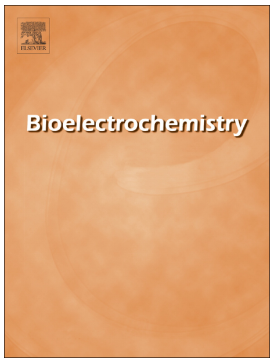

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A DNA Biosensor Based on Gold Nanoparticle Decorated on Carboxylated Multi-walled Carbon Nanotubes for Gender Determination of Arowana fish

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Abstract: Multi-wall carbon nanotubes (MWCNTs) were modified to design a new DNA biosensor. Functionalized MWCNTs were equipped with gold nanoparticles (GNPs) (~15 nm) (GNP-MWCNTCOOH) to construct DNA biosensors based on carbon-paste screen-printed (SPE) electrodes. GNP attachment onto functionalized MWCNTs was carried out by microwave irradiation and was confirmed by spectroscopic studies and surface analysis. DNA biosensors based on differential pulse voltammetry (DPV) were constructed by immobilizing thiolated single-stranded DNA probes onto GNP-MWCNTCOOH. Ruthenium (III) chloride hexaammoniate $[Ru(NH_3)_6,2Cl^-]$ (RuHex) was used as hybridization redox indicator. RuHex and MWCNT interaction was low in compared to other organic redox hybridization indicators. The linear response range for DNA determination was 1×10^{-21} to 1×10^{-9} M with a lower detection limit of 1.55×10^{-21} M. Thus, the attachment of GNPs onto functionalized MWCNTs yielded sensitive DNA biosensor with low detection limit and stability more than 30 days. Constructed electrode was used to determine gender of arowana fish.

Keywords: DNA biosensor; Arowana Fish; Differential pulse voltammetry; Carbon nanotube; Ruthenium (III) chloride hexaammoniate

1. Introduction

DNA is the carrier of genetic information and foundation material of biological heredity. Detection DNA structure involves utilization of developments in biotechnology and molecular science. Different DNA biosensing methods based on complementary base pairing have significant roles in diagnosis, medical food, pharmaceuticals, environmental monitoring, and crime and forensics [1-4]. In general, DNA sequence detection is performed using gel electrophoresing fragments of DNA and polymerase chain reaction (PCR); moreover, traditional methods, which are time consuming and labor intensive, utilize coupling of electrophoretic separation and radioisotopes (^{32}P) [5]. Various methods, including radiochemical [6], enzymatic [7], fluorescent [8], piezoelectric [9], quartz crystal microbalance [10], plasmon resonance spectroscopy [11], electrophoretic [12], electrochemiluminescence [13], atomic force microscopy [14], acoustic [15], and electrochemical means [16-19], were developed to detect DNA hybridization. Since 1960s, DNA detection was performed via direct electrochemical method based on reduction of purine bases (guanine and adenine). Electrochemical methods are preferred over other methods because of their simplicity, reliability, sensitivity, and good selectivity.

In previous investigations, carbon nanotubes (CNT) were directly used as DNA substrate [20] that DNA was attached on multi-walled carbon nanotubes (MWCNTs) through diimide-activated amidation, and results showed the covalent bond between the carboxylic acid groups on CNTs and amino groups on DNA bases [21] or modified by gold nanoparticles (GNPs) and zinc oxide nanowires on a glassy carbon electrode [22]. The materials were also combined with polyaniline [23, 24], cytochrome C [25], polypyrrole [26, 27], and chitosan [28, 29].

GNP decorate on MWCNT by direct and indirect deposition methods. GNP attach on MWCNT by using covalent or non-covalent bonds in indirect deposition [30, 31]. Non-covalent bonding includes three binding types, namely, π-π stacking [32], electrostatic interaction [33], and hydrophobic interactions [34]. In direct deposition, microwaves could assist GNP attachment to the sides of MWCNT [35]. The overall tubular structure of MWCNT is resistant against rupture and tube breakage when microwaves radiate the material during GNP solution preparation; this procedure is in contrast to the steps during functionalization under aggressive sonication and acid treatments [35]. In another method, oxygenated defects were induced on CNT surfaces using oxygen plasma treatment, in which GNPs were formed as nucleus and clusters by Radio Frequency sputtering [36]. GNPs are important in DNA biosensors because of their affinity to solid electrodes or thiol-DNA. Thiol-DNA is also one of the most suitable for biosensors because of its strong affinity between sulfur and gold atoms [37]. Various reagents containing thiol groups were also used to improve DNA attachment on GNPs [16, 38, 39]. Due to electrochemical properties of GNPs, these particles are suitable to signal amplifier in many electrochemical DNA biosensors [40, 41] and enhance sensitivity [16] and immobilization amount of single-stranded DNA (ssDNA) probes [42]. GNPs were used to decorate Bingel–thiol functionalized MWCNTs to prepare efficient and robust catalysts [43].

Arowana is as ancient beautiful and expensive monomorphic fish, which identifying arowana gender is difficult before maturity. Thus, it reasons hardship in mating and selling of arowana. It is an carnivorous fish that lives in fresh water [44]. Identify gender of arowana was exposed based on their sexual organs but it is danger for their lives. Currently, classification is performed using physical inspection, such as checking the mouth. Male arowana fish incubate eggs laid by the female fish in their mouths, which can hold 30–100 offspring. Therefore, males have slightly

larger head and droopier jaws compared with the female. Identifying and classifying this organism during the juvenile stage can improve efficiency and cost effectiveness of fish farming industry. However it still lacks accuracy [45]. Determining arowana gender is important in determining the sex ratio of brooders to maximize breeding.

The aim of this study was to construct a hybridization biosensor for the identification and detection of arowana fish gender and development detection limit by convenience hybridization indicator and further, GNP immobilization on MWCNT whereas GNP has homogeneity in small size and dispersal on MWCNT. We covered screen printed electrode surfaces with functionalized modified MWCNT by COOH group and GNPs and demonstrated that MWCNTCOOH has catalytic properties and increases electrochemical active area, repeatability, and stability of modified SPE electrodes [46].

However, the DNA detection strategy does not merely dependent on the nanomaterial properties, the DNA hybridization detection strategy plays an equally important role. In this work, the use of RuHex with that of CNT is a very useful strategy in terms of electrochemical detection of DNA hybridization. The use of CNT has not been very successful with many other organic intercalators or redox indicators mainly because of adsorption of this organic molecules by CNT, which leads to high baseline DPV current and loss of selectivity. Thus, RuHex which is an inorganic metallo-complex can reduce the unspecific adsorption of the indicator onto CNT and thus the baseline DPV current is expected to be low enough to allow a workable DNA biosensor.

Considering the unique structure of the MWCNT, ruthenium (III) chloride hexaammoniate (RuHex) was used as hybridization indicator. Moreover, label interaction time, pH, and buffer concentration were optimized, and dynamic range, life time, and real samples were investigated.

2. Materials and methods

2.1. Materials

MWCNT was purified and functionalized with H_2SO_4 and HNO_3 purchased from Aldrich. The material was modified with $AuCl_3$ and tri-sodium citrate (99.5%) purchased from Aldrich and Systern, respectively. Arowana fish DNA probe (pDNA) was designed by Freshwater Fisheries Research Division, FRI Glami Lemi via molecular biology method involving PCR using male arowana fish scale. The probe has DNA sequences that was supposed to be specific to male fish. The thiolated pDNA probe, complementary and non-complementary DNA (cDNA and ncDNA) were then synthesised by Sigma to be used for DNA biosensor construction (Table 1).

