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RESEARCH ARTICLE

Creatinine biosensing by immobilizing creatininase, creatinase and sarcosine oxidase on nanohybrid interface

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Abstract

Commercial enzymes creatininase (CA), creatinase (CI) and sarcosine oxidase (SO) were co-immobilized on the gold nanoparticles/chitosan-multi walled carbon nanotubes (AuNPs/CHIT/c-MWCNT) composite film electrodeposited on the surface of glassy carbon (GC) electrode. A creatinine biosensor was fabricated using enzymes/AuNPs/CHIT/c-MWCNT modified GC electrode as working electrode, Ag/AgCl as reference electrode and Pt wire as auxiliary electrode. The enzyme electrode was further characterized by various methods including chronoamperometric study, cyclic voltammetry study, scanning electron microscopy (SEM) and electrochemical impedance spectroscopy (EIS). After polarization of working electrode at 0.2 Vs Ag/AgCl, an optimum response of 4 s at 25 °C in pH 7.5 was achieved. The electrocatalytic response was obtained by a linear dependence on creatinine concentration ranging from 0.5 to 1000 µM with a detection limit of 0.5 µM (S/N = 3). In this study, a good correlation (r = 0.98) with a standard colorimetric method was established. The biosensor was used 100 times over the period of 180 days and only the 50% loss of its initial activity at 4 °C was observed.

INTRODUCTION

Creatinine (2-amino-1-methyl-5H-imidazol-4-one) is an end product of creatine metabolism. It is transported in the blood and removed by kidney and further secreted through urine. Creatinine determination in the biological fluids is one major challenge for diagnosis of renal diseases, thyroid malfunctioning & muscular disorders (Lad et al., 2008, Ruedas-Rama and Hall, 2010). The physiological concentration range of a healthy person in serum and urine are 45-140 µM and 0.8-2.0 g per day. Creatinine concentration is slightly higher in men compared to women (Whelton et al., 1994). The creatinine concentration may reach upto 1000 µM in serum during kidney dysfunction or muscle disorder.

There are various techniques for creatinine determination are used including HPLC (Yang, 1998), mass spectroscopy (Schwedhelm et al., 2000), IR spectroscopy (Pezzanti et al., 2001), capillary zone electrophoresis (Clark et al., 2001) and flow injection analysis systems with electrochemical and spectrometric detection (Del Campo et al., 1995). However biosensing technology provides many advantages over these techniques for creatinine analysis in clinical laboratory. Biosensing technology reduces time, complexity and the cost of routine clinical analysis. Different types of biosensors for creatinine determination are previously reported (Lad et al., 2008, Tiwari and Shukla, 2009). These reported potentiometric biosensors have certain limitations through interfering cations and endogenous ammonia circulated in blood and urine (Shih and Huang, (1999). Comparatively, the amperometric biosensors resolve these limitations but certain parameters including sensitivity, selectivity, elimination of

interferences and sensor stability has to be improved (Lad et al., 2008, Tiwari and Shukla, 2009, Yadav et al., 2011). In order to improve these parameters, the preparation of a functional film for the modification of the electrode and then immobilize enzyme directly on the film coated electrode is advised. Furthermore, an effective catalyst support carbon nanotubes (CNTs) were employed in these biosensors. CNTs posses large surface areas with exceptional structural, electrical and mechanical properties with superior biocompatibility and easy preparation for renewal of their surface (Iijima, 1991, Chauhan and Pundir, 2011). CNTs are represented by an important group of nanomaterials having attractive geometrical, electronic and chemical properties. Integration of nanoparticles (NPs) to the CNTs films will generate new nanostructures with excellent optical, electronic and electro catalysis behavior (Wang et al., 2007).

Considering the distinctive properties of gold nanoparticles (AuNPs) having high stability under atmospheric state, resistance for being oxidized and compatible with living tissues (Corti et al., 2002, Corti and Holliday, 2004, Huang and Yang, 2005), the AuNPs are used in a various industries by means of their optical and electronic properties. AuNPs are widely used in optics, electronics, medical diagnostic and hospitals by treatments through drug delivery and tissue/tumour imaging (Corti et al., 2002, Corti and Holliday, 2004, Daniel and Astruc, 2004). Recently, the 'green synthesis' of the nanoparticles are implied rapidly in nanoscience and nanotechnology (Korbekandi et al., 2009, Kim et al., 2010, Verma et al., 2010). Therefore, for the first time, a bryophyte *Taxithelium nepalense* (*Schwagr.)* broth gametophyte extract was used to reduce tetrachloroauric acid of AuNPs.

Furthermore a stabilizing agent for enzyme immobilization biopolymer chitosan (CHIT) was applied on nanoparticles. CHIT exhibits a magnificent ability to form films with mechanical solidity, non-toxicity and compatibility to living cells, water permeability, vulnerability for chemical modifications and cost-effectiveness (Narang et al., 2013).

In this proposed work, an amperometric creatinine biosensor employing co-immobilization of enzymes onto the AuNPs/CHIT/carboxylated multi-walled carbon nanotubes (c-MWCNT) composite film modified glassy carbon (GC) electrode was developed. This work successfully showed a construction of an improved category of amperometric creatinine biosensor.

Material and Methods

Chemicals and reagents

Creatinine amidohydrolase (CA, E.C. 3.5.2.10., from *Pseudomonas sp.),* creatine amidinohydrolase (CI, E.C. 3.5.3.3, from *Pseudomonas sp.),* sarcosine oxidase (SO, E.C. 1.5.3.1., from *Bacillus sp.),* tetrachloroauric acid ($HAuCl_4$, $6H_2O$) and chitosan (CHIT) were purchased from Sigma–Aldrich, USA. Carboxylated multi-walled carbon nanotubes (Functionalized MWCNT or c-MWCNT) (12 walls, length 15–30 µm, Purity 90%, Metal content: nil) from Intelligent Materials Pvt. Ltd., Panchkula (Haryana) India were used. Gametophytic extract of the bryophyte *Taxithelium nepalense* was used for eco-friendly extracellular synthesis of metallic AuNPs. Deionized water (DW) was used throughout the experiments. All other chemicals of analytical reagent grade were used.

