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Functionalized Carbon Nanotube - Modified ELISA for Early Detection of Heart Attack

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ABSTRACT

A warning issue of heart attacks in young adults needs immediate attention lately. Enzyme-linked immunosorbent assay (ELISA) is an easy and commonly used method for detecting early stages of heart attack. Cardiac troponin I (cTnI) is a responsible biomarker for acute myocardial infarction. However, the conventional ELISA system was only able to detect at 100 pM of cTnI. To improve the system, enhancements were introduced through the integration of functionalized carbon nanotube (fCNT) to amplify cTnI detection signals. By utilizing the advantage of fCNT, a noticeable improvement in results can be obtained. The detection limit was lowered down to an impressive 10 pM. Furthermore, the change of absorbance increased from 31.90% for conventional ELISA surge to 98.61 for modified ELISA system. This three-fold increase in sensitivity shows remarkable improvement through the introduction of fCNT in modified ELISA technique.

Keywords: Cardiac Troponin I, Enzyme-linked immunosorbent assay, Functionalized carbon nanotube

1. INTRODUCTION

Unhealthy lifestyles, insufficient physical activity, sleep deprivation, and conditions such as high blood pressure and obesity draw attention regarding the prevalence of heart attacks among younger individuals. Complications of heart disease occur at younger ages. Some people already have heart disease in their 20s, 30s and 40s[1]. However, this phenomenon can be reduced by early detection, prevention, and treatment. For prevention, the early detection of heart attack is usually detected by electrocardiogram (ECG) recording which is bulky and inconvenient [2]. Thus, a more straightforward method, enzyme-linked immunosorbent assay (ELISA) is introduced. This technique is highly specific as it detects only a specific biomarker through the binding of target molecules with specific antibodies. These methods also reduce false positives and enhance diagnostic accuracy.

Cardiac troponin I (cTnI), cardiac troponin T (cTnT), creatine kinase, and myoglobin are the common biomarkers for myocardial damage. In this study, cTnI is used as a detection biomarker due to its specificity and prolonged presence (6 to 8 days) in the bloodstream. As the damage to the heart grows larger, the level of troponin found is greater. The threshold value of cTnI is 0.001 ng/mL. When the level increased to 0.1 ng/mL, acute myocardial infarction (AMI) occurs [3]. However, the conventional ELISA alone is not enough to determine low levels of biomarkers, especially in the early stage of AMI. To address this, modification of the ELISA

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system was conducted to increase its performance in terms of sensitivity and accuracy. In this research, functionalized CNT (fCNT) was used to modify the ELISA system for the detection of cTnI.

2. THEORETICAL BACKGROUND

ELISA is a biochemical method that is usually used to detect or measure the presence of antigens, antibodies, and proteins in a sample [4]. In this case, ELISA was used to detect a cardiac biomarker, cTnI. The important elements of ELISA are biomarkers, antibodies, blocking agent, washing buffer, and substrate. Firstly, the biomarker, which are the molecules to be detected is immobilized on the surface of the ELISA plate. After the immobilization process, a blocking agent is coated on the plate to avoid non-specific binding in the subsequent steps of the process. Next, a primary antibody that is specific to the biomarker was introduced and binds to the biomarker to form a bioconjugation. A labeled secondary antibody is added that links to the primary antibody. This secondary antibody acts as a signal amplifier. In each step in the process, the plate is washed with washing buffer to remove unbound molecules. Lastly, a substrate is introduced to the plate to initiate the enzymatic reaction which will cause color changes corresponding to the signal intensity.

