrication of electrodes were zinc acetate dihydrate, 2-methoxy ethanol and ethanolamine from Sigma Aldrich. Chitosan (CS) from crab shells (70- 85% deacetylated, high purity, medium molecular weight~ 190- 300 kDa) and Glucose oxidase (GOx) (E.C.1.1.3.4, 151,000 unit/g) were also from Sigma Aldrich. The platinum paste was purchased from Solaronix (platinum catalyst platisol T/SP) and all the chemicals used for the experiments were of analytical grade and used without further purifications. The biosensor electrode was fabricated on fluorine doped tin oxide (FTO; 13 Ω/sq; Hartford Glass Co., Inc.).

2.2. Preparation of β-chitin from gladius

For the synthesis of chitosan, the gladius were separated from the muscle of squid, *Todarodes pacificus* and washed thoroughly with water to remove adherent proteins and soluble organic materials. The cleaned gladius were dried at room temperature and stored below 0 °C until further analysis. The samples were cut into small pieces (1 mm × 1 mm) and ground using ball mill (Retsch-model no: PM-100) for 1 h at 600 rpm to form fine powder. The β-chitin was extracted from the powdered gladius by deprotenization and demineralization process. In deprotenization process, powdered sample was added slowly to 1 M NaOH solution to obtain a ratio of solid alkaline solution of 1: 14 (w/v) at 50 °C for 5 h under constant stirring. The sediment was filtered and washed with de-ionized water until the pH reaches neutral. In addition, the sample was dehydrated twice with methanol and once with acetone and transferred to glass tray and dried overnight at 50 °C in forced air oven. The yield percent was accurately weighed immediately after the samples were cooled in the desiccators. Then, the resultant sample was subject to demineralization by adding 150 ml of 1M HCl per 10 g under constant stirring for 2 h at room temperature. The residue was filtered and washed with buffer solution (pH 7.0) to remove excess acid and also to prevent acid-alkaline reaction. Finally, the obtained β-chitin powder was dried overnight in vacuum oven at 60 °C.

2.3. Synthesis and characterization of chitosan submicron particles (GCSPs)

As prepared β-chitin powder was added to 50 % (w/v) NaOH to obtain a ratio of solid to alkaline solution of 1: 15 (w/v) at 60 °C under nitrogen atmosphere with constant stirring for 2 h. Under this deacetylation process, pure white chitosan particles were formed. The suspensions were centrifuged, filtered and dried completely at room temperature. The

obtained CS particles were further subjected to ball milling process, to form fine powder (Retsch- model no: PM- 100). Milling process was carried out in two step; in first step, the dried CS (10 mg) was placed in 500 ml strengthened Teflon lid stainless steel grinding bowl with ZrO_2 balls (10 No.) of 6 mm size and grounded at 600 rpm for 1 h at 30 °C. Secondly, an appropriate amount of absolute ethanol (99.9 %) was mixed with the powder and grounded for 2 h at 30 °C. Finally, the obtained sample was dried in a hot air oven to remove the excess ethanol content. Finely powdered sample was used for further characterization. The morphology of GCSPs were recorded using TEM (JEOL model, JEM 2011) operated at an accelerating voltage of 200 kV. The FTIR spectra of GCSPs and SCS for degree of deacetylation and the functional groups analysis were performed by Perkin Elmer US/Spectrum GX spectrometer (KBr pellet technique).

2.4. Fabrication of biosensor electrodes

Generally platinum or gold substrates were used for the fabrication of biosensor electrodes. In this present work fluorine doped tin oxide (FTO) was used as a substrate because of its compatibility towards zinc oxide films. Moreover, the FTO substrate posses comparable conductivity, durability, stability and low cost than that of gold and platinum metals. To deposit a ZnO layer on FTO substrate, ZnO precursor solution was prepared using 0.5 M of zinc acetate dihydrate ($Zn(CH_3COO)_2).2H_2O$) and 0.5 M monoethanolamine in 2-methoxyethanol ($C_3H_8O_2$) solution. The mixture was homogenously stirred at 65 °C for 1 h and the solution was left for 5 h to obtain required sol. The sol was drop casted over a selected area of (active area 0.25 cm^2) ultrasonically cleaned FTO substrate using a polymer mould and dried completely in hot air oven. Then, the drop casting was repeated few times to acquire a homogeneous thin film. The obtained film was sintered at 400 °C for 1 h to

evaporate organic impurities and acquire a good adhesion with the substrate. The Pt nanoparticle was dispersed over ZnO surface by carefully tap casting Pt paste over the ZnO thin film, dried and subsequently heat treated at 400 °C for 30 min in air to remove the organic impurities. Finally, the polymer supporting layer was coated using GCSPs solution prepared by dissolving 1 mg of fine powder in 1 % acetic acid (100 ml) solution. The ZnO/Pt film was dipped in chitosan solution for 10 seconds and dried at room temperature to form ZnO/Pt/ GCSPs electrode. Further, the electrode was rinsed in freshly prepared phosphate buffer saline (PBS, 0.1 M, pH 7.4) to remove the excess biopolymer particles and acid content. Similarly, another electrode, ZnO/Pt/SCS was prepared with standard chitosan to perform comparative studies.

2.5. Immobilization of GOx on ZnO/Pt/GCSPs and ZnO/Pt/SCS electrode

To immobilize GOx enzyme over ZnO/Pt/GCSPs and ZnO/Pt/SCS electrodes, the GOx solution was freshly prepared by dissolving 1 mg of GOx in 1 ml of phosphate buffer (0.1 M, pH 7.4). For enzyme immobilization, appropriate amount of GOx was dropped over both sets of electrode surface and dried at room temperature and stored under dark at 4 °C before performing the experiments. Scheme 1 illustrates the fabrication steps of GCSPs based glucose biosensor.

Insert Scheme. 1

2.6. Preparation of β-D- glucose solution

A glucose stock solution was prepared in 0.1 M phosphate buffer solution (pH 7.4). The solution was kept at room temperature for 24 h prior to analysis, which was to ensure the presence of β-D- glucose form. From this solution, different concentrations (50- 2000 μM) of analyte samples were prepared.

2.7. Characterization of electrodes

The crystalline nature of ZnO and ZnO/Pt thin films were analyzed by X-ray diffraction (XRD, D/Max-2400, Rigaku) using a Cu Kα source operated at 40 kV and 30 mA in the 2θ range of 20–80°. The surface morphology and energy dispersive X-ray spectrum (EDX) of ZnO and ZnO/Pt films were analyzed by field emission scanning electron microscope (FE-SEM, S-4200, Hitachi) operated at 15 kV.

2.8. Electrochemical measurements

Electrochemical studies were carried out in three electrode setup utilizing ZnO/Pt/ GCSPs/GOx and ZnO/Pt/SCS/GOx as a working electrode. A platinum wire and the saturated calomel electrode (SCE) were used as a counter and reference electrodes. The cyclic voltammetry was carried out using the BioLogic potentiostat (SP-150, France). Phosphate buffer solution of 0.1 M and pH 7.4 was used as an electrolyte for the entire electrochemical analysis. The glucose sensing properties of the electrodes were performed using various concentrations of glucose solution in PBS electrolyte. Amperometeric experiments were carried out using ZIVELAB electrochemical workstation (ZIVE SP2). The electrochemical cell consists of working electrode (ZnO/Pt/GCSPs/GOx and ZnO/Pt/SCS/GOx), reference electrode ($Ag/AgCl_2$) and counter electrode (Pt). The experiment was performed by applying constant potential of 0.6 V using various concentrations (50-2000 µM) of glucose solution in PBS electrolyte at 20 ±3 °C room temperature. The experiment was repeated 5 times to ensure the reproducibility of the electrode.