Here Table 1

Phosphate buffer solutions (PBS) were prepared based on Fisher Bio-reagent instructions using K_2HPO_4 and KH_2PO_4 from Merck. RuHex label was obtained from Aldrich. Deionized water with 18 MΩ cm conductivity was used in all experiments. $K_4Fe(CN)_6$ and $K_3Fe(CN)_6$ with 99%–100% purity were purchased from Merck and R&M, respectively.

2.2. Instrumental

Homogeneous CNT solutions were prepared using an Elma S30H sonicator bath and UV radiated using a Mega LV202E. GNPs were attached using a Panasonic NN-CD997S Microwave Oven. Samples were freeze-dried in a Christ model instrument. Deionized water was used in preparation of all solutions. Platinum rod and Ag/AgCl Metrohm

electrodes were used as axillary and reference electrodes, respectively. Potentiostate Metrohm PGSTAT10 was utilized in differential pulse voltammetry (DPV) and cyclic voltammetry (CV). Characterization was accomplished by using field emission scanning electron microscopy (FESEM) instrument models, Zeiss Supra 55 VP, and Variable Pressure Scanning Electron Microscopy (VPSEM)-Energy Dispersive Spectroscopy (EDX) Philip XL 30. Transition electron microscopy (TEM) Philips CM 12 was utilized for characterizing the CNT surface. Other characterization steps were performed using a Perkin Elmer Spectrum GX Fourier transform infrared spectroscopy (FTIR) and UV-Vis Spectrophotometer Varian Carry 50.

2.3. MWCNT preparation and modification

Concentrated H_2SO_4/HNO_3 (3:1 mole) combination was prepared and added to MWCNT. A total of 180 µl HNO_3 (conc.) was mixed with 480 µl H_2SO_4 (conc.) and 660 µl deionized water; 3.0 mg of pristine MWCNT was soaked in this solution. The solution was sonicated for oxidation for 90 min at 75 °C to 80 °C and sonicated with UV radiation for an additional 3 min. MWCNTCOOH was separated by centrifugation and washed with deionized water until pH became higher than five. Functionalized MWCNT was then freeze-dried [47, 48]. Modified MWCNT was characterized using FTIR and SEM-EDX.

A total of 1 ml $HAuCl_4$ 1% was added to 1.0 mg MWCNTCOOH. Functionalized MWCNT was dispersed in $HAuCl_4$ solution for 10 min. The suspension was diluted to 10 ml and heated for a few minutes in a microwave oven until boiling. A total of 400 µl sodium citrate solution (1%) was added to the hot solution and heated for 3–5 min in 350 W microwave radiation. Au^{3+} was reduced along the MWCNTCOOH and attached onto

the MWCNTCOOH surface. GNPCNTCOOH was separated and washed by centrifugation with deionized water. GNPCNTCOOH was dried in a freeze-drying instrument and characterized using FESEM, EDX, UV, FTIR, and TEM [49, 50]. An optimized concentration of 0.1% $AuCl_3$ was used to produce GNP with the highest immobilized amount and smallest size (15 nm).

2.3.1. CNT functionalization and characterizations of modified CNT and CNTCOOH with GNPs

For FTIR measurement, the CNT mixed with KBr was strictly controlled to avoid IR absorption caused by excess addition of the compound. To obtain the FTIR spectrum, 0.3 mg of modified MWCNT was mixed with 10 mg dried KBr and ground into fine and smooth mixture for data collection. The mixture was transferred to FTIR cell holder for determination.

To attach GNPs, 1.0 mg MWCNTCOOH was dispersed in 1.0 ml of $AuCl_3$ at different concentrations and sonicated for 5 min. Solutions were diluted to 10 ml with deionized water (10^{-3}, 10^{-2}, 0.1, 1, and 2% wt) and heated by microwave radiation for 5 min at 350 W. A total of 400μL of tri-sodium citrate 1% was added to the hot solutions, which were irradiated again in the microwave oven at the same power for 160 seconds. Modified nanoparticles were separated by centrifugation after cooling to room temperature and used after freeze-drying.

The conductive SEM-EDX samples were placed directly on a round sample holder with a sticker and fixed onto the multi-sample holder device. GNP size and distribution on CNT and CNTCOOH surfaces were determined based on a randomly selected area using an InLens detector. EDX was used to measure gold and carbon percentages.

FESEM instrument was coupled with EDX, and EDX measurements were performed on FESEM samples continuously.

The samples were dispersed in alcohol for 20 min via sonication before capturing the TEM micrographs. One drop of the dispersed solution was transferred to a Cu grid and placed in front of an electron gun to investigate microscopic images.

A cuvette of UV-Vis sample was filled with prepared GNP red-purple solutions using microwave radiation method, and the wavelength was scanned from 290 nm to 640 nm. The area of UV-Vis peak was used to confirm the shape and size of the produced GNP in the solution.

2.4. DNA biosensor preparation

MWCNT was attempted to be attached onto the SPE surface without using any polymer materials. However, using MWCNT alone, it detached from the SPE surface, therefore, MWCNT was functionalized with the COOH group so that it could adhere on SPE surfaces. GNP was attached onto CNTCOOH and then coated on SPE surfaces via solution deposition.

A total of 0.23 mg modified GNPCNTCOOH was deposited on SPE. After evaporating the water, a thin film of GNPCNTCOOH remained on the SPE surface. Injection of solution suspension was performed several times on SPE to create a homogenous thin film layer. Bare and SPEs modified with GNPCNTCOOH were immersed in 5 µM of thiolated pDNA solution and 0.05 M PBS at pH 7 overnight to immobilize the DNA probe onto the GNP. The SPEs were then washed with deionized water. The electrodes were divided to two groups and immersed in 5 µM cDNA and

ncDNA solutions with 0.01 M PBS at pH 7. Lastly, SPEs were rewashed and immersed in 0.15 mM RuHex for 1.5 h. The preparations were conducted in the refrigerator. To compare the DPV results of various constructed electrodes, measurements were performed in a mixture solution of 0.05 M PBS at pH 7 and 0.1 M KCl at a scan rate of 50 mV/S. The compared responses of electrodes showed either single- or double-stranded DNA (ssDNA or dsDNA). The advantage of the SPE-type carbon in electrochemical sensing platforms is that each microelectrode is disposable and affordable. Thus, extensive cleaning or electrode pre-treatment are not required in-between measurements.

2.4.1. Label preparation

According to type of labels and their concentrations, calculated amounts of labels were dissolved in 0.1 M PBS and kept in dark in the refrigerator. The potential peak positions of the labels were derived using CV scanning diagrams at 1 mM of label solution dissolved in 0.1 M PBS at pH 7. Several CNTCOOH- and GNP-CNTCOOH-modified SPEs were kept in 1 mM of some labels for 1 h. All determinations were performed in 0.1 M PBS at pH 7 solutions at a 50 mV/s scan rate.

2.4.2. Optimization of reaction time between RuHex and double helix DNA

A total of 0.23 mg GNP-CNTCOOH was deposited onto SPEs. These electrodes were immersed in 5 µM pDNA with 0.05 M PBS at pH 7 overnight. SPEs were then dipped in 1 µM cDNA mixed with 0.05 M PBS at pH 7 for 90 min, washed with deionized water, and immersed in 0.15 mM RuHex solution for 5, 10, 20, 30, 45, 60, 90, and 150 min. All prepared electrodes were washed with deionized water and used in measurements.

2.4.3. Response of constructed DNA biosensors with RuHex label

A bare SPE, as working electrode, was placed in 0.1 mM solution of RuHex to explore oxidation and reduction potential by using cyclic voltammogram. CV was scanned from -1.5 V to +1.5 V at a 50 mV/s scan rate. Ag/AgCl and Pt rods were used as reference and auxiliary electrodes, respectively.

