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DNA Carbon-Nanodots based Electrochemical Biosensor for Detection of Mutagenic Nitrosamines

Sristi Majumdar, Debajit Thakur, and Devasish Chowdhury*

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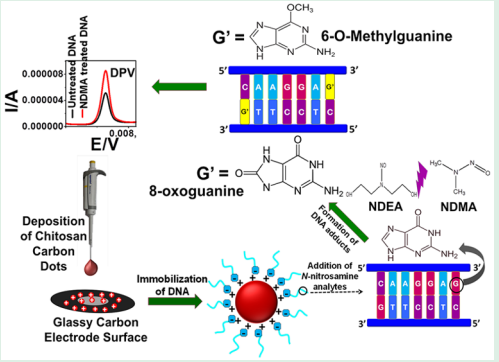
ABSTRACT: Mutagenic and Carcinogenic substances are a threat to any living organism, and its detection is of paramount importance. In this work, we fabricate for the first time a DNA-carbon dots based electrochemical biosensor for sensitive and selective detection of mutagenic nitrosamines like *N*-nitrosodimethylamine (NDMA) and *N*-nitrosodiethanolamine (NDEA). At first, on the glassy carbon electrode (GCE), chitosan carbon dot was deposited, then, DNA was electrostatically immobilizing on the surface of carbon dots to fabricate the sensing electrode (DNA/chiCD/GCE modified electrode). In the presence of NDMA and NDEA, in differential pulse voltammetry technique, the absolute peak current increases, and thus it can detect NDMA and NDEA. The system DNA/chiCD/GCE modified electrode is highly selective and sensitive toward NDMA and NDEA. The detection limit was determined to be 9.9×10^{-9} M and 9.6×10^{-9} M, respectively. The possible reason for DNA/chiCD/GCE modified electrode showing such electrochemical selectivity toward nitrosamines is investigated and discussed.



KEYWORDS: *chitosan, carbon dots, electrochemical, biosensor, N-nitrosamine, DNA sensor, differential pulse voltammetry*

INTRODUCTION

N-nitrosamines are highly toxic compounds found in foods and meats, making them ubiquitous. These potent carcinogens and mutagens form when meats are processed by smoking, curing, or salting or when processed with potassium or sodium nitrite to prevent botulism.[1,2] *N*-nitrosamines are possible carcinogens in humans as reported by the U.S. Environmental Protection Agency (USEPA).[3] They are mostly detected by spectral methods,[4,5] chromatography,[6−8] or electrochemistry. Most of the techniques have detection limits mostly in μM. Out of all these methods, electrochemistry has gained utmost praise due to accuracy, low cost, simplicity, lower detection limit, and shorter time consumption. Amperometric determination of *N*-nitrosamine at ruthenium-based polymer modified electrodes,[9] enhanced voltammetric stripping analysis of *N*-nitrosamine at sulfopolyester-modified electrodes,[10] and electrochemical detection of NDPhA using poly(diallyldimethylammonium chloride) stabilized graphene/platinum nanoparticles[11] have been reported and are sensitive analytical methods for *N*-nitrosamine detection. Furthermore, DNA adsorbed pyrolytic graphene electrodes were also used to determine *N*-nitrosamine.[12] All of these have demonstrated that modified electrodes can be used in the development of *N*-nitrosamine sensors. Use of DNA in the sensing field has also increased the opportunities leading to various sensing strategies.

One of such strategy is DNA based electrochemical sensor.[13,14] Over the past decade, there is a rapid increase in electrochemical sensing platform due to ultrasensitive and easy detection methods.[15−17] However, for accuracy, the DNA needs to get immobilized on the surface of electrode.[18] Many recent studies show the use of nanomaterials like graphene, carbon nanotubes, or gold nanoparticles as electrodes.[19−21] Nanoparticles due to the high surface area provide multiple binding sites for DNA.[22,23] For example, Tang's group electrodeposited Au nanoparticles to establish strong interaction with the thiolated DNA. This Au-thiolated DNA system was used to develop an amperometric biosensor for Pb^{2+}.[24] Korri-Yousoffis's group developed carbon nanotube immobilized DNA for the sensitive detection of human prion proteins.[25] DNA can also be immobilized on the surface of graphene oxide by $\pi-\pi$ interaction. Li et al. developed a graphene oxide-DNA based electrochemical sensing platform for the detection of methotrexate.[26] Wang et al. used DNA-modified electrode for the detection of aromatic amines.[27] Hence, the DNA based biosensor are suitable for sensing applications as base pairing interaction between complementary sequences are specific.

