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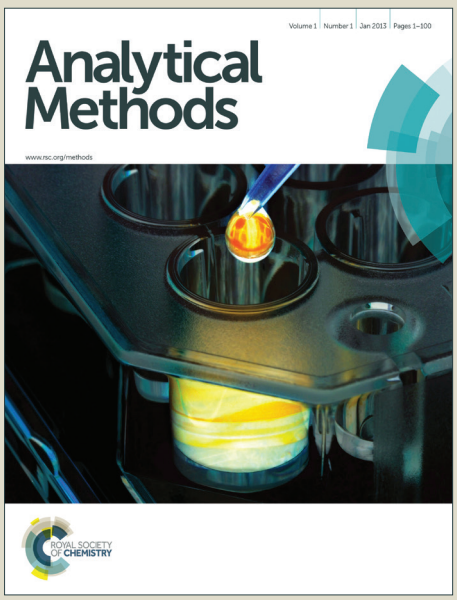

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ARTICLE TYPE

Genosensor based on Nanostructured Platinum Modified Glassy Carbon Electrode for *Listeria* Detection

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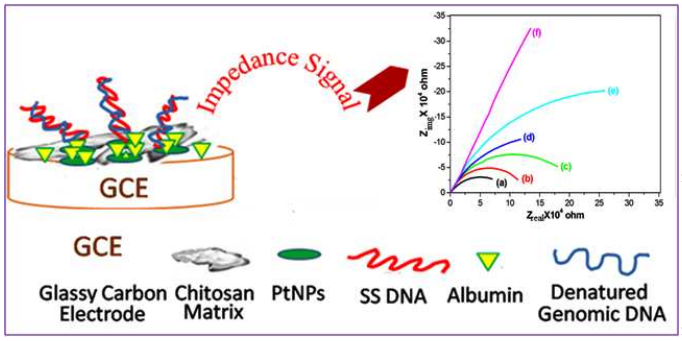
Graphical Abstract:



Herein the present article, we describe development of simple and cost effective genosensor probe based on glassy carbon modified electrode with platinum nanomaterials dispersed in chitosan matrix. Further this probe is explored for the label free detection of *Listeria monocytogenes* obtained from milk samples. DNA based interfacial interaction between target DNA with platinum nanomaterials (PtNPs) immobilized with 24 mer ssDNA is investigated using impedance spectroscopy. As prepared nanomaterials (for utilisation in electrode fabrication) have been characterized by UV-vis, FT-IR, TEM and Cyclic Voltammetry before fabricating impedimetric sensor platform. The Interfacial interaction between PtNPs and DNA results in the increase of charge transfer resistance (R_{CT}) on hybridization with consecutive increasing concentrations of target DNA. This user-friendly and simple platform is used for the detection of target DNA, which shows excellent response and specificity (even for 1 bp mismatch of target DNA). Also this sensing platform is utilized for the detection of *Listeria monocytogenes* real samples (Milk beverage) in the wide range of detection from 1x 10^{-12} M to 1x 10^{-4}. In general, our simple and user-friendly sensor probe shows potential of detection of *Listeria monocytogenes* in food samples with high specificity.

Keywords: DNA detection, Genosensor, Impedance Spectroscopy, Glassy Carbon Electrode, Platinum nanomaterials based modified electrode.

1. Introduction

Intensive research in genomics made gene diagnosis a hot spot leading to the boom in production of electrochemical gene sensors in the past decade. Electrochemical gene sensors show attractive features such as high sensitivity, fast response and cost-effective requirements which is needed for the preliminary detection of diseases, preventative therapy of genetic disorders and for the treatment of bacterial and viral infections. Great efforts have also been made to develop DNA electrochemical impedimetric based sensors. Signal amplification in DNA based sensors is usually achieved with various surface modifications or by attaching additional labels to DNA such as enzymes, quantum dots, or metals [1-4]. Metallic nanoparticles show intensive amplifiable effect on the resulting signal response. Uniqueness of nanomaterials is due to their mechanical, electrical, optical, catalytic and magnetic properties as well as their extremely high surface area to volume ratio which reflects amplified signal towards detection of analyte [5-7]. Genosensor are biosensors consisting single stranded DNA (DNA probe)

with a transducer to detect a specific gene. DNA biosensors present several advantages such as low cost, rapid analysis, simplicity and possibility of miniaturization as compared to conventional methods. As mentioned, DNA detection plays an ever-increasing role in a number of areas related to human health such as diagnosis of infectious diseases, genetic mutations, drug discovery [7-8].

