Poly-L-lysine/hydroxyapatite/carbon nanotube hybrid nanocomposite applied for piezoelectric immunoassay of carbohydrate antigen 19-9

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Hybrid composites are of special scientific interest for biochemical applications wherein the abilities to modulate the morphology and property of the hybrid material are important. In this paper, the formation of poly-L-lysine/hydroxyapatite/carbon nanotube (PLL/HA/CNT) hybrid nanoparticles is described and a general design strategy for an immunosensing platform has been proposed on the basis of PLL/HA/CNT nanocomposite adsorption of antibodies. Quartz crystal microbalance (QCM) used as a model transducer and the detection performances of the resulting immunosensor were investigated by use of the immuno-system of carbohydrate antigen 19-9 (CA19-9), an important indicator in the diagnosis of clinical cancers. The hybrid nanocomposite was characterized by the transmission electron microscope (TEM), scanning electron microscope (SEM) and Fourier transform-infrared (FT-IR) spectrum measurements. The frequency response characteristics for the processes of immobilization and immunoreaction of anchored anti-CA19-9 antibodies were studied in detail. It was found that the developed sensing interface has some advantages such as the activation-free immobilization and the high antigen-binding activities of antibodies. The as-prepared immunosensor can allow for the determination of CA19-9 in the concentration range of around 12.5–270.0 U ml^{-1}. Such an interface design with the hybrid nanocomposite should be tailored as a new alternative used for biosensor design.

1 Introduction

A considerable interest in the synthesis of novel multi-components hybrid materials with improved properties and performances such as permeability, solubility *etc.* exists in chemistry and material science. The organic–inorganic hybrid nanobiomaterials with unique physical and chemical properties, when utilized in conjunction with their remarkable biomolecular recognition capabilities, could give access to a new field of research with promising applications in biosensors. Hydroxyapatite [HA, $Ca_{10}(PO_4)_6(OH)_2$] as a potential biomedical material is a major inorganic component of bone with excellent biocompatibility and, therefore, does not stimulate the immune system directly and nor does it cause any systemic toxicity if a small amount is re-absorbed. Numerous investigations have been made on its structure properties and possible applications as biomaterials.[1,2] However, the usage of pure HA-based materials in most cases is limited due to their instability, brittleness and insolubility. Recently, the synthesis of HA using different matrices such as nanoparticles,[3,4] polymers[5,6] and nanotubes[7,8] has received much attention to overcome these problems. Moreover, since the first observation by Iijima,[9] carbon nanotubes (CNT) have been the focus of considerable research because of their special structure features and unique electronic and mechanical properties in the construction of biosensors.[10,11] They have also been widely used as a nanomatrix for the controlled synthesis of nanostructures.[7,12,13] For example, Aryal *et al.*[13] reported the synthesis of HA using carboxylated CNT and showed that the carboxylated CNT were capable of creating nucleation sites for HA with superior mechanical properties.

To extend the application of HA, we report here another facile pathway for functionalizing HA directly with CNT and hydrophilic poly-L-lysine (PLL) for the first time, to our best knowledge, as potential bioactive nanobiocomposite scaffolds. PLL, a kind of polypeptide, has plentiful active amino groups, and as the linker, it has several advantages including good biocompatibility, a flexible molecular backbone and relatively good solubility in water. Hence, the PLL-based assembly with plentiful amino groups and carboxyl functional groups will open the way to a large number of opportunities in the preparation of nanocomposite and bioactive molecular attachment.

Quartz crystal microbalance (QCM) biosensors have been widely applied to study biomolecular interactions and bioassay due to the primary advantages such as high sensitivity, real-time output, label- or radiation-free entities and simplicity.[14] The general preparation of such a device involves constructing an immunosensing platform to immobilize either antibody or antigen by chemical or physical treatments. Some interfacial designs using conventional methodologies, unfortunately, suffer from lowered sensing performance due to the difficulty in obtaining a homogeneous biocompatible film with good adhesion abilities to the transducer components. Therefore, exploring an improved immunosensing platform with high loading and bioactivity of the biomolecules for piezoelectric immunosensors is of considerable significance.

In this paper, we have initiated a program to synthesize hybrid biocomposite scaffolds of PLL/HA/CNT nanoparticles

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according to the precipitation method. The PLL/HA/CNT hybrid nanocomposite scaffolds can be utilized to act as an 'affinity template' for protein immobilization. An improved biomolecular immobilization method by using the PLL/HA/CNT hybrid particles has been developed for a QCM immunosensor. A model immunological system was tested for carbohydrate antigen 19-9 (CA19-9), an important carbohydrate tumor marker expressed in many malignancies such as pancreatic,[15] colorectal[16] and hepatic carcinomas.[17] The antibody immobilization performances of the sensing interface with the hybrid particles were investigated. The analytical characteristics and merits of the newly developed immunosensor are discussed in detail.