Carbon dots (CDs) is already established as a biocompatible and environmentally friendly material. In this work, chitosan

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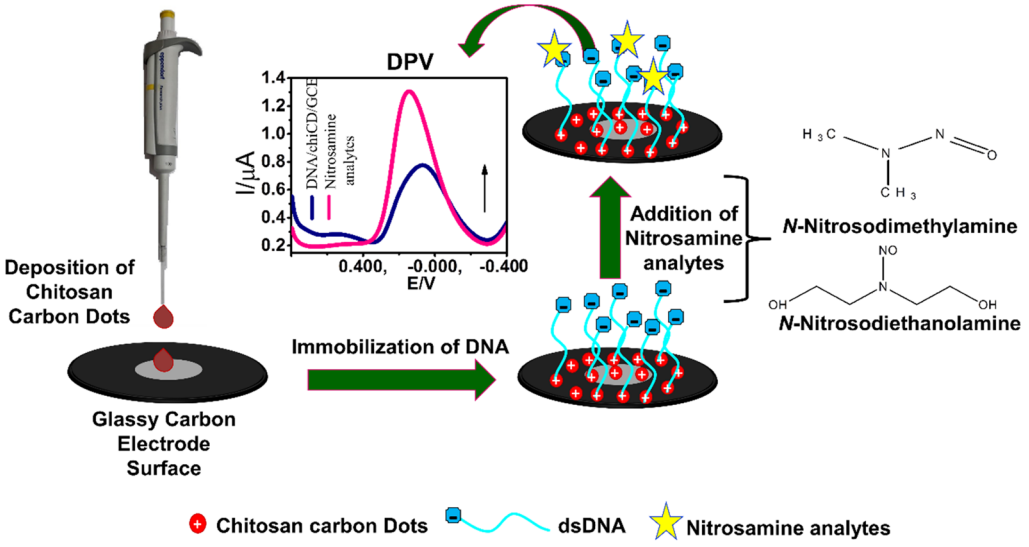
Scheme 1. Schematic Representation of the Process of Fabricating Modified Electrode and Subsequent Detection of Nitrosamine



was used to synthesize CDs, which is also naturally derived and environment-friendly sustainable material. Since food wastes are used to produce CDs, their toxicity is also very low and does not possess any threat to environment.[28] In this study, we have fabricated an electrochemical biosensor using DNA immobilized on the surface of carbon dots for sensitive and selective detection of *N*-nitrosamine. A group of *N*-nitrosamine is mutagenic, which enables them to interact with DNA easily and modify its structure. These small structural modifications can easily get detected by electrochemistry. Due to the larger surface area and various surface functionality,[29] carbon dots provide multiple binding sites for DNA. In fact, to our knowledge, this is the first report of biosensor for carcinogenic or mutagenic compound NDMA and NDEA.

EXPERIMENTAL SECTION

Materials. Chitosan was purchased from SRL India. *Bacillus thuringiensis* DNA was extracted by using QigenQIAmp DNA Mini Kit and stored at −20 °C. We purchased *N*-nitrosodimethylamine, *N*-nitrosodiethanolamine, and 1-nitrosopyrrolidine and 2-ethyl-1-hexylamine from Sigma-Aldrich, India. Aniline and 1,4-dinitrobenzene were purchased from Merck, India. Nitromethane was purchased from Himedia, India. Ultrapure water (18.2 MΩ·cm) was obtained from a Milli-Q water purification system and used throughout the experiments.

Preparation of Chitosan Carbon Dots (chiCD). We followed our already reported protocol for the synthesis of chitosan carbon dots.[30] Typically, 0.1 g of chitosan was dissolved in 10 mL of 1:3 ratio mixture of 1% glacial acetic acid: glycerol and neutralized by 5 N NaOH to form a gel which was microwaved in 0.1 M acetic acid for 5 min to get the desired bright fluorescent CDs. The prepared carbon dots were subjected to different instrumental characterization.

DNA Extraction. *Bacillus thuringiensis* (accession number: MK088287) was obtained from Microbial Biotechnology Laboratory of IASST and was grown overnight in nutrient broth at 37 °C. From the broth, 1 mL of bacterial culture was taken and centrifuged, and the pellet obtained was used to extract DNA using the protocol provided with Qiagen Amp DNA Mini Kit (protocol followed as per the Qiagen manual provided with the DNA extraction kit). The bacterial pellet was suspended in lysis buffer and incubated for 30 min at 37 °C. After addition of Proteinase K and AL buffer, the tube was again incubated for 30 min at 56 °C. RNase enzyme was added and again incubated at 37 °C for 2 min. 200 μL AL buffer was added and incubated at 70 °C for 10 min. To this 200 μL of ethanol was added and centrifuged for a few seconds. The solution was then carefully transferred to QIAamp mini spin column and centrifuged at RCF 6297×*g*. To the column 500 μL of AW1 buffer was added and again centrifuged at 6297×*g* for 1 min. To a clean 2 mL centrifuge tube, the QIAamp mini spin column was transferred, and 500 μL of AW2 buffer was added and centrifuged at 19 283×*g* for 3 min. The column was again transferred to a 1.5 mL centrifuge tube, and 50 μL of AE buffer was added and incubated for 5 min at room temperature. At 6297×*g*, the tube was again centrifuged for 1 min. The DNA was extracted in the 1.5 mL tube and stored at −20 °C for further use.