Carbon nanotube (CNT) is a commonly used nanomaterial to enhance the performance of the detection system. CNT provides a large surface area to volume ratio that gives an advantage for the immobilization of molecules. Furthermore, CNT is easy to functionalize to introduce new functional groups such as carboxyl (-C=O) and amine (-NH) groups to capture more biomolecules. A research was done on modifying ELISA by using CNT to detect a peptide biomarker (Human VEGF) [5]. They claimed that the result was more improved and stable. Miao et al., 2017 introduced an ELISA method by comparing pH as a point-of-care (POCT) application. They used synthetic melanin nanoparticles and combined them with glucose oxidase and detection antibodies for immediate detection of cTnI. Their study shows that the detection limit was 0.15 pg/mL with linear range detection of 0.5 pg/mL to 10 ng/mL. Cho et al., 2020 fabricated an ELISA-on-a-chip biosensor to detect cTnI. They utilized biotin-streptavidin interaction to capture more cTnI biomolecules. After that, a luminescent substrate was added to the mixture to generate a luminescent signal. The signal was then analyzed as the optical density value of cTnI. The advantage of this approach is it reduces the detection time, and the detection range was 0.1 ng/mL to 100 ng/mL.

3. MATERIAL AND METHODS

3.1 Chemical and Instruments

Multi-walled carbon nanotube (MWCNT) was brought from Fibremax Composites (Greece) with a diameter of 10 – 40 nm. Cardiac troponin I protein and troponin T were purchased from Abcam (USA) while monoclonal antibody was purchased from Invitrogen (USA). The conjugated secondary antibody-horseradish peroxidase (HRP) was purchased from thermo-scientific (USA). The ELISA plate was purchased from Corning Costar (USA). The chemicals used in this experiment were purchased from Sigma Aldrich (USA). Fourier Transform Infrared Spectroscopy (FTIR, Perkin Elmer Spectra 65) was used to determine the functional group of CNT, and Field Emissions Scanning Electron Microscope (FESEM, LEO SUPR 50VP) was used to study surface morphology. Nanodrop Ultraviolent-visible spectroscopy (DS-11 Series UV-Vis Spectrophotometer) was used to measure protein in Ultraviolent wavelength (450 nm).

3.2 Functionalization of MWCNT

500 mg of CNT was added in 100 mL of ethanol and 50 mL of 2% (3-Aminopropyl) triethoxysilane (APTES) and sonicated for 2 hours. The solution was then washed with distilled (DI) water. Next, 5mL of 2% glutaraldehyde (GA) was added and stirred for 1 hour. The solution was then washed with DI water and dried in oven at 70°C overnight.