2 Experimental

2.1 Apparatus

The quartz crystal microbalances (AT-cut, 9 MHz, gold electrode) were supplied by Chenxing Radio Equipments (Beijing, China). One side of the crystals was sealed with an O-ring of silicon rubber covered by a plastic plate forming an air compartment isolated from the aqueous solution in order to stabilize the oscillation frequency in the solution. The one-side-sealed QCM was mounted in a laboratory-made reaction cell, where the test solution was gently agitated by a magnetic stirrer (Model JB-2, Shanghai, China). The resonance frequency was monitored with a quartz crystal analyzer (QCA 922, Princeton Applied Research, USA). The experimental temperature was controlled with a thermostat (model CSS501, Chongqing Experimental Equipments, China). Transmission electron microscopic (TEM) images were taken by a JEM-3010 electron microscope (JEOL, Japan) and copper grids were purchased from Scientific Instruments of the Chinese Academy of Sciences (Beijing, China). Scanning electron microscope (SEM) images were carried out by a JEM-6700F electron microscope (JEOL, Japan). The FT-IR spectrum of the hybrid nanocomposite was recorded *in situ* by a Nicolet Fourier transform-infrared spectrometer using a vacuum cell capable of controlling temperature and introducing gas at a desired pressure.

2.2 Reagents and materials

3-Mercaptopropionic acid (MPA) was the product of Acros Organics (USA). *N*-Ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide (EDC), *N*-hydroxylsulfosuccinimide (NHS) and poly-L-lysine with a molecular weight of 15 000–30 000 were obtained from Sigma. Multi-wall carbon nanotubes (CNT) purchased from CNT Co. Ltd. were purified by washing with concentrated hydrochloric acid and nitric acid. Calcium nitrate tetrahydrate [$Ca(NO_3)_2{\cdot}4H_2O$], diammonium hydrogen phosphate [$(NH_4)_2HPO_4$] and ammonia [$NH_3{\cdot}H_2O$] were supplied by Henan Jiaozuo Chemicals (Henan, China). Mouse anti-CA19-9 antibody was obtained from Zhongshan Biological Products (Beijing, China). Purified CA19-9 antigen of human serum, positive serum samples of cancer patients and normal human serum were provided by the Hunan Provincial Tumor Hospital (Hunan, China). Bovine serum albumin (BSA) and human serum albumin (HSA) were purchased from Shensuo Biological Products (Shanghai, China). Phosphate buffered saline (PBS) solution with various pHs was prepared using 0.01 M Na_2HPO_4 and 0.01 M KH_2PO_4. All other reagents were of analytical grade. All the solutions were prepared with doubly distilled water.

2.3 Preparation of poly-L-lysine/hydroxyapatite/carbon nanotube hybrid nanocomposite

Poly-L-lysine/hydroxyapatite/carbon nanotube (PLL/HA/CNT) nanocomposites were synthesized by the *in situ* chemical precipitation method using PLL, $Ca(NO_3)_2$, $(NH_4)_2HPO_4$ and CNT at ambient temperature and atmospheric pressure according to the literature reports.[7,18,19] Carboxyl group-functionalized CNT (CNT–COOH) were firstly synthesized by ultrasonicating CNT with nitric acid for 5 h to get a homogeneous mixture. Then the mixture was centrifuged, washed with ethanol and distilled water, and finally dried under vacuum for 30 h at 50 °C. CNT–COOH thus obtained were used for the synthesis of the PLL/HA/CNT hybrid material. In a typical experiment, aqueous 0.5 M $Ca(NO_3)_2$ and 0.5 M $(NH_4)_2HPO_4$ solutions were prepared with pHs adjusted to 9.3 and 12.0, respectively. When 41.5 ml of $Ca(NO_3)_2$ solution was added to a round-bottomed flask and stirred at 1200 rpm at the reaction temperature, 25 mg CNT–COOH and 10 mg PLL were then added and violently stirred for 30 min. Following that, 25 ml of 0.5 M $(NH_4)_2HPO_4$ solution was added at a rate of 4 ml min^{-1}. After the addition of the solution ended, the reactants were stirred for another 24 h and the color of the reaction mixture had changed from black to grey, then the suspension was left to settle for 24 h. The resulting mixture was centrifuged, washed with ethanol and water and finally dried at 90 °C in an oven. The nano-sized PLL/HA/CNT particles were of the form observed from TEM images.