Biological synthesis of AuNPs

The gametophyte of *Taxithelium nepalense* (moss) was thoroughly washed several times with DW. Plant bodies (10 g) were quickly frozen in liquid nitrogen. These frozen tissues were powdered in a mortar in liquid nitrogen and then homogenized in 10 ml of DW using a polytron at full speed. The DW was added further at a ratio of 1:10 of tissue to water (w/v). It was then filtered through Whatman filter paper no. 1. The collected filtrate was used as reducing agent and stabilizer. Then in a typical experiment, 40 ml of that filtered broth was added to 60 ml aqueous chloroauric acid ($HAuCl_4$) solution (3 mM final concentration) and agitated for 12 h at room temperature (37 °C). Simultaneously, only the filtered broth of *Taxithelium nepalense* and only chloroauric acid solution were maintained under same conditions. Within 12 h dark red solution was obtained. The AuNPs were separated out through centrifugation for 10 min at 12000 × g and then settled nanoparticles were throughly washed with DW (three times). The purified AuNPs were resuspended in DW and ultrasonicated for 10 min. Synthesis of AuNPs was repeated for three times and subsequently utilized for characterization by transmission electron microscopy (TEM), UV-visible spectroscopy and dynamic light scattering (DLS) experiment.

Preparation of CHIT/c-MWCNT/GC electrode

The surface of GC electrode was cleaned by thorough washing with DW and further kept in ethanol and then sonicated to remove adsorbed particles and finally washed with DW for 3-4 times. The CHIT/c-MWCNT nanocomposite film was electrodeposited through cyclic voltammetry in a Potentiosatat-Galvanostat (Make: Autolab, model: AUT83785, manufactured by Eco Chemie, The Netherland) by applying 20 successive polymerization cycles at - 0.1 to 0.2 V with a scan rate of 50 mV s^{-1} by immersing the GC electrode in a solution (25 ml) containing 0.1 M phosphate buffer (22 ml), 1.0 wt% CHIT solution (2 ml) and cMWCNT (0.5 mg/ml) (Luo et al., 2005).

Preparation of AuNPs/CHIT/c-MWCNT/GC electrode

The electrodeposition of AuNPs onto the CHIT/c-MWCNT modified GC electrode was carried out by immersing the modified electrode into a mixture of 22 ml of 0.1 M KCl and 3 ml of AuNPs colloidal solution and then applied a potential field from -0.2 to +0.4 V (vs. Ag/AgCl) for 10 cycles at a scan rate of 0.1 V s^{-1}. After rinsing with DW, the AuNPs/CHIT/c- MWCNT/GC electrode was dried in air (Rawal et al., 2011).

Preparation of enzymes/AuNPs/CHIT/c-MWCNT/GC electrode

The CA, CI and SO were co-immobilized onto the AuNPs/CHIT/c-MWCNT/GC electrode surface through glutaraldehyde coupling. First 10 µl of 2.5% glutaraldehyde solution was spread over the AuNPs/CHIT/c-MWCNT/GC electrode, and kept for 5 h at room temperature, washed thoroughly in 0.1 M phosphate buffer, pH 7.5 and then 100 µl of CA (44 unit), CI (36 unit) and SO (24 unit) was spread on top of the electrode and dried. The resulting enzymes/AuNPs/CHIT/c-MWCNT/GC electrode was thoroughly washed with 0.1 M phosphate buffer of pH 7.5 to rinse off any loosely bound enzyme from the electrode. The resulting enzyme electrode was dried and then stored in the refrigerator at 4 ^{o}C, when not in use. The fabricated electrode was characterized by scanning electron microscopy (SEM).

Testing of electrochemical creatinine biosensor

An amperometric creatinine biosensor was constructed using a three-electrode electrochemical cell system, consisting of a working electrode (enzymes/AuNPs/CHIT/c-MWCNT/GC), a silver/silver chloride (Ag/AgCl) as reference electrode and Pt wire as counter electrode. These electrodes were connected through Autolab Potentiostat/Galvanostat. The electrode system was dipped into a reaction mixture containing 10 ml 0.1 M phosphate buffer solution, pH 7.5 and 0.5 ml creatinine solution (100 µM). The electrode response was measured in terms of ampere (A) applying a potential range of 0.0V to +6.0V. The steady state current response increased obviously with working potential from 0.0V to +0.2V. Therefore, +0.2V was selected as the working potential for amperometric detection of creatinine concentration. The measurement of creatinine by sensor is based on the fact that H_2O_2 is produced in conversion of creatinine into creatine, creatine into sarcosine & urea and finally sarcosine into formaldehyde, glycine and H_2O_2 produced by immobilized CA, CI and SO enzymes respectively is directly proportional to the concentration of creatinine (Scheme1A).

Applications of creatinine biosensor

Serum samples of apparently healthy persons were collected from hospital of Pt. BDS University of Health & Medical Science, Rohtak and stored at 4 ^{o}C until use. The measurement of serum creatinine was carried out in the same manner as described for testing of creatinine biosensor under optimal working condition except that the creatinine was replaced by serum sample. The concentration of creatinine in serum was extrapolated from standard curve between creatinine conc. and current in µA, prepared under optimal assay conditions of enzymes/AuNPs/CHIT/c-MWCNT/GC electrode.