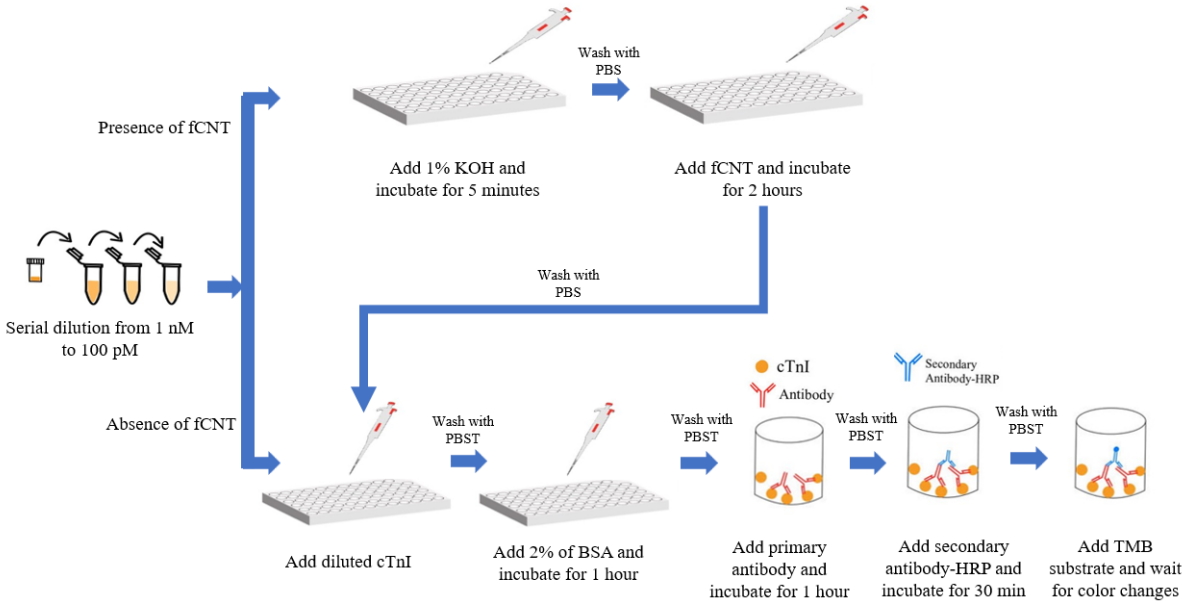

Figure 1. Schematic Diagram of conventional with absence and presence of fCNT

3.3 Absence of fCNT via conventional ELISA

Firstly, cTnI was serially diluted to different concentrations (1 nM to 1 fM) using 1X coating buffer (CB). Cardiac troponin T (cTnT) was diluted to 10 nM and 100 nM to ensure the specificity of ELISA. The diluted antigens were coated onto a 96-well ELISA plate and incubated at 4°C overnight. After that, a blocking agent, Bovine Serum Albumin (BSA) was added to each well and incubated for an hour. The diluted monoclonal cTnI antibody (1:1000) was immobilized in each well for 1 hour. Next, the secondary conjugated antibody-HPR was coated onto the plate and waited for 30 minutes. Finally, TMB substrate was added to the wells and waited for color changes. All the results were then measured with UV-Vis and photographed after 10 minutes.

3.4 Presence of functionalized CNT via conventional ELISA

In this experiment, all the procedures were similar to the previous experiment. However, the preparation on the ELISA plate was slightly different. Firstly, 1% of potassium hydroxide (KOH) was added to each well for 5 minutes to create the -OH group on the surface of ELISA plate. After that, washing of the ELISA well was performed with phosphate buffer solution (PBS). The functionalized CNT was coated onto the plate and incubated for 2 hours. The plate was then washed with PBS 3 times. The serially diluted cTnI and cTnT were added to the ELISA plate and incubated at 4°C overnight. After that, washing of the ELISA well was performed by PBS. The well was then washed with washing buffer 3 times. Finally, TMB substrate was added to the wells and waited for color changes. All the results were then measured with UV-Vis and photographed.