Advancement in nanomaterials science offered amazing opportunities of making new sensitive biosensors. Nanomaterials based platform finds wide use in many electrochemical, electro analytical and bio-electrochemical applications. Its application in biosensing has gained exciting academic research and industrial interest [9-11]. Bio molecule/enzyme modified electrode suffers some limitations such as fragility of enzyme, preservation issues, fouling of substance at the electrode surface etc. Nanomaterials based modified electrode is the solution for such problems. Electrochemical DNA sensors based on the electrochemical impedance spectroscopy (EIS) are of considerable interest these days due to their high sensitivity and simplicity. Such DNA sensors are considered as suitable candidate for direct and fast biosensing since they can convert the hybridization event into a direct impedance signal. So, there is no need for complex signal transduction equipment and the detection can be accomplished with an inexpensive measurement methodology.

Sequence-specific detection of either genetically or pathogenically associated DNA has become increasingly important for applications including point-of-care diagnostics, antiterrorism, environmental monitoring and forensic analysis. Electrochemical DNA sensing impedance based approaches covers wide areas viz. intrinsic electroactivity of DNA, electrochemistry of DNA-specific redox reporters, electrochemistry of nanoparticles and conducting polymers (CPs) [12-18].

Recently, the incorporation of NPs into a variety of matrices to form nanocomposite films is attracting major research interest. Metal nanoparticles have been utilized for enhancing the impedance signals by increasing the surface area of modified electrode thereby increasing the adsorption of immobilized probe ssDNA molecules. [19-20]. However, biocompatibility of nanoparticles in sensing is a major obstacle for efficient detection. Therefore, capping of the nanoparticles is introduced recently with some biopolymers or biocompatible materials.

In this work, we report a one-step synthesis of platinum nanomaterial (PtNPs) by using nontoxic and biodegradable polysaccharide chitosan as a stabilizing agent and sodium borohydride as a reducing agent. Chitosan, a novel biopolymer is a product of deacetylation of chitin, which is the second most abundant natural polymer after cellulose [21-22]. This biocompatible platform is modified with ssDNA to sense the target DNA. In the present study we selected the target DNA of *Listeria monocytogenes*. *Listeria monocytogenes* is a bacterial food borne pathogen responsible for listeriosis, ailment characterized by encephalitis, septicaemia, and meningitis [23-25]. The platform is modified with 24 mer oligonucleotides of *hlyA Listeria monocytogenes* based on our earlier study, which showed the selectivity of this sequence [35, 36]. The chemical immobilization of bio molecule suffers various limitations, therefore in the present work we have reported simple and user-friendly sensors probe. Nanomaterials based modified electrode 24 mer oligonucleotides of *hlyA Listeria monocytogenes* is physically immobilized on chitosan PtNPs. The hybridization even with the denatured target DNA of *Listeria monocytogenes* over modified platform was analyzed by impedance spectroscopy (change in change transfer resistance; R_{CT}). The sensor probe is further explored for the analysis of the genomic DNA of *Listeria monocytogenes* obtained from the milk beverage.

2. Experimental:

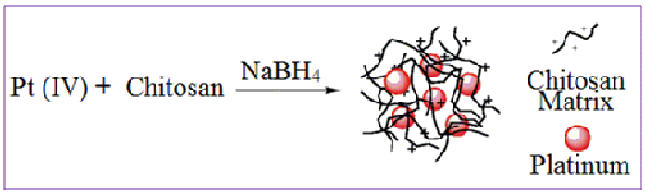
2.1. Chemicals:

The DNA products of *Listeria monocytogenes* were purchased from Eurofin, India. The 24 mer oligonucleotides of *hlyA Listeria monocytogenes* with sequence 5′-GCAACGTATCCTCCAGAGTGATCG-3′ and complementary oligonucleotide 3-‘CGTTGCATAGGTCTCACTAGC-5’ and 1 bp mismatch oligonucleotide is CAGTTGCAAGCGCTTGGAGTGAAT are used for study. Genomic DNA of *Listeria monocytogenes* was obtained from the milk beverage of sequences TATATCTCAAGTGTGGCATATGGCCGTCAAGTTTATT TGAAATTATCAACTAATTCCCATAGTACCAAAGTAA AAGCTGCTTTTGACGCTGCCGTAAGTGGGAAATCTG TCTCAGGTGATGTAGAACTGACAAATATCATCAAAA ATTCTTCCTTCAAAGCCGTAATTTACGGTGGCTCCGC AAAAGATGAAGTTCAAATCATCGACGGTAACCTCGG AGACTTACGAGATATTTTGAAAAAAGGTGCTACTTTT AACCGGGAAACACCAGGAGTTCCCATTGCCTATACA ACAAACTTCTTAAAAGACAATGAATTAGCTGTTATT AAAAACAACTCAGAATATATTGAAACAACTTCAAAA GCTTATACAGATGGAAAAATCAACATCGATCACTCT GGAGGATACGTTGCACTCC of *hlyA* gene (456 bp) Courtesy: Department of Botany, BHU, Varanasi [35, 36]. The DNA products were dissolved in MilliQ water and made of desired concentration just prior to use. Phosphate buffer solutions with various pH values were prepared with 0.1M NaH_2PO_4 and 0.1M Na_2HPO_4 (pH 6.8). Lower molecular weight chitosan (poly-(D glucosamine), <5400 g/mol) with a degree of deacetylation of 84.5% was bought from Sigma Aldrich USA. Due to the poor solubility of chitosan, the mixture was vigorously stirred in 1% glacial acetic acid and kept for about 18 hours until a transparent solution was obtained. Prior to use, the stock solution was diluted to the required concentration. H_2PtCl_6 was obtained from Sigma-Aldrich and used as received for preparation of Pt nanoparticles. Sodium dihydrogen phosphate, sodium mono phosphate and Potassium ferricyanide were purchased from SRL Pvt Ltd., India. BSA obtained from Genei with 98% purity, Chloroplatinic acid was obtained from Sigma Aldrich, USA. Glacial acetic acid and MilliQ from Merck India, $NaBH_4$ from SRL, India. All aqueous solutions were made with Milli-Q (18.3 M Ω cm) water.