Herein, five different groups were compared, namely, 1) Bare SPE, 2) CNTCOOH/SPE, 3) immobilized-pDNA-on-GNP-CNTCOOH/SPE, 4) double-helix-DNA-on-GNP-CNTCOOH/SPE, and 5) effect of ncDNA on pDNA-GNP-CNTCOOH/SPE. For comparison purposes, bare SPE was immersed in 0.15 mM RuHex solution for 90 min. In the second group, 0.23 mg CNTCOOH was deposited on SPE and immersed in 0.15 mM RuHex after drying. A total of 0.23 mg GNP-CNTCOOH was deposited onto three other SPE groups using the dropping method. All three groups were kept overnight in 5 µM pDNA solution with 0.05 M PBS at pH 7. Two groups were selected; one was immersed in 1 µM cDNA, whereas the other one was placed in 1 µM ncDNA with 0.05 M PBS at pH 7 for 1.5 h. All samples were washed with deionized water between each transfer from one DNA solution to another. Finally, all samples were immersed in 0.15 mM RuHex solution for 90 min and studied in 0.05 M PBS at pH 7 and KCl 0.1 M solution using the DPV method after washing with deionized water. Pt and Ag/AgCl electrodes have auxiliary and reference electrodes, respectively. The range and rate of the scan were +0.4 V to -0.5 V and 10 mV/s, respectively.

2.4.4. Determining linear response range of DNA biosensor using fish DNA

Various concentrations of cDNA ranging from 1 µM to 10^{-21} µM were prepared; the solutions included 0.05 M PBS at pH 7. Several GNP-CNTCOOH/SPE were constructed

and immersed in 300 µL of 5 µM pDNA with 0.05 M PBS at pH 7 overnight. The constructed materials were then washed with deionized water and immersed in different concentrations of cDNA solutions mixed with 0.05 M PBS at pH 7 for 90 min. The electrodes were immersed in deionized water and placed in 300 µL of 0.15 mM RuHex hybridation indicator for 90 min. The current for all constructed electrodes was plotted versus potential based on DPV method in 0.05 M PBS with 0.1 M KCl solution.

2.4.5. Evaluation of arowana fish DNA as actual sample

We used 1 µL of seven different arowana fish DNA samples extracted from the scale of the fish. These samples were diluted to 350 µL with 0.05 M PBS at pH 7, and sonicated them for 15 min. Three additional samples were prepared for each actual sample. A total of 21 SPEs were modified with 0.23 mg GNP-CNTCOOH and kept at 5 µM arowana fish pDNA overnight. The electrodes were washed with deionized water and immersed with target arowana fish sample DNA for 90 min. All samples were soaked in 0.15 mM RuHex hybridization indicator solution for 90 min.

For the evaluation of the DNA biosensor response, synthetic DNA probe with the sequence of the male arowana DNA was used in the study as complementary DNA. But for real sample tests, the DNA sample was extracted from arowana fish.

In common method, DNA was extracted from the Arowana tissue samples with confirmed gender using QIAGEN Blood and Tissue Kit according to the manufacturer protocol. Each arowana fish used in the study was dissected to identify its gender before the tissue sample was collected for DNA isolation. The DNA extract was then used for determination of the fish gender with DNA biosensor.

2.4.6. Determining the lifetime of DNA biosensors

Several pDNA-GNP-CNTCOOH/SPE were constructed. Three electrodes were immersed in 300 µL of 1 µM arowana fish cDNA for 90 min. After washing with deionized water, pDNA-GNP-CNTCOOH/SPE were kept at 300 µL of 0.15 mM RuHex label for 90 min. These electrodes were detected using the DPV in mixed 0.05 M PBS at pH 7 and 0.1 M KCl. Other constructed electrodes were applied to the arowana pDNA, and their activities were measured at various dates for one month.

3.Results and discussion

3.1. Functionalization of MWCNT via sonication and UV irradiation

UV irradiation could enhance both strength and electrical conductivity of macroscopic bundle in CNTs. Moreover, such type of radiation can generate crosslinks between tubes without damaging the CNT wall structure because of the free radicals on the CNT surface. Crosslinking decreases resistivity, because it facilitates electronic charge percolation through CNT tubes. UV irradiation is more effective in improving conductivity than electron irradiation [51]. UV light also acts as oxidation accelerator of CNTs. effects of chirality, number of walls, and nanotube diameter appear to be less significant on oxidation [52, 53]. Ultrasonication improves numerous chemical reactions, including acid oxidation of CNTs. Ultrasonic vibrations caused defects at the internal and external CNT walls, facilitated oxidation reaction, and broke down CNTs into shorter segments [54]. Compared with previous methods, this method requires less preparation time, and less risk because of experimental skills, creates stable and broken CNT nets, and induces higher oxidation through radical production on the surface.

3.2. MWCNT characterization

Sonication and UV radiation were used to immediately functionalize MWCNT. Mixing H_2SO_4 and HNO_3 can generate NO_2 (Eq.1). This method was proposed because of the ability of NO_2 to produce active $O^{\cdot}$ radicals upon exposure to UV radiation (Eq. 2), whereas the $O^{\cdot}$ radical produces O_3 to react with O_2 (Eq. 3). Therefore, strong oxidants, such as O_3, can be possibly produced in the reaction vessels (Eq. 4), as shown by the chemical equations below:

$H_2SO_4 + HNO_3 \rightarrow NO_2$ Eq. 1

$NO_2 + \text{UV radiation} \rightarrow NO + O^{\cdot}$ Eq. 2

$O^{\cdot} + O_2$ (inside sample tube) $\rightarrow O_3$ Eq. 3

$CNT + O_3 \rightarrow$ HOOC-CNT (In acidic solution) Eq. 4

Results of FTIR spectroscopy imply the presence of –OH and CO functional groups on CNT (Figure 1). The sharp peak at 1385 cm^{-1} is probably associated with the nitro group formed during high-pressure HNO_3 treatment under heating conditions [55]; such peak is also related to the bending mode of O–H in carboxylic acids. Stretching vibration of aliphatic ether appeared at 1101 cm^{-1}. Apparently, two CNTs can be attached together by ether bonding using UV radiation as cross linker (because of the free radicals on carbon nanotube surfaces), or two OH groups can release H_2O and create an ether bond at the ends or defects of MWCNT. Occasionally, different sharp peaks related to impurities or unwanted reactions appear at the spectrum. For instance, in certain spectra, peaks appeared at 1054 cm^{-1}, as shown in Figure 2. The strong peak at 1054 cm^{-1} corresponds to the stretching of sulfoxide bond (S=O), which is sometimes created in defective CNT

areas. Given the high H_2SO_4 concentration, the sulfoxide group can stay between two free carbons at the end or a defect of CNTs or between two different MWCNTs.

Here Fig. 1.

Here Fig. 2.

3.3. Immobilization of GNP on MWCNT surface

Compared with other reported methods, this approach was conducted under intermediate temperature, is organic and surfactant free (in aqueous solution), and with a short reaction time. The presence of hydrophilic functional groups, such as carboxylic acid, can help in attaching nanoparticles to CNTs. Citric acid was proposed to be capable of reducing Au^{3+} and functionalizing CNT into carboxylic groups. The residual negative charges on the functional groups are regarded as nucleation centers that absorb metallic nanoparticles onto CNT surfaces.

According to reports on Sn and Pt nanoparticle formations on CNT [56, 57], microwave irradiation induces vibrational excitations near the functional groups, called local hot spots, on CNT surfaces. These hot spots can support nucleation and nanoparticle growth at functionalized sites. The uniform coverage of GNP in this research indicated that the functional groups are distributed evenly throughout the length of CNTs (Figure 3). As shown in the SEM image, the white spots, which were observed on CNTCOOH surface, indicate the presence of incubated GNP. Different concentrations of $AuCl_3$ (2-1-0.1-0.01-0.001 %) were used to immobilize GNP on CNT surfaces using a fixed concentration of sodium citrate and similar amount of CNTCOOH.

