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Development of Amino Functionalized Carbon Coated Magnetic Nanoparticles and Their Application to Electrochemical Detection of Hybridization of Nucleic Acids

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Abstract

In our study, the development of amino functionalized carbon coated magnetic nanoparticles (NH_2-CC-MNPs) and their usage for electrochemical detection of hybridization of nucleic acids have been aimed. Firstly, NH_2-CC-MNPs were prepared by coating of pristine Fe_3O_4 nanoparticles with two layers via caramelization and silanization processes respectively. After the morphological characterization with scanning electron microscopy (SEM) it was seen that NH_2-CC-MNPs was spherical shaped and in 28 nm sized. Investigation of chemical composition with the help of scanning electron microscopy/energy dispersive X-ray spectroscopy (SEM/EDX) and fourier transform infrared spectroscopy (FTIR) was showed incorporation of carbon and APTES to the structure of NH_2-CC-MNPs. Magnetic property of NH_2-CC-MNPs after two layered coatings was demonstrated with electron spin resonance (ESR) technique and g factor was calculated as 2.6. In the second part of this study, optimization studies have carried out onto the surface of NH_2-CC-MNPs prepared in saltless phosphate-tween 20 buffer (PBTw) for the analysis of DNA hybridization. The thiol linked DNA probe sequence representing to the Hepatitis B virus (HBV) concentration, target DNA sequence concentration, the most productive hybridization time and the selection of the nanoparticle surfaces have been researched. The electrochemical detection of DNA hybridization was investigated using PGE in combination with differential pulse voltammetry (DPV) technique by measuring the guanine oxidation signal. The detection limit was calculated in the linear target DNA concentration range of 5-25 µg/mL and it was found to be 1.15 µg/mL (20 pmol in 110 µL solution). It has been intended to be more reproducible, more sensitive and faster results with developed biosensor technology.

Keywords: Amino functionalized magnetic nanoparticles; disposable graphite electrode; electrochemical biosensor; differential pulse voltammetry; HBV DNA.

1.Introduction

Nanotechnology is one of the most important research area for many biomedical and environmental applications such as enzyme immobilization [1,2], enzyme and protein separation [3,4],antibody separation [5], nucleic acid immobilization and isolation [6-8], drug delivery [9,10] and biosensors [11,12] due to their unique physicochemical properties of nanoparticles.For immobilizing and separating proteins, enzymes, and other bioactive agents in biotechnology, medicine and analytical biochemistry, magnetic particles possessed a great growing popularity among the spherical nanoparticles [13-17]. As an example, the iron oxide core could be used as nanometer sized magnetic particles and presented superparamagnetic behavior. These nanoparticles were capable of forming stable aqueous suspensions and may be easily redispersed after agglomeration in the presence of a magnetic field [18]. However, pristine nanoparticles of iron oxides tend to aggregate into large clusters and thus lose the specific properties associated with single-domain magnetic nanostructures as a result of anisotropic dipolar attraction. Because of their metalic properties they had ionic/electronic charges and they could show oxidation/reduction reaction electrochemically on their surfaces in the located medium and their stability could be affected [19]. In addition, nanoparticles have large surfaces area. Whereas, their application required the nanoparticles to be uniform in size, chemically stable and well dispersed in the media. So in order to promote the performance of nanoparticles as drug carrier and bioseparator surface modification of pristine magnetic nanoparticles (MNPs) is a commonly used method.Thus MNPs have holded hydrophilic groups which help them to overcome their agglomeration in aqueous media after these modifications and as a result of these modifications MNPs are protected from some undesirable reactions such as acid and base corrosion and release of metal ions and oxidation. In order to enhance the absorption efficiency of MNPs, they could be coated with a number of molecules such as a polymer, a silica, a corbohydrate or a surfactant without loosing their magnetic properties [13,20]. So, desired target can be easily extracted via on of the driving forces fort he adsorptions such as electrostatic, hydrophobic and ligand binding interactions [19]. Carbohydrates have been employed as coating material over the surface of iron oxide nanoparticles. In the hydrothermal carbonization process, the biomass can dehydrate, polymerize and carbonize, thus forming the carbon materials with a wide variety of special properties. This coated MNP is chemically inert and the nonmagnetic shell of carbon can weaken the particle–particle magnetic bipolar interaction and prevents them from aggregating and agglomerating. And also carbon coated magnetic particles draw interest for biomedical applications due to their magnetic property, nontoxicity, biocompatibility, biodegradability and high stability [21]. For the functionalization of surface of nanoparticles APTES is a common amino-silan coupling agent and forms a monolayer of amino-silan. The active amino groups ($–NH_2$) on the surface of nanoparticles enables further bio-functionalization by binding varied biomolecules for wide ranging applications [22].

Nucleic acid based recognition surfaces is becomed more interesting topics in analytical chemistry with each passing days. Electrochemical DNA biosensors based on nucleic acid analysis is thought to play a crucial role in the analyzes to be made at the bedside in a doctor's supervision in the future. In the field of biotechnology, the studies based on nucleic acid analysis performed using magnetic particles [23-43] including the detection of sequence

specific nucleic acid hybridization with electrochemical sensor have increased in recent years. Particles used in these studies, are paramagnetic particles, easily can be separated from the liquid phase with the aid of a small magnet and when the magnet removed from solution, the particles can easily be dispersed again in a liquid phase [18,21]. Magnetic particles are versatile, portable and allow you to create wieldy products. Magnetic particle based electrochemical DNA biosensors have a lot of advantages such as high selectivity and low detection limit. Magnetic particle based analyzes allow cheap cost, short detection time and high sensitivity for nucleic acid hybridization. Hassen et al. [38] used non-faradic electrochemical impedance spectroscopy in order to detect HBV DNA hybridization. Biotin linked DNA probes were immobilized on streptavidin modified magnetic nanoparticles by biotin–streptavidin interaction and consequently, a layer of functionalized nanoparticles was directly immobilized onto bare gold electrode using a magnet. Erdem et al. [27] reported the potential use of these magnetic nanoparticles and their applications on development of novel electrochemical based nucleic acid sensors. In our study, the sequence-selective nucleic acid hybridization using amino functionalized carbon coated magnetic nanoparticles was performed and the label-free voltammetric detection of DNA hybridization was then explored by using disposable electrochemical sensor (PGE). Firstly, magnetic nanoparticles were synthesized, and then they were modified amino molecules by silanization process effectively. Under the optimum experimental conditions, electrochemical detection of sequence-selective HBV DNA hybridization based on amino functionalized carbon coated magnetic nanoparticles was investigated by measuring the guanine oxidation signal.