All glassware used was cleaned properly prior to use. All the experiments were done at ambient temperature (25±2 °C).

2.2. Synthesis of platinum nanomaterials capped with chitosan (CS-PtNPs): Before the preparation of platinum nanoparticles, the stock solutions of 2.5% chitosan were prepared by dissolving 1.8 mg of chitosan in 1.0 % glacial acetic acid solution. Synthesis of PtNP is carried out as following:

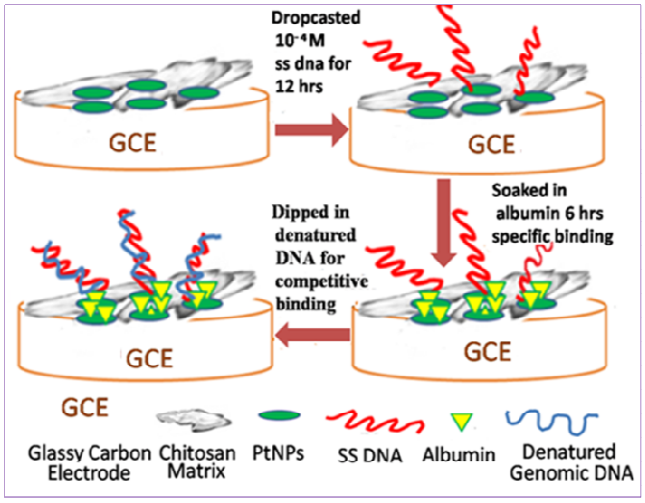
2 mL of aqueous solution of H_2PtCl_6 (4 mM) was mixed 4 mL of chitosan solution (as prepared in acetic acid) and stirred for 2hrs. Aqueous (0.03 M) of $NaBH_4$ (80µL) was then added drop wise to the stirred solution of precursor. The resulting solution was centrifuged at 4000 rpm for 6 min, to obtain the colloidal solution of nanoplatinum protected with chitosan. Chitosan has lots of functional groups such as hydroxyl, charged amino, so it has a strong ability to absorb transition metals and form metal complexes [5, 22, 23, 26]. Chitosan is positively charged and shows electrostatic attractive force between NH_3^+ of chitosan and $PtCl_6^{2-}$. All of these factors possibly favour the nucleation and growth of platinum nanoparticles in the chitosan matrix which is proposed in the reaction scheme 1. This process is repeated for twice. The obtained content was washed thrice with mixture of Milli-Q-water to remove any unreacted H_2PtCl_6, chloride ions and other impurities. Then, the obtained content is re-dispersed in desired amount of water so that can be used for further sensing applications.



Reaction Scheme 1: Schematics of Synthesis of Platinum Nanomaterials.

2.3. Electrode pre-treatment and DNA hybridization: Three electrode cell configuration were used for electrochemical experiment with glassy carbon electrode (GCE) modified with CS-PtNPs as working electrode, Pt counter and Ag/AgCl as a reference electrode. Before modification, the glassy carbon electrodes were polished with 0.05µm alumina and then rinsed thoroughly with double distilled water, followed by consecutive ultrasonication in ethanol and distilled water for 5 minutes as electrode preparation step for biosensor is shown in Scheme 2. DNA is physically immobilized in the matrix of nanoplatinum chitosan via C-0, N-H groups. This interaction is supported by FT-IR. In order to get the modified electrode, very firstly PtNPs capped with chitosan was drop casted onto bare glassy carbon electrode and kept for drying in closed environment. Further 5 µL of 100 nM probe ssDNA solution was dropped onto the surface of freshly prepared GCE modified with PtNPs and incubated at 4 ºC for 12 h to obtain the GCE/PtNPs/ssDNA electrode.

