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Impedimetric biosensor for early detection of cervical cancer†

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An impedimetric biosensor based on PEGylated arginine functionalized magnetic nanoparticles for early detection of cervical cancer is reported. The cervical cancer cells could be selectively and sensitively detected down to 10 cells mL^{-1} on the modified electrode, which is promising for advancement in clinical diagnosis and monitoring of tumors.

Cervical cancer, caused by the uncontrolled growth of cells in the cervix, is among the most serious diseases which demands immense research in the area of diagnosis and treatment. This cancer is usually treatable if detected at an early stage, however at a later stage, the cancer metastasizes to the rest of the uterus, bladder, rectum and abdominal wall and eventually reaches pelvic lymph nodes, thereby invading other organs and leading to death. Thus, diagnosis of the cancer at an early stage as well as planning of the appropriate therapy is highly desirable. Conventional histological methods not only lack accuracy and efficiency to detect cancer at early stage but also are time consuming. Therefore, the importance of development of new techniques to rapidly identify and develop the therapy is realized.[1] Electrochemical methods are finding new ways for rapid and selective operation, due to the fact that the cells attached to the electrode can produce electrochemical signals, which can be very useful in development of biosensors. Any living cell can thus be treated as an electrochemical dynamic system, because the redox reaction and ionic changes which occurs due to various cellular processes, leads to electron generation and electron transfer at the interface of living cells.

Electrochemical impedance spectroscopy (EIS) is a powerful method in biosensing as the impedance measurements are well suited for characterizing surface modifications and detection of binding/immobilizing of biomolecules on the electrode surface.[2–4] The impedance is investigated either as a function of the concentration of the analyte in solution or as a function of time. Alternatively, the biological component is immobilized on the working electrode and the interaction with an analyte molecule is detected. Therefore, the fabrication of an impedimetric sensing platform is a major challenge towards the development of a biosensor. Prior to cancer cell recognition at the electrode surface, one needs to have an electrode with high efficiency for capturing and immobilizing the cells. The performance of the biosensor usually depends on the physicochemical properties of the biomaterial immobilized over the electrode. Direct immobilization of living cells onto an electrode surface requires specific biocompatible materials and surface modification. Guo *et al.*[5] monitored the growth of human hepatocarcinoma cell line BEL7404 and evaluated the cytotoxicity of $HgCl_2$ on optically transparent indium–tin oxide electrode. Hao *et al.*[6] prepared an impedance sensor for quantifying the cell number of leukemia K562A cells based on the immunoreaction of P-glycoprotein. Liu *et al.*[7] employed a cell-based biosensor with micro-electrode arrays to investigate the culture behavior of human oesophageal cancer cell lines and evaluate the chemosensitivity of cisplatin.

In this work, we report the fabrication of an impedimetric sensor based on PEGylated arginine functionalized magnetic nanoparticles (PA-MNPs) for early detection of cervical cancer. Polyethylene glycol (PEG) is known for its good biocompatibility and PEG–protein complexes have high catalytic activity at a neutral pH. It preserves the biological activity of the protein adsorbed on the electrode surface and facilitates electron transfer between the protein and the electrode.[8]

PEGylated arginine was synthesized by adding di(ethyleneglycol)ethyl ether acrylate (4.35 g, 23 mmol) to dried methanolic suspensions of L-arginine (2 g, 11.5 mmol) under inert atmosphere. The reaction mixture was stirred at room temperature for 4 days. Solvent was removed under vacuum and an oily viscous product was obtained. The product was then further purified using ball-tube distillation at 60 °C (till no traces of acrylates were found). The magnetic nanoparticles (MNPs) were prepared by the conventional co-precipitation method and stabilized *in situ* by PEGylated arginine.[9,10] The surface morphology of PA-MNPs, as shown by transmission electron micrographs (TEMs) appears to be spherical and smooth, with mean diameters in the range of 6–12 nm and an average size of ~9 nm. Field dependence magnetization (20 kOe, 300 K) values of bare MNPs and PA-MNPs were found to be 60.3 and 32.9 emu g^{-1}, respectively. The nanoparticles exhibit superparamagnetic behavior without magnetic hysteresis and remanence. X-Ray diffraction showed characteristic diffraction peaks that is well indexed to the inverse cubic spinel structure of Fe_3O_4 (crystallite size ~8.7 nm). The zeta potential of PA-MNPs was measured as a function of the pH and a high negative value indicated a stable

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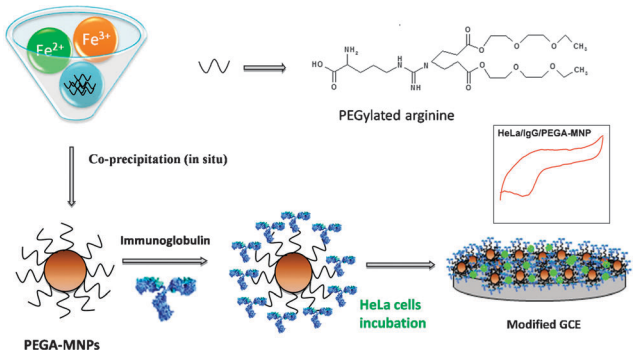