Under this goal, the pristine Fe_3O_4 magnetic nanoparticles were coated with two different layer in order to overcome stability in aquaous solution and surface functionalization for DNA binding. Firstly carbon coating was realized with caramelization of glucose via well known low-hydrothermal reaction, and secondly APTES coating was realized for adding NH_2 terminal. Pristine Fe_3O_4 magnetic nanoparticles rendered stable firstly and NH_2 functional secondly by these coating processes respectively. The Amino Functionalized Carbon Coated Magnetic Nanoparticles (NH_2-CC-MNPs) were characterized by using SEM, SEM-EDX, FTIR and ESR. The results indicated that NH_2-CC-MNPs had 28 nm size in narrow size distribution and spherical morphology, caoting steps were successfully realized by incorporating carbon, silicium and nitrogen to the structure of NH_2-CC-MNPs and NH_2-CC-MNPs still preserved their magnetic properties after two modification steps. As we known from literature there were not any reported study which combined these two modification steps like that and this was the first study which included these caramelization and silanization steps at the same time. Furthermore this study was the first example for application of NH_2-CC-MNPs to develop of electrochemical nucleic acid biosensor. In the second part of this study, the optimization studies on DNA hybridization have been carried out at the surface of NH_2-CC-MNPs prepared in saltless phosphate-tween 20 buffer as well as DNA probe concentration, target DNA concentration and hybridization time. After the sequence-selective DNA hybridization detached from the surfaces of these nanoparticles using alkaline treatment, disposable PGEs were dipped into these samples for DNA immobilization. The electrochemical detection of full DNA hybridization was investigated

using PGE in combination with differential pulse voltammetry (DPV) technique by measuring the guanine oxidation signal.

2. Experimental

2.1. Apparatus

The oxidation signal of DNA guanine base was investigated by using DPV with an µAUTOLAB-TYPE III electrochemical analysis system and GPES 4.9 software package (Eco Chemie, The Netherlands). DPV measurements were performed with a traditional three-electrode system using a disposable PGE, a platinum wire and an Ag/AgCl/3M KCl (BAS, Model RE-5B, W. Lafayette, USA) as working, counter and reference electrode, respectively.

2.2. Chemicals

D-glucose, sodium hydroxide (NaOH), Aminopropyltriethoxysilan (APTES) (%98), Magnetit nanopowder (Fe_3O_4, average diameter: 20 nm) were supplied by Sigma Chemical Co (St.Louis,MO,USA). Potassium Bromide (KBr) IR Grade, was purchased from Merck, Germany. All the chemicals used herein were of analytical grade and were used without further purification.

The synthetic oligonucleotides were purchased from TIBMOLBIOL (Berlin, Germany). Their stock solutions (500 µg/mL) were prepared with sterilized and ultra pure water and kept frozen state. Their base sequences are:

Hepatitis B virus (HBV) probe:

5'-SH– C_6-AAT ACC ACA TCA TCC ATA TA-3'

HBV Target:

5'-TAT ATG GAT GAT GTG GTA TT-3'

Single-base mismatch (MM):

5'-TAT CTG GAT GAT GTG GTA TT-3'

Non-complementary (NC):

5'-ATC TCC TGC AGC CCT CGC TC-3'

All DNA stock solutions (500 µg/mL) were prepared with Tris-EDTA solution (TE, 10 mM Tris–HCl, 1 mM EDTA, pH 8.0) and kept frozen. More dilute solutions of DNA oligonucleotides were prepared in 0.5 M acetate buffer containing 20 mM NaCl (ABS; pH 4.8). More diluted target/MM/NC solutions were prepared in 0.02 M Tris-HCl buffer containing 20 mM NaCl (pH 7.0; TBS). Ultrapure water was used in preparation of all stock solutions of buffers.

2.3. Preparation of Amino Functionalized Carbon Coated Magnetic Nanoparticles (NH_2-CC-MNPs)

2.3.1.Carbon coating as the first layer

First step for preperation of bilayered amino functionalized carbon coated magnetic nanoparticles was carbon coating onto magnetic Fe_3O_4 core. Carbon coated magnetic Fe_3O_4 nanoparticles (CC-MNPs) was prepared via low-temperature hydrothermal reaction and the method was modified from literature described before by Jiang et al. [21]. Briefly, 0.1 g Fe_3O_4

was dispersed in 40 ml of 0.5 M glucose and 10 mM NaOH solution. Solution was sonicated for 15 minutes through the instrument of ultrasonic bath to ensure better distribution of Fe_3O_4 in glucose solution. Caramelization step was realized that Fe_3O_4 distributed thoroughly in alkali glucose solution was kept for 5 h at 130 ^{0}C in autoclave. At the end of caramelization step, the carbon coated magnetic nanoparticles were separated from the solution phase magnetically. The carbon coated magnetic nanoparticles were washed three times with distilled water for removal non-coated caramel from its surface.

2.3.2. APTES coating as the amino functional second layer

As a amino functional second layer, carbon coated magnetic nanoparticles coated with APTES using the silanization method reported by Cao et al. after some modification [22]. For that, the nanoparticles were dispersed in 200 mL ethanol and 1 mL water containing 40 µL of APTES (98%). Solution was sonicated for 15 minutes in ultrasonic bath so particles were dispersed well in APTES solution. Silanization process was provided by stirring solution with an orbital shaker overnight (18 h) at room temperature. After silanization process, amino functionalized carbon coated magnetic nanoparticles (NH_2-CC-MNPs) were washed three times being 2 times with ethanol and 1 times with water in 100 mL washing volume. In all washing steps, separation was done magnetically and the nanoparticles were well distrubuted via sonication. At the end of these steps obtained amino functionalized carbon coated magnetic nanoparticles were prepared for characterization as described below.

Synthesized NH_2-CC-MNPs were distributed in salt-free phosphate buffer (PBwS), salt-free phosphate-glycine buffer (PBG) and the salt-free phosphate-Tween 20 buffer (PBTw) for investigating the effect of the stability of NH_2-CC-MNPs. The stabilities of NH_2-CC-MNPs in different buffers were investigated for 1.day, 7.day and 14.day.