Solution of the blocking agent albumin is prepared as 1mg/ml using a PBS buffer solvent and the electrodes was soaked in this albumin solution for 6 hrs and rinsed twice with phosphate buffer solution before recording its EIS.



Scheme 2. Schematic illustration for fabrication of the genosensor electrode (CS-PtNPs) assembly for DNA detection.

Thereafter, the hybridization reaction was performed by immersing the different concentrations of denatured target DNA solution onto the surface of modified electrode with configuration GCE/ PtNPs/ ssDNA/ albumin under optimized conditions. The optimized conditions were obtained by repeating same experiment several times under various temperature ranges, pH, ionic strength etc as we have done earlier [10, 12, 35]. Hybridization with complementary probe was carried out by immersing the modified electrode into phosphate buffer (pH 6.8). This process was achieved in glass vial by taking target DNA followed by denaturation of the target dsDNA (if genomic DNA). The dsDNA solution was first sonicated and then heat treatment was given at 92-94 °C for 3-5 minutes followed by cooling for denaturation. During cooling step, ssDNA immobilized probe electrode (temperature > 75 °C) was dipped into the solution and temperature was brought down to 40-42 °C for hybridization for 15 minutes hybridization time (Fig. 1d). The hybridized GCE/ PtNPs/ssDNA/ albumin/denatured DNA electrode was then rinsed to remove the unhybridized DNA using PBS buffer. EIS is performed with this platform and similarly for various successive increasing concentrations of target DNA (genomic DNA- real sample milk beverage).

3. Instrumentation

UV–vis detection and FTIR was carried out on Lambda-25 UV-visible Spectrophotometer in the wavelength range

from 200-800nm, Perkin Elmer, Germany and 8400 FT-IR, Shimadzu, Japan respectively. The microscopic views of the samples were examined by using a Transmission Electron Microscope (Technai G2, 20 FEI Corporation Netherlands) operating at 200 kV. A few µL of the colloidal platinum suspension was dropped onto carbon-coated copper grids (Pelco International, USA) and used for TEM images. All electrochemical experiments were performed using a CHI708C instrument (CH Instruments, Austin, TX). Impedance measurements are performed in 0.1M PBS buffer solution (pH 6.8) at a potential of +0.35V vs. Ag/AgCl using modulation amplitude of 10 mV in the frequency range from 0.10 Hz to 0.1MHz.

4. Results and discussion

4.1. UV–vis absorption study of as prepared PtNPs:

The formation of the chitosan capped PtNPs is confirmed by UV-visible spectroscopy. The precursor H_2PtCl_6 solution has a pale-yellow colour and shows a peak in between 200-300 nm corresponding to the ligand to metal charge transfer (LMCT) transition as shown in Fig 1. The colour of the precursor solution changed to brownish black and peak at 200-300 nm disappears upon addition of reducing agent, indicating the reduction of the $PtCl_6^{2-}$ ions to colloidal Platinum and reaction continued under magnetic stirring using a water bath until a blackish-brown colloidal solution is obtained. The reaction time is adjusted by the concentration of chitosan, and 2-2.5 hrs reaction time is found to be appropriate.

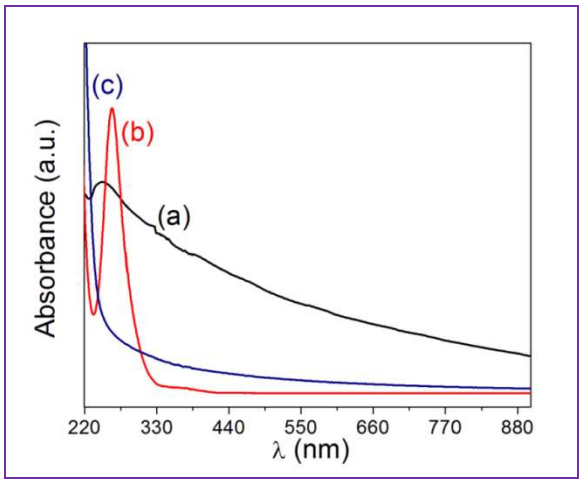


Fig 1. UV-vis absorption spectrum of (a) Chitosan (b) H_2PtCl_6 (c) Chitosan capped Pt nanoparticles.