Reuse and storage of enzymes/AuNPs/CHIT/c-MWCNT/GC electrode

The storage and stability of enzymes/AuNPs/CHIT/c-MWCNT/GC electrode was studied by storing it in 0.1 M reaction buffer, pH 7.5 at 4 °C, when not in use.

Result and Discussion

In this study, the intensive cross-linkages formed between the -COOH groups in c-MWCNTs and the hydroxyl (-OH) and amino ($-NH_2$) groups in CHIT were exploited. The CHIT molecular chains and c-MWCNTs both chemically react and physically knot with each other, leading to form a three-dimensional interlaced CHIT/c-MWCNT composite coating. In order to form a stable, reproducible and rigid c-MWCNTs composite coating, a co-electrodeposition method was used (Luo et al., 2005).

In this study, a method for construction of an amperometric biosensor using AuNPs/CHIT/c-MWCNT/GC electrode was applied. Firstly, CHIT/c-MWCNT composite film was electrodeposited onto GC electrode using cyclic voltammetry. An electrodeposition method was selected to produce AuNPs on modified electrode surfaces because it is easy to be carried out and the layer thickness could be controlled. Secondly, commercially available creatinine amidohydrolase (CA, E.C. 3.5.2.10., from *Pseudomonas sp*), creatine amidinohydrolase (CI, E.C. 3.5.3.3., from *Pseudomonas sp*) and sarcosine oxidase (SO, E.C. 1.5.3.1., from *Bacillus sp*) enzymes were co-immobilized on AuNPs/c-MWCNT by CHIT coupling (Scheme 1B).

Characterization of AuNPs

Transmission electron microscopic (TEM)

The characterization of AuNPs was performed by TEM images (Fig. 1). These observations revealed that spherical structure of the AuNPs were formed in the reaction solution. The size of the AuNPs was 40 to 60 nm confirming their synthesis.

UV-visible spectroscopic analysis

The characteristic red color of the reaction solution was appeared by the excitation of the surface plasmon vibration of Au^0 particles which provided a convenient spectroscopic signature of their formation. No significant color change in the same experimental conditions in both the control set was observed. The reduction of chloroauric acid was subjected to spectral analysis by using the UV-visible spectrophotometer. An absorbance peak at 540 nm is shown (Fig. 2), which was specific for AuNPs (Verma et al., 2011).

Particle size measurement

Particle size was determined by dynamic light scattering (DLS) measurement. The particle size in the range of 41 to 62 nm (Fig. 3) with an average diameter of 51 ± 5 nm was appeared when performed by Laser diffraction method.

Surface characterization using SEM

The surface morphologies of bare GC electrode, CHIT/c-MWCNT/GC, AuNPs/CHIT/c-MWCNT/GC and enzymes/AuNPs/CHIT/c-MWCNT/GC were investigated using SEM (Fig. 4). The surface of bare GC electrode (Fig. 4a) was smooth. Fig. 4b shows SEM micrographs of CHIT/c-MWCNT/GC electrode which was observed by the uniform and cable-like morphology of the nanostructure of CHIT/c-MWCNT composite film. After AuNPs deposition, sub-nanosized Au clusters were sparsely distributed over the surfaces of the CHIT/c-MWCNT, indicating the formation of AuNPs/CHIT/c-MWCNT nano-biocomposite (Fig. 4c). However, after the immobilization of enzymes onto AuNPs/CHIT/c-MWCNT nanocomposite, sporadic appearance of globular/beaded structure was obtained (Fig. 4d).

Impedance measurements

The electrochemical impedance spectra of the bare GC electrode, AuNPs/CHIT/c-MWCNT/GC electrode and enzymes/AuNPs/CHIT/c-MWCNT/GC electrode was shown in Fig. 5. An Rct value of 2.5 KΩ of bare GC electrode was observed which was decreased to 1.7 KΩ once treated with AuNPs/CHIT/c-MWCNT indicating an easy electronic transport at the electrode surface interface. An increased Rct values of 2.9 KΩ after subsequent immobilization with the enzymes was appeared. This increase in Rct is attributed to the fact that most biological

molecules, including enzymes, are poor electrical conductors at low frequencies (at least <10 kHz) and cause hindrance to the electron transfer.

Response towards creatinine onto the enzymes/AuNPs/CHIT/c-MWCNT/GC electrode

To evaluate the catalytic activity of enzymes at the AuNPs/CHIT/c-MWCNT/GC electrode, the modified electrode was characterized by a cyclic voltammogram in the presence of creatinine between the potential range of -0.1 V to +0.35 V. Fig. 6 shows CV of the enzymes/AuNPs/CHIT/c-MWCNT/GC electrode in 0.1 M phosphate buffer (pH 7.5) without (curve a) and with (curve b) creatinine solution at scan rate 20 mVs^{-1}. It was observed that with the addition of 0.5 ml creatinine (100 µM), oxidation current was increased, while reduction current decreased, revealing the improved catalytic properties of modified electrode to the oxidation of creatinine. A well defined oxidation peak (0.2 V vs Ag/AgCl) was observed, which was clearly indicated by the catalytic properties of modified electrode. Thus, for further amperometric study of enzymes/AuNPs/CHIT/c-MWCNT/GC electrode, a potential of +0.2 V (vs Ag/AgCl) was applied.

Chronoamperometric response of enzymes/AuNPs/CHIT/c-MWCNT/GC electrode

Chronoamperometric response study was done using enzymes/AuNPs/CHIT/c-MWCNT/GC electrode as the working electrode at a bias voltage of 0.2 V vs. Ag/AgCl in 0.1 M phosphate buffer (pH 7.5) containing 2 mM $Fe(CN)_6]^{3-}$ as the redox mediator. In Fig. 7, a chronoamperometric response of the enzymes/AuNPs/CHIT/c-MWCNT/GC electrode was shown, which was appeared as a function of creatinine concentration in 0.1 M phosphate buffer (pH 7.5) in the presence of a redox mediator. An increasing order of amperometric response was observed with increasing creatinine concentration (0.5 to 1000 µM) (Curve a to h in Fig. 7) with a sensitivity of 4.6 $\mu A/\mu M/cm^2$. The 95% steady-state current response to creatinine was obtained at 4 s.

