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A simple MWCNTs@paper biosensor for CA19-9 detection and its long-term preservation by Vacuum Freeze Drying

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ABSTRACT

This paper introduces a cheap simple MWCNTs@paper biosensor for the detection of CA19-9, which is a biomarker of pancreatic cancer. By adding the CA19-9 antibody to the surface of MWCNTs which are deposited on the microporous filter paper, the correlation between the concentration of CA19-9 and resistance of biosensor element was linear due to the site-specific binding of antigen and antibody. The detection range is wide (0U/mL~ at least 1000U/mL), and even in the low concentration of CA19-9, the linearity remains satisfying. Based on this property, it could be used for the detection of early-stage pancreatic cancer. Besides, this research originally introduces a vacuum freeze-drying method for the long-term preservation of biosensor, prolonging its storage time from 3h to at least 7 days, which signifcantly promoted its value in practical application. One piece of the MWCNTs@paper biosensor only cost $2 (about 30 times cheaper than ELISA) approximately, and the detection speed is satisfying (2 hours, 12 times faster than ELISA), which will possibly increase its opportunity of mass production and clinical practice.

Keywords

MWCNTs; paper biosensor; CA19-9; long-term preservation; vacuum freeze-drying

1 Introduction

Early detection of tumour is essential to improve the survival rate of its patients. for example, the pancreatic tumour is a gastrointestinal malignancy that could be hardly diagnosed and cured, and the 5-year survival rate of pancreatic cancer is less than 1%, making it one of the malignant tumours with the highest mortality.

Currently, the most popularized early detection methods of pancreatic cancer are based on the detection of one tumour marker, pancreatic-specific-antigen (CA19-9). Tumour marker CA19-9(Carbohydrate Antigen 19-9, also known as cancer antigen 19-9), can be found in blood, urine, or body tissue. Among the reports about the sensitivity of CA19-9 detection, the detection rate of pancreatic cancer ranks first (up to 90.2%) [1], followed by the biliary duct cancer (67%~86%) [2], the stomach cancer (31.5%~68%) [3,4], the liver cancer (49%~60.9%) [5], and the specificity of detection of pancreatic cancer is also considerably high. These two reasons enable CA19-9 detection to become the widespread detection of pancreatic cancer, even the preferred method in the clinical application. Basically, the normal concentration-level of CA19-9 in healthy human serum is less than 40 kU/L. When the concentration reaches above 120 kU/L, it indicates a high risk of pancreatic cancer.

Generally, the gold standard for detecting CA 19-9 is considered as ELISA [6]. However, this traditional method of CA19-9 detection is time-consuming (about one day), money-burning, and require well-trained personnel. Meanwhile, as for other newly developed technologies, such as the LFI technology combining the technology of thin-layer chromatography and immune recognition reaction, the inspection process requires to be carried out in a well-equipped laboratory because the procedures of detecting have to be performed with high-tech laboratory equipment by professionals due to their complexity[7]. In this way, the diagnosis of pancreatic cancer is tough and unfriendly to possible patients in some undeveloped countries and regions at present.

To solve the problem, electrochemical biosensor, as an efficient and simple method, is now being rapidly developed. It can measure the concentration of target analytes, such as CA 19-9, by converting a biological response into a detectable electrical signal [8]. In comparison with traditional chemical immunoassay methods, recent studies show the high specificity [8,9], speed [9], portability [8~11], and low cost [8,9,11] characteristics of nanomaterials based biosensors offer exciting and promising opportunities for numerous applications, especially clinical applications [12].

According to the reports, CNTs possess unique biochemical characteristics and excellent biocompatibility to be the scaffold

for additional adsorptive biomolecules, which endows CNTs with exceptional biochemical properties. On account of the original electrical conductivity of CNTs, these CNT-based biomolecules combinations possess highly sensitive and selective simultaneous electrochemical characteristics meeting the very needs of biosensors.

The precedented researches have validated the utilization of CNTs in multiple fields of biosensors. For example, one approach is assembling electrochemiluminescence (ECL) of quantum dots with CNTs [13]; another approach to deploying CNTs into biosensors is recognizing and amplifying the electrical perturbation of the combination of SWNTs and biomolecules by field-effect-transistor (FET) [14]. An inspiring innovation that evolved the approach was claimed by Sungkyung Ji [15] taught by Andraka — filtering out the solution of prepared MWNTs-based biomarkers, which leaves residuals turning the filter paper into the sensor. It is budget-saving as well as procedure-simplified. However, the method provided by Sungkyung requires the biosensor to stay wet until the completion of the site-specific reaction between the antibody and antigen [15], which, in other words, means that the biosensor can only be kept for about 3 hours in the typical environment before adding the antigen to be detected. This would be the main disadvantage for putting the experiment result in the market.

Vacuum freeze-drying technology, in this situation, may help to solve the problem. It has widespread valid applications in food and medicine preservation while maintaining the nutrition in the preserved food and the biological activity in some vaccines. During the vacuum freeze-drying process, the water in the sample is rapidly frozen into ice crystals and then sublimated to keep dry, and its physical, chemical and biological properties are unchanged. When the sample needs to be used, adding an appropriate amount of water can recover its functions, which lays a good foundation for the preservation of the paper-based biosensors.

This work, inspired by the work done by Sungkyung Ji et al. [15], introduces a sensitive, inexpensive, and straightforward biosensor designed to detect early-stage pancreatic cancer by conjugating CA19-9 antibodies to MWCNTs, which utilizes the characteristics of antigen-antibody binding and the electrical properties of MWCNTs. When antigens pair with antibodies, the electrical properties of MWCNTs will be altered , thus converting a biochemical signal to an electronic signal. The concentration-resistivity response of the sensor is satisfactorily verified to be relatively linear. The whole process of detecting process only costs two hours, while the cost of production for one sensor element is only two dollars. It achieves accurate, low-cost and rapid detection of pancreas cancer.