3. Results and discussion

3.1. Characterization of GCSPs

The average weight of each gladius collected from the squid, *T. pacificus* was 0.345 ± 0.082 g. Almost, 42-43 % water insoluble white fibrous β- chitin without any pigment was obtained after demineralisation and deprotenization treatments [29]. Approximately, 0.078 g of β- chitin was obtained from a gladius and the amount of chitin obtained in this study was comparable with the reported result [5, 8, 30]. Further, the β-chitin was converted into chitosan by deacetylation procedure which was followed by ball milling to form ultra fine white powder[6].

As prepared chitosan were then subjected to morphological observation using TEM images. The TEM images of well dispersed CS samples (Fig. 1) revealed the presence of GCSPs with spherical morphology of diameter range from 100 to 500 nm [10, 11, 15]. From these observations, it was clear that the ball milled CS sample from gladius composed of large number of spherical submicron particles in agglomerated form. While ball milling, bulk structure of CS can easily deduced to form submicron particles [31].

Insert Fig. 1

The functional groups present in these GCSPs were compared with commercially obtained standard chitosan (SCS) using FTIR spectroscopy. The FTIR spectrum (Fig. 2) clearly explains that there were no chemical changes between GCSPs and SCS. The OH- and CH stretching groups involved in hydrogen bonding were occurred in 3460 and 2880 cm^{-1} with intensity variation is independent of deacetylation. The characteristic peaks of CS was observed at 1660 cm^{-1} (amide I) and 1580 cm^{-1} (amide II) [8]. The specific band for N-acetyl- glucosamine appears at 1322 cm^{-1} [32].

Insert Fig. 2

The Degree of Deacetaylation (DD %) of GCSPs was estimated by choosing the bands at 1655 and 3450 cm^{-1} of the FTIR spectrum. The DD % was calculated using the modified baseline technique of Domszy and Roberts [33] (Baseline (1)) by Baxter et al. [34] (Baseline (2)). The two baselines were computed using the equation:

$$DD = 100 - [(A_{1655}/A_{3450}) \times 100/1.33] \quad \text{Baseline (1)}$$

$$DD = 100 - [(A_{1655}/A_{3450}) \times 115] \quad \text{Baseline (2)}$$

where, A_{1655}/A_{3450} is the ratio of absorbance at 1655 cm^{-1} and 3450 cm^{-1}, the factor 1.33 denote the value of A_{1655}/A_{3450} ratio. The DD were calculated for GCSPs samples using the modified baseline (2) revealed 80 ± 2.5 %. FTIR spectra clearly explain that the milling treatment did not cause any change in chemical structure of chitosan [35]. The obtained degree of deacetaylation is comparable to the values reported previously for gladius of squid [6, 36]. According to various literatures, the β- chitin from gladius has weaker intermolecular hydrogen bonds, which reinforces the susceptibility of β-chitin to deacetylation reaction and resulted in high degree of deacetylation to from β- chitosan [5, 6, 8].

3.2. Morphological and structural studies of electrodes

The surface morphology of ZnO/Pt/GCSPs and ZnO/Pt/SCS electrodes were observed using FE-SEM images. The microscopic image (Fig. 3A) clearly displays the porous spherical granular morphology of ZnO/Pt with size ranges from 30 to 50 nm. Moreover, there is no considerable change in the surface morphology of electrode due to the influence of GCSPs. But, the SCS show decrease in porosity of the electrode surface and shows the existence of polymeric structure of SCS over ZnO/Pt nanostructure (Fig. 3B). The porous structure of ZnO/Pt nanocomposites with GCSPs can effectively promotes the immobilization

of enzyme and may provide comparatively higher conductive surface and active site for the GOx molecules.

Insert Fig. 3 A&B

The crystallinity and structure of ZnO/Pt film was analyzed by XRD and EDX method. Fig. 4 depicts the resultant XRD and inset shows the EDX spectrum of the ZnO/Pt electrode surfaces. The X-ray diffraction patterns of platinum coated ZnO films on FTO substrate shows the hexagonal wurtzite phase of ZnO structure of crystallization reported as in JCPDS card (no. 36-1451). Besides, the reflections from ZnO wurtzite and FTO phases, a reflection observed at 39.8° representing the reflection of face centered cubic (111) plane of Pt nanoparticles representing the existence of Pt nanoparticles. Apart from this, the EDX analysis confirms the presence of Pt nanoparticle in the ZnO electrodes.

Insert Fig. 4

3.3. Electrochemical behavior of electrodes

The cyclic voltammograms (CV) of bare ZnO/GOx, ZnO/Pt/GCSPs/GOx and ZnO/Pt/SCS/GOx electrodes recorded using 0.1M PBS (pH 7.4) containing 2 mM of glucose solution at 50 mVs^{-1} scan rate is shown in Fig. 5. From the resultant CVs, the bare ZnO/GOx without chitosan and Pt nanoparticles has no significant result compare to ZnO/Pt/GCSPs and ZnO/Pt/SCS electrodes with GOx. This may be due to lack of binding sites in ZnO for the attachment of GOx. But, in other electrodes there are two possible interactions may happen, (i) based on the electrostatic interaction between the positively charged GCSPs and negatively charged GOx and (ii) a simple physical adsorption between GOx and biopolymer [37]. The two electrodes show excellent profile of redox reaction due to the enzymatic catalysis occurred between physically immobilized GOx and glucose. The redox reaction

generates H_2O_2, which was indicated as redox peak (at 0.6 and -0.1 V) in voltammograms. The GCSPs based electrode shows comparable redox profile with high reduction peak current than that of SCS electrode. The reduction peaks in both the electrodes are possibly due to the incorporation of Pt nanoparticles, owing to its high catalytic property [26, 27].

Insert Fig. 5

The cyclic voltammograms of GOx immobilized ZnO/Pt/GCSPs and ZnO/Pt/SCS electrodes for various concentrations of glucose were shown in Fig. 6 (A) and (B). From the obtained voltammograms, it is clear that both the electrodes show nearly same profile of oxidation and reduction reactions in various concentrations of glucose solution. In case of modified electrode ZnO/Pt/GCSPs/GOx, the reduction (-0.1 V) at negative potential showed high peak current while, the oxidation at positive potential (0.6 V) increased slightly with increase in glucose concentration for catalyzes reaction [38, 39]. Among these two electrodes, ZnO/Pt/GCSPs immobilized GOx shows improved cathodic peak current for all glucose concentrations than that of ZnO/Pt/SCS electrode. The variation in these GCSPs electrode may be due to structure and conductivity of the electrode[40]. The porous GCSPs based electrode allows excess glucose molecules to react with the GOx immobilized on the surface as well as inside the pores of electrode. Since, GCSPs from squid have better quality, reactivity, solubility and swelling than that of SCS due to its low molecular hydrogen bonding. But in the case of SCS, which blocks conducting surface and pores of the electrode and restrict the electron transfer.