Optimization of experimental conditions of biosensor

A maximum response of this biosensor was observed at pH 7.5. The optimum temperature of biosensor was determined at 25 ºC. Therefore, the subsequent experiments were performed at pH 7.5 and 25 ^{o}C. An optimum response time this biosensor was shown in 4 s.

Evaluation of creatinine biosensor

The detection limit of biosensor was 0.5 µM at a signal to noise ratio of 3, which is lower than previously reported creatinine biosensor based on thick film hydrogen peroxide electrode (Kim et al., 1999), sulfonate-hydrogel with nafion (Tombach et al., 2001), carbon paste electrode (Stefan-van et al., 2006), iron oxide nanoparticles/chitosan-g-polyaniline modified Pt electrode (Yadav et al., 2012) and polymer film (polyanion polypyrrole) (Khan and Wernet, 1997). It is comparable to ZnO-NPs/CHIT/c-MWCNT/PANI composite film based creatinine biosensor (Yadav et al., 2011). Analytical recovery of exogenously added creatinine in serum (0.5 mg/dl and 10 mg/dl) was 97.69% and 98.02% showing a reliability of this method. The results of batch coefficient of variation (CVs) (within and between) for serum creatinine determination were <3.6% and <4.5% indicating that the method was reproducible and consistent. A comparative account of the analytical performance of creatinine biosensors is given in Table 1.

Interference study and selectivity

The interference study of the present creatinine biosensor was performed through comparing the amperometric response (before and after) by adding some interferents such as uric acid, glycine, ascorbic acid, acetaminophen and creatine at their physiological concentration of 100 µM creatinine in 0.1 M phosphate buffer at pH 7.5. No significant effect was observed after adding uric acid, ascorbic acid, glycine and acetaminophen. However, an obvious interference was observed when creatine coexisting with creatinine, which is consistent with the results of previous mediator biosensors (Marcel et al., 1996). To avoid creatine interference, total creatinine (creatinine + creatine) using the tri-enzyme electrode (CA/Cl/SO) were determined and then the concentration of creatine in the

same sample was determined by bienzyme electrode (Cl/SO). The true creatinine was calculated by subtracting the creatine value from total creatinine.

Application of creatinine biosensor

The creatinine level in apparently healthy individuals serum as measured by the current biosensor was in the range of 0.63 to 1.32 mg/dl (Table 2). When these results were compared with the results of chemical spectrophotometric method (Jaffe, 1886), a good correlation (r = 0.989) was observed.

Stability of enzyme electrode

The stability of the enzyme electrode was investigated every week under storage conditions at 4 ^{o}C. The current response of the sensor was maintained by 50% of the initial current response even after 180 days of regular 100 uses. This is better than polymer film (polyanion polypyrrole) (Khan and Wernet, (1997), iron oxide nanoparticles/chitosan-g-polyaniline modified Pt electrode (Yadav et al., 2012) and ZnO-NPs/CHIT/c-MWCNT/PANI composite film (Yadav et al., 2011) suggesting a good stability of AuNPs/CHIT/c-MWCNT composite film.

Conclusion

In the present work, a novel amperometric creatinine biosensor was constructed by immobilizing three enzymes (CA, CI and SO) onto AuNPs/CHIT/c-MWCNT modified GC electrode. Immobilization of these enzymes into nanocomposite structure forming matrix facilitates biosensor to keep higher bioactivity and stability in the operational conditions resulting in fast, stable and sensitive responses. A linear range of 0.5 to 1000 µM with a lower detection limit of 0.5 µM was observed with this biosensor when AuNPs/CHIT/c-MWCNT/GC electrode was used. The response time of 4 s reaching a 95% steady-state current value was calculated. This study provides a feasible approach for developing a new kind of AuNPs based amperometric biosensors.