Meanwhile, considering that the method provided by Sungkyung cannot offer long-term preservation for the biosensors, in this study, we originally applied vacuum freeze-drying technology to the preservation of MWCNTs@paper biosensor. With the help of freeze-drying technology, the basic structure and functional properties of the freeze-dried materials remain well kept, thus maximizing the activity of the biological tissue and greatly extending the storage time of the biosensor. It will significantly contribute to the mass production, transportation, and storage of the produced biosensor and make its application value greatly improved.

2 Materials and Methods

2.1 Materials

MWCNTs (Multi-walled carbon nanotubes) (20nm in diameter and 5μm in length)
Concentrated nitric acid
Concentrated sulfuric acid
MES (M 2-(N-morpholinyl) ethane sulfonic acid) solution
EDC (N-(3-dimethylaminopropyl)-N'-ethyl carbodiimide hydrochloride) solution
NHSS (N-hydroxysulfosuccinimide sodium salt)
PBS (phosphate buffer) buffer
Tween 20 (polyoxyethylene (20) sorbitan monolaurate) solution
BSA (bovine serum protein) solution
CA19-9 monoclonal antibody
CA19-9
Several microporous filter papers (with a pore size of 0.45μm)

2.2 Methods

This research is based on MWCNTs and microporous filter paper to produce an inexpensive, straightforward and sensitive detector of early pancreatic cancer antigen, CA19-9.

For the preparation of biosensors, carboxylated carbon nanotubes should be dispersed in MES solution containing EDC and NHSS at the beginning, which would activate MWCNTs to bind to CA19-9 antibody. The CA19-9 antibody bound MWCNTs got at this stage was named Ab-MWCNTs. Meanwhile, BSA solution and Tween 20 solution was added for preventing all non-characteristic combinations, which would ensure the valid function of the biosensor. The sample was deposited on the microporous filter paper to obtain carbon nanotube cake and sensor elements, which were cut from the cake. Define the result of this step as air-dried samples to distinguish the sample after freeze-drying.

In order to extend the sensor storage time, the sensor element was processed with the vacuum freeze-drying method, involving freezing, sublimation drying, and desorption drying process. The low-temperature freezing quickly crystallized the solution, and the vacuum condition sublimated the crystal, preserving the structure and the biological activitiesof the samples. The result of this step was named as freeze-dried samples.For the detection, either air-dried samples or freeze-dried samples had the same functional properties. After dropping the CA19-9 antigens on sensors and incubate for a certain time, the electrical resistance of the biosensor changed, demonstrating a proportional correlation to the antigen concentration, which was the detecting basic principle for the MWCNTs@paper biosensor produced in this study.

3 Experiments

The main experimental steps involve the carboxylation of carbon nanotubes, attachment of CA19-9 antibody to MWCNTs, a combination of CA19-9 and Ab-MWCNTs, filtering and cutting process and vacuum freeze-drying. These steps are complicated and time-consuming. Care must be taken during the experiment to accurately weigh solid materials such as MWCNTs and liquid substance such as MES solution. At the same time, safety issues are particularly crucial during the experiment, and experiments must be carried out in strict accordance with the experimental requirements and laboratory safety requirements.

3.1 Carboxylation of carbon nanotubes

The use of MWCNTs to obtain Ab-MWCNTs (with BSA) requires a series of chemical reactions and physical treatment processes, which is shown in Fig. 1. The first step is to the prepare carboxylated carbon nanotubes.

Put 300 mg of MWCNTs into a 100 mL beaker, then stir it and add 7 mL of concentrated nitric acid and 21 mL of concentrated sulfuric acid to it. Then, seal the beaker containing the mixed solution with a parafilm and place it in a water bath sonicator (Xinzhi SB-3200 OTD) for sonic oscillation at 50℃ for 2 hours. After the solution cooled to room temperature, add 300 mL of deionized water into another large beaker, pour the mixed solution slowly into this large beaker. The functionalized MWCNTs were washed and filtered more than ten times with distilled water until the pH was 7. This step allows MWCNTs to be made into MWCNTs (with -COOH) which is shown in the first step of Fig. 1. For determining the acidification of the results, and then use Fourier transform infrared spectrometer (Nicolet iS50) to analyse the MWCNTs (with -COOH) which have been dried for 24 hours in the condition of 100 ℃.

The formation mechanism of carboxylated carbon nanotubes can be illustrated as follows: When a carbon nanotube breaks, its fractured carbon atom is chemically active and will be easily oxidized to a carboxyl group or other groups. Carbon nanotubes with carboxyl groups can be surface-attached to various structural groups or macromolecules after a series of chemical reactions. The addition of nitric acid can not only refine the carbon nanotubes but also make the carbon atoms at the fracture of the carbon nanotubes become more active due to the unsaturated state and can form carboxyl or hydroxyl groups, namely the carboxylation process of carbon nanotubes.

3.2 Binding to the CA19-9 antibody

As shown in the second step of Fig. 1, 2 mg of the MWCNTs (with -COOH) were dispersed in 4 mL of 0.1 M MES solution (containing 0.4 mmol EDC and 0.1 mmol NHSS), and the solution was sonic oscillated in a water bath for 1 min. Then the dispersion liquid was mixed evenly (SCI LOGEX ICC 8). After mixing, the mixture was centrifuged (Plain Instrument TD6) for 10 min (3000 rpm), and the supernatant was removed. Then PBS buffer solution was added to the precipitate to remove excess EDC and NHSS. The above PBS washing process should be repeated for at least five times (this process must clean all the extra EDC and NHSS; otherwise it will cause cracks in the carbon nanotube cake obtained by suction filtration). After the washing process, the mixture was put into the ultrasonic cell crusher for full dispersion for a period of time. In this way, the added 0.5 mL (0.01 mg/mL) of CA19-9 antibody could bind well to MWCNTs. And the combination should be further promoted by magnetic stirring at room temperature for one night.

10 μL of 0.05%Tween 20 solution was mixed in 100 μL of 1% BSA, which would be added to the solution obtained after stirring overnight later. After the mixture solution stood for 1.5 hours at room temperature, centrifuge it for 5 minutes (3000 rpm). Perform the following steps at least 5 times to remove excess CA19-9 antibodies and BSA: remove supernatant, add PBS buffer, and then centrifuge. The product of this process is called is called Ab-MWCNTs. This is shown in the second step of Fig. 1.