Insert Fig. 6 A & B

3.4. Amperometric response of ZnO/Pt/GCSPs/GOx and ZnO/Pt/SCS/GOx electrodes

The amperometric response of GOx immobilized ZnO/Pt/GCSPs and ZnO/Pt/SCS electrodes (Fig. 7) were analyzed with sequent injection of glucose into PBS (pH 7.4, under stirring) at applied potential of 0.6 V. An obvious increase in the current was observed upon successive addition of glucose. A well defined amperometric response was obtained within short response time, ≤ 2s which indicates the rapid and sensitive response with the change of glucose concentration. The response time was significantly higher than earlier reported amperometric glucose biosensor [24, 41]. The resulting calibration curve (Inset fig. 7) of two electrodes displayed linearly from 0.05 to 1 mM with a correlation coefficient of 0.99. Furthermore, the linear glucose detection ranges for developed electrodes are higher or comparable to other reported glucose sensor based electrodes with immobilized GOx [40, 42, 43].

Insert Fig. 7

From the slope of calibration curve, the sensitivity of both electrodes were determined. The ZnO/Pt/GCSPs/GOx sensor shows an excellent sensitivity of 88.36 $\mu AmM^{-1}cm^{-2}$ and ZnO/Pt/SCS/GOx sensor with 62.73 $\mu AmM^{-1}cm^{-2}$ sensitivity. The sensitivity of these electrodes were comparable or higher than that of other reported electrodes, for eg., Nafion/ZnO-hollow nanospheres immobilized GOx on glassy carbon electrode (65.82 $\mu AmM^{-1}cm^{-2}$) [44], ZnO/PVP/GOx nanofiber (70.2 $\mu AmM^{-1}cm^{-2}$)[45] and GOx on graphene/chitosan (37.93 $\mu AmM^{-1}cm^{-2}$) [43]. The GOx immobilized ZnO/Pt/GCSPs showed higher sensitivity than ZnO/Pt/SCS electrodes. These improvements were mainly attributed to the large effective working area of porous electrode. Since the sensor signal mainly dependent on the amount of immobilized enzyme on the surface of electrodes. Thus, the high

sensitivity of GCSPs of sensor confirmed that large quantity of GOx was immobilized on the porous electrode than that of SCS electrode. Moreover, the addition of Pt over ZnO nanoporous has improved the catalytic nature and acts as an excellent electron mediator and which accelerates the electron transfer between GOx and electrode [21].The detection limit (LOD) for ZnO/Pt/GCSPs/GOx was 0.03 mM and 0.09 mM for ZnO/Pt/SCS/GOx at a signal to noise ratio of 3 which was comparable with already reported enzyme based glucose biosensors[22, 42, 43, 46], the detailed comparisons are listed in Table 1.

Insert Table 1

From the immobilized GOx, the apparent Michaelis–Menten constant (K_m^{app}) were calculated for the enzyme activity over electrodes using the Linweaver–Burke plots. The obtained Linweaver–Bruke plots (reciprocal response current density vs. reciprocal of glucose concentration) for biosensors were shown in Fig. 8. The value K_m^{app} was calculated from the intercept and the slope of the Linweaver–Bruke plot. The apparent Michaelis–Menten constant (K_m^{app}) were 0.22 mM for ZnO/Pt/GCSPs/GOx and 0.31 mM in case of ZnO/Pt/SCS/GOx electrode. The value represents the enzyme loading and the enzymatic activity of the electrodes towards glucose[37]. Overall, the low values of Michaelis–Menten constant for the electrodes offered a friendly environment and porous structure for the immobilization of more glucose oxidase and enhance good biocatalysis towards glucose and substrate. The value of K_m^{app} is significant in line with the reported results for other GOx based biosensors [20, 47-49] .

The reproducibility of the biosensor was investigated by measuring the peak current for 0.1 mM glucose. With a series of 5 experiments, the relative standard deviation (R.S.D.)

of 3.2 % was achieved. The overall results indicated that the immobilized GOx possesses high enzymatic activity, and the ZnO/Pt/GCSPs film provides favorable microenvironment for GOx.

Insert Fig. 8

4. Conclusion

Chitosan submicron particles synthesized from the gladius of squid, *Todarodus pacificus* was used as a biomaterial for the immobilization of glucose oxidase enzyme in ZnO/Pt based biosensor electrode. The electrode fabricated using this chitosan, showed a fine porous granular morphology which provide an effective biocompatible environment for enzyme. The ZnO/Pt/GCSPs/GOx shows high sensitivity towards glucose than ZnO/Pt/SCS electrodes immobilized with GOx. The results made two inferences; the enhanced sensitivity may be due to the chitosan submicron particles from gladius with low surface area, high biocompatibility and the porous granular structure allow the analyte easily and thereby enhance catalytic activity. Another reason, the Pt nanoparticles embedded ZnO electrode demonstrated an excellent electronic conductivity as well as good biocompatibility and enhances the electron transfer between glucose and the electrode surface. In addition, fabricated glucose biosensor exhibits a low detection limit and low Michaelis–Menten constant indicates a high affinity of the GCSPs based electrode towards GOx. The proposed method of fabricating biosensor system using chitosan from gladius of squid could broadly be explored with more wide application for other biosensing techniques.

Acknowledgement

The authors would like to thank Pusan National University, Busan and Dongguk University, Seoul, South Korea for providing research facilitates. We also thank the Management of Loyola College, Chennai, India for their support and encouragement.

References

[1] A. Tiwari, Recent developments in bio-nanocomposites for biomedical applications, Nova Science Publishers, Hauppauge, N.Y., 2010.

[2] S.P. Davis, Chitosan : manufacture, properties, and usage, Nova Science Publishers, Hauppauge, N.Y., 2011.

[3] M. Rinaudo, Chitin and chitosan: Properties and applications, Progress in Polymer Science, 31 (2006) 603-632.

[4] M.N. Belgacem, A. Gandini, Monomers, polymers and composites from renewable resources, 1st ed., Elsevier, Amsterdam ; Boston, 2008.

[5] D.K. Youn, H.K. No, W. Prinyawiwatkul, Preparation and characteristics of squid pen β-chitin prepared under optimal deproteinisation and demineralisation condition, Int. J. Food Sci. Technol. 48 (2013) 571-577.

[6] D.K. Youn, H.K. No, W. Prinyawiwatkul, Preparation and characterisation of selected physicochemical and functional properties of β-chitosans from squid pen, Int. J. Food Sci. Technol. 48 (2013) 1661-1669.

[7] S. Li, Smart polymer materials for biomedical applications, Nova Science Publishers, New York, 2010.

[8] L.L. Reys, S.S. Silva, J.M. Oliveira, S.G. Caridade, J.F. Mano, T.H. Silva, R.L. Reis, Revealing the potential of squid chitosan-based structures for biomedical applications, Biomedical Materials, 8 (2013) 045002.

[9] J.L. Chen, Y. Zhao, Effect of molecular weight, acid, and plasticizer on the physicochemical and antibacterial properties of beta-chitosan based films, J. Food Sci. 77 (2012) E127-136.

[10] W. Anderson, D. Kozak, V.A. Coleman, A.K. Jamting, M. Trau, A comparative study of submicron particle sizing platforms: accuracy, precision and resolution analysis of polydisperse particle size distributions, J. Colloid Interface Sci. 405 (2013) 322-330.

[11] A. Bernkop-Schnurch, A. Heinrich, A. Greimel, Development of a novel method for the preparation of submicron particles based on thiolated chitosan, Eur. J. Pharm. Biopharm. 63 (2006) 166-172.