4. RESULTS AND DISCUSSION

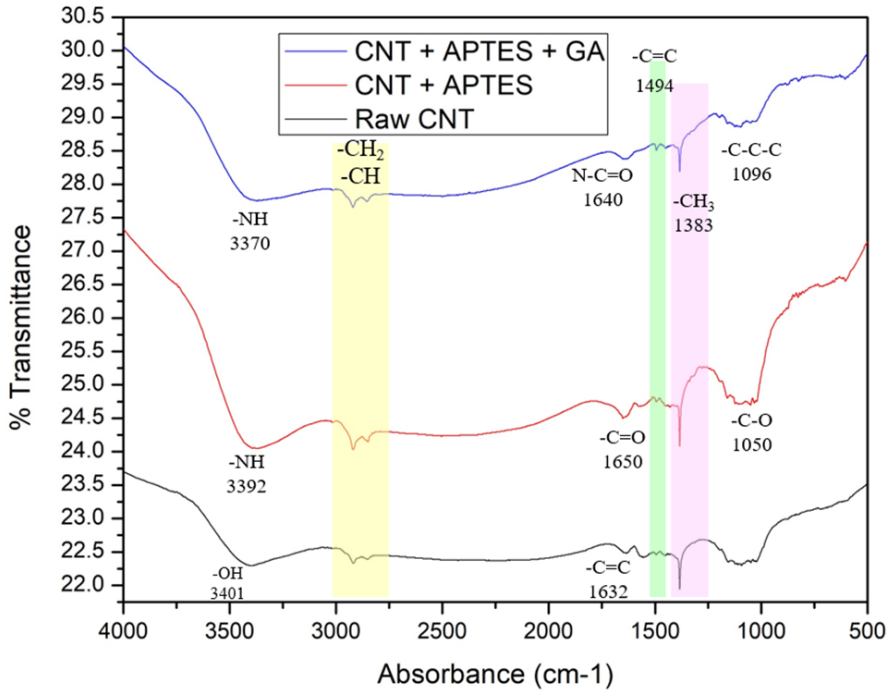

Figure 2. FTIR of Raw CNT and fCNT.

Figure 2 shows the KBr-FTIR spectra of CNT and fCNT from the range of 4000 to 500 cm^{-1}. Peaks are observed at 3401 cm^{-1} for CNT and 3392 cm^{-1} for CNT coated with APTES indicating the hydroxyl stretch (-NH) from the amine group [8]. The methylene groups ($-CH_2$ and -CH) shown at 2921 cm^{-1} and 2849 cm^{-1} represent the -CH and -CH2 of the CNT, respectively[9], [10]. The same goes for the peak at 1494 cm^{-1} responsible for the alkene groups (-C=C) of the CNT. Besides, methyl group ($-CH_3$) is also found at peak 1383 cm^{-1}. After the functionalization of CNT with APTES, carboxylic group (-C=O) is spotted at 1650 cm^{-1} from the alkene group (1632 cm^{-1}) of raw CNT [11], [12]. The shifted peak at 1640 cm^{-1} represents N-linked C=O groups, indicating that the amine groups of GA were successfully functionalized onto CNTs and APTES [13]. In addition, the -NH group shown at 3370 cm^{-1} also indicates the successful functionalization of CNTs by GA [14]. Moreover, the peaks observed at 1050 cm^{-1}, and 1096 cm^{-1} represent -C-O stretching vibration and -C-C-C bending vibrations, respectively [15], [16]. The -NH and -C=O from the functionalization contributed to capturing more cTnI biomolecules and enhancing the performance of ELISA.