The explanation of the role of BSA and Tween 20 in the preparation process is as follows: BSA blocks all non-characteristic binding , thus preventing unwanted surface reactions of MWCNTs (such as random attachment or accidental binding of chemicals on the surface of MWCNTs). In this way, the prepared Ab-MWCNTs can be much purer and will be easily to be combined with CA19-9 validly. As for tween 20, it is a polysorbate-type nonionic surfactant, which serves as an emulsifier to boost the dispersion ability of the MWCNTs in water.

3.3 Test of the air-dried sample

The test of samples involves the following steps:

First, we multiplied a specific kind of membrane which is of 40mm diameter and has an array of 4 18mm×5mm square holes cut out. The upper surface of the membrane is water proof, and the lower surface of the membrane is adhesive to the filter paper, hence preventing the solution percolating through the gaps between the membrane and the filter paper. In the process of suction filtration, the cutouts of the membrane will circumscribe the deposit of the MWCNTs in the cutouts, forming 4 identical sensor elements each filter.

Second, drop a drop of CA19-9 (100 μL) with PBS solution (0 to 1000 ng/mL) on the top of the sensor element and attach it to a printed circuit board (PCB), and then insert the assembly into a 3D printed PCB holder which can evenly press the contact surface between the sensor elements and the PCB, which alleviates the variance of the contact resistance. The sensor element was incubated at 37 ℃ for 1.5 hours and dried in air at room temperature for more than 30 minutes. The sample prepared at this stage was named Ag-Ab-MWCNTs. Then, we used a digital multimeter (KEITHLEY 2000) to measure the resistance from the output of the PCBs, as shown in Fig. 3. Morphological information of MWCNTs, Ab-MWCNTs and Ag-Ab-MWCNTs was observed through scanning electron microscope (SEM), respectively.

3.4 Test of the freeze-dried samples

According to the traditional method, after the sensor elements were prepared, these elements could only be stored in the ambient condition for less than 3h, because the dehydration and other environmental factors that could possibly affect the functioning of sensors will inactivate a large number of antibodies, thus depriving the biosensor of its original valid performance. In this way It is required to initiate a new round of preparation of the testing papers 24 hours ahead of the test, which is both time-consuming and effort-demanding. To address this problem, the vacuum freeze-drying method could be used to enhance the long-time preservation of the sensors. Freeze the sample at a low temperature so that the water in the sample could be crystallized quickly, and the structure of antibodies could be well preserved.

For the freeze-dried sample, the filtering and cutting procedure is consistent with that in the air-dried sample preparation. The main phases of the procedure are shown in Fig. 4, and the additional steps are as follows:

First, we added a usual amount of protein protectant (e.g., trehalose) to prevent protein denaturation in the freezing process ,which could be caused by a variety of stresses, such as low-temperature stress, frozen stress and so on. Next, wait until the temperature of cold trap drops to -30 ℃, and then transfer the samples directly from the room temperature to cold trap to achieve rapid crystallization. Subsequently, keep a suitable average cooling speed (e.g., 1.5℃/min) until the sample was cooled to - 60 ℃, and keep that temperature for at least one night.

Second, remove the frozen sample from the cold trap and place it on the iron frame above the cold trap. Lower the pressure and elevate the temperature at the same time. The suitable average speed of raising temperature should about 0.5 ℃ / min until the temperature got closed to the glass transition temperature. The pressure should drop to a relatively low level at this time. Then the sublimation drying process is finished, which might last approximately two hours.

Third, slowly rose the temperature to room temperature at a slightly higher speed (e.g., 1.5 ℃ / min) while kept the ambient pressure of the sample at about 6 Pa. This desorption drying process should go on for more than 8 hours. At the end of this step, the sensor elements could be encapsulated and stored in a dry environment at an appropriate temperature, keeping away from light and ready for use.

When it is needed to use the samples, add CA19-9 (100 μL) with the PBS solution on the element. The sample should be incubated at 37 ℃ for 1.5 hours and dried in air at room temperature for more than 30 minutes. The next test procedure for the freeze-dried sample was consistent with that for the air-dried samples.

4 Result and Discussion

4.1 Surface morphology

Figs. 5 (A), (B), (C) show SEM images of carboxylated MWCNTs, Ab-MWCNTs, and Ag-Ab-MWCNTs under SEM (scanning electron microscope), while Figs. 5 (D), (E), (F) show TEM images of them. It can be seen from the figure that the morphology of these three samples is significantly different due to the difference in surface functional groups.

These images were obtained by depositing samples on filter paper and then drying under ambient conditions for more than 2 hours. Fig. 5(A) and Fig. 5(D) show surface morphology of carboxylated carbon nanotubes with no significant damage on the surface, which have an average diameter of 20 nm.

The image in Fig. 5 (B) is an SEM of Ab-MWCNTs layer coated with a layer of CA19-9 antibody and BSA (4 nm in diameter). The observed average diameter of Ab-MWCNTs was approximately 25 nm, which is smaller than the expected diameter (~28 nm) because the protein shrinks under dry conditions. This is also the reason why the morphology of the antibody-bound multi-walled carbon nanotube shown in Fig. 5 (B) shows similarity to the carboxylated carbon nanotube in Fig. 5 (A). Clusters appear in the middle of the SEM image were assumed to be caused by antibody concentration. As for Fig. 5 (E), the MWCNTs in the TEM image were covered by a layer of organic macromolecules, which further proved the combination of MWCNTs and the added CA19-9 antibody.

Figs. 5 (C) and (D) are images of Ag-Ab-MWCNTs, which was added with a high level of CA19-9 (1000 U/ml). In Fig. 5 (C), there is visible arachnoid surface on the membrane. Since the adhesion of CA19-9 on MWCNTs will increase the thickness of the protein layer between nanotubes, the diameter of MWCNT samples will also increase, which is consistent with SEM and TEM images.