[12] S.P. Chakraborty, S.K. Sahu, P. Pramanik, S. Roy, Biocompatibility of folate–modified chitosan nanoparticles, Asian Pac. J. Trop. Biomed. 2 (2012) 215-219.

[13] A. Enriquez de Salamanca, Y. Diebold, M. Calonge, C. Garcia-Vazquez, S. Callejo, A. Vila, M.J. Alonso, Chitosan nanoparticles as a potential drug delivery system for the ocular surface: toxicity, uptake mechanism and in vivo tolerance, Invest. Ophthalmol. Vis. Sci. 47 (2006) 1416-1425.

[14] O.K. Krasil□nikova, Porous structure and adsorption behaviours of chitosan, Nova Science Publishers, New York, 2011.

[15] K. Konecsni, N.H. Low, M.T. Nickerson, Chitosan-tripolyphosphate submicron particles as the carrier of entrapped rutin, Food Chem. 134 (2012) 1775-1779.

[16] D.W. Kimmel, G. LeBlanc, M.E. Meschievitz, D.E. Cliffel, Electrochemical sensors and biosensors, Anal. Chem. 84 (2012) 685-707.

[17] C. Shan, H. Yang, D. Han, Q. Zhang, A. Ivaska, L. Niu, Graphene/AuNPs/chitosan nanocomposites film for glucose biosensing, Biosens Bioelectron. 25 (2010) 1070-1074.

[18] N.S. Oliver, C. Toumazou, A.E. Cass, D.G. Johnston, Glucose sensors: a review of current and emerging technology, Diabetic medicine : a journal of the British Diabetic Association, 26 (2009) 197-210.

[19] J. Wang, Glucose Biosensors: 40 Years of Advances and Challenges, Electroanalysis. 13 (2001) 983- 988.

[20] S.A. Miscoria, J. Desbrieres, G.D. Barrera, P. Labbe, G.A. Rivas, Glucose biosensor based on the layer-by-layer self-assembling of glucose oxidase and chitosan derivatives on a thiolated gold surface, Anal Chim Acta. 578 (2006) 137-144.

[21] S.K. Shukla, S.R. Deshpande, A. Tiwari, Fabrication of a tunable glucose biosensor based on zinc oxide/chitosan-graft-poly(vinyl alcohol) core-shell nanocomposite, Talanta. 99 (2012) 283-287.

[22] Z.W. Zhao, X.J. Chen, B.K. Tay, J.S. Chen, Z.J. Han, K.A. Khor, A novel amperometric biosensor based on ZnO:Co nanoclusters for biosensing glucose, Biosens Bioelectron. 23 (2007) 135-139.

[23] J. Ren, W. Shi, K. Li, Z. Ma, Ultrasensitive platinum nanocubes enhanced amperometric glucose biosensor based on chitosan and nafion film, Sens. Actuators, B. 163 (2012) 115-120.

[24] H. Chen, R. Yuan, Y. Chai, J. Wang, W. Li, Glucose biosensor based on electrodeposited platinum nanoparticles and three-dimensional porous chitosan membranes, Biotechnol Lett, 32 (2010) 1401-1404.

[25] N. Chauhan, J. Narang, Meena, C.S. Pundir, An amperometric glutathione biosensor based on chitosan-iron coated gold nanoparticles modified Pt electrode, Int. J. Biol. Macromol. 51 (2012) 879-886.

[26] X. Kang, Z. Mai, X. Zou, P. Cai, J. Mo, A novel glucose biosensor based on immobilization of glucose oxidase in chitosan on a glassy carbon electrode modified with gold-platinum alloy nanoparticles/multiwall carbon nanotubes, Anal Biochem. 369 (2007) 71-79.

[27] Y. Wang, Y. Zhu, J. Chen, Y. Zeng, Amperometric biosensor based on 3D ordered freestanding porous Pt nanowire array electrode, Nanoscale, 4 (2012) 6025-6031.

[28] J. Du, X. Yu, J. Di, Comparison of the direct electrochemistry of glucose oxidase immobilized on the surface of Au, CdS and ZnS nanostructures, Biosens Bioelectron, 37 (2012) 88-93.

[29] R.L. Lavall, O.B. Assis, S.P. Campana-Filho, Beta-chitin from the pens of Loligo sp.: extraction and characterization, Bioresour. Technol. 98 (2007) 2465-2472.

[30] J. Jung, Y. Zhao, Characteristics of deacetylation and depolymerization of beta-chitin from jumbo squid (Dosidicus gigas) pens, Carbohydr. Res. 346 (2011) 1876-1884.

[31] W. Zhang, J. Zhang, Q. Jiang, W. Xia, Physicochemical and structural characteristics of chitosan nanopowders prepared by ultrafine milling, Carbohydr. Polym. 87 (2012) 309-313.

[32] J.L. Chen, Y. Zhao, Effect of molecular weight, acid, and plasticizer on the physicochemical and antibacterial properties of beta-chitosan based films, J. Food Sci. 77 (2012) E127-136.

[33] G.A.F.R. Julian G. Domszy, Evaluation of infrared spectroscopic techniques for analysing chitosan, Makromol. Chern, 186 (1985).

[34] M.D. Alasdair Baxter, K. D. Anthony Taylor and George A. F. Roberts, Improved method for i.r. determination of the degree of N-acetylation of chitosan Int. J. Biol. Macromol., 14 (1992) 166-169.

[35] A. Chandumpai, N. Singhpibulporn, D. Faroongsarng, P. Sornprasit, Preparation and physico-chemical characterization of chitin and chitosan from the pens of the squid species, and, Carbohydr. Polym. 58 (2004) 467-474.

[36] J. Huang, D. Zhao, S. Hu, J. Mao, L. Mei, Biochemical activities of low molecular weight chitosans derived from squid pens, Carbohydr. Polym. 87 (2012) 2231-2236.

[37] J.R. Anusha, H.-J. Kim, A.T. Fleming, S.J. Das, K.-H. Yu, B.C. Kim, C.J. Raj, Simple fabrication of ZnO/Pt/chitosan electrode for enzymatic glucose biosensor, Sens. Actuators, B. 202 (2014) 827-833.

[38] O. Yilmaz, D.O. Demirkol, S. Gulcemal, A. Kilinc, S. Timur, B. Cetinkaya, Chitosan-ferrocene film as a platform for flow injection analysis applications of glucose oxidase and Gluconobacter oxydans biosensors, Colloids Surf., B. 100 (2012) 62-68.

[39] Y. Zhang, Y. Liu, Z. Chu, L. Shi, W. Jin, Amperometric glucose biosensor based on direct assembly of Prussian blue film with ionic liquid-chitosan matrix assisted enzyme immobilization, Sens. Actuators, B. 176 (2013) 978-984.

[40] T. Kong, Y. Chen, Y. Ye, K. Zhang, Z. Wang, X. Wang, An amperometric glucose biosensor based on the immobilization of glucose oxidase on the ZnO nanotubes, Sens. Actuators, B. 138 (2009) 344-350.

[41] Y. Fang, Y. Ni, G. Zhang, C. Mao, X. Huang, J. Shen, Biocompatibility of CS-PPy nanocomposites and their application to glucose biosensor, Bioelectrochem. 88 (2012) 1-7.