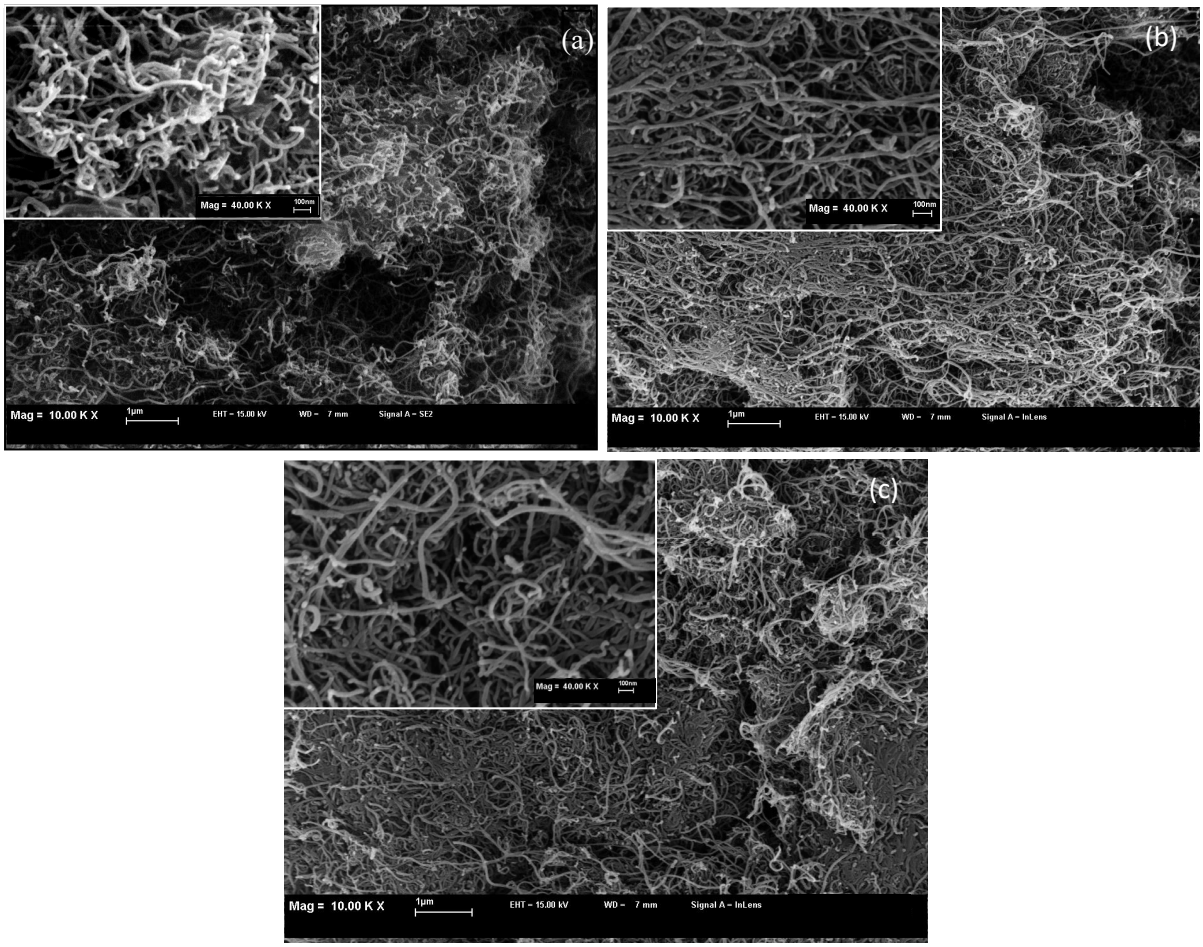

Figure 3. SEM image of (a) raw CNT; (b) CNT with APTES functionalization; (c) CNT with APTES/GA functionalization.

Figure 3 shows images of raw CNT and fCNT obtained by FESEM at 10KX and 40KX. From Figure 3 (a), it can be seen that raw CNT were agglomerated due to the hydrophobic and Van der Waals forces as well as the amorphous carbon region of individual tube [17]. In addition, there are also large free spaces observed within the tubes. The average diameter of raw CNT is approximately 45 to 50 nm. After the functionalization of CNT with APTES, the surface morphology remains unchanged, and no defects are found as shown in Figure 3.1(b). The average diameter of CNT with APTES functionalization is approximately 37 to 40 nm. After coating GA on APTES (Figure 3.1(c)), the average diameter of APTES/GA is 35 to 37 nm. The free space decreases between the tubes and tightly fit to each other. This shows that higher interaction between the fCNT [18], [19]. This indicates that APTES and GA were successfully functionalized on CNT.