4.2 Infrared spectrum

As shown in Fig. 6, it is an infrared spectrum of the carboxylated carbon nanotubes and the Ab-MWCNTs measured by Fourier transform infrared spectroscopy. Apparently, the peak of 1720 cm^{-1} in Fig. 6 corresponds to the C=O bond of the carboxyl group produced by the nitric acid treatment of MWCNTs. This peak value decreased after the CA19-9 antibody was combined with MWCNTs because a simple substitution reaction that forms an amide group will reduce the C=O bond of some carboxyl groups on the surface of the MWCNTs. This substitution reaction between antibodies and the hydroxyl group in the carboxyl group was induced by EDC/NHSS[20], which was added to carboxylated carbon nanotubes before preparing Ab-MWCNTs. The amount of N-H band of the amid group will also increase for the same reason, and the N-H band happens around 1560 cm^{-1}, of which the peak value was increased indeed in Fig. 6.

4.3 Possible detection mechanism

Table 1 Sample resistance test

Three kinds of samples, which are MWCNTs, Ab-MWCNTs, and Ag-Ab-MWCNTs, were deposited through the dedicated filter papers and assembled into the PCB for the resistance test. Their resistances are listed in Table 1.

From the table, we can see that the resistance of MWCNTs, Ab-MWCNTs, and Ag-Ab-MWCNTs are of different magnitudes. The binding of antibody CA19-9 increased the electrical resistance of MWCNTs to 237%.

First, the detection mechanism is based on the sp^2 pi-band of carbon. Since the charge transport occurs through sp^2 pi-band of carbon, the formation of carboxyl groups on the surface of the MWCNTs will decrease the sp^2 pi-band of carbon, leading to an increase of electrical resistance.

Second, the mechanism that the overall resistance elevated takes the tunnelling resistance of the nanotube layer into account. Before dropping the antigens, the resistance of the Ab-MWCNTs samples are already considerably larger than the MWCNTs ones, and it is because the attached proteins inserted the gap between the MWCNTs, which enlarged the gap, and consequently elevated the potential barriers. Furthermore, since the charge beholden by the antibodies are identical within the same solution and same charges are repulsive to each other, the antibody increasingly loosened the structure of the MWCNTs, which impeded the potential barriers [21]. Therefore, after linking proteins (CA19-9 antibody and BSA) on the carboxylated MWCNTs, the overall resistance increased. When the antigens percolated through the porous MWCNTs, by the same reason, the binding with antigens and antibodies would also produce a significant change in the electrical resistance of MWCNTs. This is consistent with our experimental principles and assumptions.

As shown in Fig. 7, the possible sensing mechanism is the site-specific reaction between the attached CA19-9 antibody and CA19-9 in the sensor, while any other random interaction between the MWCNT surfaces is protected by BSA blockade [22].

Site-specific reactions forbid the direct charge transfer between the MWCNTs framework and CA19-9, which ensures the concentration of the charge carrier to be constant [23]. At the same time, the thickness of the insulator layer between the crossed MWCNTs increases because of the attachment of CA19-9. The tunnelling resistance between filaments of MWCNTs occupies the most notable position in the conductivity of MWCNTs, which depends on the thickness and dielectric constant of the insulator layer between crossed MWCNTs. And a small change in the thickness of the insulating layer can lead to a substantial change in the value of the resistance [24]. Therefore, the difference of the concentration of the attached CA19-9 will leads to the variety of the resistance in Ag-Ab-MWCNTs, because the insulating distance between the crossed MWNCTs changes.

4.4 Resistance test at different antigen concentrations for the air-dried samples

As shown in Fig. 8, 0.5 mL of CA19-9 antibody (0.01 mg/mL) was used, different concentrations of antigen were combined, and then the electrical resistances were tested. The UI feature curve of each sample was well linear, while the concentration of antigens which was added to the sensor showed a good linear relationship with the resistance of the sensor, which is shown in Figs. 8(A), (B). From this, it can be seen that within a certain range, the changes of element resistance are positively correlated with the antigen concentration.

The resistance is tested with Ag-Ab-MWCNT elements produced from different batches. Six identical sensor elements were made from one batch at a time and resistance tests were conducted on different concentration levels of CA19-9. In order to exclude the effect of other factors between different batches on resistance value, such as the thickness of the carbon layer and the moisture content, we choose relative electrical resistance (R/R0) as the index of test, where R is the resistance exposed to CA19-9 and R0 is the resistance exposed to PBS buffer solution (zero level CA19-9). Fig. 8(A) shows that the change in relative electrical resistance varies almost linearly over the concentration of CA19-9 in the range of 0 to 1000 U/mL.

For the practical steps in detecting antigen concentrations, in order to eliminate the difference caused by moisture and loss of ingredient in the preparation process, 3-5 pieces of sensor elements should be taken in one batch. By adding antigen of known concentration and measuring resistance, the linear relation curve could be established and used as internal standard, so that sensor elements can detect more accurately in different test environments.

Fig. 8(B) shows the detectability of the sensor at a low CA19-9 concentration range (0 to 40 U/mL). The concentration of CA19-9 in normal organisms should be less than 40 U/mL, and when the concentration is above 120 U/mL, it means high risks of having pancreatic cancer. The area between 40-120 U/mL should be called suspected area. In the figure, the relative resistance is good linear-related with the CA19-9 concentration at a low concentration range. This means that the sensor can detect low concentrations of CA19-9 to detect early stages of pancreatic cancer.

The selectivity of the sensor for CA19-9 is critical in the practical application of the diagnosis of pancreatic cancer, which can be ensured by the usage of monoclonal antibody and BSA. When the CA19-9 coated multi-walled carbon nanotubes were exposed to CA19-9, however, without the CA19-9 active site, their resistance did not change as the normal samples. This again confirmed that the random adsorption of CA19-9 on the walls of multi-walled carbon nanotubes could be prohibited. As reported by others, DNA-

Antibody concentration 0.01 mg/mL		**Antigen concentration** 800 U/mL
Sample	**Resistance (kΩ)**	**Average Resistance (kΩ)**
MWCNTs 1	1.14	
MWCNTs 2	0.99	1.04
MWCNTs 3	1	
Ab-MWCNTs 1	3.64	
Ab-MWCNTs 2	3.45	3.84
Ab-MWCNTs 3	4.43	
Ag-Ab-MWCNTs 1	27	
Ag-Ab-MWCNTs 2	22	25.66
Ag-Ab-MWCNTs 3	28	

(apurinic or apyrimidinic site) lyase, APEX nuclease's influence on the selectivity of the active site of the sensor was tested. The APEX can be accessed from the bulk solution by a simple diffusion process without any additional barriers to the active site of the BSA-coated multi-walled carbon nanotubes [25,26].