[42] Z. Dai, G. Shao, J. Hong, J. Bao, J. Shen, Immobilization and direct electrochemistry of glucose oxidase on a tetragonal pyramid-shaped porous ZnO nanostructure for a glucose biosensor, Biosens Bioelectron, 24 (2009) 1286-1291.

[43] X. Kang, J. Wang, H. Wu, I.A. Aksay, J. Liu, Y. Lin, Glucose oxidase-graphene-chitosan modified electrode for direct electrochemistry and glucose sensing, Biosens Bioelectron, 25 (2009) 901-905.

[44] B. Fang, C. Zhang, G. Wang, M. Wang, Y. Ji, A glucose oxidase immobilization platform for glucose biosensor using ZnO hollow nanospheres, Sens. Actuators, B. 155 (2011) 304-310.

[45] C.P. Mashkoor Ahmad, Zhixiang Luo, Jing Zhu, A single ZnO nanofiber-based highly Sensitive amperometric glucose biosensor, J. Phys. Chem. C, 114 (2010) 9308- 9313.

[46] J. Jung, S. Lim, ZnO nanowire-based glucose biosensors with different coupling agents, Appl. Surf. Sci. 265 (2013) 24-29.

[47] J. Li, R. Yuan, Y. Chai, X. Che, W. Li, Construction of an amperometric glucose biosensor based on the immobilization of glucose oxidase onto electrodeposited Pt nanoparticles-chitosan composite film, Bioprocess Biosyst. Eng. 35 (2012) 1089-1095.

[48] V. Krikstolaityte, J. Kuliesius, A. Ramanaviciene, L. Mikoliunaite, A. Kausaite-Minkstimiene, Y. Oztekin, A. Ramanavicius, Enzymatic polymerization of polythiophene by immobilized glucose oxidase, Polymer, 55 (2014) 1613-1620.

[49] C. Deng, J. Chen, X. Chen, C. Xiao, L. Nie, S. Yao, Direct electrochemistry of glucose oxidase and biosensing for glucose based on boron-doped carbon nanotubes modified electrode, Biosens Bioelectron, 23 (2008) 1272-1277.

[50] W. Shi, Z. Ma, Amperometric glucose biosensor based on a triangular silver nanoprisms/chitosan composite film as immobilization matrix, Biosens Bioelectron, 26 (2010) 1098-1103.

Table 1. Comparison of glucose sensors constructed based on different modified electrode materials.

Glucose Biosensor[#]	Sensitivity ($\mu AmM^{-1}cm^{-2}$)	Limit of Detection (mM)	Linear range (mM)	K_m^{app} (mM)	Ref.
PET/Ti/Au/ZnO:Co/GOx	14.3	0.02	0-4	21	[22]
GOx/GA/APS/ZnONW	17.72	0.02	0.01- 0.7	1.3	[46]
GOx/ZnONT	21.7	0.001	0.05- 12	19	[40]
Nafion/CHIT/GOx@ PtNC/Pt	35.92	0.005	0.001-8	-	[23]
GOx/Chit/IL/PB/Pt	37.8	0.005	0.01- 4.2	-	[39]
GOx/Graphene/CHIT/GCE	37.93	0.02	0.08-12	4.4	[43]
Nafion/ZnOHNSPs/ GOD/ GCE	65.82	0.001	0.005- 3.15	-	[44]
CHIT/GOD@AgTNPs/Pt	67.17	0.001	0.003- 3	3.84	[50]
ZnO/PVPNF	70.2	0.001	0.25-19	2.19	[45]
ZnO/Pt/SCS/GOx	62.73	0.09	0.05- 1	0.31	Current work
ZnO/Pt/GCSPs/GOx	88.36	0.03	0.05- 1	0.22	Current work

Chit- chitosan, PET- poly(ethylene terephthalate), GA- gluteraldehyde, APS- (3-aminopropyl) methyldiethoxysilane, ZnONW- ZnO nanowire, ZnONT- ZnO nanotube, HNSPs- hollow nanospheres, AgTNPs- silver triangular nanoprisms, GOD- glucose oxidase, GCE- glassy carbon electrode, PVPNF- poly(vinyl pyrrolidone) nanofibers

Figure captions

Scheme 1. Schematic representation of the fabrication of GCSPs based glucose biosensor

Fig. 1. TEM microphotographs of spherical submicron particles synthesized from the gladius of *Todarodus pacificus*

Fig. 2. FTIR spectra for the comparative analysis of (a) SCS and (b) GCSPs

Fig. 3. FE-SEM images of (A) ZnO/Pt/ GCSPs and (B) ZnO/Pt/SCS electrodes

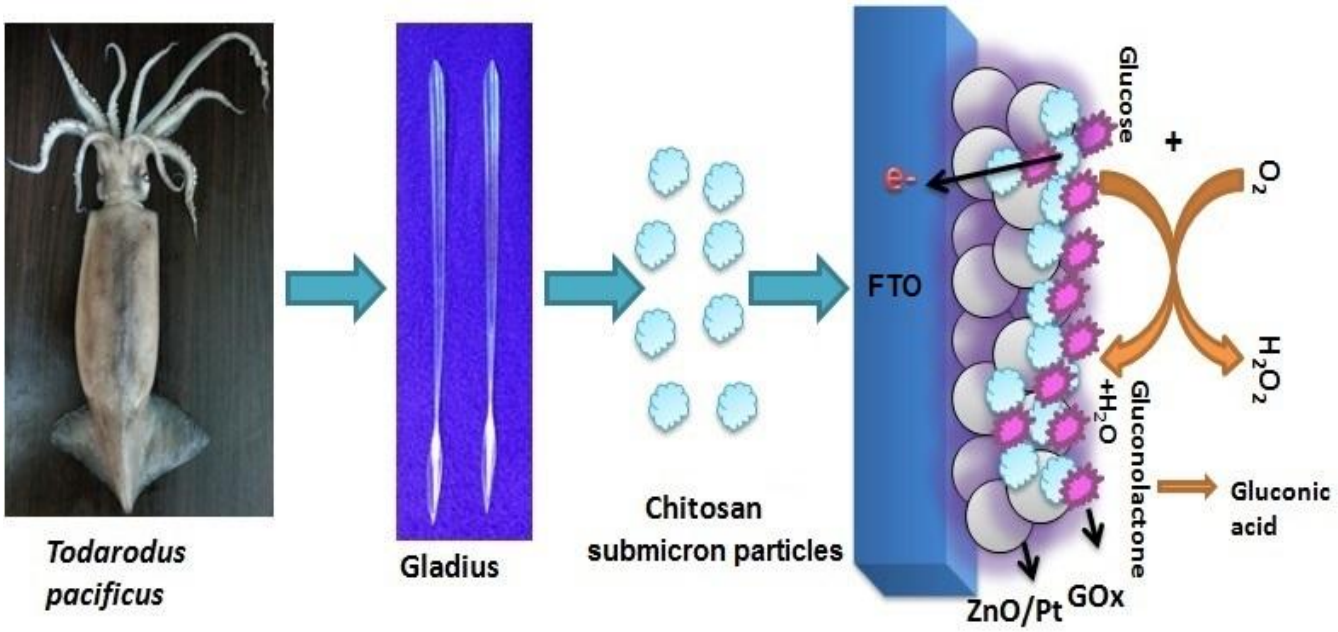
Fig. 4. XRD pattern and EDAX spectrum (inset image) of ZnO/ Pt electrode.