4.2. FTIR spectroscopy characterization

The interaction between PtNPs with the amine group of chitosan was confirmed by Fourier Transform infrared spectroscopy analysis. Fourier-transform infrared (FT-IR) spectroscopy (Fig. 2) was used to characterize the CS-PtNPs, and some major changes occur to the bands assigned to chitosan upon formation of chitosan capped PtNPs, as shown in the highlighted spectra in the region of 500–3800 cm^{-1}. Band observed in the spectrum are further compared with the literature was assigned from the literature [27-30]. 919 cm^{-1} and 1040 $cm^{-1}$is due to saccharide structure due C-O-C group and CH_3COH group. vibrations of OH and CH in the ring, symmetric stretching vibration of CH_3 in amide group, and NH-bending vibration in amide group, NH group stretching which appear at 1436 cm^{-1}, 1340 cm^{-1}, and 1556 cm^{-1}, 3465 cm^{-1} respectively. 1639 cm^{-1} and 2473 cm^{-1} corresponds to the N-H and C-H stretching vibrations In the FTIR spectrum of PtNPs@chitosan, the saccharide peak 869 cm^{-1} and 1074 cm^{-1} these peaks are observed at 1124 cm^{-1}, 1304 cm^{-1}, 1536 cm^{-1} , 3421 cm^{-1} ,1621 cm^{-1} and 2894 cm^{-1} respectively.

The shift of these peaks to lower wavenumbers indicates a decrease in the bond strength in assigned frequency of chitosan capped PtNPs. The decrease in the assigned stretching frequencies is attributed to the transfer of electron density from the C and N atoms to Pt atom, resulting in electrostatic interactions between C or N atoms and PtNPs.

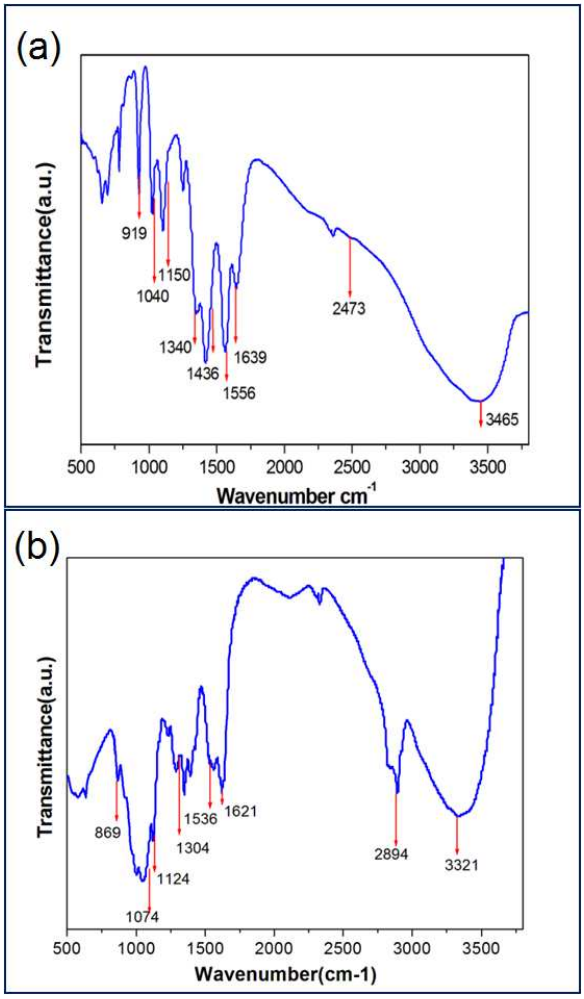


Fig 2. FT-IR Spectra of (a) Chitosan and (b) CS-PtNPs

This experimental FT-IR interpretation shows that the PtNPs are encapsulated in the chitosan matrix through electrostatic interaction with C and N atoms.

4.3. Electrochemical study of CS-PtNPs:

PtNPs@chitosan modified electrode was characterized by its characteristic CV. It shows Pt oxidation at 0.45 V and its corresponding Pt oxide reduction at 1.1 V which indicates that surface is modified with Pt nano crystal as shown in Fig. 3.

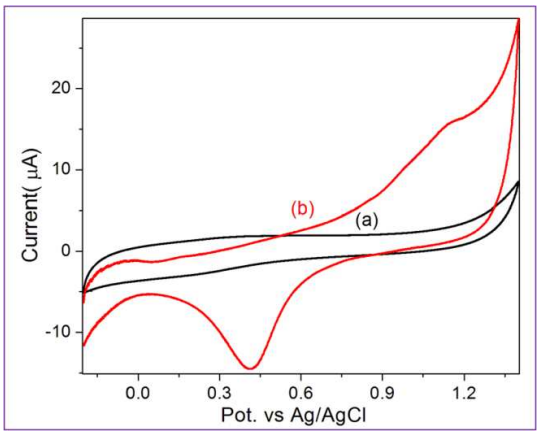


Fig 3. Cyclic voltammetric responses obtained for (a) Bare GCE in 0.1M PBS of pH 7.0 (b) GCE modified with PtNPs showing characteristic CV.