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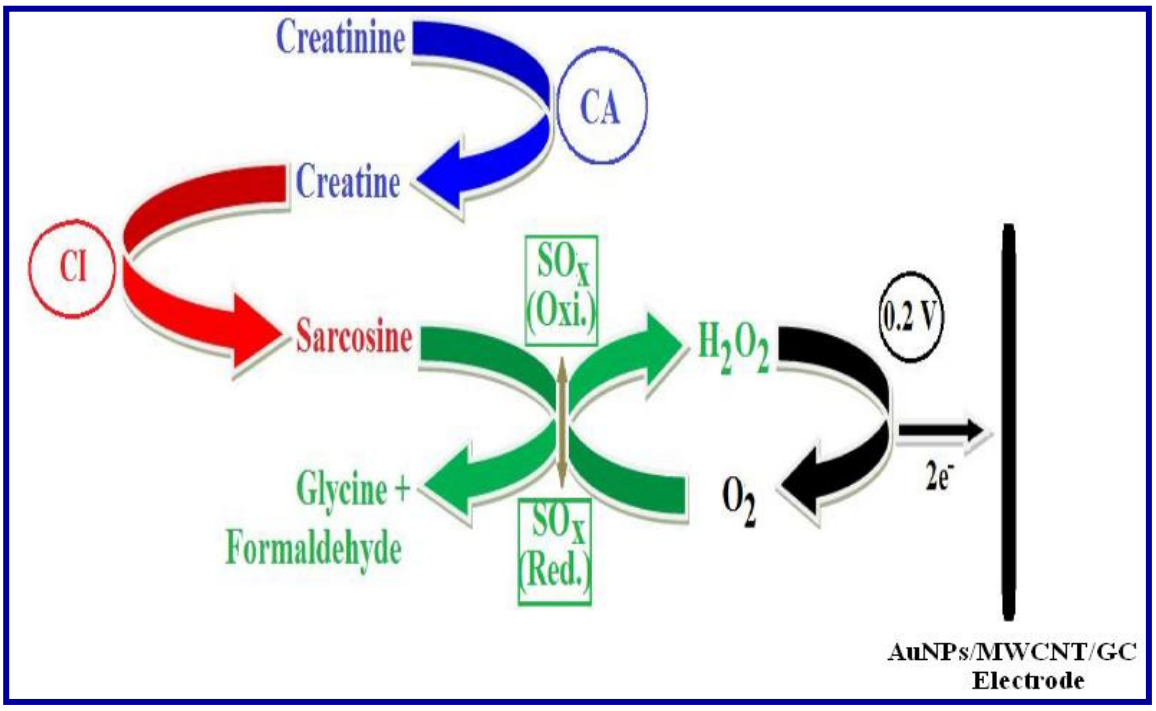
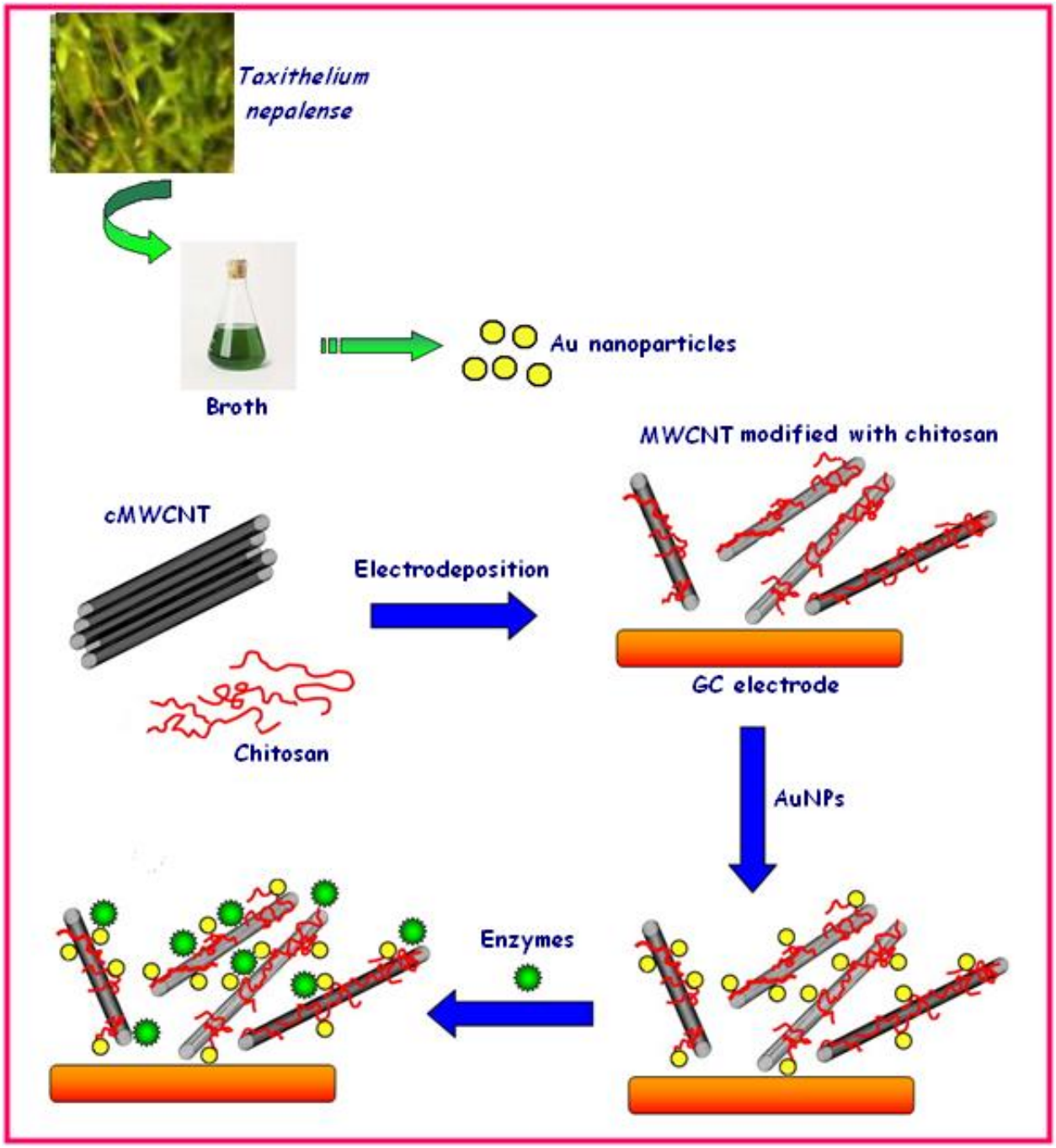
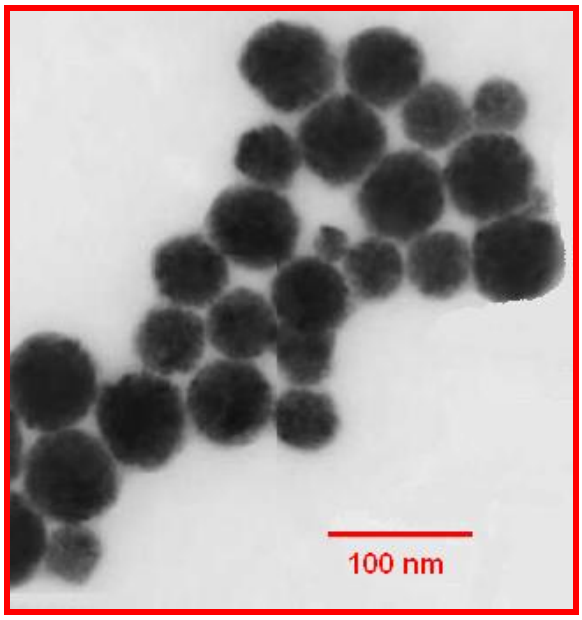