Therefore, it can be drawn by analogy that the selectivity of the sensor is derived from a site-specific reaction in which the active site of CA19-9 (antibody-multi-walled carbon nanotubes) interacts only with CA19-9, which, in another word, is selective. However, in practical applications, selectivity for other types of clinical markers should be taken into consideration.

4.5 Resistance test at different antigen concentrations for freeze-dried samples

As showed in Fig. 9(A), freeze-dried samples were stored for seven days, different concentrations of antigen were combined, and then the electrical properties were tested. The curves acquired are well linear related, and within a certain range, the changes of element resistance are still positively correlated with the antigen concentration, just like the traditional samples. This result proved that the samples were still functional after long-time preservation, and the vacuum freeze-drying method is feasible for the preservation of biosensor. As the curve for freeze-dried samples shown in Fig. 9(B), the lower limit of the sensor for antigen concentration detection does not change, while the upper limit was slightly decreased from at least 1000 U/mL to about 400 U/mL due to possible contamination in the preservation environment and partial inactivation of antibodies, but this did not affect the function of the sample in the actual detection for human. Because the CA19-9 concentration in normal human body is at 0-40 U/mL, and when the concentration is above 120 U/mL, it means high risks of having pancreatic cancer. When the detection value reached the current upper limit of 400 U/mL, the high possibility of cancer of the test subject had already been characterized, and further examination should be conducted in the hospital. In the process of sample testing, the non-linear region above 400 U/mL would not interfere with the test results. Therefore, vacuum freeze-drying could be used for the long-term preservation of biosensor samples.

In the Fig. 9 (B), it also demonstrates the difference of the test result between the freeze-dried samples and air-dried samples. The air-dried samples were kept in the normal environment for a long time (24h) and dry naturally (RT). In the vacuum freeze-drying process, samples are frozen directly into solid state, and then the ice crystals are sublimed at the appropriate temperature and vacuum degree to make samples dry. As the fact reveals, the air-dried samples totally lost its detecting function while the freeze-dried samples still have, which means the vacuum freeze-drying method is suitable for biosensors. Because the free water in the freeze-dried sensor is firstly frozen into ice crystals, and pores will be left in the sensor after drying, to ensure that the structure of the sensor will not be damaged. The physical, chemical and biological properties of biosensors are basically unchanged after rehydration. In addition, the freeze-dried paper biosensor can be revived by simply adding antigen to moisten it that shows a good rehydration performance and convenience of usage.

Conclusions

This paper introduces the preparation and the preservation a cheap, straightforward, and sensitive paper-based biosensor for the detection of early-stage pancreatic cancer and illustrates the possible detection mechanism. By adding antibodies of pancreatic cancer marker to the surface of MWCNTs and latterly being deposited on microporous filter paper, this research utilize the characteristics of antigen-antibody binding and electrical propriety of MWCNTs. Antigen concentration can be detected to diagnose early stage pancreatic cancer by using site-specific binding of antigen and antibody to induce resistance changes. In the experiment, the linear relationship between the relative resistance and the concentration of antigen can be confirmed. Its qualitative analysis can be induced by two possible mechanisms mentioned before, but the quantitative interpretation still needs to be further explored. In addition, the freeze-drying method is used to prolong the storage time of paper-based biosensor.

This biosensor has several advantages: With a wide detection range (0U/mL~ at least 1000U/mL) and a wide application range, it can well detect early-stage pancreatic cancer and be extended to other types of cancer. The preparation step is suitable for mass production and is conducive to productization and marketization. The price is quite low: Every carbon nanotube cake costs about $13.77 in Table 2, which means the price of each biosensor is around $2.41. As fpr ELISA, a 96-strip-well ELISA kit, which is around $300, could be used for at (most) about 96-5=91 detections. Hence the average cost of per detection is about $300/91=$3.3, which is higher than that of our proposed biosensor ($2.41).

Table 2 Estimated cost of a biosensor chip

Elements	Material	Price Per Unit	Dosage	Prize	Sum
Senor	MWCNTs (with -COOH)	$54/5g	2mg	$0.0216	$13.77/8 =$1.72
	EDC	$14/5g	77mg	$0.2156	
	NHSS	$233/1g	22mg	$5.1260	
	MES	$14/25g	85.3mg	$0.0478	
	PBS	$68/1L	50mL	$3.4000	
	Tween 20	$59/1L	10μL	$0.0006	
	BSA	$29/5g	1mg	$0.0058	
	Anti-CA19-9 McAb	$99/0.1mg	5μg	$4.9500	
PCB parts	/	/	/	$0.519	$0.519
3D printing parts	ABS	$19.61/kg	8.5g	$0.1652	$0.1652
Total					$2.41

Besides, the short detection period (2 hours, 12 times faster than ELISA), simple operation and long storage time (at least 7 days) make it suitable for the early screening of cancer in less developed areas, with high practical application value and promotion prospect.

Therefore, we believe that this approach could become a new and powerful tool for cancer prevention in the near future.

Conflicts of interest

The authors report no conflicts of interest in this work.