2.4. Characterization of Amino Functionalized Carbon Coated Magnetic Nanoparticles (NH_2-CC-MNPs)

Characterization of amino functionalized carbon coated magnetic nanoparticles was done as morphologically with scanning electron microscopy (SEM), as chemically with scanning electron microscopy/energy dispersive X-ray spectroscopy (SEM/EDX), fourier transform infrared spectroscopy (FTIR) and as magnetically with electron spin resonance (ESR) techniques. The samples were initially dried in oven at 37 °C for 2 days before being analyzed.

For morphological characterization, the dry sample was mounted on an SEM carbon paste sample mount and was sputtered under vacuum with a thin layer of gold for 1 minute before imaging under SEM. The sample was then mounted in a scanning electron microscope (SEM, FEI Quanta 250 S FEG). The surface of the sample was then scanned at the desired magnification to study the morphology of the beads. For qualitative chemical characterization the sample was scanned right after by using EDX dedector (Oxford Azteck).

FTIR spectra of nanoparticles were obtained by using a FTIR spectrophotometer (Perkin Elmer 100 FTIR). The dry sample (about 2 mg) was thoroughly mixed with potassium bromide (KBr) (98 mg, IR Grade, Merck, Germany), and pressed into a tablet, and the spectrum was then recorded.

Magnetic property was investigated with an electron spin resonance (ESR) spectrometer (EL 9, Varian, Palo Alto, CA).

2.5. Studies with Electrochemical Sensor System

2.5.1. Electrode preparation

The disposable PGE was used in voltammetric measurements. A Tombow pencil was used as a holder for the graphite lead. PGEs were pretreated by applying a potential of +1.40 V for 30 s in acetate buffer solution (ABS; 0.5 M, pH 4.8) containing 20 mM NaCl. This pretreated electrodes were used for further experiments.

2.5.2. Electrochemical Detection of Hybridization Event

The thiol linked probe immobilization was performed by the procedure reported in the literature [23-28,42,43]. 5 µL of NH_2-CC-MNPs were transferred into a 1.5 mL centrifuge tube and washed twice for 5 min with 90 µL of 50 mM phosphate buffer (PBS, pH 7.4). After washing steps, 50 µL of NHS solution was transferred into the tubes and incubated for 15 min for activation of NH_2-CC-MNPs. And then nanoparticles were washed twice for 5 min with 90 µL of PBS. After washing steps, 30 µL of PBS (pH 7.4) buffer containing 100 µg/mL thiol linked HBV probe were transferred into the tubes and incubated for 60 min at room temperature with gentle mixing, then, thiol linked probe immobilized NH_2-CC-MNPs were seperated and washed with 90 µL of PBS (pH 7.4). The NH_2-CC-MNPs carrying the probe were resuspended in 30 µL of 50 mM phosphate buffer (PBS, pH 7.4) containing required amount of complementary target, non-complementary (NC) or single-base mismatch (MM) solution. The hybridization reaction was carried out at room temperature. The hybridized NH_2-CC-MNPs conjugates were then washed twice with 90 µL of PBS (pH 7.4) and resuspended in 25 µL of 0.05 M NaOH solution for alkaline treatment for 5 min. Then, 25 µL of the resulted sample was transferred into 85 µL of 0.5 M acetate buffer (ABS, pH 4.8). 110 µL of resulted sample was mixed during 1 min. The samples were immobilized at the surface of PGEs by using passive adsorption during 15 min, then, electrodes were rinsed in 0.5 M ABS (pH 4.8). The PGEs were connected to the three electrode system of the electrochemical cell containing ABS (pH 4.8) for DPV measurements. The guanine oxidation signal was evaluated in our study due to its better reproducibility comparison to the adenine oxidation signal. Since HBV DNA probe sequence does not contain any guanine bases (26,27), this important detail brings a benefit to our study in order to direct electrochemical detection in the case of full match HBV DNA hybridization by measuring guanine oxidation signal measured at +1.00 V that is indicating of HBV sequence-selective DNA hybridization. Note that, we have reached an effective electrochemical detection of guanine oxidation signal by using DPV in the samples by scanning from +0.40 V to +1.40 V at 50 mV pulse amplitude and 50 mV/s scan rate. A new PGE surface was used in each electrochemical detection cycle. The analytical signals represent the differences in the magnitudes of guanine oxidation signal. All the experiments were performed at room temperature (25.0 ± 0.5 ^{0}C).

3. Result and discussion

Preparation of NH_2-CC-MNPs was shown in Figure 1. In carbon coating step, it was expected that oligosaccarides formed by glucose molecules caramelized and turned into acidic carbon units. Then the acidic carbon units adsorbed around magnetic Fe_3O_4 core in alkali condition and formed a carbon shell by crosslinking [21]. After that, in the silanization process, Si-O-Si bonds occured between OH groups on sugar molecules and Si ions on APTES molecules. In this reaction alkoxide groups ($-OC_2H_5$) are replaced by hydroxyl groups (OH) to form reactive silanol groups, which condense with other silanol groups to produce siloxane bonds (Si–O–Si). Alcohol C_2H_5OH and water H_2O are produced as by-products of condensation [18].

Figure 1

3.1. Characterization of NH_2-CC-MNPs

When SEM image as seen in Figure 2.(A) was viewed, it was understood that NH_2-CC-MNPs had regular spherical morphologies with about 28 nm sizes. It could be said that these smooth morphology provided regular binding kinetics in contrast to irregular, rauph shaped functional particles, so it gained great advantages to their alternatives. Moreover, it could be thought that their sizes also provided effective binding of analytes onto bioactive layer in compared to smaller sized nanoparticles because of less sterical hinderance between bonded macromolecules like DNA onto the nanoparticle, and in compared to larger sized nanoparticles because of larger surface/volume ratio so larger specific surface area. In addition to these, the amino silan agent APTES used for second layer of the nanoparticles, had tripropyletoxy chains which could be thought as spacer arms were able to overcome these possible sterical hinderance [44-46]. On the other hand, size of the nanoparticles was in the suitable range below 30 nm in order to show superparamagnetic behaviour at room temperature [47].

Figure 2

After coating steps, it was observed that the nanoparticles showed visibly differences at colour and smell. Colour of nanoparticles turn into black from brown and the nanoparticle gained characteristic smell of caramel. And also the nanoparticles preserved their magnetization when used a magnet (Figure 2.(B)). These differences also showed the successfully realizing of coating steps but surely these was proved with the help of futher characterization techniques.