Here Fig. 3.

The optimized concentration of $AuCl_3$ at 0.1% w/v produced homogenous GNP in dispersion and in size of approximately 15 nm. Moreover, the size of assembled GNPs along MWCNT can be controlled using the concentration of sodium citrate [30]. The most efficient power is 350 W through microwave irradiation. Furthermore, TEM results confirmed that GNP was attached on the MWCNT surface and the average size was less than 25 nm (Figure 4a). In Figure 4b, the attached GNPs MWCNTCOOH peaked at 1053 cm^{-1} in the FTIR spectrum and is in agreement with the results of Geng et al. [58]. Moreover, the peaks at approximately 3431 and 1638 cm^{-1} are related to stretching vibrations of carboxylic functional group in MWCNTs, whereas those at approximately 2350 cm^{-1} represent CO_2 molecules in the material's surrounding. GNP immobilization on MWCNT was confirmed using the UV-Vis method. According to the GNP size and shape and color of the gold solution, wavelength position changed within the UV-Vis spectrum [59, 60]. UV-Vis spectroscopy was used to characterize the GNP deposition solution produced by microwave irradiation at different concentrations of $AuCl_3$ solution. In Figure 5, the peak at 518 nm is related to the produced reddish-purple GNPs, and observation is consistent with other experimental results [60]. At high $AuCl_3$ concentrations (2% w/v), the peak at 330 nm is relative to Au, which comprised a yellow colored solution. Per the wavelength position, the constructed GNPs are spherical.

Here Fig. 4.

Here Fig. 5.

3.4. Redox peaks of RuHex label

In CV voltammogram, oxidation and reduction peak currents of Ru(III) in the solution appeared at -0.079 and -0.17 V. The experiments were performed on different DNAs based on the achieved reduction peak current.

3.5. Comparison response of ss- and dsDNA of arowana male fish using a RuHex hybridization indicator

Arowana fish DNA was selected as a real sample. To discern the roles of individual components, the electrochemical response of RuHex at different modified electrodes were examined using the DPV. The reduction peak current of RuHex slightly increased in CNTCOOH/SPE-modified electrodes, in contrast to the bare SPE because of the larger surface area and fast electron-transfer ability of CNTCOOH. When AuNPs were attached onto the surface of CNTCOOH/SPE, the RuHex reduction peak current improved, whereas AuNPs enhanced the electrode area. When the dsDNA of male arowana was immobilized onto the surface of AuNP/MWCNTCOOH/SPE-modified electrodes, the negatively charged phosphate backbone of the DNA on the electrode surface adsorbed the positively charged RuHex, further increasing the reduction peak current of RuHex [22]. The specificity of the DNA biosensor can be seen from the very obvious different in the DPV currents for the response from the hybridization with complimentary DNA when compared with non-complimentary DNA and DNA with 4 base-pair mismatched, where the DPV current was much lower. The results are shown in Figures 6A and 6B.

Here Fig. 6A.

Here Fig. 6B.

3.6. Characterization of constructed DNA biosensor for arowana fish gender

The dynamic range of the DNA biosensor was prepared from various complementary DNA concentrations ranging from 10^{-15} µM to 10^{-3} µM. Because of the negative charge on the CNT surface provided by the carboxylic groups, this creates repulsion with the phosphate groups from the DNA, thus preventing folding of DNA around CNT, and this allows better hybridization between the DNA probes and sample DNA. The constructed DNA biosensor has improved dynamic range compared with other DNA biosensors. With low cDNA concentrations, hybridization of ssDNA is minimal given the low absorption of RuHex on the double helix DNA, so, the current is low. At high cDNA concentrations, double-stranded DNA saturates the biosensor surface. Thus, after adding 10^{-3} µM DNA, the response should be similar. Figures 7 show the linear response range of the DPV current with the concentrations of DNA complimentary to the male arowana DNA probe sequences.

Here Fig. 7.

Table 2 presents the comparison with other biosensors prepared based on CNTs. This constructed biosensor has wider dynamic range and lower detection limit in comparison with others.

Here Table 2

As illustrated in Figure 8, the optimum time for RuHex and dsDNA interaction is approximately 90 min. The kinetics of interaction is slow, and need around 90 min to be completed so that RuHex could be adsorbed onto the DNA surface. Kinetic interaction was initially high but gradually decreased. In addition, the high negative charge of phosphate could adsorb the positive charge of RuHex. However, given desorption

between positive charges of RuHex and the less negative phosphate charge positions, adsorption gradually decreased, and the current eventually became constant.

Here Fig. 8.

According to results in Figure 9, the DNA biosensor sensitivity is constant after approximately one month. The operational life of biological components, such as protein, DNA, and bacteria, is an important aspect in practical application of biosensors. Moreover, deactivation and denaturation caused by environmental conditions, such as temperature, pH, leaching, and storage methods, can shorten the lifetime of biosensors [61].

Here Fig. 9.

3.7. Determination of arowana fish gender using the DNA biosensor

Different current values in DPV method depend on the DNA sequences of the male and female fish. Such values can assist in gender identification of arowana fish. In general, DNA can be extracted from the scales of the arowana fish and the DNA biosensor developed could be used for determination of the male gender because the male DNA sequence which was used as the gender detection DNA probe. Thus, when the DPV current is higher than the baseline values, DNA hybridization was expected and the gender of the fish was confirmed as male. Any DPV current same or below that of the baseline value indicated no hybridization and thus female fish is concluded. Table summarizes the DNA biosensor responses of several DNA samples from the scale of the arowana fish. Therefore, when the current produced was higher than baseline, the real sample belonged to a male arowana fish (sample numbers: 85, 153, 232, 384, and 428)

but when the current was lower than baseline, female arowana fish was indicated (sample numbers: 431, 437, and 531). The results showed 67% and 60% agreement with gender determination for the male and female arowana fish via anatomical studies on the fish killed for the study respectively. The accuracy of the gender determination is very much dependent on the DNA probe design from the fish. It is unlikely to be due to the DNA biosensor design itself in this case. The DNA probe used here is not that specific to the gender determination and this has been confirmed by earlier molecular biology studies, which attained about 70% accuracy in terms of male arowana fish gender identification. Actually, highly specific DNA probe for the gender of the male arowana fish is still not obtained. However, currently 2-3 probes that are less specific are used to increase the chances of accuracy of male fish gender determination. In this work, one of these three probes were used in the biosensor. Furthermore, using synthetic DNA such as base-pair mismatched DNA and non-complimentary DNA, the DNA biosensor demonstrated good DPV current discrimination. This confirms that the original DNA probe may not be highly specific to the arowana fish real samples.

Here Table 3

4. Conclusions

In conclusion, the strategy of developing a DNA biosensor employing decorated GNP on MWCNT and a hybridization detection label with RuHex has been successful in creating a DNA biosensor of improved analytical performance. The success was attributed to the immobilization of DNA probe on decorated GNP, good electrical conductivity provided by the MWCNT and less unspecific response from RuHex as a hybridization redox indicator. RuHex is an organometallic complex with a linear structure

ligand that no have interact with CNT surface. Detection limit, dynamic range, and sensitivity were increased by optimizing the amount and size of GNPs, surface of CNT and proper hybridization redox indicator.