Acknowledgements

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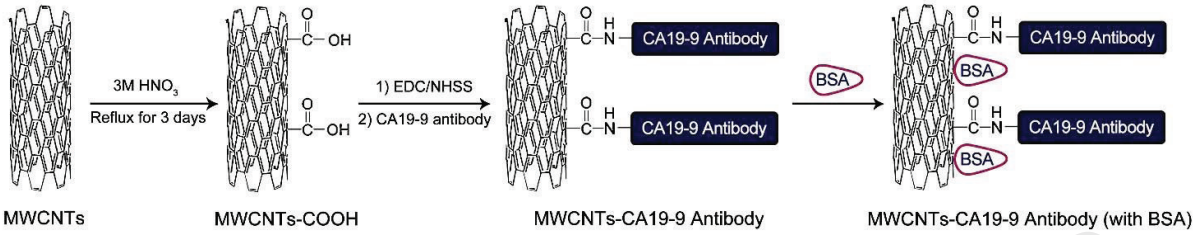


Fig. 1. Preparation of Ab-MWCNTs (with BSA)

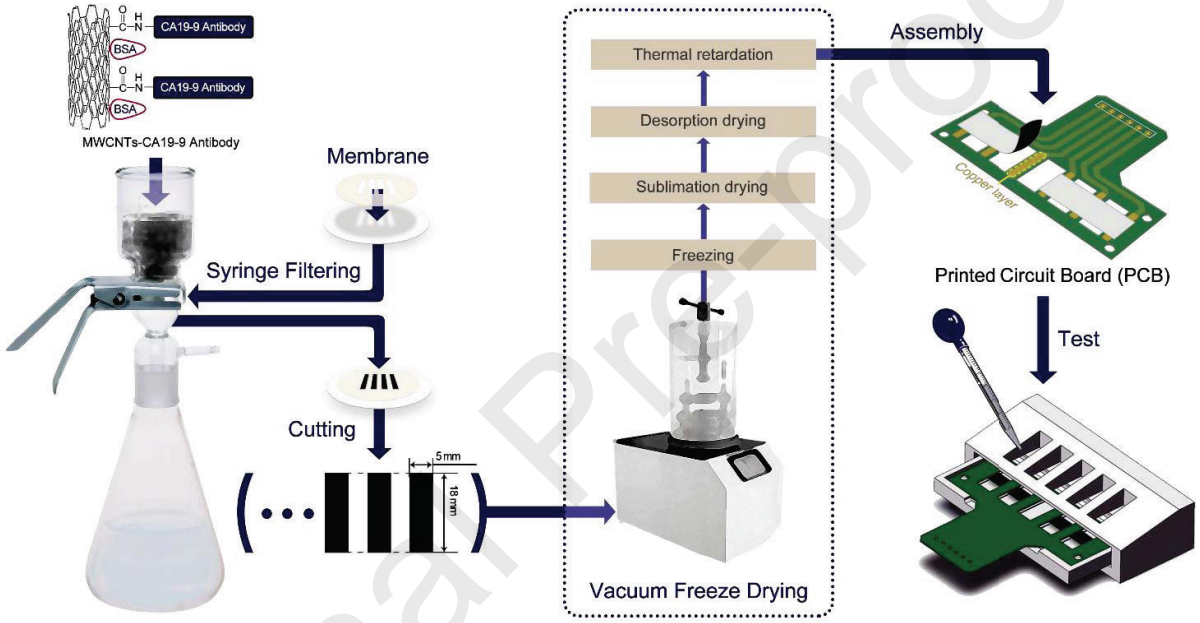


Fig. 2. Preparation of sensing elements and CA19-9 testing procedure

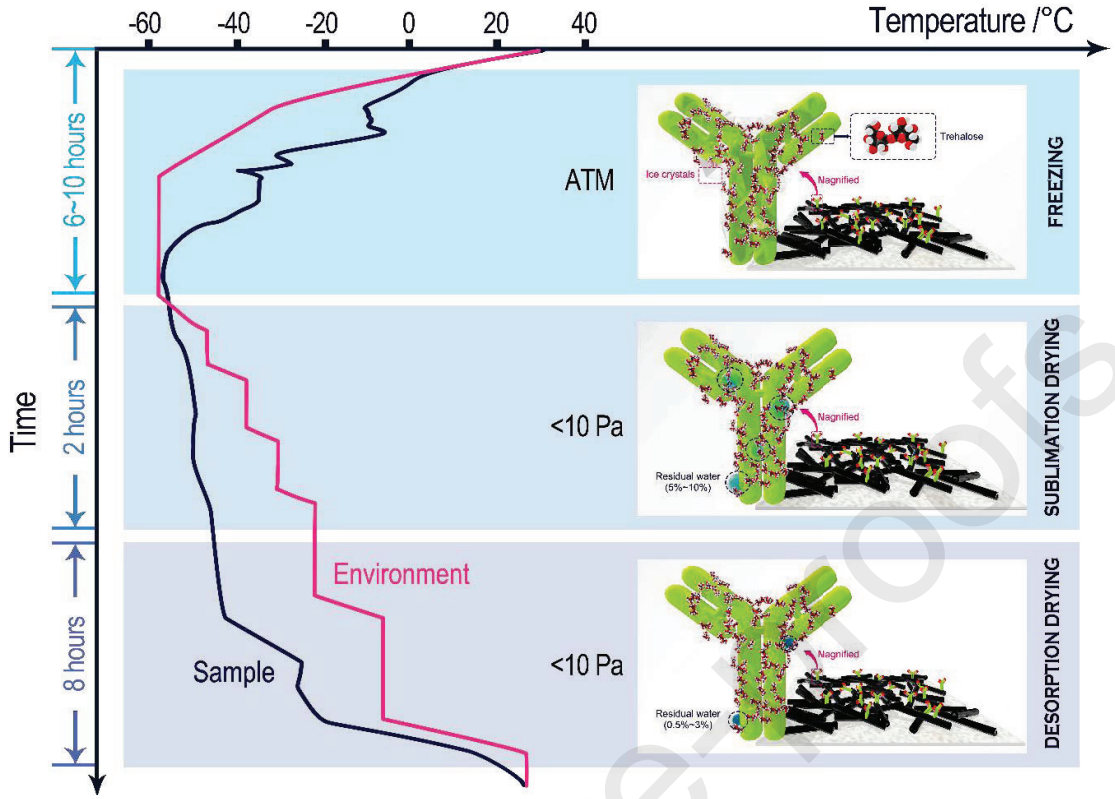
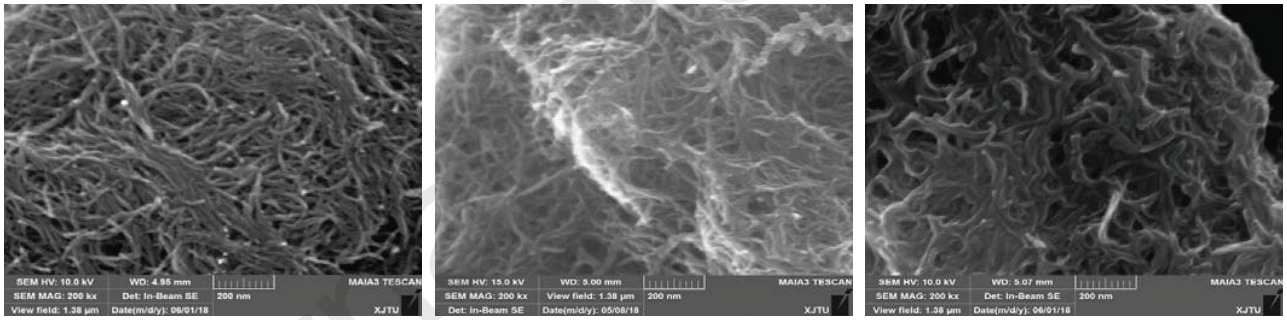