As seen in Figure 3.(A) in the EDX results, dedection of carbon (C) was proof of carbon coating, and detection of nitrogen (N) and silisium (Si) were proofs of APTES coating onto the Fe_3O_4 MNPs. These results could be evaluated as qualitative in spite of given atomic ratios. In order to examine surface coating of the nanoparticles FTIR spectra were employed (Figure 3.(B)). The black line showed Fe_3O_4 spectrum and the characteristic absorption band of Fe_3O_4 appeared at ~630 and 563 cm^{-1} [22,48] and the residual –OH bands were seen around 3400 cm^{-1}. After coating with glucose in the blue line, the band at around 2900 cm^{-1} could be assigned to the CH stretch and the characteristic peak of aldehyde group of glucose was seen

at 1622 cm^{-1} (mainly CO stretch due to the absorption bands). Also strong peak of hydroxyl was in existence, suggesting that there were large quantities of hydroxyl all the same. APTES coated final nanoparticles, NH_2-CC-MNPs, were seen in red line indicated that coating was succesfull with existence of many peaks. For example, the presence of the anchored propyl groups of APTES were confirmed by increased C–H stretching vibrations that appeared at 2900 cm^{-1}. In addition, the band at around 3470 cm^{-1} could be attributed to N–H stretching vibration and hydrogen-bonded silanols could also absorb at around 3470 cm^{-1}. The peak at 1625 cm^{-1} could be referred to NH2 bending mode of free NH2 group and the peak at 1512 cm^{-1} could be related to C–N stretch [18,49]. Furthermore, APTES caoting was also showed by bands at 1124, 1064 and 978 cm^{-1} assigned to the Si-O–H and Si–O–Si groups. The Si-O-H vibration band appeared at 978 and Si-O-Si stretching vibration bands appeared in the spectrum at 1064 [18,48]. As it understood from chemical characterizations coating both of layers firstly carbon and finally amino silan, were realized successfully.

Figure 3

Figure 4

Magnetic behaviour of the NH_2-CC-MNPs was investigated by ESR spectroscopy. Gauss Curve obtained from ESR data was given in Figure 4. It was observed from that 26000 mAU signal intensity obtained around 2200 G magnetic field. That indicated of magnetic property of the NH2-CC-MNPs. As a quantity characteristic of the molecules in which the unpaired electrons are located, g factor was calculated by help of equation:

$$g=hv/(BHr) \qquad \text{Eq.(1)}$$

Here, h is the Planck constant and its value was 6.626 x 10^{-27} erg/s, B is the universal constant and its value was 9.274 x 10^{-21} erg/G, v is frequency and its value was 9.707 x 10^{9} Hz and Hr is the resonance of the magnetic field and obtained from Gauss curve as 2663 G. When these data were used in equation .. the g factor calculated as 2.6. The measurement of the g factor for an unknown signal can be a valuable aid in the identification of a signal is origin. In the literature, the g factor for Fe^{+3} (low spin and high spin complexes) was determined to be between 1.4–3.1 and 2.0–9.7, respectively [50,51]. So it was seen that, g factor of NH_2-CC-MNPs was compatible for Fe^{+3} and after two coating modifications, obtained NH_2-CC-MNPs, still had a local magnetic field of magnetite in its structure.

3.2. DNA Hybridization Studies

Fig. S1shows the oxidation signal of guanine measured at +1.012 V in the presence of 100 µg/mL thiol linked DNA ODN after immobilization at the surface of NH_2-CC-MNPs prepared in PBwS, PBG and PBTw buffers. The average guanine signal obtained after immobilization thiol linked DNA ODN at the surface of NH_2-CC-MNPs in PBwS,NH_2-CC-MNPs in PBG and NH_2-CC-MNPs in PBTw were measured as 12.7 ± 1.8 µA, 13.1 ± 0.6 µA and 13.15 ± 0.2 µA with the relative standard deviations % (RSD%) as 13.9%, 4.9% and 1.6% (n=3), respectively. The stability of NH_2-CC-MNPs was also investigated (Fig. S1.B) and the guanine signal was obtained using NH_2-CC-MNPs in PBwS,NH_2-CC-MNPs in PBG and NH_2-CC-MNPs in PBTw at their 1^{th}, 7^{th} and 14^{th}synthesis day. The decreases at the guanine signals were calculated as as 63.52%, 69.51%, 62.54% using the NH_2-CC-MNPs in

PBwS,NH_2-CC-MNPs in PBG and NH_2-CC-MNPs in PBTw at 14thsynthesis day. The less decrease at guanine signal was obtained using NH_2-CC-MNPs in PBTw. Moreover, the highest and more reproducible guanine signal was obtained using NH_2-CC-MNPs in PBTw as 4.9 ± 0.1 µA with the RSD% 2.1% (n=2) even its 14thsynthesis day. Therefore, PBTw buffer was used for further experiments for preparation of NH_2-CC-MNPs.

In the next step, the voltammetric detection of sequence selective DNA hybridization related to Hepatitis B virus (HBV) was performed using NH_2-CC-MNPs. Firstly, the effect of thiol linked HBV DNA probe concentration was studied (shown in Fig. 5). In the case of full hybridization between HBV DNA probe at the concentration level of 25 to 125 µg/mL and 100 µg/mL of HBV DNA target onto the surface of NH_2-CC-MNPs. The average guanine signals (*n*=3) were measured as 0.97 ± 0.073 µA, 1.48 ± 0.17 µA, 1.73 ± 0.480 µA, 2.17 ± 0.19 µA and 2.07 ± 0.014 µA with the RSDs% as 7.52%, 11.47%, 27.79%, 8.92% and 0.68%, respectively. 100 µg/mL thiol linked HBV DNA probe concentration was chosen as optimum due to the highest guanine oxidation signal was obtained at this concentration level (Fig.5 A,B-f).

Figure 5

The effect of hybridization time was studied in the case of full hybridization occured at the surface of NH_2-CC-MNPs between 100 µg/mL of SH-HBV probe with 100 µg/mL of HBV target by applying different hybridization times varying from 5 to 60 minutes (Fig. S2).After hybrization at 5, 15, 30 and 60 min, the average guanine signal was measured respectively as 1.39 ± 0.21 µA, 1.44 ± 0.21 µA, 2.16 ± 0.19 µA and 1.08 ± 0.16 µA with the RSDs % as 15.26%, 14.30%, 8.92% and 14.57%, (*n*=3). Since the highest guanine signal was obtained after 30 min hybridization (Fig.S2 A,B-c), 30 min hybridization time was chosen as optimum for our further studies.