Preparation of Modified Electrode. The glassy carbon electrode (GCE) surface was cleaned by micro polish powder to a mirror-like smoothness and air-dried. Then GCE was then coated with 50 μL of chitosan Carbon Dot (chiCDs) and air-dried at room temperature. The cross-section area of the glassy carbon electrode (GCE) electrode was 7.07 mm^2. DNA was extracted from nonpathogenic soil bacteria as described. After deposition of chiCD on the GCE surface, 10 μL of DNA deposition was carried out on the chiCD modified electrode followed by air drying.

Instrumentation. The characterization of the chiCD was done using UV−visible spectroscopy on a Shimadzu 2600 UV−vis spectrophotometer and Fourier transform infrared (FTIR) studies were done using a Nicolet-6700 FTIR spectrophotometer. Particle size measurement was carried out using a Malvern Zetasizer Nano series, Nano-ZS90. JEOL 2100 Plus was used for transmission electron microscopy images. Electrochemical sensing studies were carried out in CHI660E electrochemical workstation (CH Instruments, Austin, TX) and a standard three-electrode cell contained a platinum wire auxiliary electrode, a saturated calomel reference electrode (SCE) and the modified electrode as working electrode (CH Instruments) were employed for electrochemical studies.

RESULT AND DISCUSSION

In this work, we endeavor to fabricate a DNA-carbon dots based electrochemical sensor for the detection of *N*-nitrosamines. Differential pulse voltammetry (DPV) technique was used to detect *N*-nitrosamines. We modified the glassy carbon electrode (GCE) with chiCD. Subsequently, DNA was immobilized to obtain DNA/chiCD/GC electrode. This modified electrode was finally used to detect *N*-nitrosamines. Scheme 1 depicts the fabrication of a modified electrode and its use in the detection of *N*-nitrosamines.

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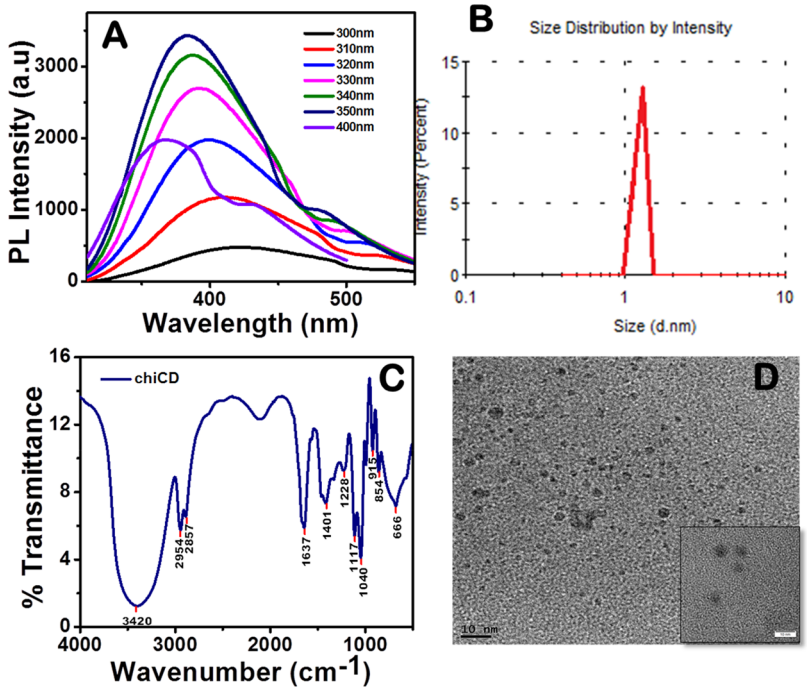

Figure 1. (A) Photoluminescence spectroscopy of chiCD. (B) Dynamic light scattering studies. (C) Fourier transformed infrared spectroscopy of chitosan carbon dots (chiCD). (D) TEM image of carbon dots, magnified image showing few carbon dot particles (inset).