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DNA constraction	sequence of nucleotide bases
Probe Deoxyribonucleic acid (pDNA)	5’-GGGGCAGAGCCTCACAACCT-thiol C3]3’
Complementary Deoxyribonucleic acid (cDNA)	5’-AGGTTGTGAGGCTCTGCCCC
Non-complementary Deoxyribonucleic acid (ncDNA)	5’-GTACCCACTCATGGAATAGG
Mismatched Deoxyribonucleic acid	5’-GGATGGACGAAGCGCTCAGG

Table 1. Sequence of nucleotide bases used at fabrication DNA biosensor

Material	Detection Methods	Dynamic Range (M)
Electrodepositing zirconia on composite films of SWCNT and poly (2,6-pyridinedicarboxylic acid) [60]	EIS	1.0×10^{-11} to 1.0×10^{-6}
Acetylcholinesterase immobilized on polyaniline deposited on CNTs wrapped with ssDNA [6]	Voltammetry	1.0×10^{-11} to 1.0×10^{-6}
Ionic liquid supported CeO_2nanoshuttles–carbon nanotubes composite [61]	EIS	1.0×10^{-12} to 1.0×10^{-7}
polypyrrole/ss-DNA/multi-wall carbon nanotubes paste electrode [13]	DPV	1.0×10^{-10} to 1.0×10^{-8}
Platinum nanoparticles combined carbon nanotubes [62]	DPV	2.25×10^{-7} to 2.25×10^{-11}
Au nano–CNT/PANI film [17]	EIS	1.0×10^{-12} to 1.0×10^{-6}
GNP-CNTCOOH/SPE (This Work)	DPV	1.0×10^{-21} to 1.0×10^{-9}

Table 2. Comparison table with other biosensor prepared based on CNT

Sample No	$i_1 \times (10^{-5})$ A	$i_2 \times (10^{-5})$ A	$i_3 \times (10^{-5})$ A	Ave.I $\times (10^{-5})$ A	SD	RSD %	Inference (male/female)
85	2.211	2.188	2.044	2.147	0.090	4.21	Male
153	2.447	2.349	2.308	2.368	0.071	3.01	Male
232	3.428	3.190	3.622	3.413	0.216	6.33	Male
384	2.893	2.593	2.623	2.703	0.165	6.11	Male
428	3.129	3.044	2.673	2.948	0.242	8.22	Male
431	1.256	1.302	1.263	1.273	0.024	1.94	Female
437	1.727	1.737	1.782	1.748	0.029	1.675	Female
531	1.879	1.858	1.849	1.862	0.015	0.826	Female
Base line	2.051	2.011	1.839	1.967	0.113	5.726	-

Table 3. The determination of arowana fish gender using the DNA biosensor constructed from immobilised male arowana specific DNA probe. DPV Current (A) that higher than the baseline value is an indication of the male gender.

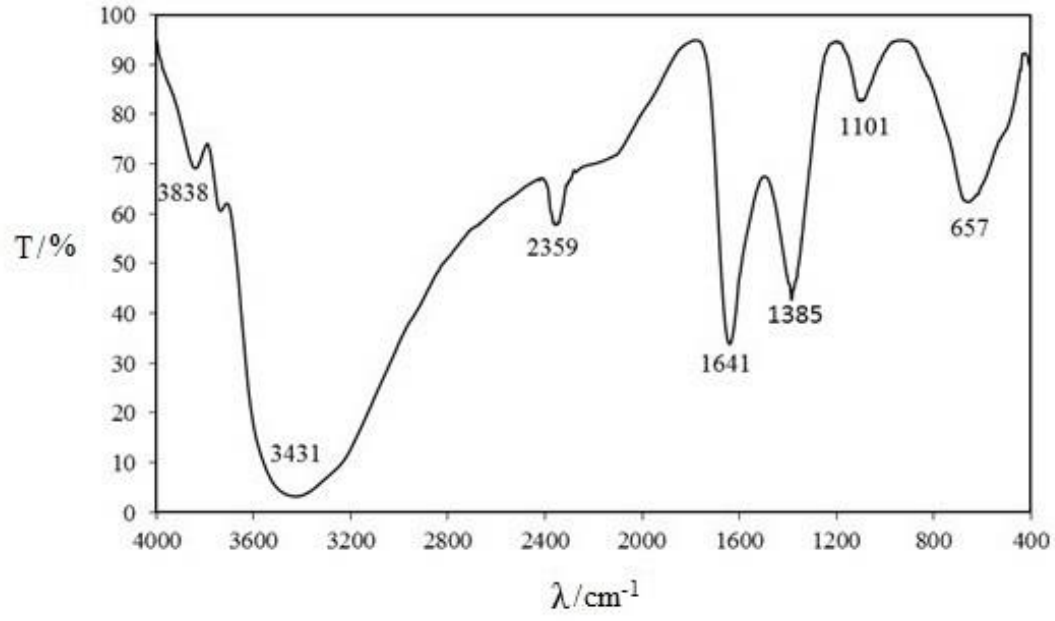

Figure 1. FTIR spectrum of carboxylateded MWCNT by mix of H_2SO_4/HNO_3 (3:1) under sonication and UV radiation

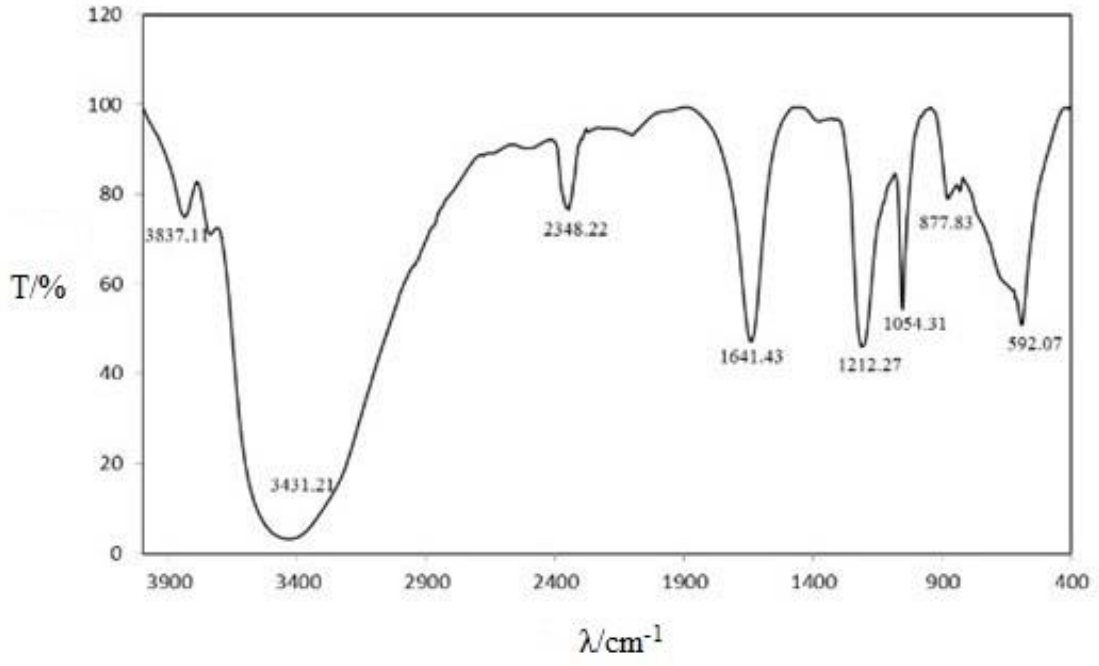

Figure 2. FTIR spectrum of functionalized MWCNT with carboxylic group by sonication and UV irradiation