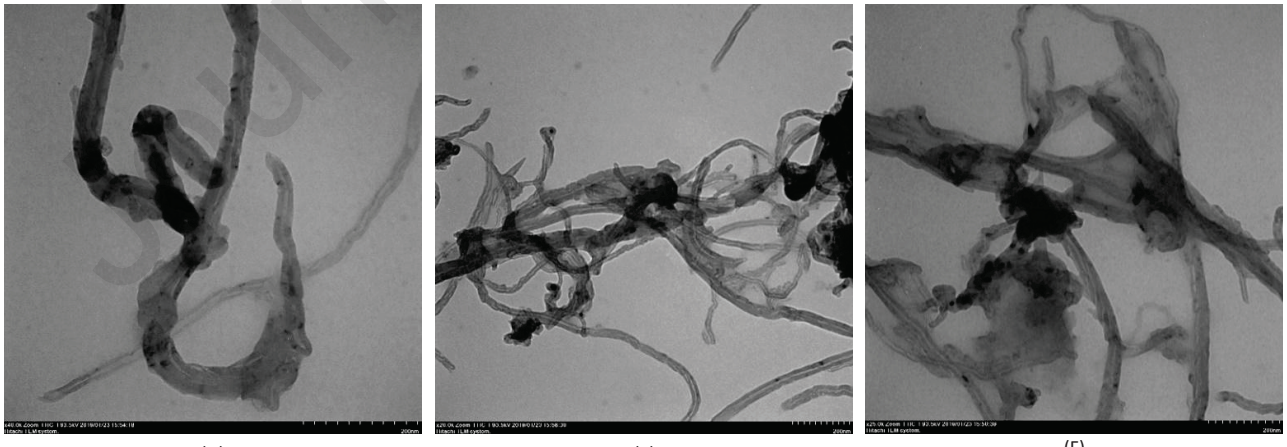


Fig. 3. The procedure of the vacuum freeze drying



(A) (B) (C)



(D) (E) (F)

Fig. 4. The morphology of MWCNTs based sample under SEM and TEM, (A) and (D) are SEM and TEM images of carboxylated MWCNTs, (B) and (E) are SEM and TEM images of Ab-MWCNTs, (C)and (F) are SEM and TEM images of Ag-Ab-MWCNTs