2.4 Preparation of CA19-9 immunosensor

Prior to the modification and measurements, each of the piezoelectric quartz crystals was cleaned in fresh piranha solution (70% H_2SO_4, 30% H_2O_2) [**warning:** piranha solution reacts violently with organic solution] followed by rinsing with water. Then, a self-assembled monolayer was formed on the surface of the quartz crystal by immersing the crystal into a solution of 0.01 M MPA for 12 h. Thereafter, the MPA-coated crystal was immersed in a mixed solution of 0.2 M EDC and 0.05 M NHS for 1 h at 37 °C to ensure that the carboxyl groups on the crystal surface were fully activated. Then, the crystal was washed with distilled water and air-dried. Following that, 30 μl of 7.5 mg ml^{-1} PLL/HA/CNT solution (pH 7.0) was dropped onto the crystal surface to be incubated for 2 h at 37 °C. After that, the crystal was washed with PBS of pH 7.0 and water. Subsequently, 30 μl of the anti-CA19-9 antibody solution (2 : 5 dilution ratio) were incubated on the crystal surface for 1 h at 37 °C, washed again and dried in air, then 30 μl of 10 mg ml^{-1} BSA were introduced accordingly to block the non-specific binding sites on the crystal surface. After that, the device was washed and air-dried.

2.5 QCM measurement procedure

Each of the prepared QCM probes was inserted into the reaction cell containing 3.0 ml of buffer solution (PBS, pH

7.0). Under gentle stirring and thermostatic control at 25 °C, each 30 μl aliquot of CA19-9 analytes of different concentrations was introduced into the reaction cell after the stabilization of the resonance frequency (shift less than 2 Hz min^{-1}). In order to avoid the error resulting from different additions of CA19-9 serum, the frequency changes of the crystal were started to be recorded as the immunoreaction proceeded 30 s after the addition of analytes until equilibrium was reached. The control test using HSA and the sample analysis were performed accordingly. The frequency changes in all the experiments referred to the frequency changes of the immunoreaction (ΔF), unless otherwise indicated.

3 Results and discussion

3.1 Preparation and characterization of poly-L-lysine/hydroxyapatite/carbon nanotube hybrid nanocomposite

According to the literature,[20] modification of CNT by chemical oxidation generally results in the introduction of various functional groups, such as –COOH and –OH, which can act as nucleation sites for metal ions such as Ca^{2+}. Similarly, PLL containing carboxyl groups can also provide nucleation sites for Ca^{2+}. In addition, PLL has plentiful active amino groups, some of which could form surface hydrogen bonds with the P–OH groups of HA. Therefore, HA was synthesized by reacting $(NH_4)_2HPO_4$ with Ca^{2+} bound to carboxylate ions of the CNT and PLL matrix.[19,20] TEM images of the CNT (Fig. 1A) and PLL/HA/CNT hybrid composite (Fig. 1B) were taken by dipping copper grids coated with carbon film in a dispersion of CNT and PLL/HA/CNT in acetic acid, respectively. Comparing Fig. 1A with Fig. 1B, we can find that multilayer HA crystals were formed over the surface of the carboxyl-functionalized CNT and PLLs were entangled on the matrices. The present biocomposite scaffolds have a well-interconnected pore network structure and a large surface area necessary for biomolecular attachment.

In addition, the formation of the PLL/HA/CNT hybrid material was identified by analyzing its FT-IR spectrum (Fig. 2). From Fig. 2, it can be seen that the peaks were attributed to the v_2 (876, 1039, 1095 cm^{-1}), v_3 (569, 604, 633 cm^{-1}) and v_4 (470 cm^{-1}) stretching modes of the phosphate group.[13] Besides, the amide I band with a characteristic frequency at 1645 cm^{-1} was mainly associated with the stretching vibrations on carbonyl groups along the polypeptide backbone and was a sensitive marker of polypeptide secondary structure, which indicated that PLL was successfully covalently modified on the hybrid material.[19,21] Moreover, the vibrations of the hydroxyl group of HA and the carboxylate ion of CNT were found at 3440, 3570 and 1456, 1452 cm^{-1}, respectively. These bands and their positions in the FT-IR spectrum indicated the formation of the PLL/HA/CNT hybrid composite.