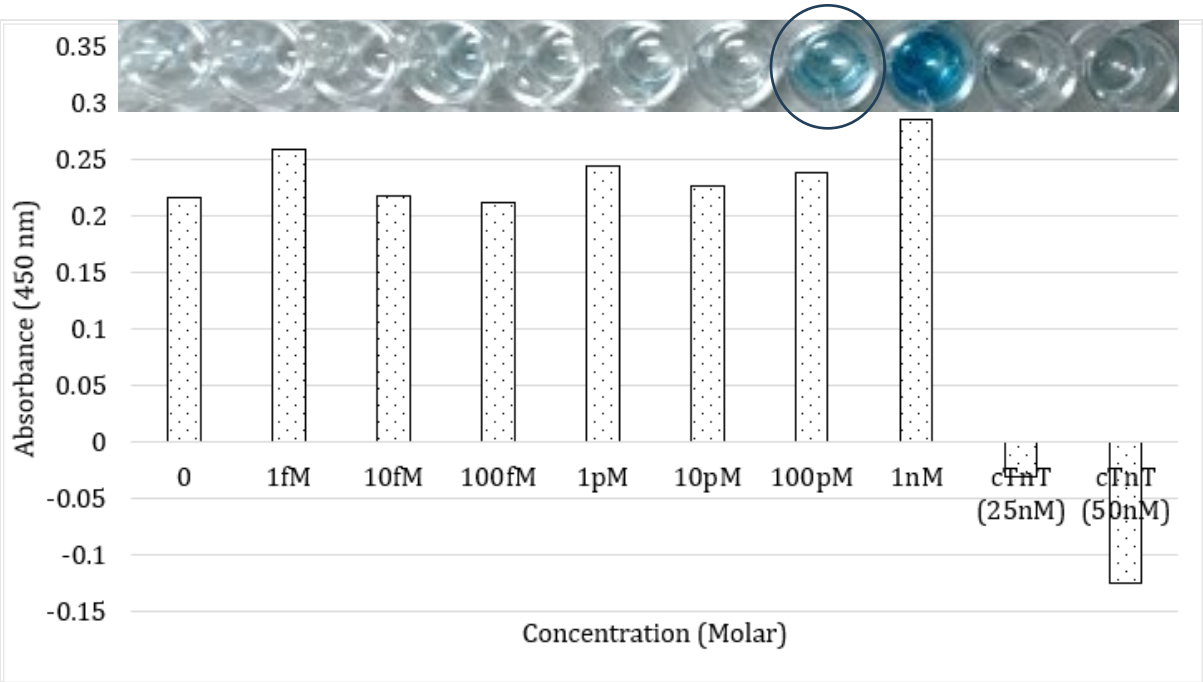

Figure 4. Absorbance analysis with the absence of fCNT via conventional ELISA.

An analysis for the detection of cTnI without fCNT was carried out by conventional ELISA methods. This method was set up without fCNT to compare the ELISA analysis with the presence of fCNT. Figure 4 illustrates the results of absorbance and color change after cTnI detection. As shown in Figure 4, a noticeable color change is observed at 100 pM. Hence, the limit of detection (LOD) without fCNT is 100 pM without fCNT. However, the final color changes are not noticeable at lower concentrations (< 10 pM) and the consistency of the absorbance value is less pronounced too. The negative absorbance of cTnT that serves as a control shows that the interaction is highly specific for cTnI.