Fig. 5. Cyclic voltammograms of GOx immobilized (a) bare ZnO, (b) ZnO/Pt/GCSPs and (c) ZnO/Pt/SCS electrodes in PBS solution containing 2 mM concentration of glucose at 50 mVs^{-1} scan rate.

Fig. 6. Cyclic voltammograms of GOx immobilized (A) ZnO/Pt/ GCSPs and (B) ZnO/Pt/SCS electrodes in PBS solution containing various concentrations of glucose, (a) 100 µM, (b) 200 µM, (c) 400 µM, (d) 600 µM, (e) 800 µM, (f) 1000 µM, (g) 2000 µM at 50 mVs^{-1} scan rate

Fig. 7. Chronoamperometric response of (a) ZnO/Pt/SCS/GOx and (b) ZnO/Pt/GCSPs/GOx modified electrode in 0.1 M PBS (pH 7.4) on injecting various concentrations of glucose at working potential of 0.6V. The inset shows the resulting calibration curve.

Fig. 8. Linweaver-Bruke plot of GOx immobilized (a) ZnO/Pt/SCS and (b) ZnO/Pt/GCSPs electrodes



Scheme 1.

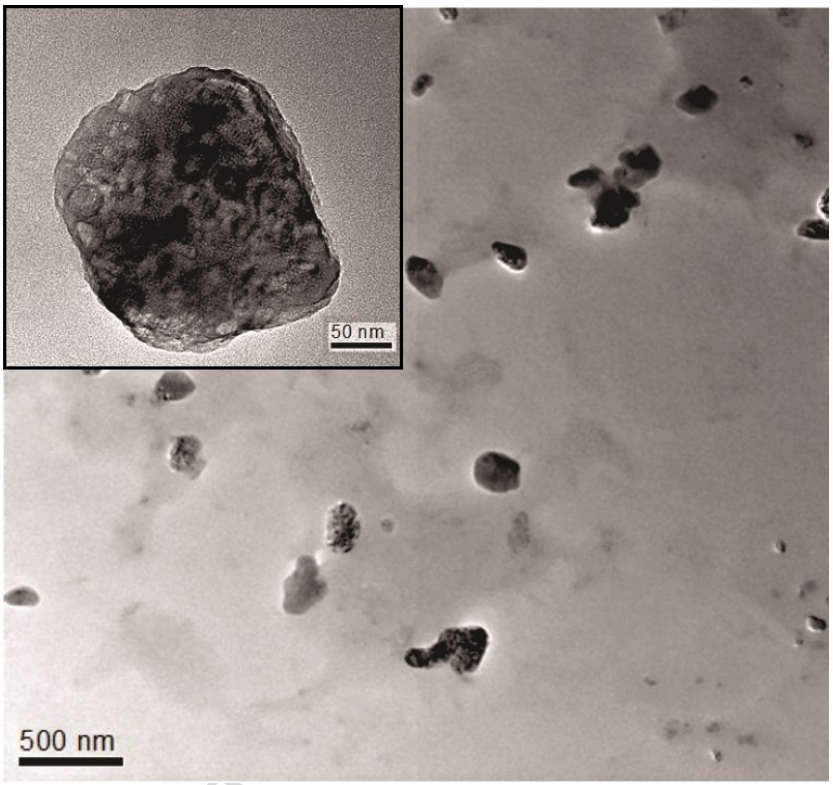


Fig. 1.

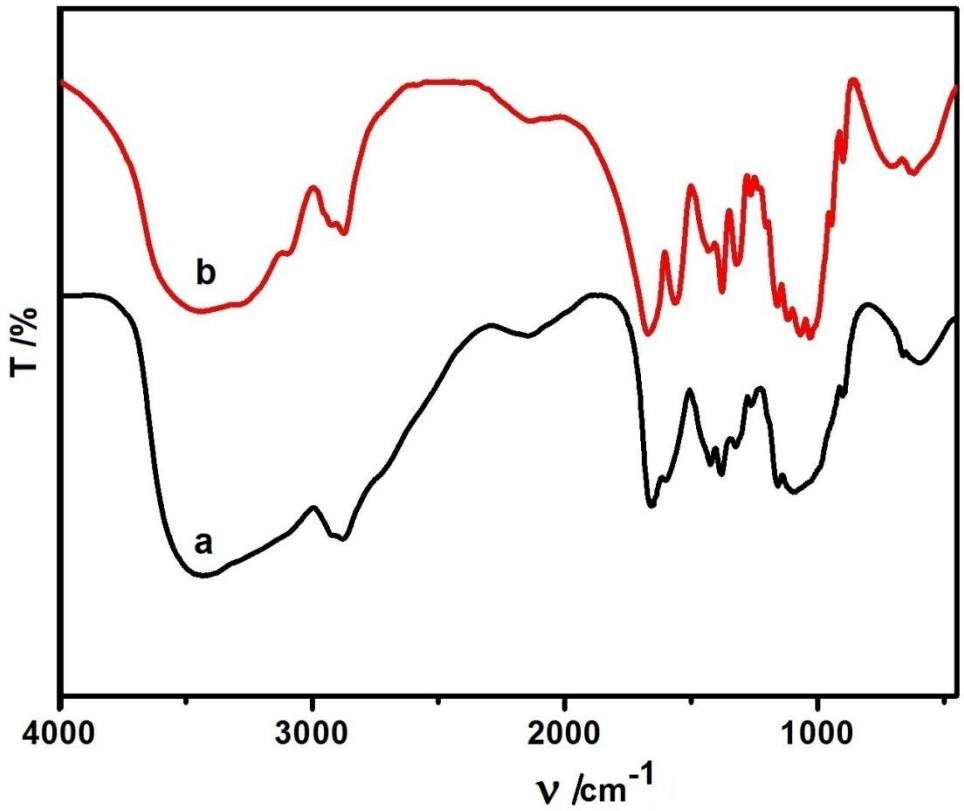


Fig. 2.

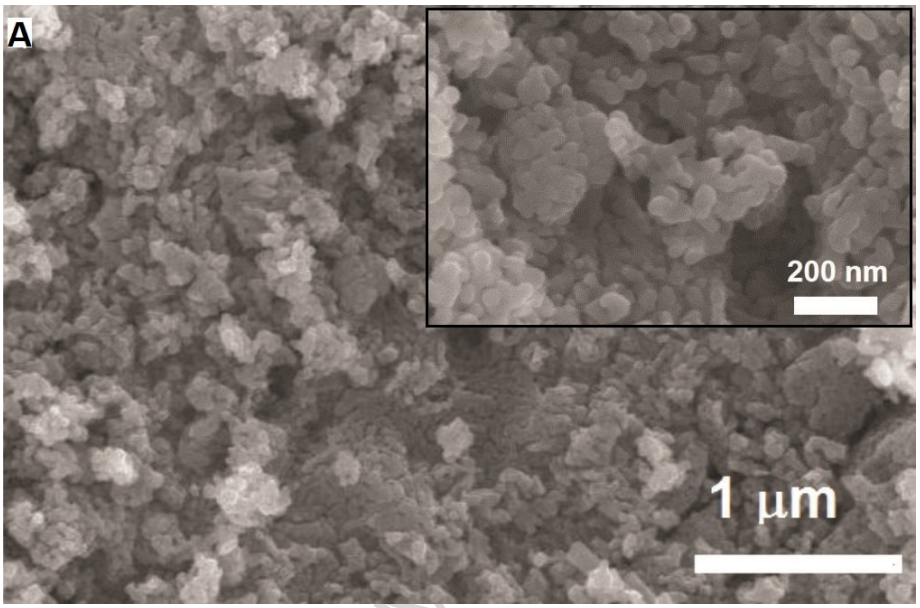


Fig. 3A.