The surface topologies of the PLL/HA/CNT hybrid composite and the PLL/HA/CNT-antibodies modified crystal were investigated using a scanning electronic microscope (SEM) with images shown in Fig. 3. It can be seen in Fig. 3A that the particles of PLL/HA/CNT are rod-like and close-packed on the surface of crystal, with a very narrow particle size distribution. Fig. 3B represents the SEM of antibodies

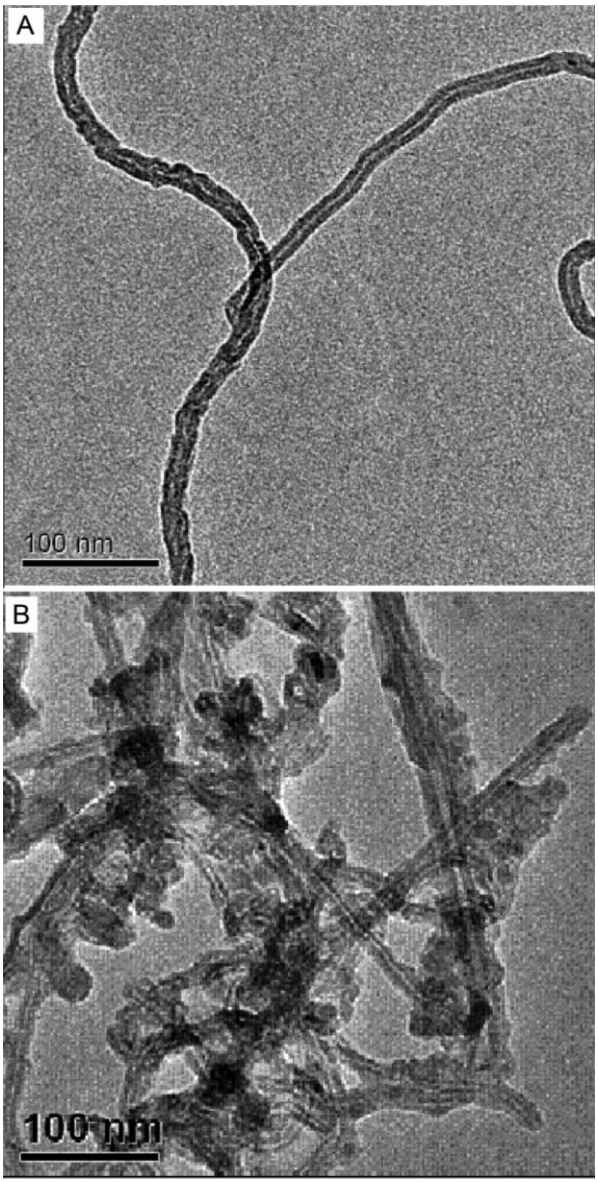


Fig. 1 TEM images of (A) CNT and (B) the poly-L-lysine/hydroxyapatite/carbon nanotube hybrid composite.

immobilized on the PLL/HA/CNT crystal. Compared with Fig. 3A, a considerable change in morphology has been observed, which demonstrated a lot of antibodies immobilized on the PLL/HA/CNT surface.

3.2 Preparation of an antigen-functionalized probe

In current work, the QCM crystal was initially assembled with MPA to generate carboxyl groups on the surface, available to capture PLL/HA/CNT hybrid particles from the solution by covalently linking the amino groups on the surface of the PLL/HA/CNT hybrid particles. Furthermore, with the unique adsorption capability and bioactive properties of HA and the synergic action of the PLL/HA/CNT nanocomposite, it was used to bind CA19-9 antibody molecules, as schematically illustrated in Scheme 1.The interaction between the antibodies and the substrate is mainly by the special

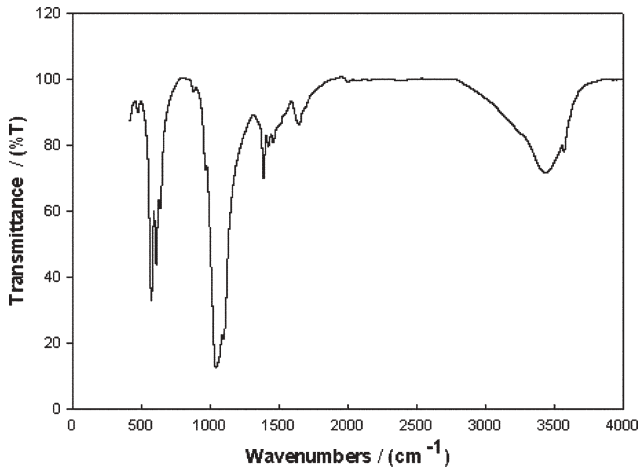


Fig. 2 FT-IR spectrum of the PLL/HA/CNT hybrid composite synthesized at room temperature.

adsorption performance existing between the antibody molecule and hydroxyapatite. This is because the hydroxyapatite and poly-L-lysine possess excellent biocompatibility, and therefore, it does not cause denaturation of the adsorbed antibodies.