The DNA hybridization related to HBV was then performed between 100 µg/mL HBV DNA probe and its complementary target at various concentration level from 25 to 125 µg/mL and the results representing the guanine signals were given in Figure 6A. After full match hybridization, guanine oxidation signals were increased gradually until HBV target concentration increased to 125 µg/mL. There was a gradual increase at the guanine signal and it levelled off at 100 µg/mL target concentration (Fig. 6A-e). After the hybridization between 100 µg/mL HBV DNA probe and 100 µg/mL HBV DNA target, the average guanine signal was measured as 0.22 ± 0.19 µA (RSD% = 8.92%, *n*=3). Thus, 100 µg/mL concentration level of HBV DNA target was chosen as optimum value for selectivity studies.

The detection limit (DL) was also calculated in the linear concentration range of HBV DNA target as 5-25 µg/mL (Fig. 6B). According to the method described by Miller and Miller [52], DL was calculated and found to be 1.15 µg/mL (20 pmole in 110 µL sample) using the regression equation y=17.77x +361.35 (R^2=0.9976).

Figure 6

The selectivity of the magnetic nanoparticle based voltammetric DNA biosensor was then tested in the presence of the hybridization of 100 µg/mL HBV DNA probe and 100 µg/mL MM, NC or the mixture sample containing of target: MM or target:NC (1:1) and accordingly,

the guanine signals obtained in these studies that were shown given in Fig. S3. The response obtained in the presence of full hybridization between HBV DNA probe and its target was defined as 100 unit. After the hybridization of probe with MM , or probe with target in the case of mixture sample containing MM: HBV DNA target (1:1), there was a decrease at guanine signal about 68.4% (Fig.S3-a) and 16.9 % (Fig.S3-b) respectively. Similiarly, in the case of hybridization of probe with NC, or probe with target in the case of mixture sample containing NC: HBV DNA target (1:1), there was a decrease at guanine signal about 97.9 % and 25.4% respectively. These results claimed that our magnetic nanoparticles assay based on voltammetric genosensor shown a selective behaviour even in the presence of unwanted substituents (MM or NC, especially, one base MM DNA sequence).

The stability of NH_2-CC-MNPs was also tested after thiol linked DNA ODN was immobilized onto the surface of NH_2-CC-MNPs at 1., 4., 7., 14., 21., 28. day of particle synthesis and accordingly, the guanine signal was measured at 1., 4., 7., 14., 21., 28. day of nanoparticle synthesis (shown in Fig. S4). The average guanine signals were measured as 13.15 ± 0.21 µA, 10.00 ± 1.69 µA, 8.37 ± 0.18 µA, 4.93 ± 0.1 µA, 2.62 ± 0.41 µA and 1.86 ± 0.31 µA with the RSDs% as 1.61%, 16.89%, 2.20%, 2.15%, 15.65% and 16.73%, (n=3) using 1., 4., 7., 14., 21., 28. day of nanoparticle synthesis. The % decrease ratio at guanine oxidation signals was calculated and were given in Table S1.

It has been intended to be more reproducible, more sensitive and faster results with developed biosensor technology. After designing of electrochemical sensor technologies with materials such as magnetic nanoparticles which superior properties, low detection limit and more selective analysis of sequence-selective nucleic acid hybridization in low-determination would be provided, thus, these technologies could be a model for analysis of hereditary or contagious diseases in the future. In aspects of analysis time and detection limit (DL) presented herein, the results were compared to earlier reports [23-30, 36-40, 53] , and consequently, some of these literatures were summarized in Table 1.

Table 1.

In our study, disposable PGE provided advantages such as simple to use in contrast to the other studies performed by other traditional electrodes; such as, GE, HMDE, GCE, CPE and M-GECE [25, 29, 38, 39,53]. Additionally, a more sensitive detection was obtained herein with a lower DL in contrast to the ones reported by earlier studies [36,38].

It has been planned to provide novel applications with in addition benefits such as results of multiple analysis acquired more fast, more selective, more sensitive, more reproducible, cheaper, lower determination limit, less amount of sample and without the use of any reagents by the developed new sensor designs contrast to other reported in literatures [27,38,41].

4. Conclusion

Herein, an electrochemical assay on the readout coming from guanine oxidation in presence of the label- free HBV DNA hybridization by disposable sensor technology was introduced for the first time using the amino functional carbon coated magnetic nanoparticles, NH_2-CC-MNPs tailor-made by our group. Accordingly, it provides a chemically/electrochemically stable, powerful, inexpensive, selective, sensitive, rapid, less time-consuming electrochemical

technique resulted in low detection limit. These DNA biosensors have been coupled successfully with magnetic separations in order to perform highly sensitive and selective hybridization detection. Favorable features of both methods have been combined. Such signal amplification after magnetic separation will be useful in highly selective label-free HBV DNA hybridization. Magnetic separation technique is very useful to transfer immobilized molecules from one solution to another, i.e., into the appropriate optimum environment. The reported amino functional carbon coated magnetic nanoparticles, NH_2-CC-MNPs obtained via novel combination of two modification techniques in order to enhance of pristine Fe_3O_4 magnetic nanoparticles and were in narrow size range (about 28 nm) with preserved superparamagnetic properties after two layer coating. Hence, NH_2-CC-MNPs had desired properties which provided many advantages for valuable magnetic separation in target containing aquaous media like biocompatibility, well-dispersing, nano-sized, strong magnetization, having spesific binding sides. It was also important that not to need non requiring of any pre-treatment before separation was also gained to costing advantages to related systems because of decreasing number of process steps.

PGEs based electrochemical biosensor platform had some advantages, such as easy-to-use, inexpensive, disposable and portable which were crucial properties for fabrication of DNA chip technology. Moreover, there was a higher guanine signal measured in our study in the presence of full HBV DNA hybridization comparison to the ones performed by magnetic particles based assays [26, 27]. Such coupling of magnetic hybridization surfaces in combination with renewable transducers and label-free electrical DNA detection eliminates herein the needs for external indicators and further steps of advanced surface modification. The use of magnetic beads for detection of DNA hybridization could be developed furtherly in flow detection systems for DNA microarray design in diagnosis or drug discovery.

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Declaration of interest

The authors report no declarations of interest.

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The captions of figures:

Figure 1. Preparation scheme of amino functionalized carbon coated magnetic nanoparticles (NH_2-CC-MNPs).

Figure 2. (A) Macroscopic images of NH_2-CC-MNPs **(a)** dispersed form after caramelizetion process, **(b)** separation of NH_2-CC-MNPs from the washing medium after caramelization with the help of a magnet **(B)** Scanning electron microscope (SEM) image of NH_2-CC-MNPs.