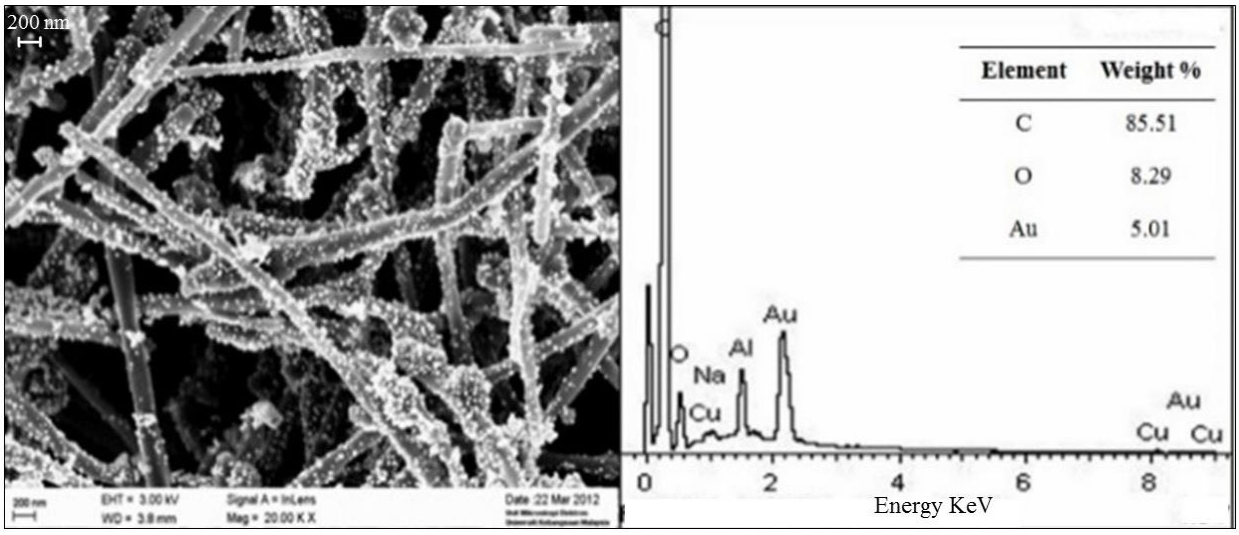

Figure 3. FESEM micrographs of attached uniform GNPs on MWCNTCOOH (left) and EDX spectrum (right)

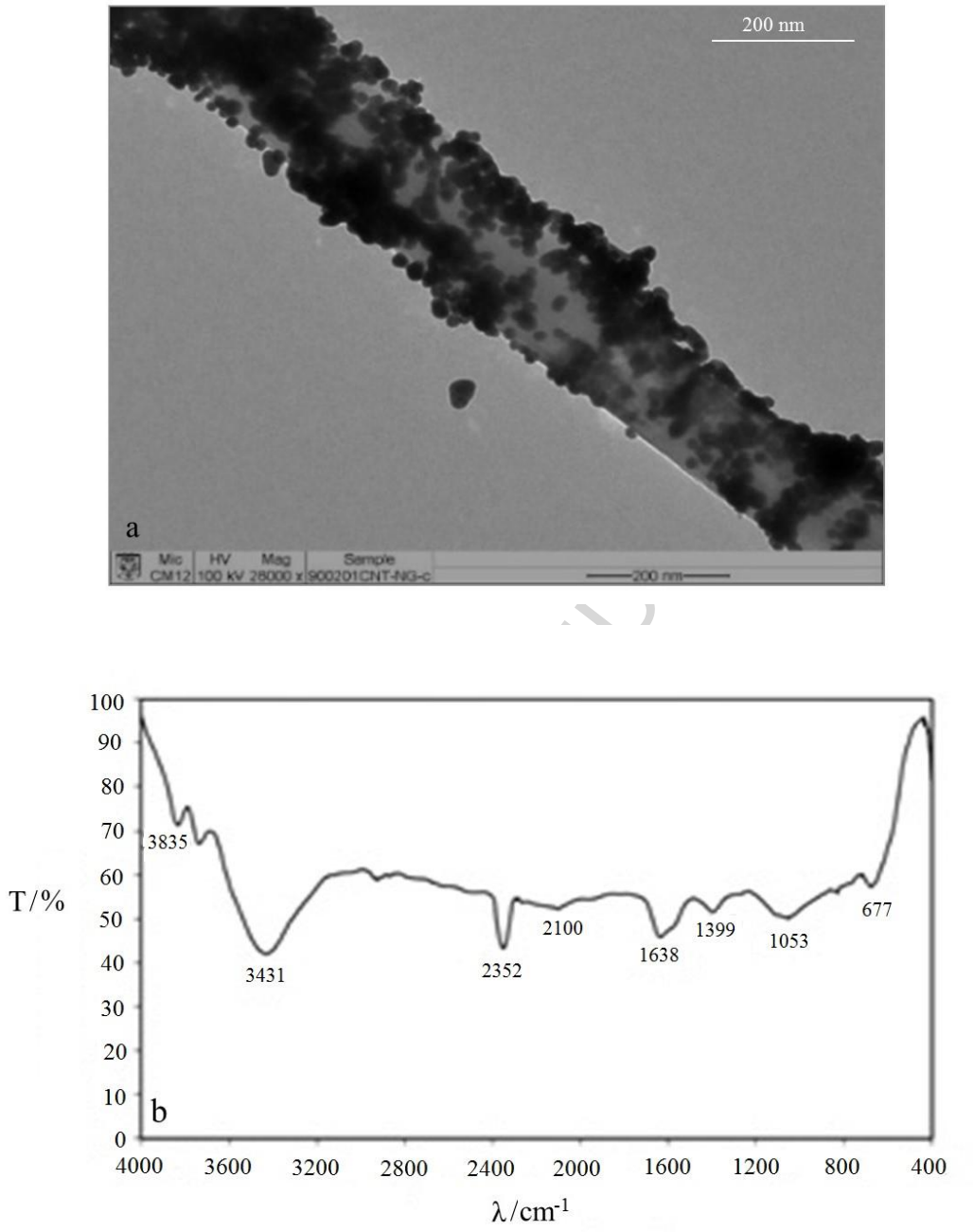

Figure 4. a) TEM and b) FTIR of GNPs immobilized on MWCNTCOOH

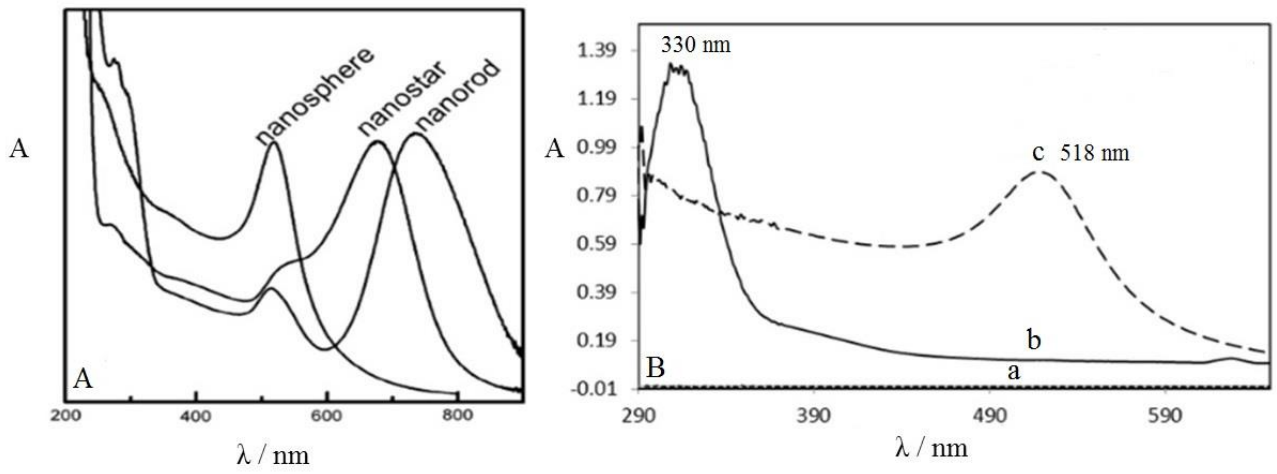

Figure 5. A) The UV-Vis wavelength position of GNPs change based on their size and shape [60] B) UV-Vis spectrum of prepared GNP solution, a) Blank b) Gold c) Gold nanoparticles