3.3 Characteristic frequency responses to immunoreaction

Fig. 4 presents the typical characteristic *in situ* frequency responses to immunoreaction of the sensor prepared using the PLL/HA/CNT particle immobilization procedure. When a portion of 100.0 U ml^{-1} of CA19-9 antigen was tested, the frequency change tended to increase as the immunoreaction proceeded towards the equilibrium (2500 s). The overall frequency change of the probe was about 312 Hz (curve b). Moreover, as is manifested in Fig. 4, when 700 μg ml^{-1} of HSA was detected using the PLL/HA/CNT sensor immobilized with CA19-9 antibodies as the control test, the frequency response caused by non-specific adsorption was trivially low (only about 12 Hz), indicating a favorable selectivity of the proposed immunosensing system.

3.4 Optimization of main experimental conditions

As the concentration of the PLL/HA/CNT hybrid particles immobilized on the surface of the quartz crystal increases, the amount of bound anti-CA19-9 antibodies is expected to increase, thus possibly enhancing the amount of antibody–antigen immuno-complexes deposited on the surface of the crystal. The effect of the concentration of the PLL/HA/CNT particles was investigated over the range of 2.0–10.0 mg ml^{-1}. Fig. 5 indicates that an ideal value of 7.5 mg ml^{-1} could be recommended. This may be attributed to the fact that the lower particle concentration might result in fewer binding sites for antibodies; however, too high a concentration will cause a great increase in the density of the particles, which might result in an increased steric hindrance or inhibition of immobilization of the antibodies. In addition, we chose 2 h for the immobilization time of the PLL/HA/CNT particles to subsequently capture the antibodies by investigating the immobilization time-dependence of the PLL/HA/CNT particles, though the data are not shown here.

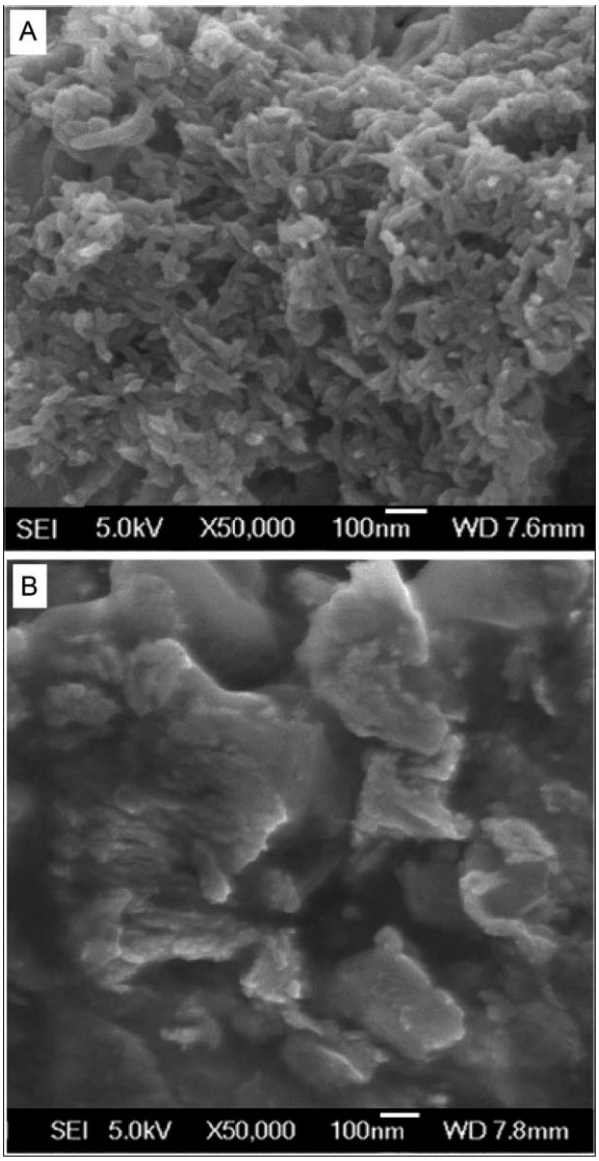
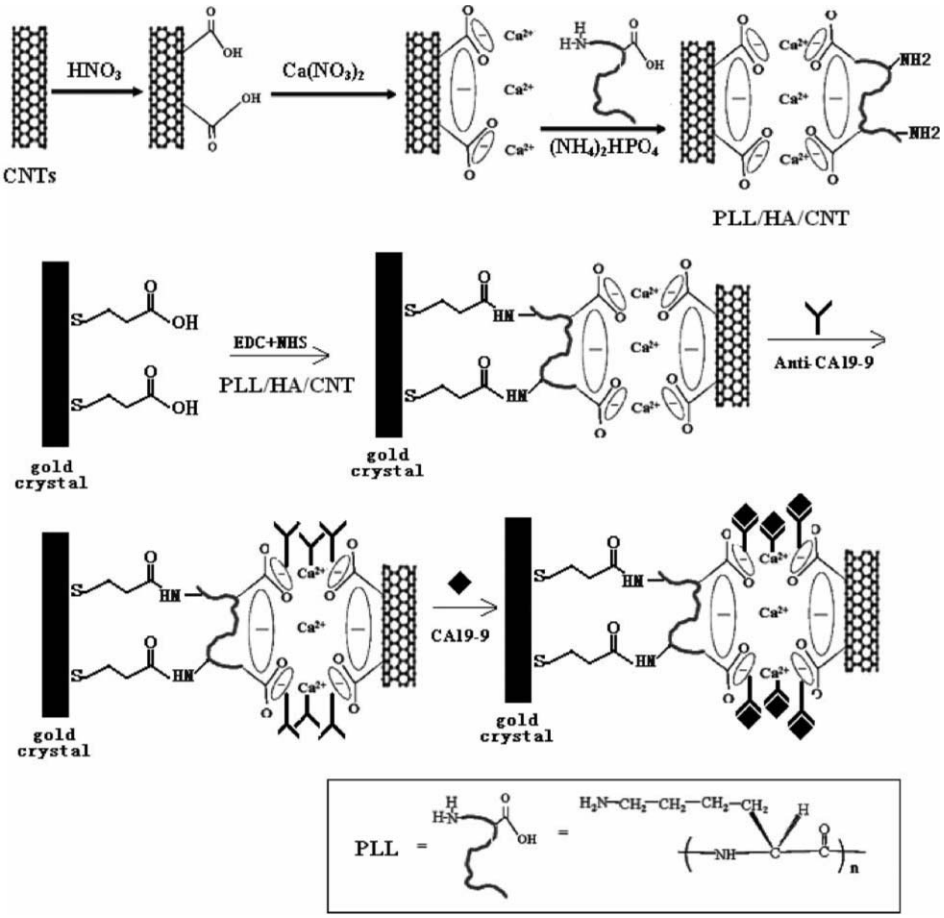