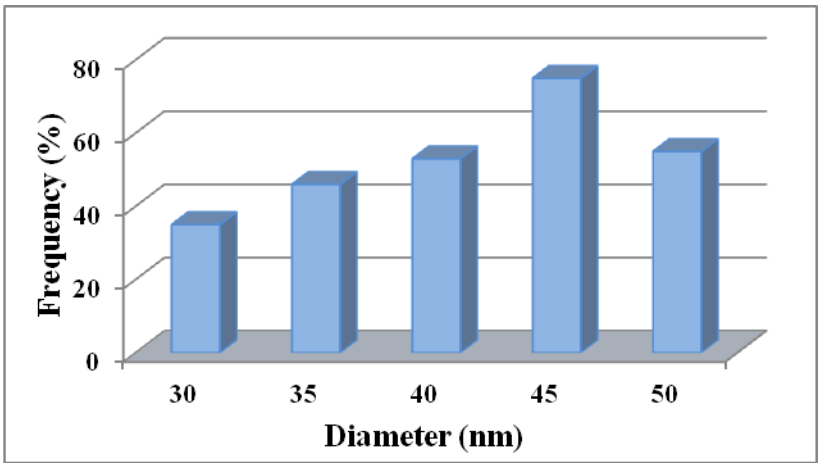
Scheme 1A. Schematic representation of chemical reaction involved in the fabrication of enzymes/AuNPs/CHIT/c-MWCNT/GC electrode.



Scheme 1B. Schematic illustration of the stepwise amperometric creatinine biosensor fabrication process.



(A)



(B)

Fig. 1 Transmission electron microscopic (TEM) images of AuNPs (A). Particle size distribution histogram of AuNPs (B).

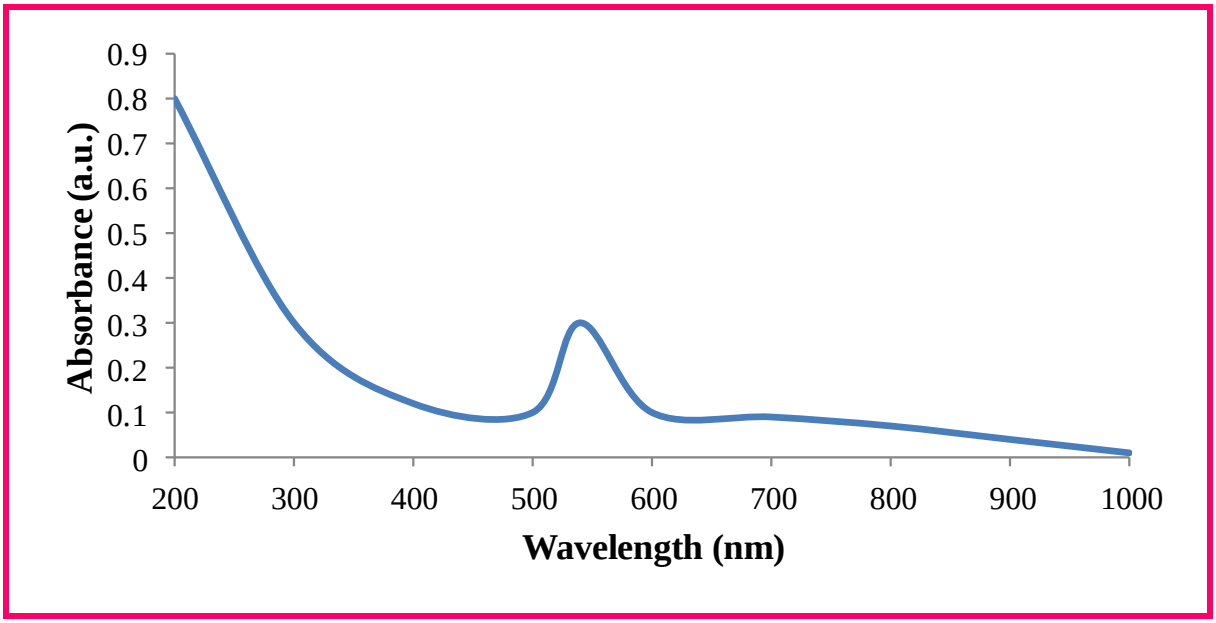


Fig. 2 UV–visible spectra recorded of an aqueous solution of 3 mM $HAuCl_4$ with the bryophyte gametophyte extract.