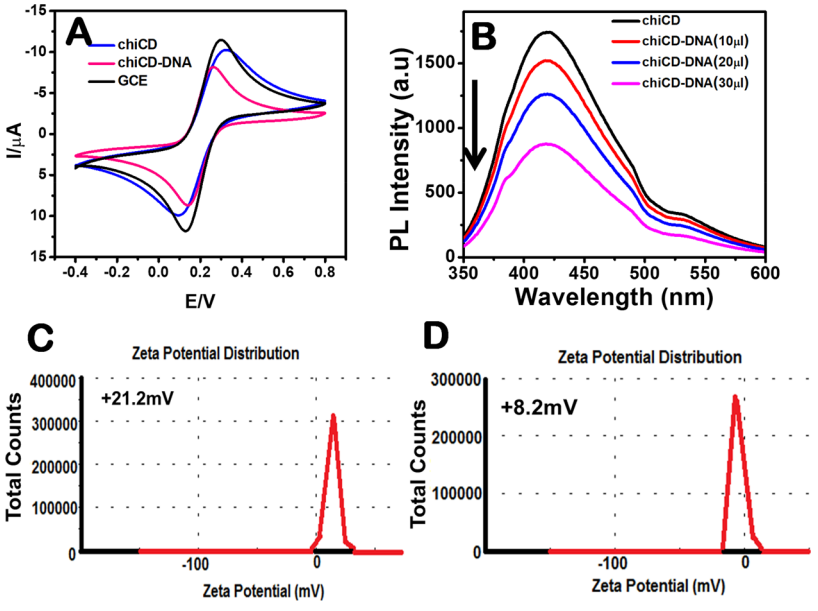

Figure 2. (A) Cyclic voltammogramsof GCE, chiCD/GCE, and DNA/chiCD/GCE modified electrode in 0.1 M KCl solution containing 5 mM $K_3[Fe(CN)_6]$ and $K_4[Fe(CN)_6]$. The scan speed used was 0.1 mV/s. (B) Photoluminescence spectra of chiCD and chiCD-DNA (DNA immobilized chitosan carbon dots). (C) Zeta potential value of chiCD. (D) Zeta potential value of DNA immobilized chitosan CD (chiCD-DNA).

Characterization of Chitosan Carbon Dots (chiCD). First, we prepared carbon dots by already established protocol from chitosan hydrogel. Various instruments were used to characterize chitosan carbon dots. The UV−visible spectrum of chiCD shows a single peak at 260 nm corresponding to $\sigma \rightarrow \sigma^*$ transition (Supporting Information (SI) Figure S2). Figure 1(A) shows the photoluminescence (PL) spectra of the carbon dots at various wavelengths. It is evident from the emission spectrum that chiCDs shows the highest fluorescence at 350 nm. DLS study of carbon dots were carried out in glass cuvette at 25 °C in 0.1 M acetic acid with refractive index 1.371 at 10 s duration. The study shows particles of ∼1 nm (Figure 1 (B)). The size of the chiCDs is well supported by transmission electron microscope (TEM) images, which shows the particles, are in the range of 1−4 nm with an average size of 3 nm. Figure 1D shows the TEM analysis of Chitosan CDs. Magnified TEM image depicting few particles is shown in inset figure.

The carbon dots were also subjected to FTIR analysis to determine the various functional groups present on their surface. Figure 1(C) shows the FTIR spectrum of chitosan carbon dots. The spectrum shows a peak at 3420 cm^{-1}

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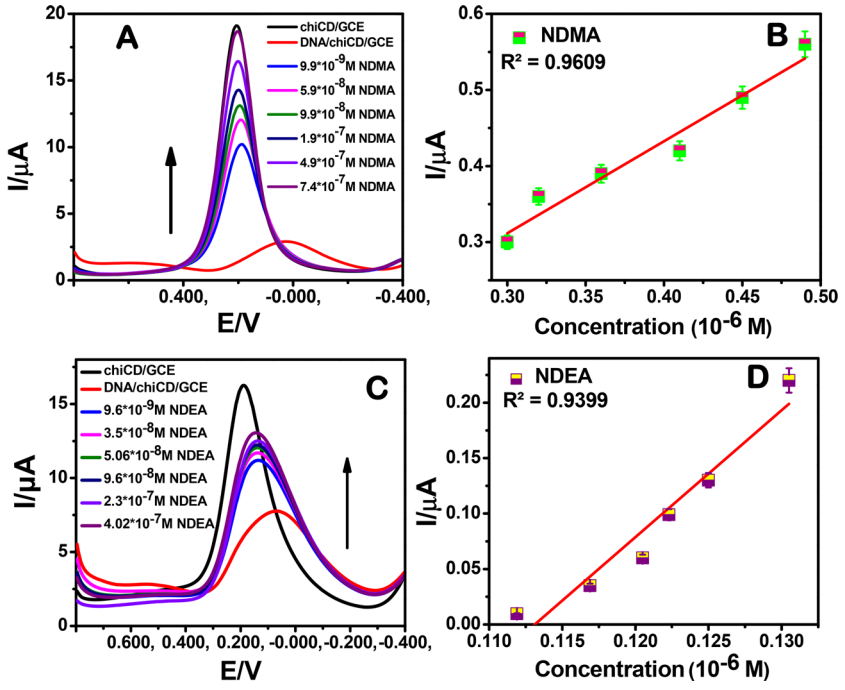