Fig. 3 SEM micrographs of (A) the PLL/HA/CNT composite and (B) antibodies immobilized on the surface of the PLL/HA/CNT modified crystal.

The antibody concentration-dependence of the anti-CA19-9 antibody immobilization was also investigated under the condition of 7.5 mg ml^{-1} of the nanocomposite. The results are illustrated in Fig. 6. As can be seen from Fig. 6, the frequency change increased with an increasing free antibody dilution ratio up to 2 : 5, where a plateau level or 'saturation' appears. Higher titers of antibody might presumably result in increased steric hindrance of the immunoreaction. Therefore, the use of a 2 : 5 dilution ratio of free anti-CA19-9 antibody is preferred. Additionally, it is important to emphasize that a relatively high titer of free antibody in the incubating solution would substantially shorten the time necessary for the adsorptive equilibrium, including an appropriately high temperature, *i.e.* 37 °C.



Scheme 1 Schematic illustration of the synthesis of the PLL/HA/CNT hybrid material and immunosensor design for detection the CA19-9 antigen.

3.5 Analytical performance characteristics

The capability of detecting CA19-9 in human serum with the developed PLL/HA/CNT-based immunoassay was investigated under the optimized conditions. Fig. 7 represents the calibration curve of the relationship between the frequency change (ΔF) of the immunoassay and the concentration of CA19-9. As shown in Fig. 7, the linear detection range for the probe produced using the PLL/HA/CNT hybrid particle

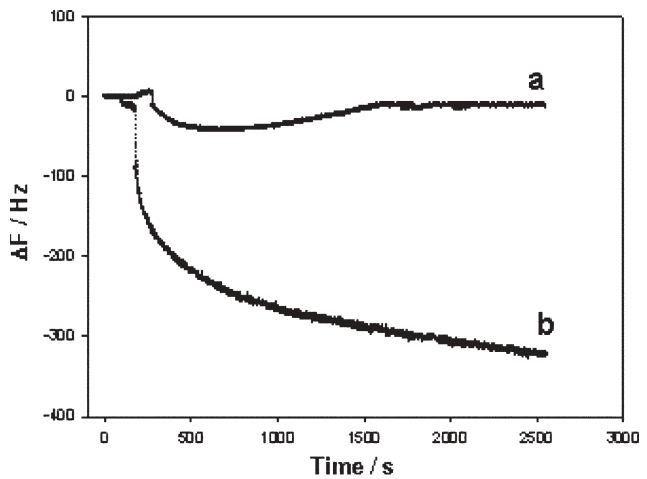


Fig. 4 Typical characteristic frequency responses to CA19-9 serum (100.0 U ml^{-1}) of the sensor prepared using the PLL/HA/CNT immobilization procedure (curve b), with HSA examined as the control test (curve a).