Figure 3. Chemical characterizations of NH_2-CC-MNPs; (A) SEM-EDX results, (B) FTIR spectra; black line: pristine Fe_3O_4 MNP, blue line: CC-MNP, red line: NH_2-CC-MNP.

Figure 4. Magnetic characterizations of NH_2-CC-MNPs, Gauss Curve obtained from ESR.

Figure 5. (A) Voltammograms, **(B)** histograms representing the guanine oxidation signals after hybridization between thiol linked HBV DNA probe and its DNA target at the surface of PBTw-(NH_2-CC-MNPs). Control experiments of **(a)** PBTw-(NH_2-CC-MNPs), **(b)** SH-HBV DNA probe. Hybridization was carried out between **(c)** 25, **(d)** 50, **(e)** 75, **(f)** 100, **(g)** 125µg/mL of SH-HBV DNA probe and 100 µg/mL its complementary target (n=3).

Figure 6. (A) Voltammograms representing the guanine signals obtained after hybridization between 100 µg/mL of HBV DNA probe and **(a)** 0, **(b)** 25, **(c)** 50, **(d)** 75, **(e)** 100, **(f)** 125 µg/mL HBV DNA target at the surface of PBTw-MB-NH_2s **(B)** Calibration graph based on the guanine signals obtained after hybridization of 100 µg/mL of HBV DNA probe and HBV DNA target at the concentration level of 5 to 25 µg/mL.

Figure 1.

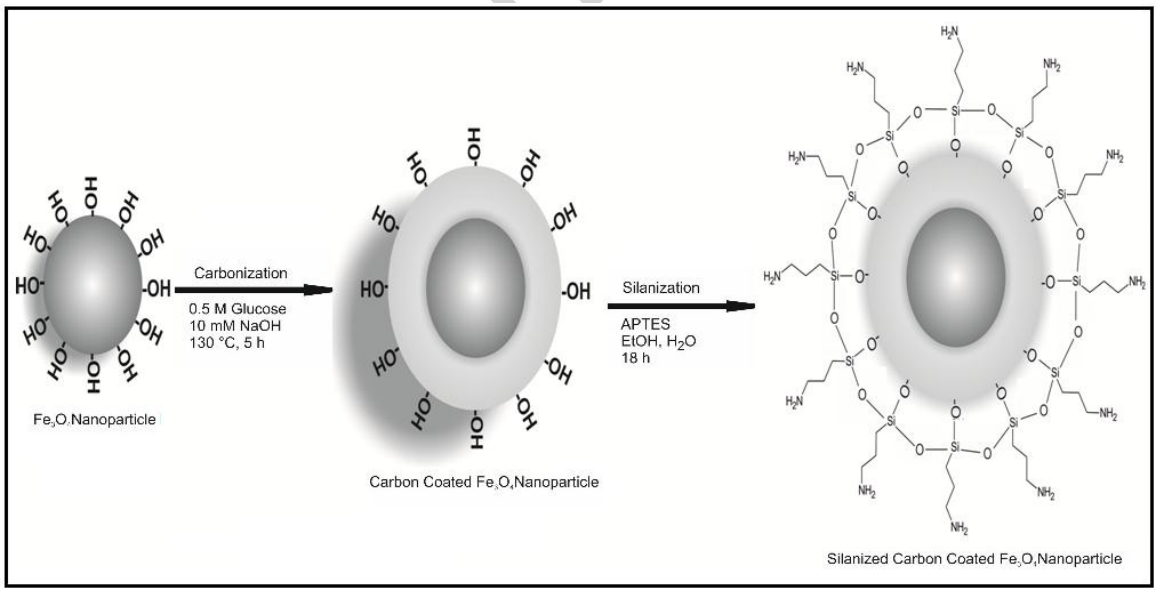

Figure 2.

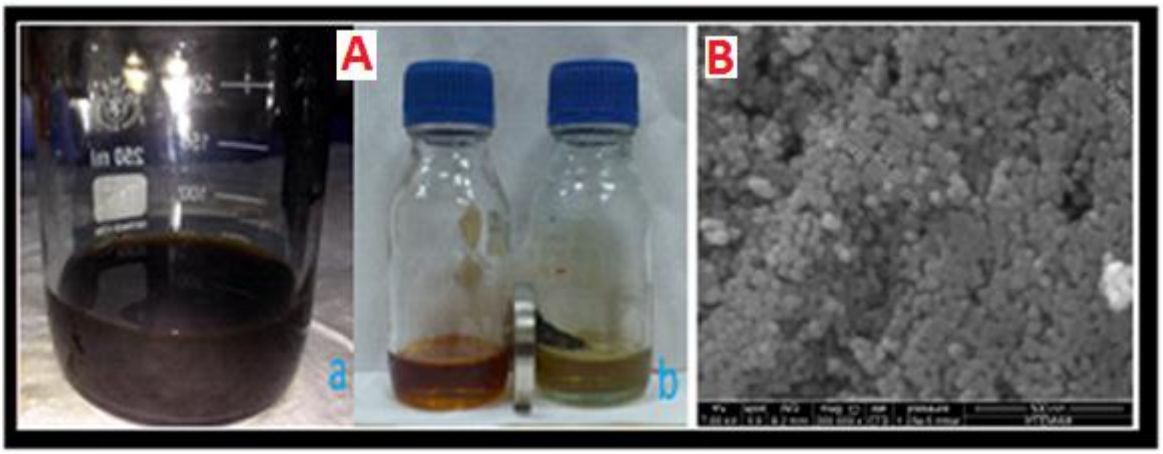

Figure 3.