Scheme 1 Schematic representation of fabrication of PA-MNPs based biosensor.

suspension which originates from the binding of PEGylated arginine on the surface of iron oxide nanoparticles. The electrochemical impedance (EI) measurements of the fabricated electrodes were measured by a (Gamry EIS 300) instrument in a single compartment three-electrode cell where bare or modified GCE was working electrode, platinum foil was counter electrode and Ag/AgCl (satd. KCl) was the reference electrode. Prior to electrode modification, PA-MNPs were incubated with immumnoglobulin G (IgG) for 6 h at room temperature. HeLa cells were also incubated for 24 h in Minimum Essential Media (MEM). Fluorescence-activated cells sorting (FACS) was used to determine the distribution of cells in each phase of the cell cycle (Fig. S1, ESI†). The electrodes were then modified to obtain PA-MNPs/GCE, IgG/PA-MNPs/GCE, HeLa/IgG/GCE and HeLa/IgG/PA-MNPs/GCE electrodes. The synthesis of PA-MNPs and modification of electrodes are depicted in Scheme 1. After immobilization, the electrodes were taken out; air-dried and electrochemical measurements (cyclic voltammetry and electrochemical impedance spectroscopy) were performed. The selective response of the modified electrodes was also studied with breast cancer cells (MCF-7) and normal mouse fibroblast cells (L-929) (Fig. S2, ESI†). To see the effect of drugs on the cancer cells, cyclic voltammetry were carried out with various anticancer drugs *viz*., doxorubicin (DOX), methotrexate (MTX) and hydroxyurea (HU).

The inoculated HeLa cells were allowed to attach and grow for 24 h on the IgG/PA-MNPs/GCE electrode surface and the effect of varying concentrations of DOX onto the HeLa cells immobilized electrode was studied. It was observed that the reduction peak at +0.3 V (corresponding to the reduction of the disulfide bonds of the cell proteins[11]) disappeared on addition of 100 μg of DOX. With further addition of DOX, a shift in the peak potential with decrease in peak current was observed (Fig. 1).[12] This suggests an efficient uptake of the DOX by the cells on application of external potential.[13] Confocal imaging of the cells incubated with DOX-PA-MNPs showed internalization of the DOX in the cytoplasm as well as in the nuclei of the cells within 3 h of incubation (Fig. 1 (inset)).

Similarly, the effect of methotrexate (MTX) and hydroxyurea (HU) on the cancer cells was also studied electrochemically. The positive shift of the peak current of the immobilized Hela electrodes along with a decrease in peak current of the control

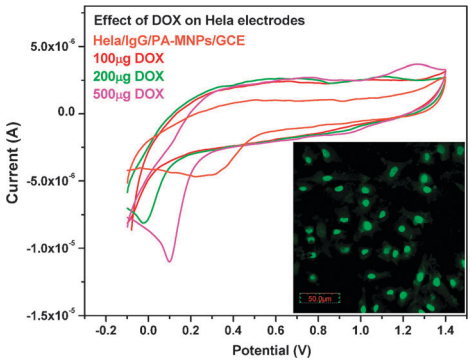

Fig. 1 CVs of HeLa cells treated with varying concentration of DOX and confocal image of cellular internalization of DOX-PA-MNPs (inset); Magnification is 60×.

MTX (reduction of the amino group appears at +0.2 V[14]) depicts specific binding of the MTX with the Hela cells (Fig. S3, ESI†). This also indicates a reduction in cell viability, probably due to the fact that MTX decreases the expression of guanine.[15] Similar observations were noticed in the cyclic voltammograms of HU (Fig. S4, ESI†). Internalization of drug-loaded magnetic nanoparticles within the cells was observed with a confocal microscope. The images (insets of Fig. S3 and S4, ESI†) clearly show that MTX-PA-MNPs and HU-PA-MNPs are uptaken by the nuclei as well as the cytoplasm in sufficient quantities within initial 3 h of incubation. In both the cases, the strong fluorescence also shows change in cellular morphology, with disruption of cell membrane ultimately leading to cell death.

EI measurements were carried out at open circuit voltage in PBS (pH 7.0) solution in the frequency range of 0.1–10^5 Hz by applying an alternating current signal of 10 mV amplitude and the resulting impedance plots were fitted using a REAP2CPE suitable model. The diameter of the depressed semicircle in the high frequency region of the Nyquist plot of bare GCE increases following each modification from PA-MNPs/GCE to IgG/PA-MNPs/GCE to Hela/IgG/PA-MNPs/GCE (Fig. 2).