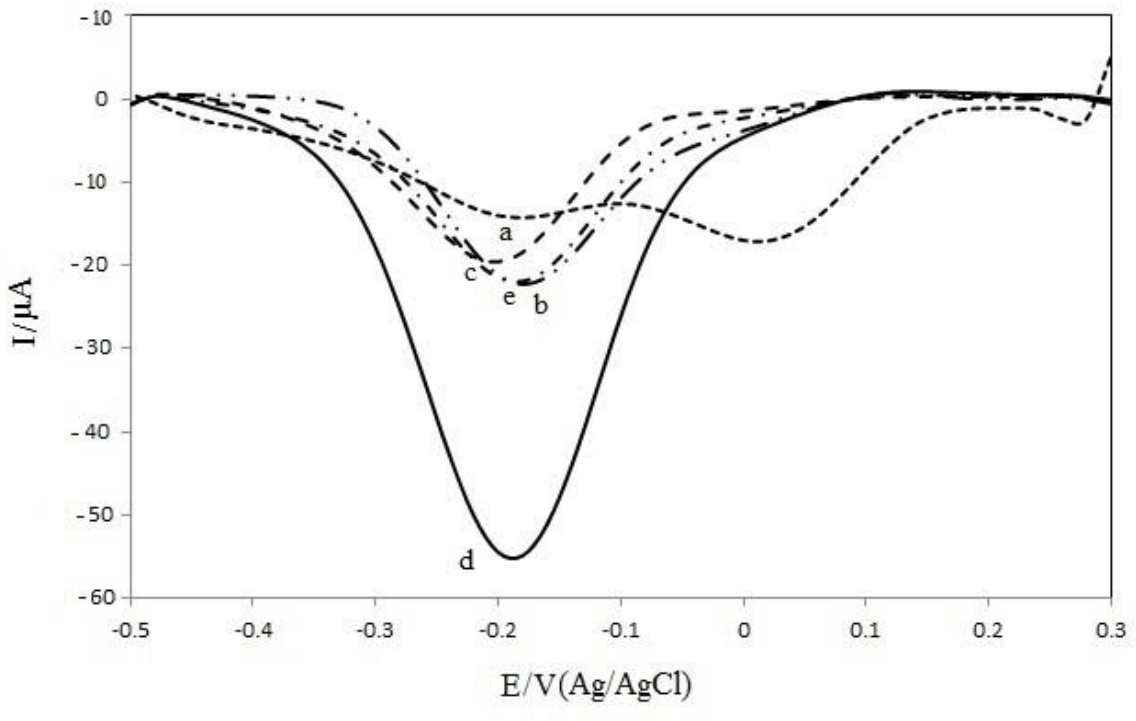

Figure 6A. DPV responses of different modified SPE performed in 0.05 M PBS pH 7 solution when RuHex was utilized as label. a) CNTCOOH/SPE, b) GNP-CNTCOOH/SPE, c) pDNA/GNP-CNTCOOH/SPEs, d) cpDNA/GNP-CNTCOOH/SPEs, and e) ncDNA/GNP-CNTCOOH/SPEs

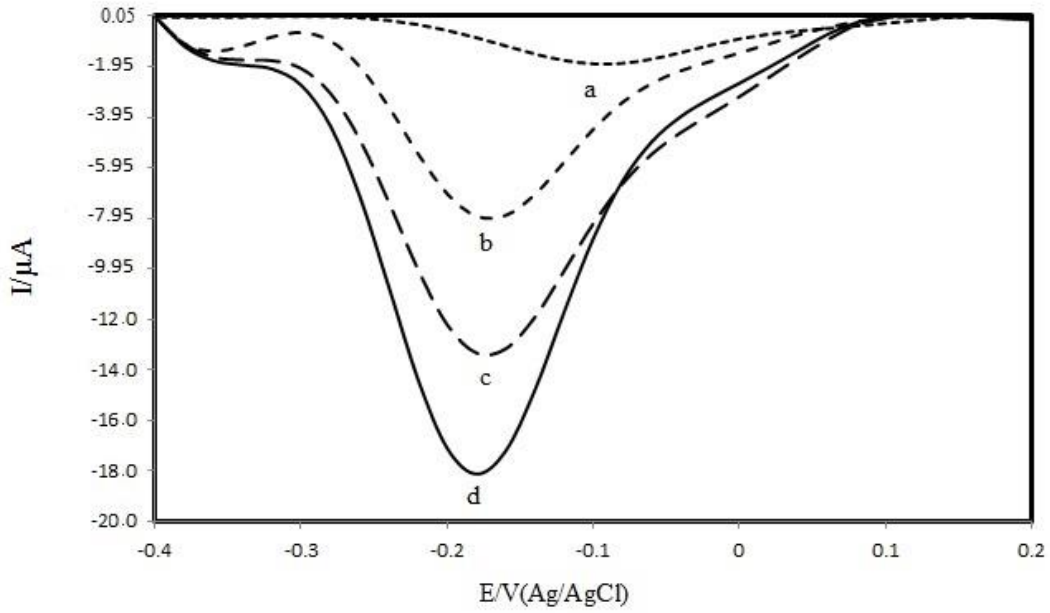

Figure 6B. DPV responses of different modified SPE performed in 0.1 M PBS pH 7 solution when RuHex was utilized as label. a) bare SPEs, b) pDNA/GNP-CNTCOOH/SPEs, c) 4 base pair DNA mismathch for DNA/GNP-CNTCOOH/SPEs, and d) cDNA/GNP-CNTCOOH/SPEs

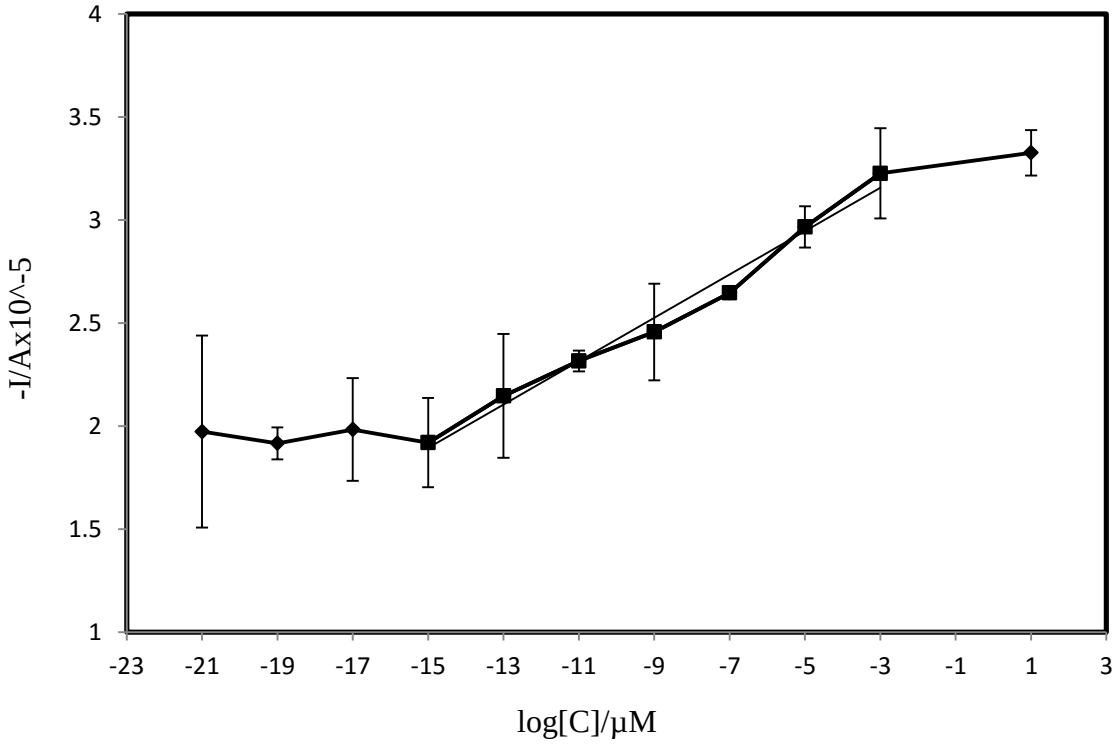

Figure 7. Dynamic range of constructed DNA biosensor based on RuHex hybridation indicator for DNA arowana fish. The line equation and regression are y=0.1052x + 3.4723 and R^2= 0.9838 respectively