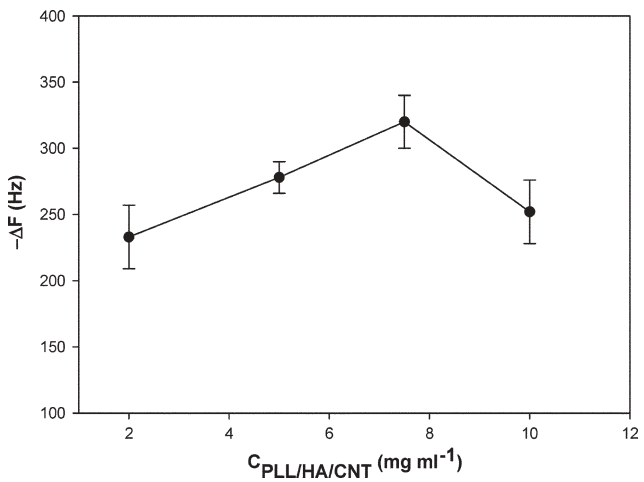


Fig. 5 Effect of PLL/HA/CNT hybrid particle concentration on the frequency change of the immunoassay. The assay for the anti-CA19-9 antibody diluted 2 : 5 with PBS solution using 100.0 U ml^{-1} of the CA19-9 antigen was carried out.

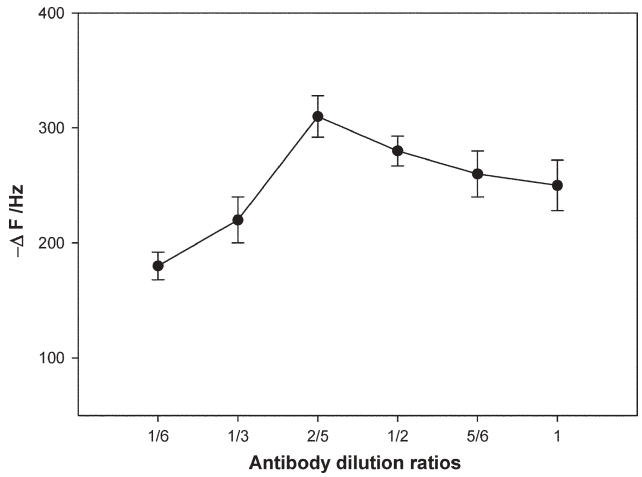


Fig. 6 Effect of antibody concentration in the dilution ratio on the anti-CA19-9 antibody immobilization by the PLL/HA/CNT immobilization procedure under the following conditions: varying anti-CA19-9 antibody dilution ratios at pH 7.0, incubated for 1 h at 37 °C.

immobilization procedure is around 12.5–270.0 U ml^{-1} with a detection limit, estimated according to the 3σ (standard deviation) rule, of about 8.3 U ml^{-1}. In addition, 100.0 U ml^{-1} of CA19-9 serum was repeatedly determined by the PLL/HA/CNT immobilization procedure five times. The relative standard deviation among these five runs is 6.8%.

3.6 Sample analysis

In order to investigate the possibility of the newly proposed immunoassay to be applied for clinical analysis, several real serum samples obtained from tumor patients were examined using the developed piezoelectric immunoassay system. The background value, namely the frequency change of normal human serum corresponding to 50 Hz, has been subtracted from the frequency responses of the positive serum samples. The comparison of results between the proposed piezoelectric immunoassay and the chemiluminescence immunoassay (CLIA) method, which is a conventional method for serum analysis in this region, is shown in Table 1. From Table 1, it can be seen that the results of both methods were in reasonable agreement. Therefore, the developed piezoelectric immunoassay may provide an alternative tool in the detection of CA19-9 in clinical laboratory diagnostics.

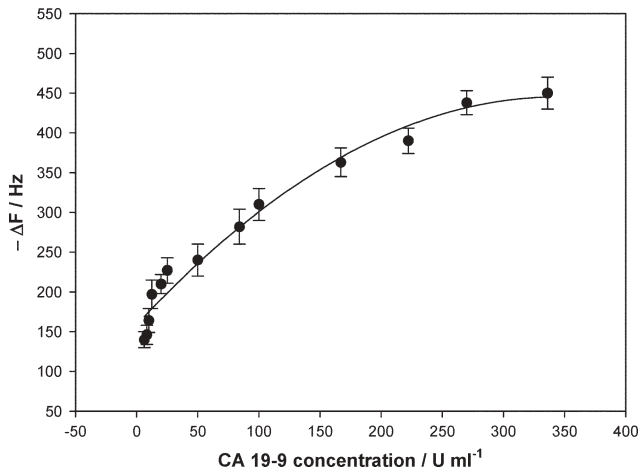


Fig. 7 Calibration profile of the sensor prepared using the PLL/HA/CNT hybrid material binding procedure, corresponding to the fitted linear detection range of *ca.* 12.5–270.0 U ml^{-1}.