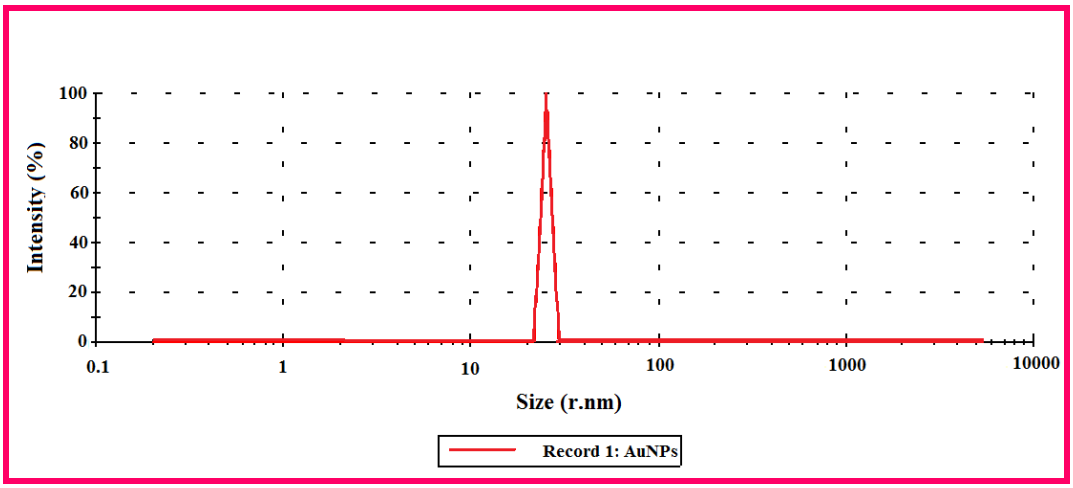


Fig. 3 Size distribution of AuNPs measured by the DLS technique.

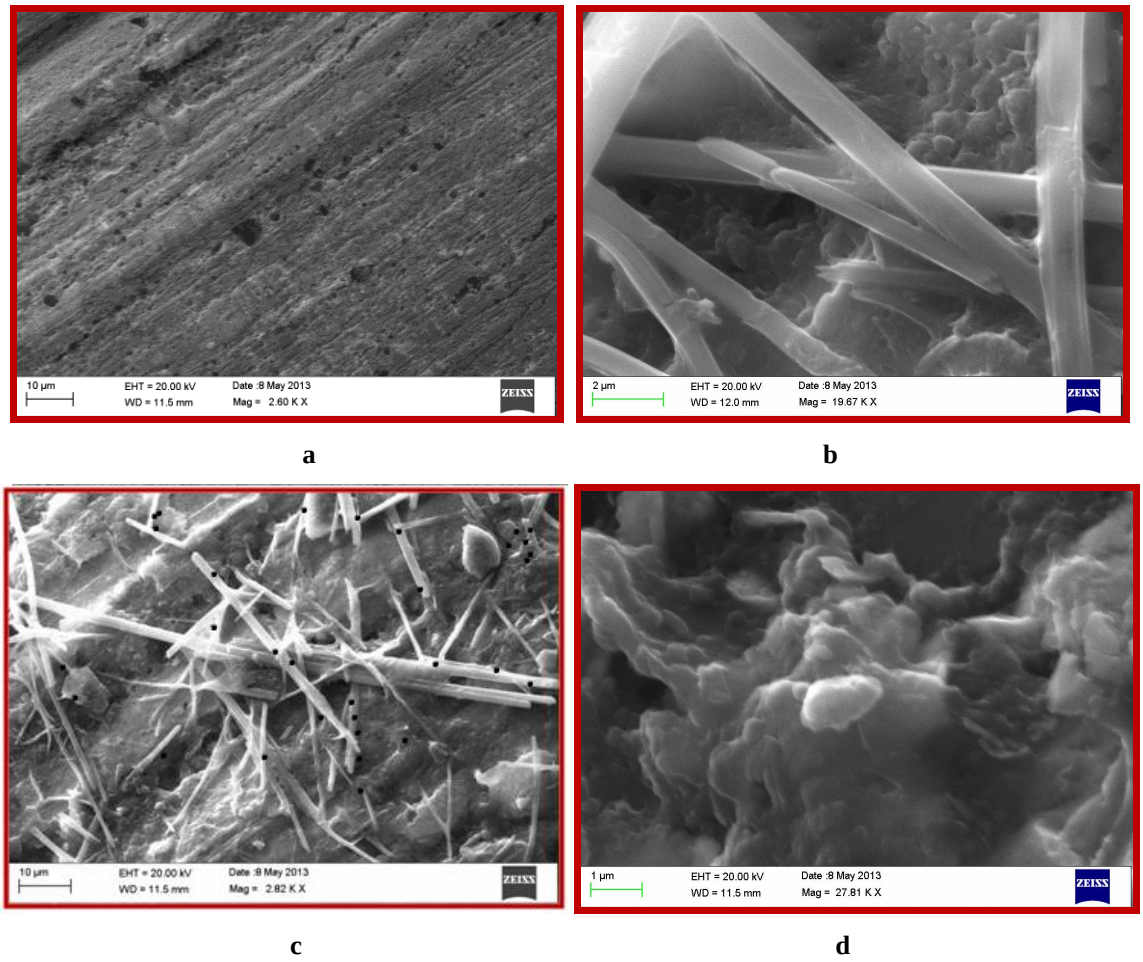


Fig. 4 Scanning electron microscopic (SEM) images of (a) bare GC electrode, (b) CHIT/c-MWCNT/GC electrode, (c) AuNPs/CHIT/c-MWCNT/GC electrode and (d) enzymes/AuNPs/CHIT/c-MWCNT/GC electrode.

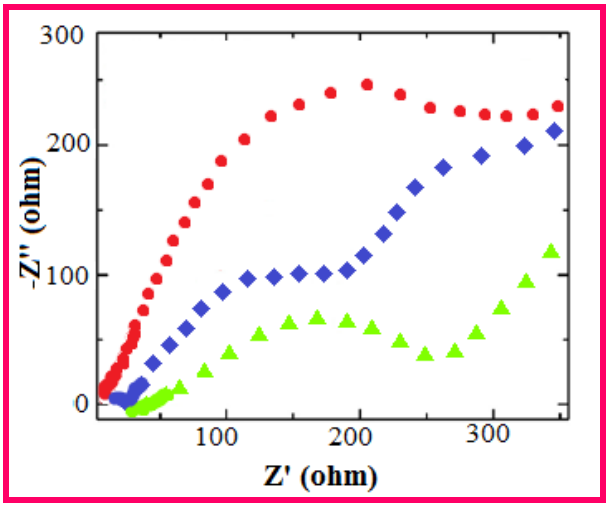


Fig. 5 Nyquist plot obtained for bare GC electrode △, AuNPs/CHIT/c-MWCNT/GC electrode ◆ , and enzymes/AuNPs/CHIT/c-MWCNT/GC electrode ● in 0.1 M KCl solution containing 2 mM $[Fe(CN)_6]^{3-}$.