Figure 3. (A) Differential pulse voltammetric detection of *N*-nitrosodimethylamine (NDMA) with increasing concentration range 9.9×10^{-9} M to 7.4×10^{-7} M. The potential window scanned was +0.7 V to −0.4 V Vs. Ag/AgCl at 0.1 V/s in 0.1 M KCl containing 5 mM $K_3[Fe(CN)_6]$ and $K_4[Fe(CN)_6]$ (B) Calibration curve for NDMA. Absolute peak current was used in the plot. (C) Differential pulse voltammetric detection of *N*-nitosodiethanolamine (NDEA) with increasing concentration range 9.6×10^{-9} M to 4.02×10^{-7} M. The potential window scanned was +0.7 V to −0.4 V Vs. Ag/AgCl at 0.1 V/s in 0.1 M KCl containing 5 mM $K_3[Fe(CN)_6]$ and $K_4[Fe(CN)_6]$. (D) Calibration curve for NDEA. Absolute peak current was used in the plot.

(symmetric and asymmetric stretches of O−H group), 2954 cm^{-1} (C−H stretch), 1637 cm^{-1} (N−H bend), 1401 cm^{-1} (C−H bend), 1228 cm^{-1} (C−N stretch), 915 cm^{-1} (N−H wag), and 666 cm^{-1} (C−H bend).[29]

Characterization of Modified Electrodes. As described above, the modified electrode was fabricated by first coating with chiCD followed by immobilization of DNA to obtain DNA/chiCD/GC electrode. chiCD/GCE electrode and DNA/chiCD/GCE electrode were characterized by cyclic voltammetry (CV). The electrochemical behaviors of chiCD/GCE and DNA/chiCD/GCE electrodes carried out in 0.1 M KCl solution containing 5 mM $K_3[Fe(CN)_6]$ and $K_4[Fe(CN)_6]$. Figure 2A shows the cyclic voltammogram of GCE, chiCD/GCE, and DNA/chiCD/GCE. The oxidative redox potential and reduction redox potential of bare GCE was determined to be +0.30 V and +0.13 V, respectively. It is evident from the figure that while chiCD/GCE shows an oxidative redox potential at +0.096 V and reduction redox potential at +0.32 V. However, after immobilization of DNA, there is a shift in the redox potential. The oxidative redox potential changes to +0.14 V. The reductive potential also changes to +0.26 V. The respective peak anodic current (*Ip,a*) and peak cathodic current (*Ip,c*) were determined to be 8.14×10^{-5} and 8.7×10^{-5} μA respectively. The change in redox potential signifies the successful immobilization of DNA over chiCD/GCE electrode. The firm attachment of the DNA to the surface of the chiCD facilitates fabrication of modified electrode for detection of *N*-nitrosamines.

The stability of DNA/chiCD/GCE modified electrode was determined by measuring DPV of the electrode at various time intervals. SI Figure S3 shows that the electrode is stable up to 3 h. and there is no significant change in the DPV peak demonstrating that the modified electrode fabricated is stable.

The photoluminescence properties of DNA immobilized carbon dots also studied (Figure 2B). Upon immobilization of DNA on chiCD results in a decrease in PL intensity. This too gives information about the possible interaction between DNA and chiCD. Moreover, zeta potential value was also checked of chiCD-DNA to determine the surface charge upon immobilization with DNA. The zeta potential (ζ) of chiCD is determined to be +21.2 mV. The ζ value changes to less positive to +8.2 mV upon immobilization by DNA (Figure 2C,D). This change in zeta potential also shed a light of the possible interaction of chiCD and DNA.

SI Figure S4 shows the confocal microscopic images of chiCD and chiCD-DNA. It is evident from the pictures that while chiCD shows more bright spots, arising from the clusters of chiCDs, however only few fluorescence spots visible reiterating quenching of fluorescence upon immobilization with DNA. The few fluorescent spots are may be due to chiCD uncoated with DNA.