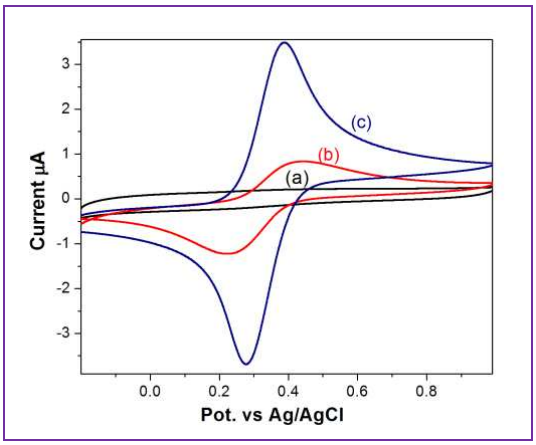


Fig 4. Cyclic voltammetric responses obtained for (a) CV for a bare glassy carbon electrode (GCE) in blank buffer pH 7.0 (b) CV responses for bare GCE containing the Fe(II)/Fe(III) redox couple. (c) GCE modified electrodes with PtNPs in buffer containing the Fe(II)/Fe(III) redox couple.

Cyclic voltammetric responses of the bare glassy carbon electrode and the electrode modified with chitosan capped platinum nanomaterials were examined by using 0.1 mM Fe(II)/ Fe(III)in phosphate buffer solution. A significant increase in the peak current was observed for the same amount of redox couple Fe(II)/ Fe(III) for the modified electrode compared to the unmodified bare glassy carbon electrode, as shown in a Fig 4.indicating that the electrode modified with chitosan capped platinum nanomaterials favours the faster electron-transfer reaction.

4.4 Microscopic characterisation: The microscopic views of (CS-PtNPs) as shown in Fig. 5 reveals that the average particle size of platinum is approximately 14 nm. The Scherrer ring of the selected area electron diffraction (SAED) pattern obtained for the PtNPs, as shown in Fig. 5 can be indexed as the (111), (200), (220) and (311) are planes of a face-centered cubic lattice of Pt atoms [27-28].

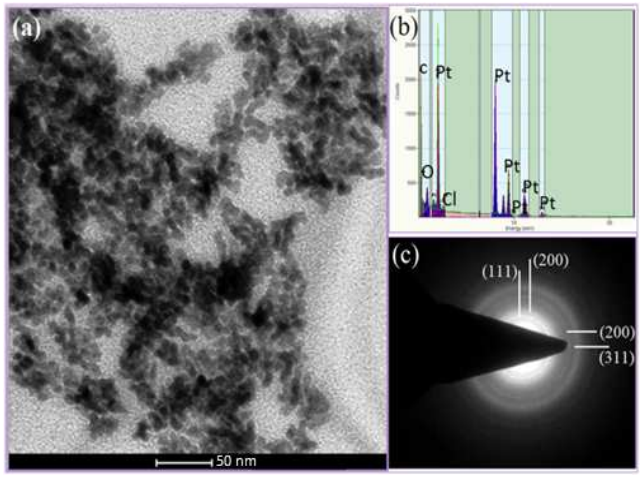


Fig 5. TEM image of as prepared PtNPs and corresponding EDAX and SAED pattern.

DNA interaction with platinum nano crystal destroys its nanocrystalline surface morphology and SAED pattern disappear as shown in Fig. 6. Interaction of DNA with PtNPs makes significant changes in PtNPs morphology which is also consistent with electrochemical impedance spectrum i.e. nano platinum interacts with DNA which covers the surface of nano conducting platinum, resulting significant changes in the R_{CT} value of EIS (which is further discussed in the electrochemical impedance section) [30-33].

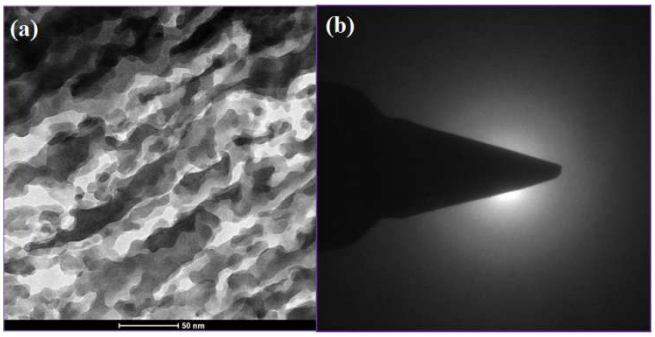


Fig 6. TEM image of (CS-PtNPs) and DNA (after interaction) and corresponding disappeared SAED pattern *c.f.* fig 5.