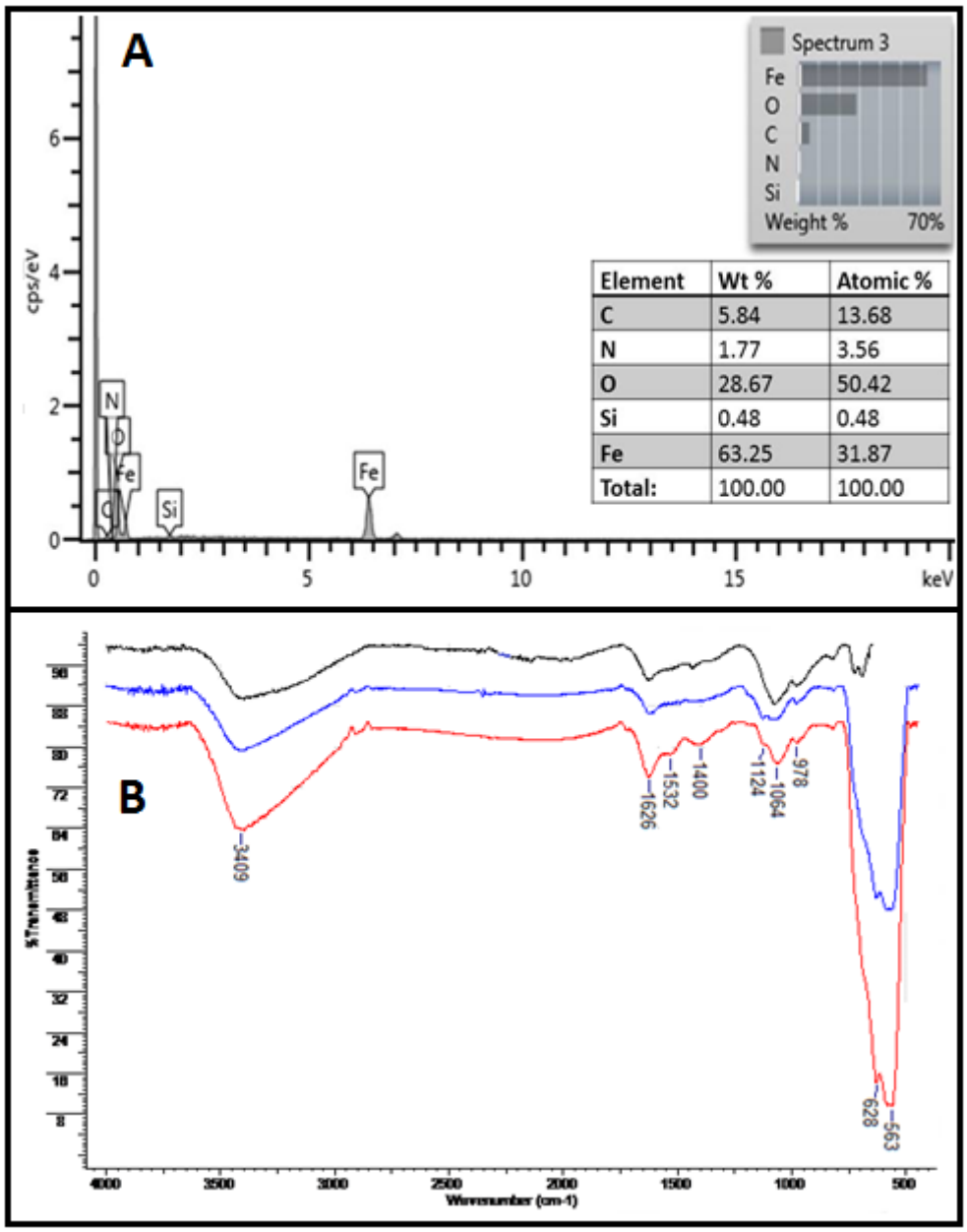
A
Spectrum 3
Fe
O
C
N
Si
Weight %
70%
Element | Wt % | Atomic %
C | 5.84 | 13.68
N | 1.77 | 3.56
O | 28.67 | 50.42
Si | 0.48 | 0.48
Fe | 63.25 | 31.87
Total: | 100.00 | 100.00
cps/eV
keV
B
%Transmittance
Wavenumber (cm-1)
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Figure 4.

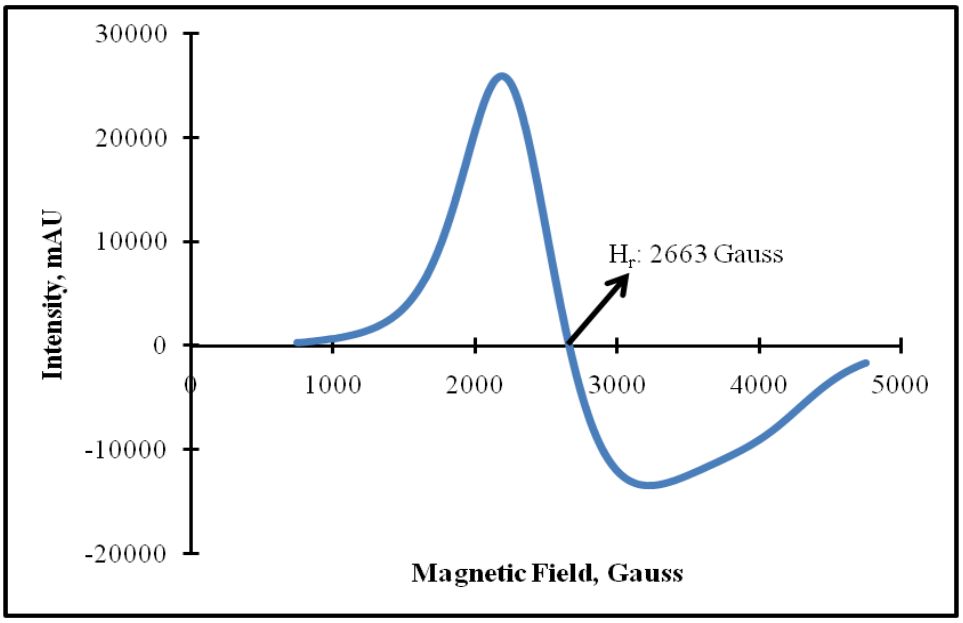


Figure 5.