Electrochemical Biosensor for Nitrosamine. DNA/chiCD/GCE modified electrode was subsequently used for the detection of *N*-nitrosamine using differential pulse voltammetry. The different derivatives of *N*-nitrosamine that was used for detection were *N*-nitrosodimethylamine (NDMA) and *N*-nitrosodiethanolamine (NDEA). Figure 3A shows the differential pulse voltammograms of DNA/chiCD/GCE modified electrode in the presence of a different concentration of NDMA. The concentration range checked was 9.9×10^{-9} M − 7.4×10^{-7} M. In the presence of NDMA, the peak current increases substantially, thus able to detect NDMA. In the studied concentration range, the peak current increases from

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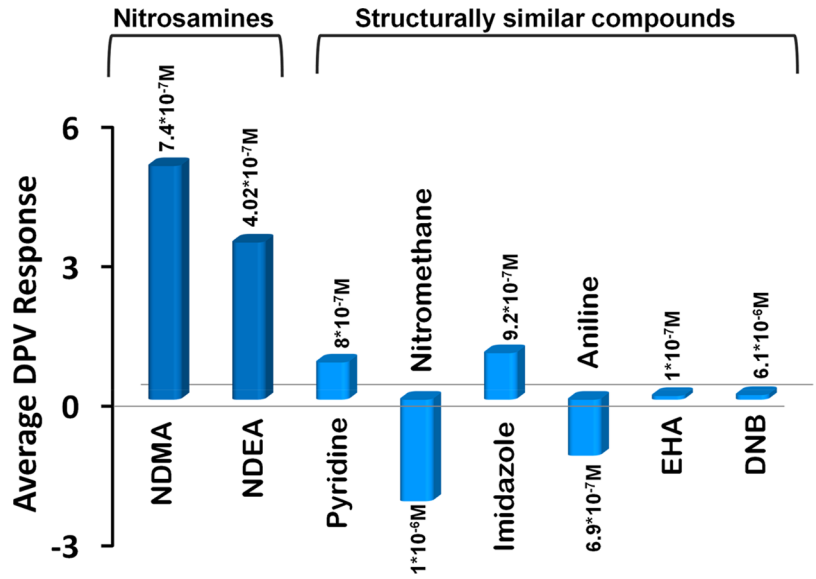

Figure 4. Average DPV response DNA/chiCD/GCE modified electrode in the presence of different analytes.

2.8 μA to 10.1 μA. The calibration plot, depicted in Figure 3B, demonstrates the near-linear increase of peak current with increase in the concentration of the analyte. Thus, DNA/chiCD/GCE modified electrode can detect NDMA in very low concentration. NDMA, a carcinogenic mutagen, can bind easily with the DNA which helps in the detection of the mutagen. We also studied the effects of another *N*-nitrosamine derivative *N*-nitrosodiethanolamine (NDEA).

Figure 3C shows the differential pulse voltammograms DNA/chiCD/GCE modified electrode in the presence of different concentration of NDEA. In this case, the absolute peak current of chiCD/GCE modified electrode was 16.2 × 10^{-6} μA. After DNA was immobilized, DNA/chiCD/GCE modified electrode showed a decrease in the absolute peak current and was determined to be 7.7 × 10^{-6} μA. On the presence of NDEA, the absolute peak current increases with increase in the concentration of NDEA. In the studied concentration range, the 9.6 × 10^{-9} M to 4.0 × 10^{-7} M the peak current increased from 11.1 × 10^{-6} to 13 × 10^{-6} μA. Figure 3D shows the Calibration plot for NDEA. The calibration plot also demonstrates the near-linear increase of peak current with increase in the concentration of NDEA. Thus, change in peak current in DPV of the modified electrode, that is, DNA/chiCD/GCE in the presence of NDMA and NDEA demonstrates that redox peak of the probe changes due to the modified electrode.

The change in electrochemical response of the modified electrode in the presence of the mutagenic NDMA and NDEA was also established by Nyquist plot. SI Figure S5 shows the Nyquist diagram of Nyquist plot for chiCD-DNA modified electrode in the presence of NDMA and NDEA. In Nyquist plot, Z cos θ is plotted against Z sin where Z is the impedance and θ is the phase angle. Z cos θ is the real part and Z sin θ is the imaginary part and denoted by Z' and Z'' respectively. It is evident from the Nyquist plot that DNA/chiCD/GCE modified electrode in the presence of NDMA and NDEA, the system becomes more conductive.