4.5. Impedimetric study for DNA detection:

The hybridization between the probe ssDNA and denatured target DNA would significantly change the conformation of DNA and charge transfer resistance over the modified electrode probe. These changes can be easily reflected through the electrochemical impedance signal [31-35]. Firstly, we performed the EIS studies of hybridization event of 24 mer oligonucleotides of *hlyA Listeria monocytogenes* with sequence 5′-GCA ACG TAT CCT CCA GAG TGA TCG-3′ and complementary oligomer 3-'CGT TGC ATA GGT CTC ACT AGC-5'. This experiment was done at various temperature ranges, pH, ionic strength of buffer and time period to optimise the condition and demonstrate the capability of our developed platform for genosensing. EIS study for hybridization event was carried out at physiological pH, which showed higher amplification signal than graphite and bare glassy carbon electrodes. In order to avoid non specific absorption of DNA we tend to increase the specificity of the electrode by soaking the electrode (GCE\CS-PtNPs\ssDNA) in blocking agent albumin solution. This avoids the interference of unwanted signal generation due to the unbounded target DNA. The impedance measurement of the GCE\CS-PtNPs\ssDNA\ albumin electrode showed relatively higher electron transfer and comparatively smaller R_{CT}. Further on hybridization with consecutive addition of increasing target DNA (complementary 24 mer oligonucleotide) concentration prominent rise in R_{CT} was observed in comparison to GCE\CS-PtNPs\ssDNA electrode.

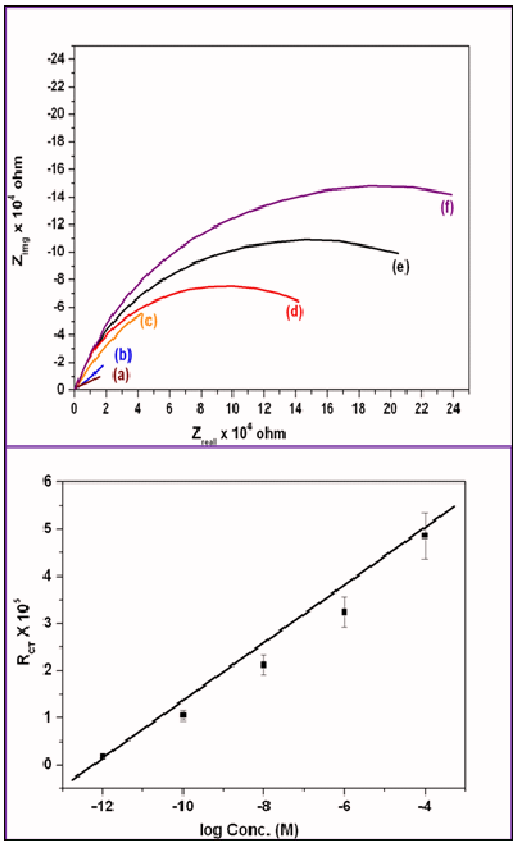


Fig 7. (a) Nyquist plot for (a) GCE\CS-PtNPs\24mers ssDNA\albumin and after hybridization with complementary oligomer of concentration ranging from (b) $1x10^{-12}$M (c) $1x10^{-10}$M (d) $1x10^{-8}$M (e) $1x10^{-6}$ M (f) $1x10^{-4}$M. **(b)** Corresponding calibration plot for 24 mer oligonucleotide of *hlyA Listeria monocytogenes* R_{ct} vs. log concentration in M.

In above EIS studies, we observed almost straight line for nanoplatinum modified GCE, due to the direct transfer of electrons as shown in Fig 7 a. After the immobilization of 24 mer oligonucleotides (ssDNA) onto the electrode surface, straight line response changed to semicircle suggesting the immobilization of DNA on the electrode surface. Further corresponding (increasing semicircle) changes were observed w.r.t. varying concentration of complementary 24 mer oligonucleotide (from 10^{-4} M to 10^{-12} M) due to insulating/blocking properties of DNA layer [34, 35]. Such electrode displayed good stability, selectivity and anti-fouling properties. Calibration plot for 24 mer oligonucleotide of *hlyA Listeria monocytogenes* R_{ct} vs. log mol concentration is plotted which showed excellent response in wide range of concentration. Further we explored the utilization of modified electrode for real samples (milk beverages). The hybridization event was performed in the same way as we did for the 24 mer oligonucleotides using the target DNA of real sample.