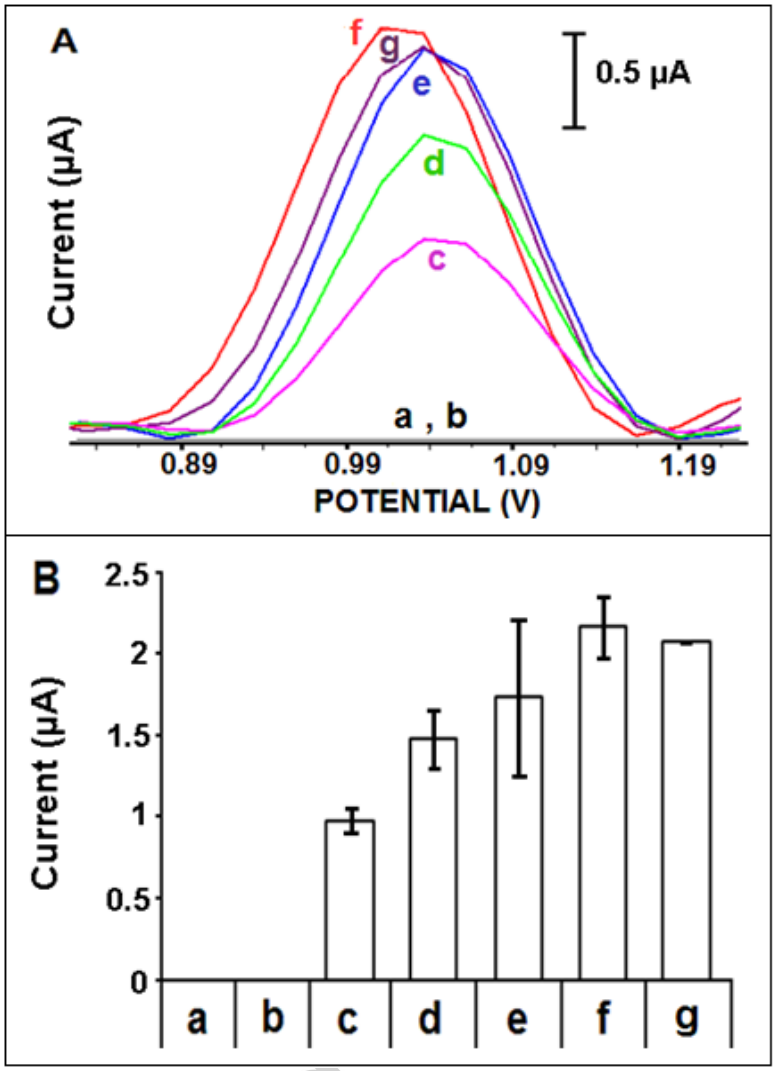
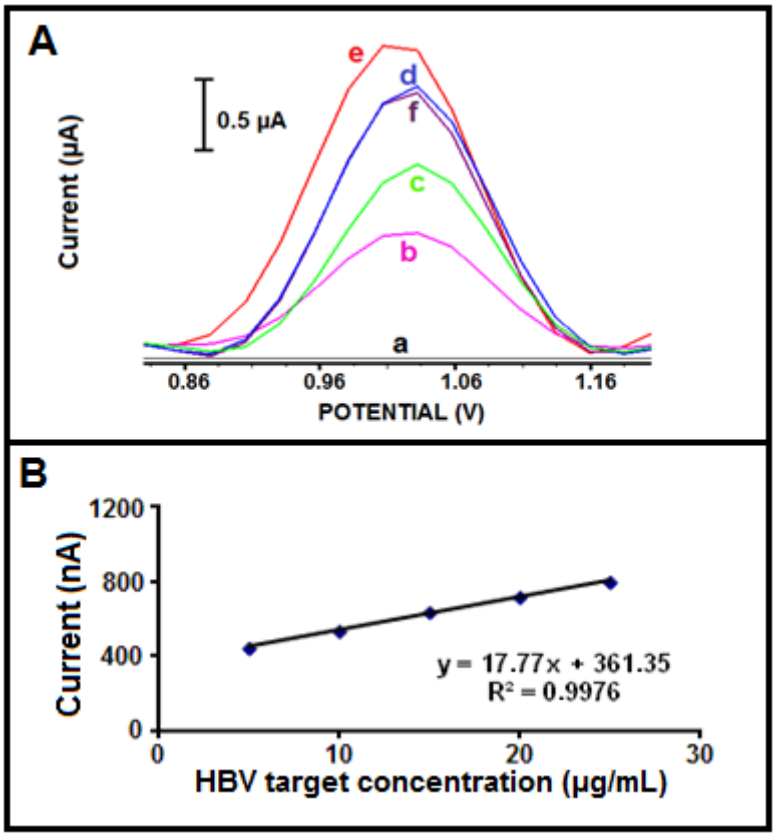
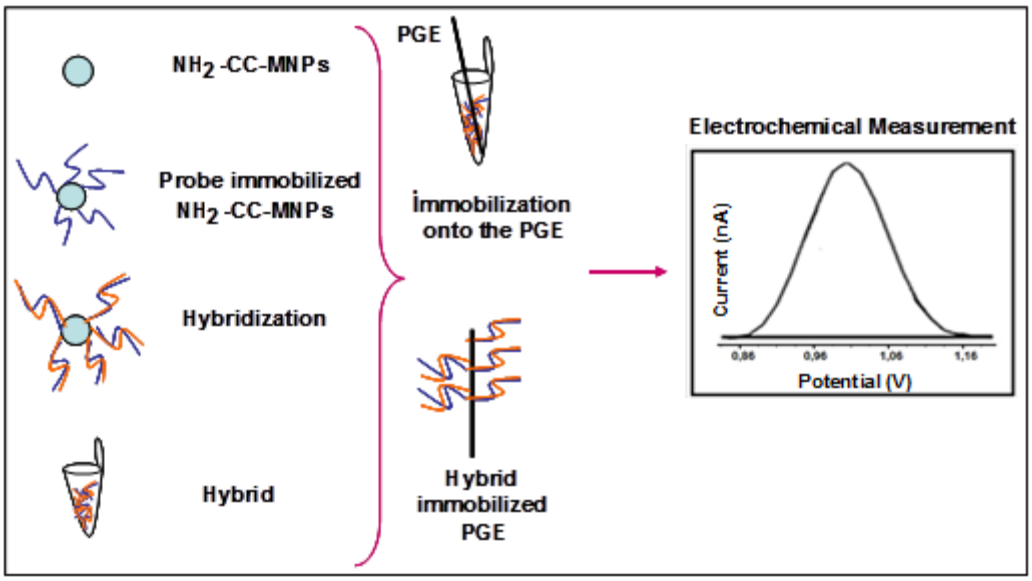


Figure 6.



Graphical Abstract



Highlights

- The Amino Functionalized Carbon Coated Magnetic Nanoparticles (NH_2-CC-MNPs) were synthesized.
- NH_2-CC-MNPs were characterized by SEM, SEM-EDX, FTIR and ESR techniques.
- Hybridization of nucleic acids was performed at the surface of NH_2-CC-MNPs.
- Electrochemical detection of sequence-selective HBV DNA hybridization was investigated by DPV and PGE.
- NH_2-CC-MNPs were used for first time on development of genomagnetic assay in combination with single-use electrochemical sensor for nucleic acid hybridization.