Selectivity Study. One of the criteria of an efficient sensor is that the system should be selective to a particular analyte. To access the selectivity of by DNA/chiCD/GCE electrode, structurally similar analytes were investigated via DPV response. We studied the electrochemical response of DNA/chiCD/GCE in the presence of nitromethane (NM), aniline, 1,4-dinitrobenzene (DNB), pyridine, imidazole, and 2-ethyl-1-hexylamine (EHA). SI Figure S6A shows the differential pulse voltammograms of DNA/chiCD/GCE modified electrode in the presence of a different concentration of pyridine. The experiment used the concentration range 1 × 10^{-8} M to 8 × 10^{-7} M to generate a DPV response. We observed that the absolute peak current increases and then decreases in that concentration range. SI Figure S6B shows the plot of peak current in the presence of a different concentration of pyridine. SI Figure S6C shows the differential pulse voltammograms of DNA/chiCD/GCE modified electrode in the presence of a different concentration of nitromethane. DPV response was checked in the concentration range 1 × 10^{-8} M to 1 × 10^{-6} M. It is observed that in this concentration range like pyridine, the peak current increases then decreases. SI Figure S6D shows the plot of peak current in the presence of a different concentration of nitromethane.

Similarly, SI Figure S7A shows the differential pulse voltammograms of DNA/chiCD/GCE modified electrode in the presence of a different concentration of Imidazole. DPV response in the concentration range 1 × 10^{-8} M to 9.2 × 10^{-7} M show inconsistent peak current with increase in the concentration of imidazole. SI Figure S7B shows the plot of peak current in the presence of a different concentration of imidazole. SI Figure S7C shows the differential pulse voltammograms of DNA/chiCD/GCE modified electrode in the presence of a different concentration of aniline. Here too the absolute peak current decreased with increase in the concentration of aniline. The concentration ranges of aniline in which the DPV response was checked was 9.9 × 10^{-9} M to 6.9 × 10^{-7} M. The plot of peak current in the presence of a different concentration of aniline is shown in SI Figure S7D.

Similarly, studies were conducted to determine the DPV response in the presence of 2-ethyl-1-hexylamine (EHA). SI Figure S8A shows the differential pulse voltammograms of DNA/chiCD/GCE modified electrode in the presence of a different concentration of EHA. We observed that the peak current was inconsistent with an increase in the concentration of EHA. SI Figure S8B shows a plot of peak current in the

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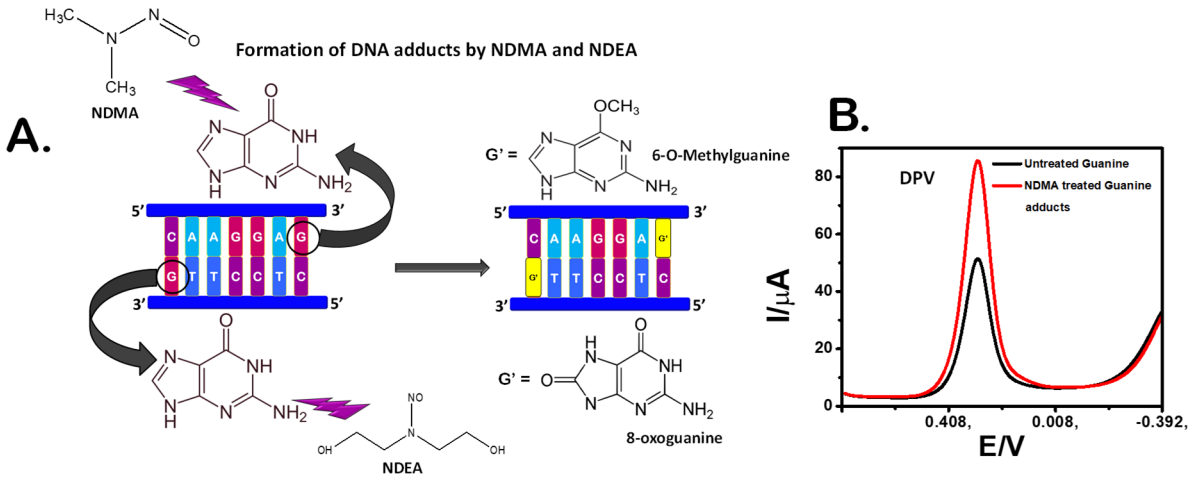

Figure 5. (A) Schematic representation of the mechanism and possible formation of DNA adduct. (B) Differential pulse voltammogram of NDMA treated DNA and untreated DNA.

presence of a different concentration of EHA. SI Figure S8C shows the differential pulse voltammograms of DNA/chiCD/GCE modified electrode in the presence of a different concentration of 1,4-dinitrobenzene (DNB). Again, we observed that the peak current was inconsistent with an increase in the concentration of DNB. SI Figure S8D shows a plot of peak current in the presence of a different concentration of DNB. Thus, the DPV response of structurally similar molecule viz. nitromethane (NM), aniline, 1,4-dinitrobenzene (DNB), pyridine, imidazole, and 2-ethyl-1-hexylamine (EHA) demonstrates that DNA/chiCD/GCE modified electrode is selective toward mutagenic *N*-nitrosamine analytes like NDMA and NDEA.