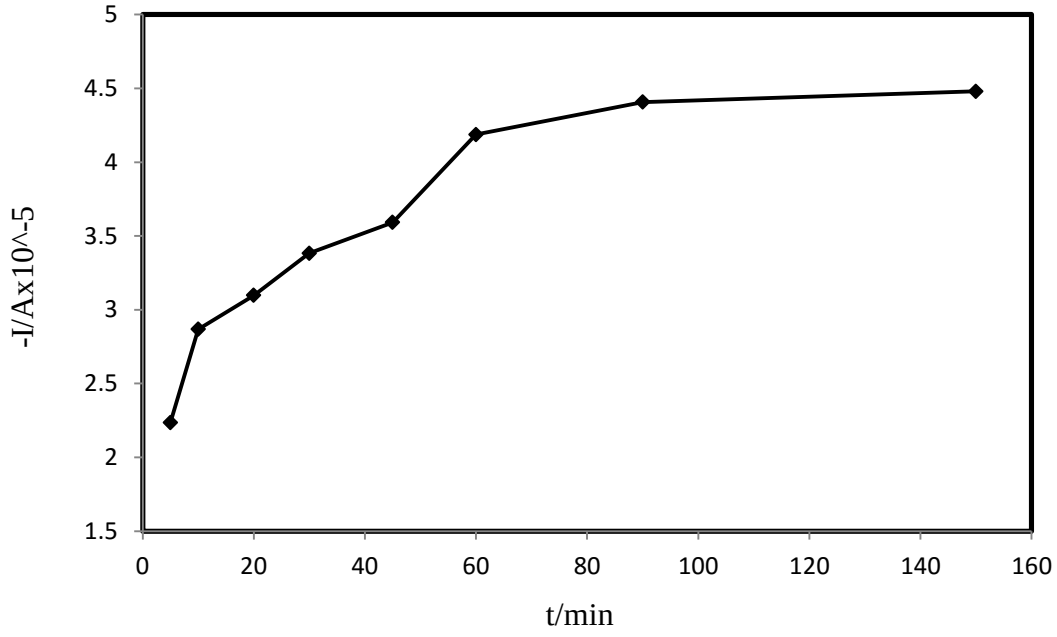

Figure 8. Change of current versus various immersing time in 0.15 mM of RuHex solution

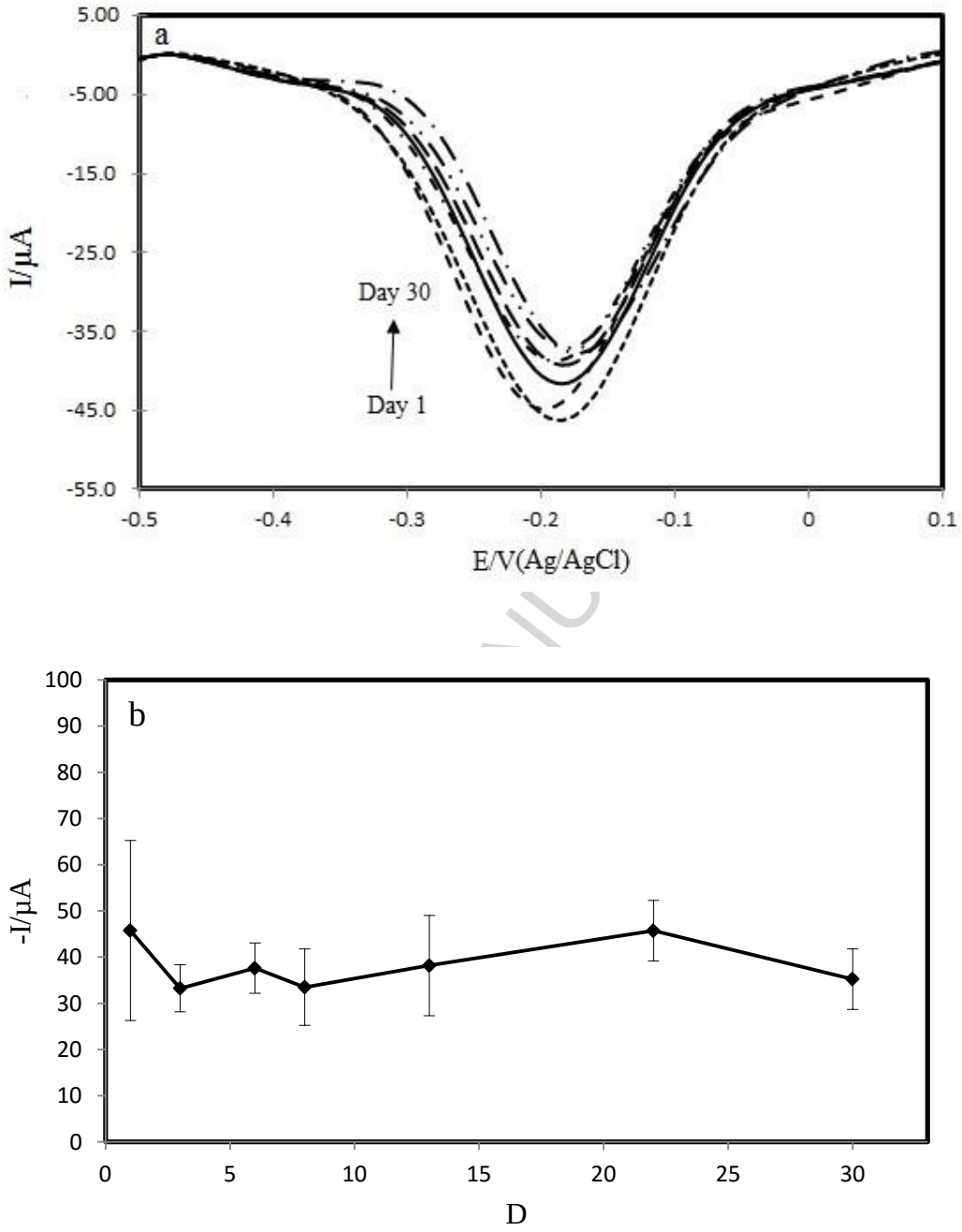

Figure 9. Shelf life of arowana fish DNA biosensor (a) DPV voltamogram (b) change in current in 28 days duration

Highlights

1) Gold nano particles were immobilized on functionalized CNT using microwave irradiation

2) DNA was detected using biomarker that prevented interaction between CNTs to improve response.

3) The DNA detection limit was 10^-21 M and linear range 10^-21-10^-9M

4) High accuracy gender recognition gender recognition was achieved for Arowana fish