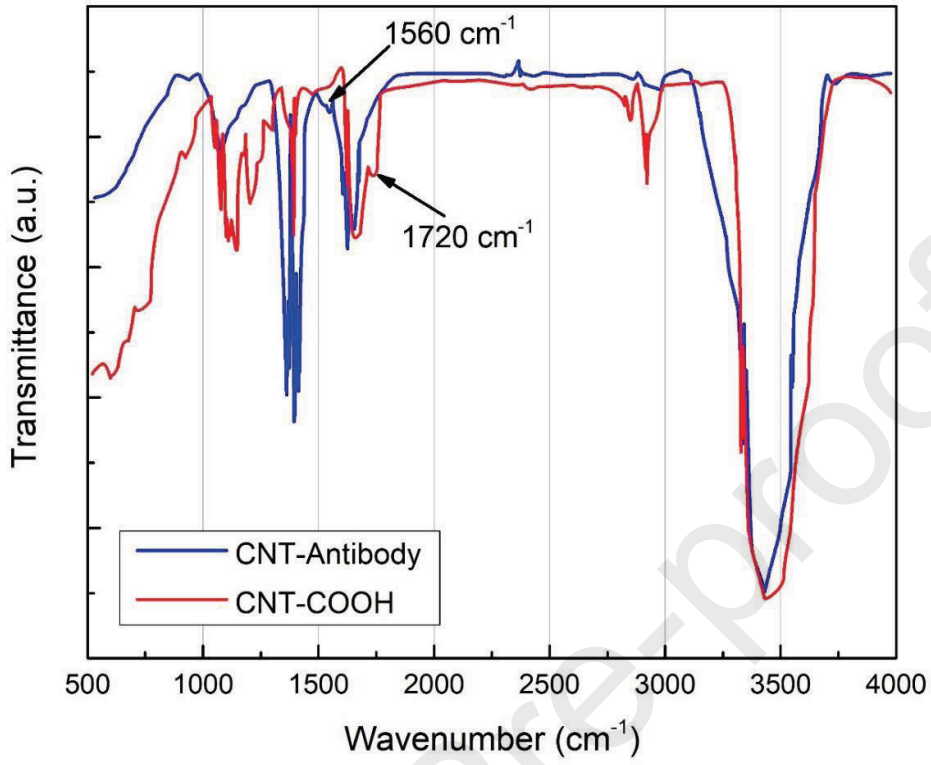


Fig. 5. Infrared spectrogram of CNT-Antibody and CNT-COOH

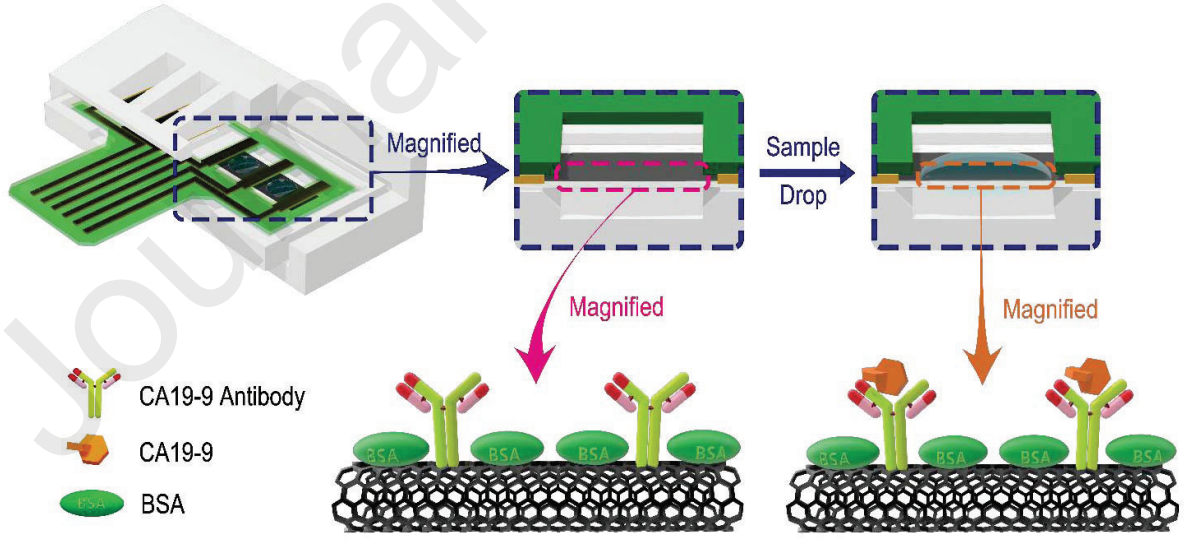
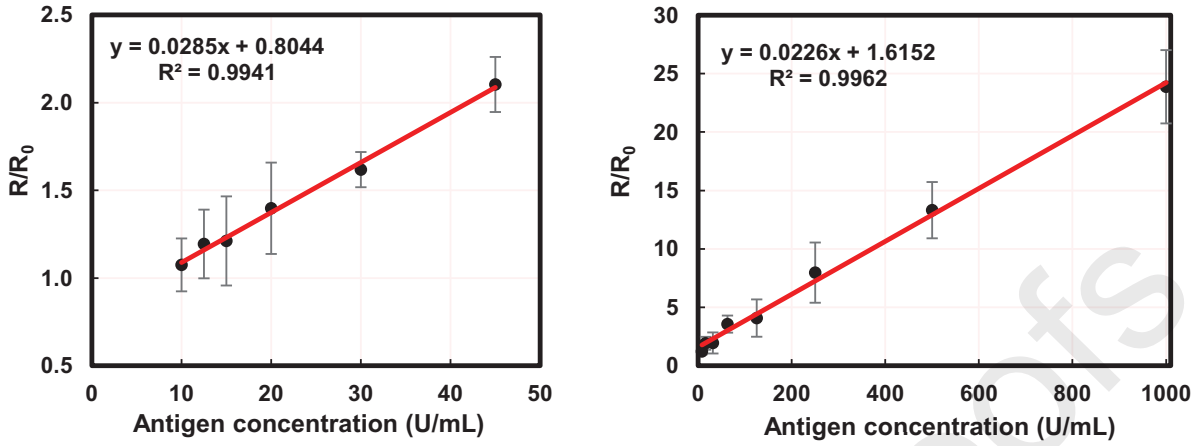
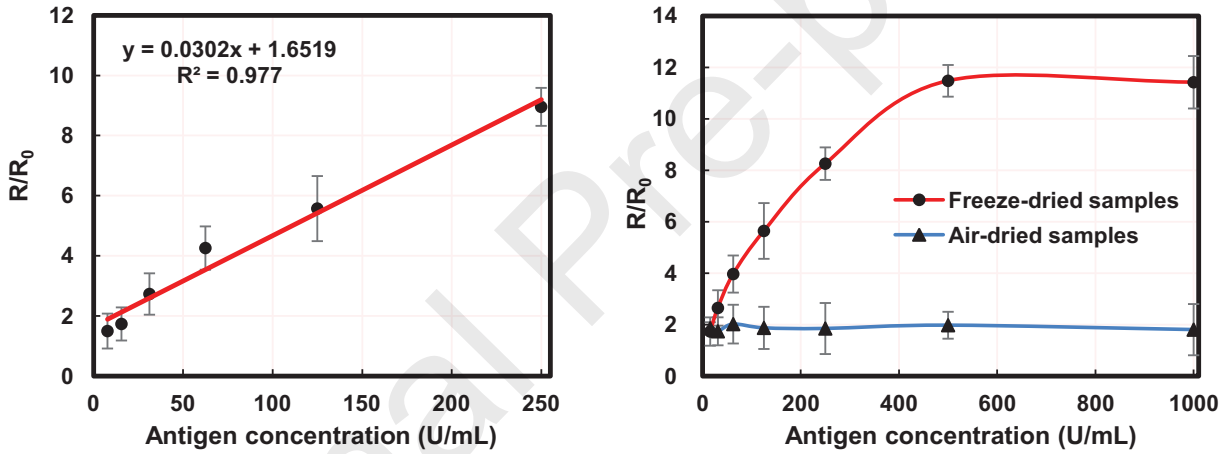


Fig. 6. Highly specific antigen-antibody combination process on the CA19-9 sites in biosensor



(A) (B)

Fig. 7. Results of resistance test for different batches of air-dried samples



(A) (B)

Fig. 8. Results of resistance test, (A) shows results of freeze-dried samples, (B) makes a comparison between freeze-dried samples and air-dried samples