Figure 4, the histogram, represents the average DPV response toward different analytes tested with DNA/chiCD/GCE modified electrode. The plot demonstrates the fact that this system is sensitive to *N*-nitrosamine analytes like NDMA and NDEA.

Mechanistic Insight. We also try to investigate the mechanistic insight for the DPV response of DNA/chiCD/GCE modified electrode in the presence of Nitrosamine analytes like NDMA and NDEA. It is now well-known that out of the base pairs A, T, G, C, Guanine (G) is electrochemically active.[31] In the presence of NDMA guanine is modified to 6-O-methylguanine or 7-methyl guanine and with NDEA modifying guanine to 8-oxoguanine to form DNA adducts.[32,33] The DNA adducts formed are electrochemically active, which ultimately leads to an increase in peak current in DPV. Such adducts are not formed in the presence of structurally similar compounds, namely pyridine, nitromethane, aniline, imidazole, 1,4-dinitrobenzene, and 2-ethyl-1-hexylamine.

We further studied the electrochemical activity of formation of guanine adduct to reconfirm above explanation. For this study, DNA extracted from *Bacillus thuringiensis* was incubated with 0.1 mM NDMA for 10 min. The treated DNA was then subjected to neutral hydrolysis.[31] To obtain exclusively the guanine base by the cleavage of glycosidic bonds, the treated DNA was heated at 100 °C in 5 mM phosphate buffer (pH 7.4) for 30 min. The DNA was precipitated with ice-cold ethanol and centrifuged. The supernatant was collected and then subjected to DPV analysis. The supernatant contain guanine adducts of NDMA as it was incubated with NDMA.

Similarly, neutral hydrolysis and DPV analysis of untreated DNA was carried out by similar protocol. Figure 5B shows the DPV of NDMA treated hydrolyzed DNA and untreated hydrolyzed DNA. The differential pulse voltammogram clearly show a higher peak current for the NDMA treated hydrolyzed DNA due to the formation of guanine adducts. This establish that the change in DPV signal in the presence of NDMA and NDEA is due to the formation of DNA adducts.

CONCLUSION

DNA based electrochemical sensor has come out as a stand out technique for detecting a very low concentration of an analyte. In this work, we develop a novel DNA-carbon dots based electrochemical sensor for detection of mutagenic *N*-nitrosamine analytes like *N*-nitrosodimethylamine (NDMA) and *N*-nitrosodiethanolamine (NDEA). The sensing element was fabricated by depositing chitosan carbon dots followed by electrostatically immobilizing DNA on a glassy carbon electrode. In the presence of NDMA and NDEA, the absolute peak current increases, and thus, it can detect NDMA and NDEA. The system DNA/chiCD/GCE modified electrode is highly selective toward NDMA, and NDEA as DPV response for other structurally similar molecule is either reverse or inconsistent, making the system highly selective. Development of such a system will lead to a sensitive and selective detection system for mutagenic *N*-nitrosamine analytes.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsabm.0c00073.

The structural formula of structurally similar analytes used in the experiment. UV−visible spectrum of chiCD, stability of DNA/chiCD/GC modified electrode Confocal microscope image of chiCD and chiCD-DNA. Nyquist plot for chiCD-DNA modified electrode in the presence of (A) NDMA; (B) NDEA Differential pulse voltammetric response against structurally similar molecule viz. Nitromethane (NM), aniline, 1,4-dinitrobenzene (DNB), pyridine, imidazole and 2-ethyl-1-hexylamine (EHA) (PDF)

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AUTHOR INFORMATION

Corresponding Author

Devasish Chowdhury − *Material Nanochemistry Laboratory, Physical Sciences Division, Institute of Advanced Study in Science and Technology, Garchuk, Guwahati 781035, India;* orcid.org/0000-0003-4829-6210; Phone: +91 361 2912073; Email: devasish@iasst.gov.in; Fax: +91 361 2279909

Authors

Sristi Majumdar − *Material Nanochemistry Laboratory, Physical Sciences Division, Institute of Advanced Study in Science and Technology, Garchuk, Guwahati 781035, India*

Debajit Thakur − *Life Sciences Division, Institute of Advanced Study in Science and Technology, Garchuk, Guwahati 781035, India*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsabm.0c00073

Notes

The authors declare no competing financial interest.

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