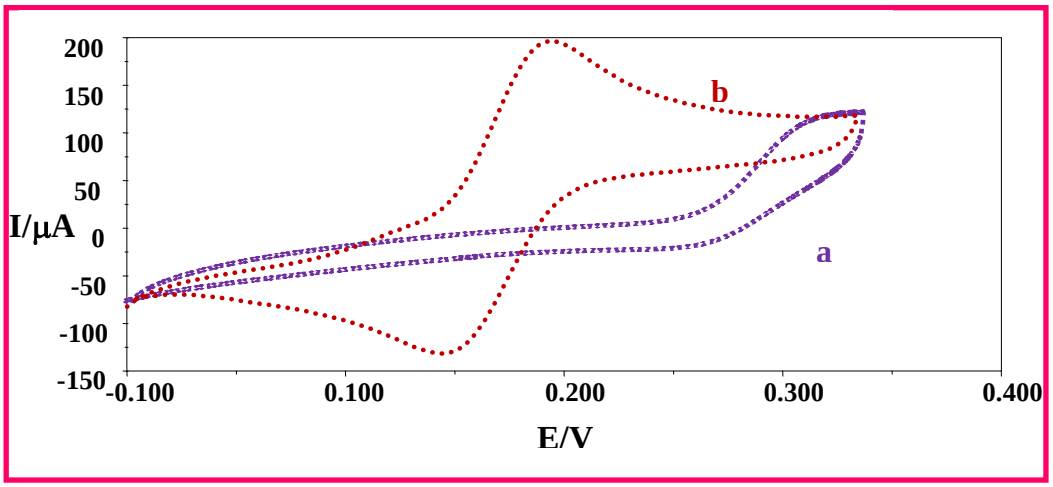


Fig. 6 Enzymes/AuNPs/CHIT/c-MWCNT/GC electrode without (curve a) and with 0.5 ml creatinine (curve b) solution (100 μM) in 0.1 M sodium phosphate buffer pH 7.5; Scan rate: 20 mV s^{-1}.

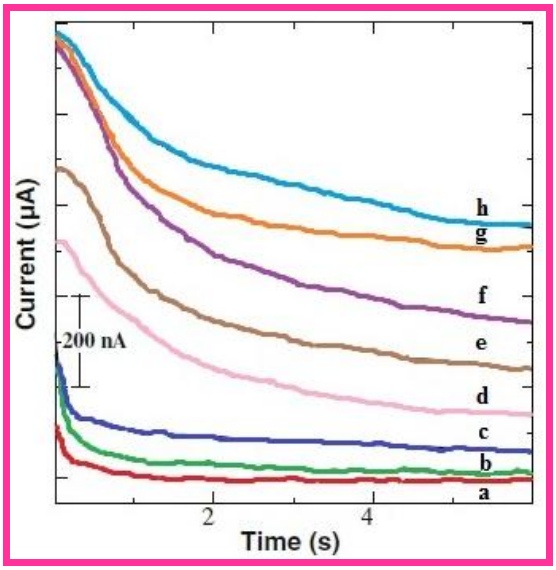


Fig. 7 Chronoamperometric response curve of enzymes/AuNPs/CHIT/c-MWCNT/GC electrode with different concentrations of creatinine.

Table 1. Comparison of analytical properties of nanomaterials based amperometric creatinine biosensors.

Sr. No	Support for immobili-zation	Enzymes	Working electrode	Method of Immobili-zation	Detection limit (μM)	Linear range (μM)	Response time (s)	Sensitivity μA/μM/cm^2	Potential applied (V)	Storage stability (Days)	Reference
1	sulfonate-hydrogel with nafion	CA, CI, SO	Pt	Entrapment	5	5-150	NR	0.005	0.6	NR	Tombach et al., 2001
2	Platinized -SEC (shapable electrocon ductive) film	CA, CI, SO	Pt	Crosslinking	1-2	10-5000	NR	0.023	0.4	30	Khan and Wernet, 1997
3	Carbon paste electrode	CA, CI, SO	Ag wire	Absorption	0.6	0.6-40	NR	NR	0.42-0.65	NR	Stefan-van Staden et al., 2006
4	ZnO-NPs/CHIT/c-MWCNT/PANI	CA, CI, SO	Pt	Covalent	0.5	10-650	10	0.030	0.5	120	Yadav et al., 2011
5	Fe_3O_4-NPs/CHIT-g-PANI	CA, CI, SO	Pt	Covalent	1.0	1-800	2.0	3.9	0.4	200	Yadav et al., 2012
6	AuNPs/CHIT/c-MWCNT	CA, CI, SO	GC	Covalent	0.5	0.5 to 1000	4	4.6	0.2	180	Present report

*NR= Not reported.

Table 2. Serum creatinine level by creatinine biosensor based on enzymes/AuNPs/CHIT/c-MWCNT/GC electrode.

S. No.	Age	Sex	Serum creatinine (mg/dL)
1.	34	F	0.87
2.	46	M	1.32
3.	41	M	1.10
4.	37	M	0.95
5.	42	F	0.89
6.	23	F	0.67
7.	18	M	0.77
8	25	M	0.82
9	16	F	0.63
10	29	M	0.97
11	24	F	0.83
12	20	M	0.92
			Mean for Males=**0.97** mg/dL
			Mean for Females=**0.77** mg/dL

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