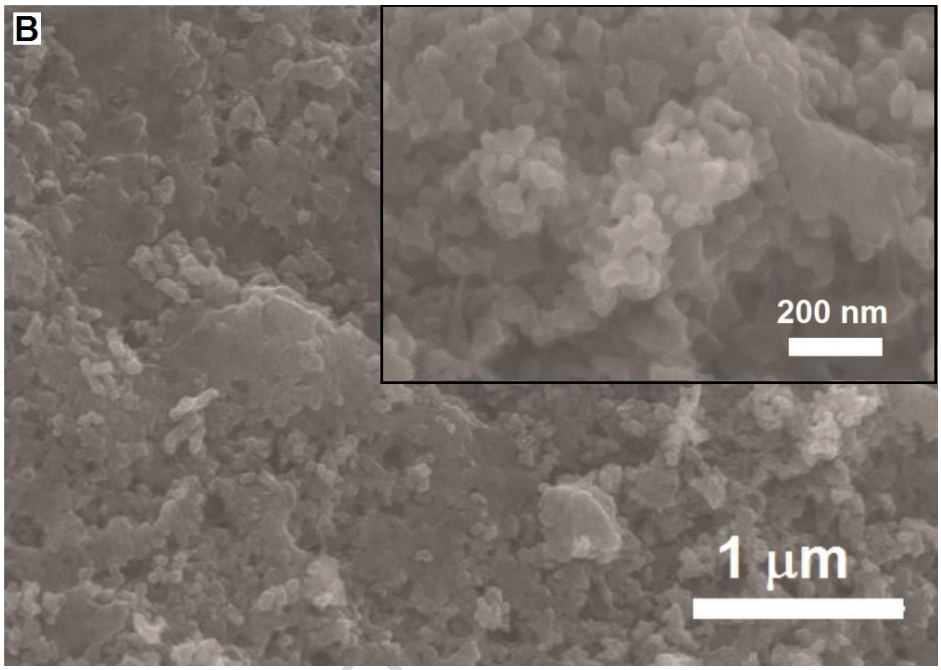


Fig. 3B.

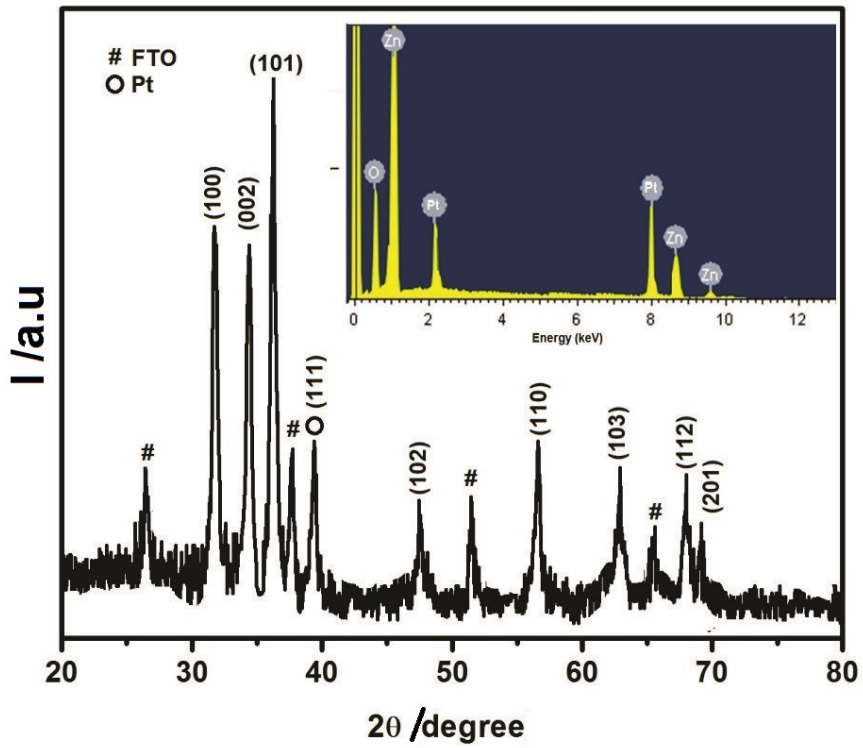


Fig. 4.

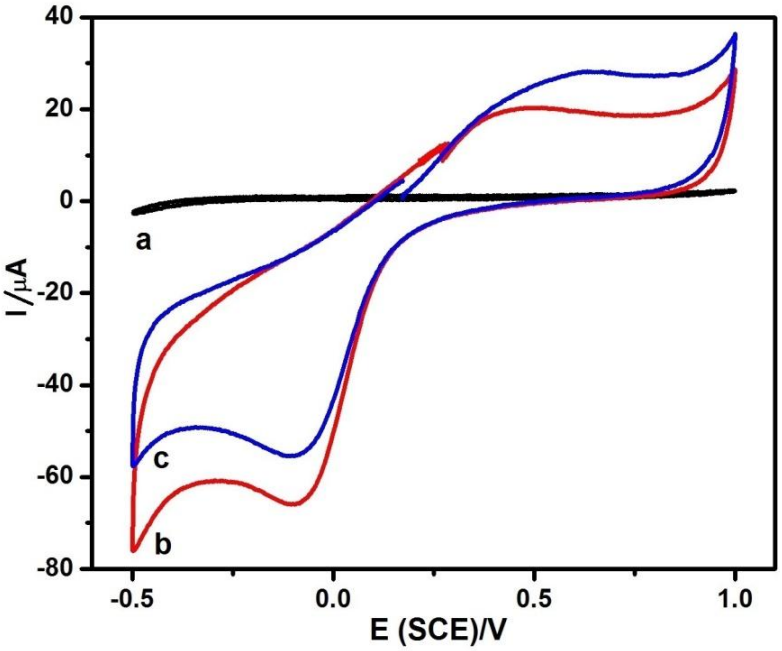


Fig. 5.