The bilayer capacitance (C_{DL}) and polarization resistance of the electrode (R_P) depend on the dielectric and the insulating features at the electrode/electrolyte interface, respectively, which changes with electrode modification. R_P increased from

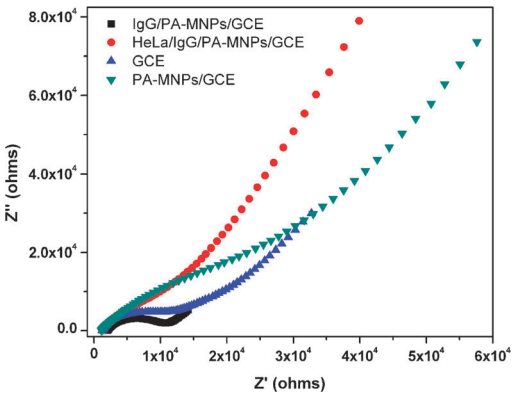

Fig. 2 Nyquist plots of (EIS) for different modified electrodes in 0.01 M PBS (pH 7.4); 100 kHz –0.1 Hz.

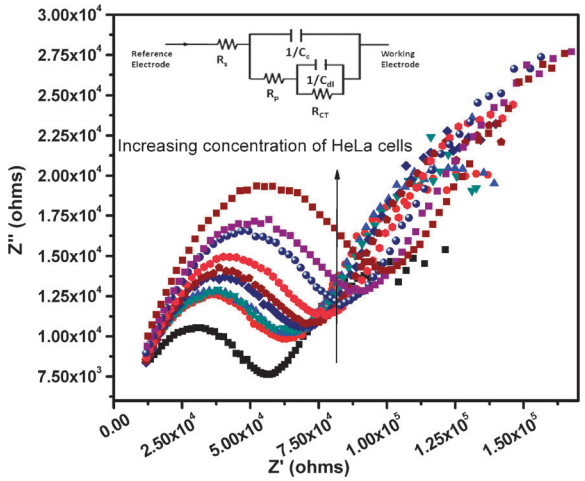

Fig. 3 Nyquist plots of IgG/PA-MNPs modified electrode with varying concentration of HeLa cells in 0.01 M PBS (pH 7.4); the inset shows equivalent circuit used to model impedance data.

9.39 to 22.35 kΩ when PA-MNPs were coated on the bare GCE implying higher blocking effect. However, electrodes coated with IgG incubated PA-MNPs showed a decreased value of R_{CT} which may be due to the formation of IgG–PA-MNP complexes on the electrode surface, perturbing the double charged layer. Further, the increase in R_P and C_{DL} values of HeLa/IgG/PA-MNPs/GCE show increase in specific binding of the IgG with the HeLa cells.[16] As the cells grow adherently onto the electrode, the available electrode area for electric current to pass is reduced, thereby increasing the interface impedance.[17] Possibly, the capture of the HeLa cells on the IgG/PA-MNPs electrodes creates a barrier for the electrochemical process which increases the electron transfer resistance. Thus, it suggests that the recognition bilayer is successfully immobilized and the bioactivity in the system is well-retained.

EIS was also applied to investigate the interfacial properties at the modified electrode with different concentrations of HeLa cells. The IgG incubated PA-MNPs were deposited on the GCE and impedance measurements were carried out to detect HeLa cells in suspension in PBS at a frequency ranging from 10^5 Hz to 0.1 Hz. Known volumes of HeLa cells (stock solution 1×10^5 cells mL^{-1}) were added to the PBS suspension and impedances were measured at a regular interval of time.

The Nyquist diagram (Fig. 3) of the modified electrode showed an increase in R_P values (from 57 to 98 kΩ) with increase in concentration of HeLa cells in solution. Increase in R_P values indicates higher attachment of HeLa cells on to the electrode surface. The inset of Fig. 3 shows the generalized equivalent circuit of the modified electrode where the cancer cell-IgG/PA-MNPs/GCE interface is represented by the double-layer capacitance (C_{dl}) in parallel connection with a charge-transfer resistance (R_{CT}). The electrochemical impedance signal was found to be directly related to the amount of cells attached to the electrode and the R_P value was proportional to the logarithm of the HeLa cell concentration ranging from 4×10^3 to 4×10^4 cells mL^{-1}. A good linear relationship was obtained with a correlation coefficient of 0.97404 while the limit of detection was calculated to be 10 cells mL^{-1} (S/N = 3). The R_{CT} values were found to increase (from 89 to 175 kΩ) with increasing concentration of cells from 5×10^2 to 5×10^4 cells mL^{-1} and a linear dependence was observed between the R_{CT} values and the logarithmic values of cell concentration. The broad detection range, low detection limit, low cost and simple fabrication process of the novel biosensor made it well suited for early detection of HeLa cells.

The electrochemical observations demonstrated that the PEGylated arginine functionalized magnetic nanoparticles could accelerate the electron transfer thereby enhancing the electrochemical detection of cancer cells. After HeLa cells are exposed to antitumor drugs, the response of the immobilized HeLa cells show obvious change, which can be used for monitoring the growth of tumor cells and evaluating the cytotoxicity of antitumor drugs. The developed impedimetric biosensor provides a simple, yet highly sensitive and selective strategy for the early detection of cervical cancer, in terms of broad detection range and low detection limit.

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