Table 1 Comparison of serum CA19-9 levels determined using two methods with an immunosensor and a chemiluminescence immunoassay (CLIA)

Serum sample	1	2	3	4	5	6
Immunosensor/U ml^{-1}	29.3	61.1	113.3	153.7	165.3	213.5
CLIA/U ml^{-1}	33.54	55.83	106.59	167.69	148.61	222.92
Relative deviation (%)	−12.6	9.4	6.3	−8.3	11.2	−4.2

4 Conclusion

In this paper, a novel hybrid material of PLL/HA/CNT has been synthesized by the chemical precipitation method. The hybrid particles were efficient for improving an antibody immobilization methodology. Use of the PLL/HA/CNT nanocomposite scaffolds as the functionalized substrate for the capture of antibodies can guarantee the activity of antibodies in the immobilization matrix. The developed QCM immunosensing system can allow for the detection of CA19-9 serum in the dynamic concentration range of around 12.5–270.0 U ml^{-1}. Remarkably, such an immobilization procedure for antibodies may circumvent most limitations on the substrate size/shape of transducers, indicating an ideal and general immobilization strategy for immunological sensor or chip array design.

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References

1 J. Wilson and S. B. Low, *J. Appl. Biomater.*, 1992, **3**, 123.
2 R. G. T. Geesink and M. T. Manley, *Hydroxylapatite Coatings in Orthopaedic Surgery*, Raven Press, New York, 1993, pp. 1–23.
3 Y. Gupta, G. N. Mathur and S. Verma, *Bioorg. Med. Chem. Lett.*, 2006, **16**, 363.
4 R. Debabrata, S. Mandal and M. Sastry, *Langmuir*, 2005, **21**, 5185.
5 T. Furukawa, Y. Matsusue and T. Nakamura, *Biomaterials*, 2000, **21**, 889.
6 T. Kasuga, M. Noqamic and M. Naqamic, *Biomaterials*, 2001, **22**, 19.
7 S. Aryal, B. K. C. Remant, N. Dharmaraj, K. W. Kim and H. Y. Kim, *Scr. Mater.*, 2006, **54**, 131.
8 A. A. White, S. M. Best and I. A. Kinloch, *Int. J. Appl. Ceram. Technol.*, 2007, **4**, 1.
9 S. Iijima, *Nature*, 1991, **354**, 56.
10 J. Wang and M. Musameh, *Anal. Chem.*, 2003, **75**, 2075.
11 S. Hrapovic, Y. L. Liu, K. B. Male and J. H. T. Luong, *Anal. Chem.*, 2004, **76**, 1083.
12 Y. Chen, Y. Q. Zhang, T. H. Zhang, C. H. Gan, C. Y. Zheng and G. Yu, *Carbon*, 2006, **44**, 37.
13 S. Aryal, S. R. Bhattarai, B. K. C. Remant, K. M. Seob, D. R. Lee and H. Y. Kim, *Mater. Sci. Eng., A*, 2006, **426**, 202.
14 G. Z. Sauerbrey, *Z. Phys.*, 1959, **155**, 206.

15 N. Tanaka, S. Okada, H. Ueno, T. Okusaka and M. Ikeda, *Pancreas*, 2000, **20**, 378.
16 T. Nakayama, M. Watanabe, T. Teramoto and M. Kitajima, *Anticancer Res.*, 1997, 17.
17 T. Uenishi, S. Kubo, K. Hirohashi, H. Tanaka, T. Shuto, T. Yamamoto and S. Nishiguch, *Br. J. Cancer*, 2003, **88**, 1894.
18 L. P. Zhao and L. Gao, *Carbon*, 2004, **42**, 423.
19 Y. J. Zhang, J. Li, Y. F. Shen, M. J. Wang and J. H. Li, *J. Phys. Chem. B*, 2004, **108**, 15343.
20 Z. J. Liu, Z. Xu, Z. Y. Yuan, W. X. Chen, W. Z. Zhou and L. M. Peng, *Mater. Lett.*, 2003, **57**, 1339.
21 V. Thomas, R. D. Derrick, V. J. Moncy, B. Mathew, S. Chowdhury and K. V. Yogesh, *Biomacromolecules*, 2007, **8**, 631.