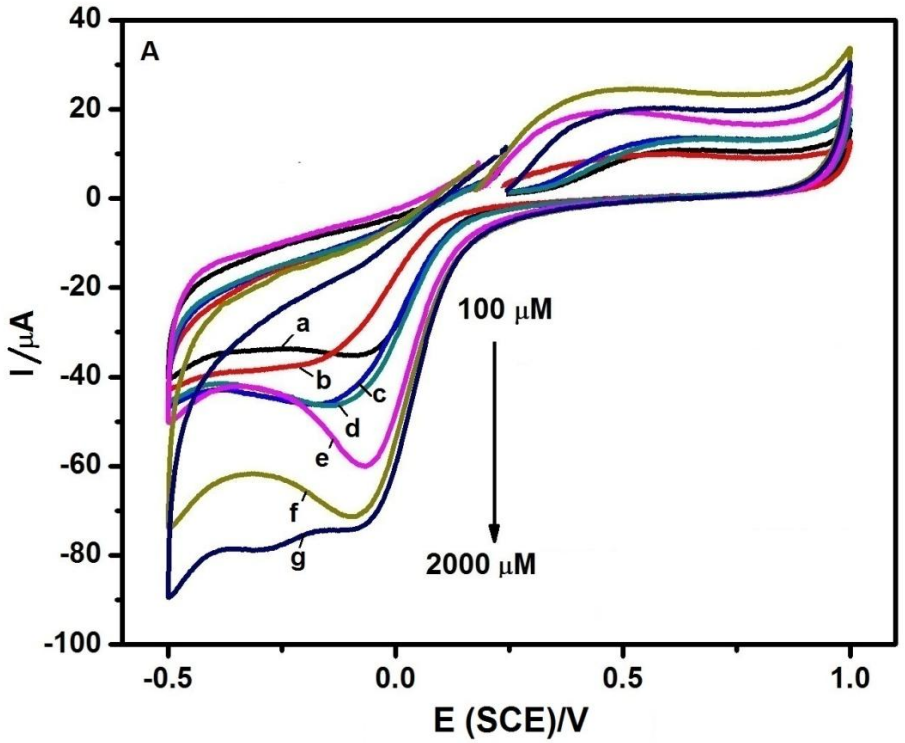


Fig. 6A.

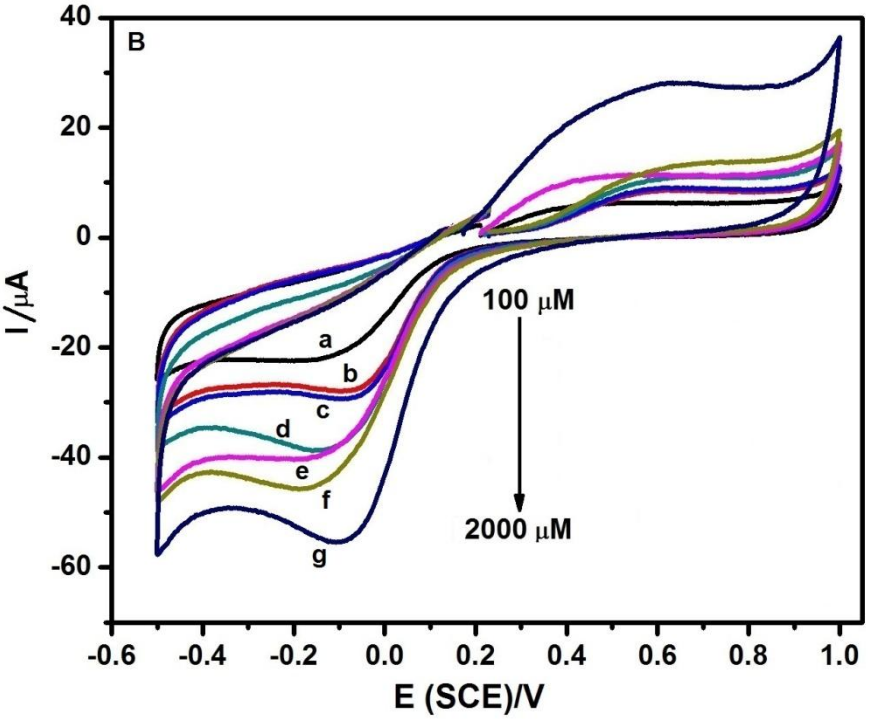


Fig. 6B.

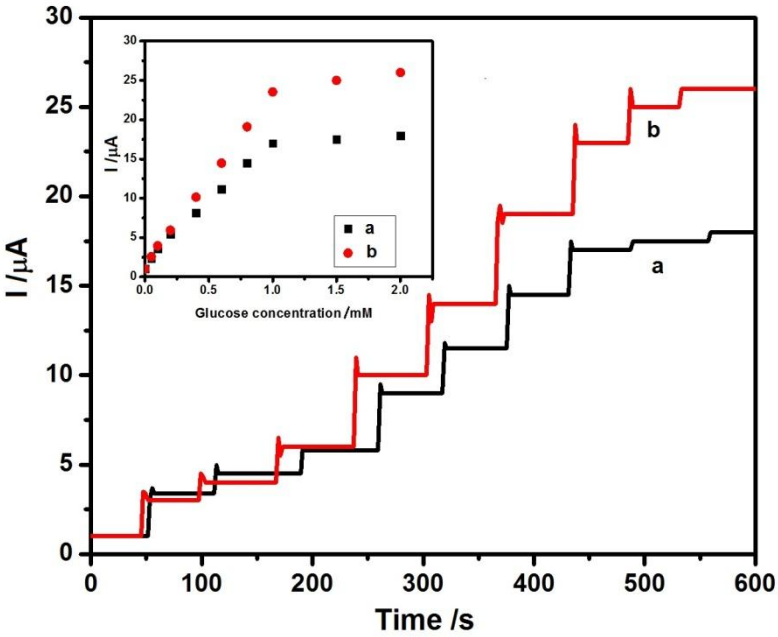


Fig. 7.

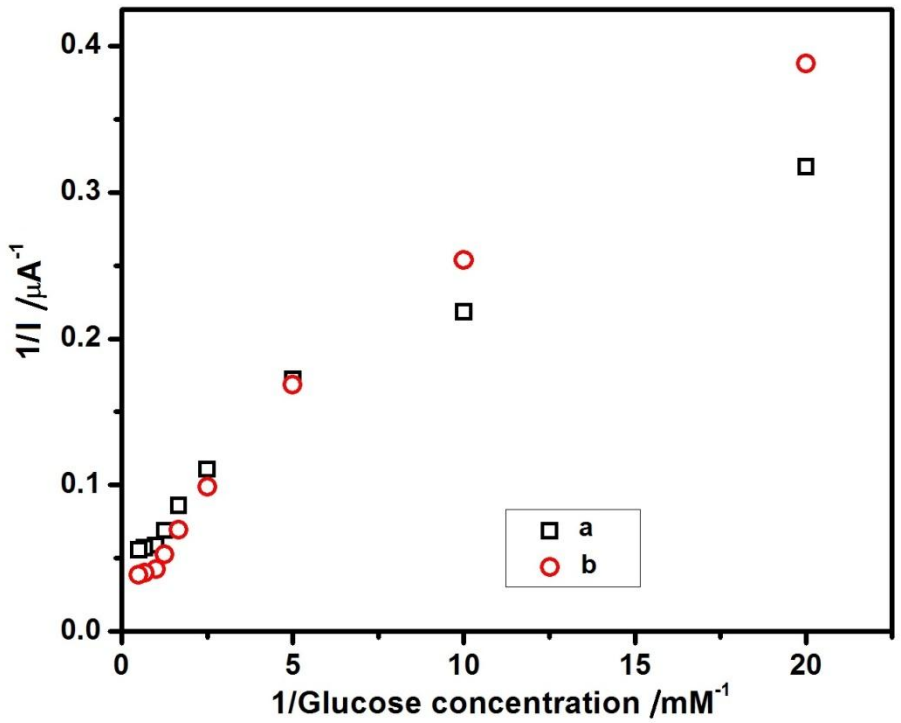


Fig. 8.

Highlights

- Effective immobilization of GOx by chitosan submicron particles for amperometric glucose biosensor
- Chitosan submicron particles from galdius of *Todarodes pacificus* provide good biocompatibility for enzyme.
- Biosensor showed excellent electrochemical performance with high sensitivity in short response time.
- Low detection limit and Michaelis- Menten constant indicates high enzyme affinity.