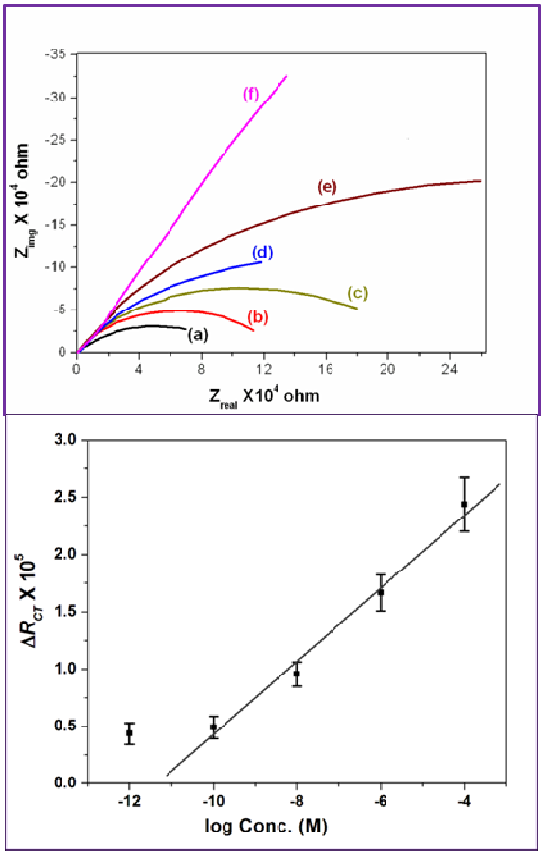


Fig. 8 (a) Nyquist plot for (a) GCE\CS-PtNPs\24mers ssDNA\albumin and after hybridization with real sample

(genomic DNA from Milk sample) of concentration range from (b) $1x10^{-12}$M (c) $1x10^{-10}$M (d) $1x10^{-8}$M (e) $1x10^{-6}$ M (f) $1x10^{-4}$M.

(b) Corresponding calibration plot for real sample (genomic DNA. R_{ct}. vs. log concentration (M).

The real sample (milk beverage) genomic DNA R_{ct} showed excellent response in wide range of concentration with saturation in detection after $1x10^{-10}$ M. The reproducibility of the detection was excellent with the 10 percent of variation of R_{ct}. In comparison to other methods and probes with chemically immobilized genes the present method showed ease in detection with better specificity.

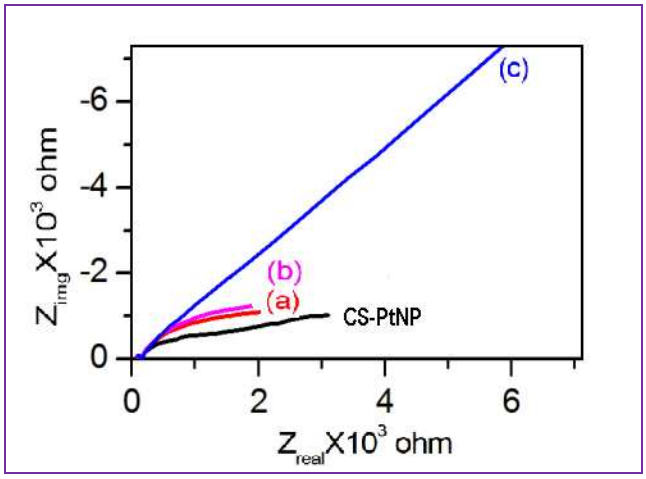


Fig. 9 Nyquist plot for modified electrode GCE/PtNp@CS immobilized with (a) ssDNA, hybridized with (b) complementary DNA (c) 1 base mismatch DNA.

Further to check the specificity of the sensor 1 bp mismatch DNA sequence as CAGTTGCAAGCGCTTGGAGTGAAT was taken as target for the probe immobilized with the oligonucleotide of sequence GCAGTTGCAAGCGCTTGGAGTGAA. Nyquist plot for 1 bp mismatch DNA sequence was compared with the matching target as shown in the Fig. 9. A significant difference in the R_{ct} for matched and 1bp mismatched targets clearly showed specificity of the sensor probe for the target DNA.

5. Conclusions:

This article demonstrates simple, easy-to-control procedure, preparation and characterization of platinum nanoparticles capped with chitosan for the development of modified electrodes for genosensing. We developed electrodes based on (CS-PtNPs)/ssDNA to detect the specific DNA on the basis of sequence specificity. The variation in interfacial properties of electrode upon binding of target DNA is studied by electrochemical impedance spectroscopy. Additional blocking with albumin has been done for reduction of unspecific DNA binding which caused enhancement in signal sensitivity. This methodology has also been tested in real samples (milk beverages). The method described in this work offered a simple, cost effective sensitive, enzyme and mediator less electroanalytical determination of gene sequence. This study opens up new vistas for making use of biocompatible chitosan capped PtNPs for various types of genosensors. Further research is targeted to the application of this system to clinical samples and in the form of field deployable screen printed electrodes.

6. Acknowledgement:

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