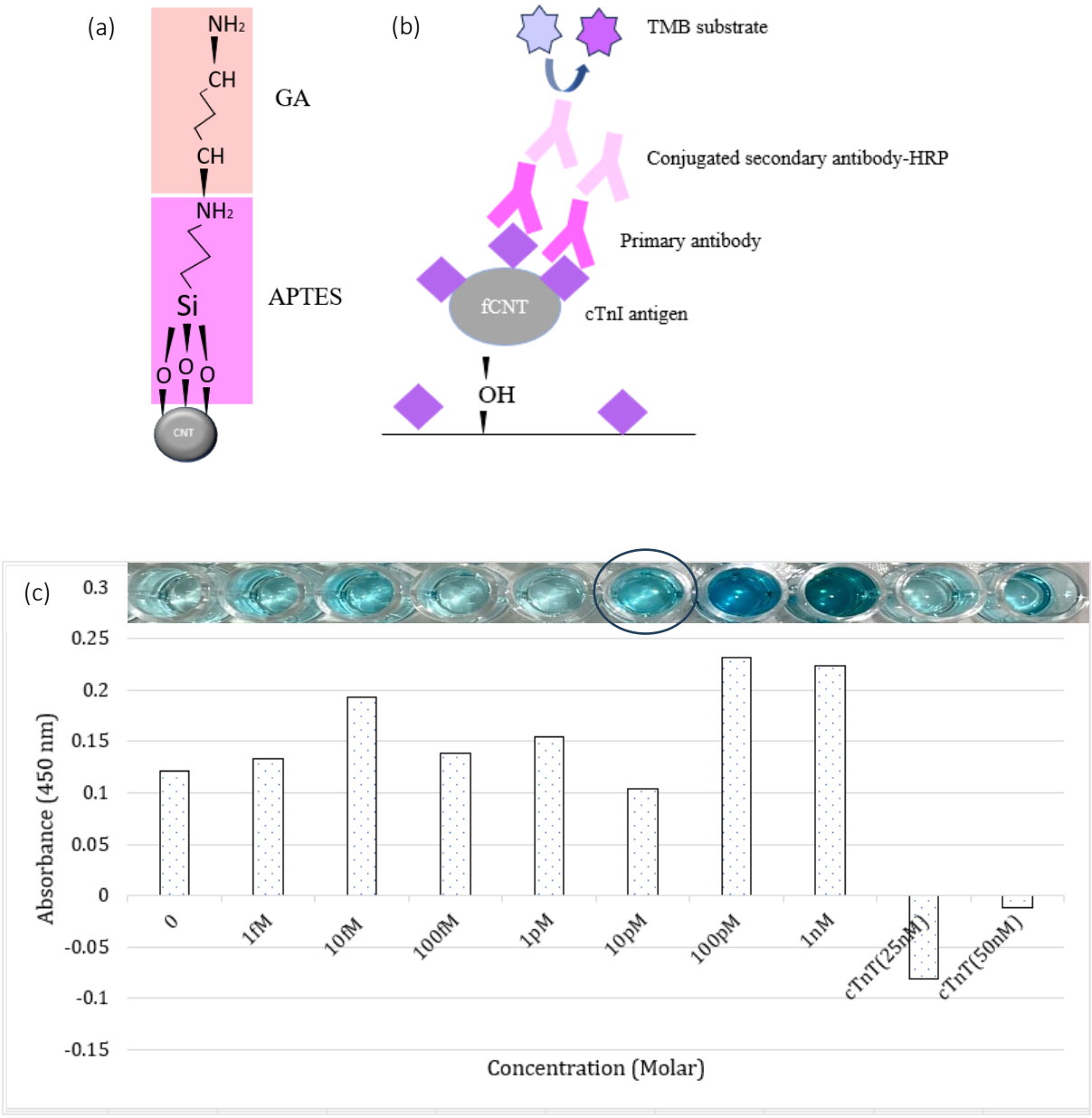

Figure 5. Schematic diagram of (a) Functionalization of CNT with APTES and GA; (b) Detection of cTnI with fCNT; (c) Absorbance analysis with the presence of fCNT via conventional ELISA.

Figure 5(a) shows the schematic diagram of the functionalization of CNT with APTES and GA. The existing hydroxyl group of CNT interacts with the oxoethyl side of the APTES. The associated functional group on another side of APTES is the amine group. Glutaraldehyde, as a crosslinker, is used to attach APTES and target. The amine group of APTES then interacts with the carbonyl group of GA. The chain is opened to aldehyde groups and covalently bonded to the amino groups of the target. In the conventional ELISA method, this technique involves direct attachment of the target onto the existing group on the surface of the ELISA plate. Figure 5(b) demonstrates a schematic diagram of cTnI detection with the presence of fCNT. This study employed an indirect ELISA approach. Modification of ELISA plate was done by incorporating fCNT as a signal enhancement onto the polystyrene (PS) plate. Firstly, incubation of 1% KOH contributed to the hydroxyl group on the PS plate to ease the fCNT attachment. Additionally, the -NH group of fCNT plays a part in enhanced attachment of cTnI. After incubation of the target, the added primary antibody was linked to the captured target. Subsequently, more secondary conjugated antibodies

bind to the primary antibodies. In addition, TMB substrate was added to show a more pronounced result. The absorbance values increase with the capture of more antibodies leading to a more pronounced result. The absorbance value increases with increasing biomarkers concentrations, indicating the presence and quantity of the target molecules.

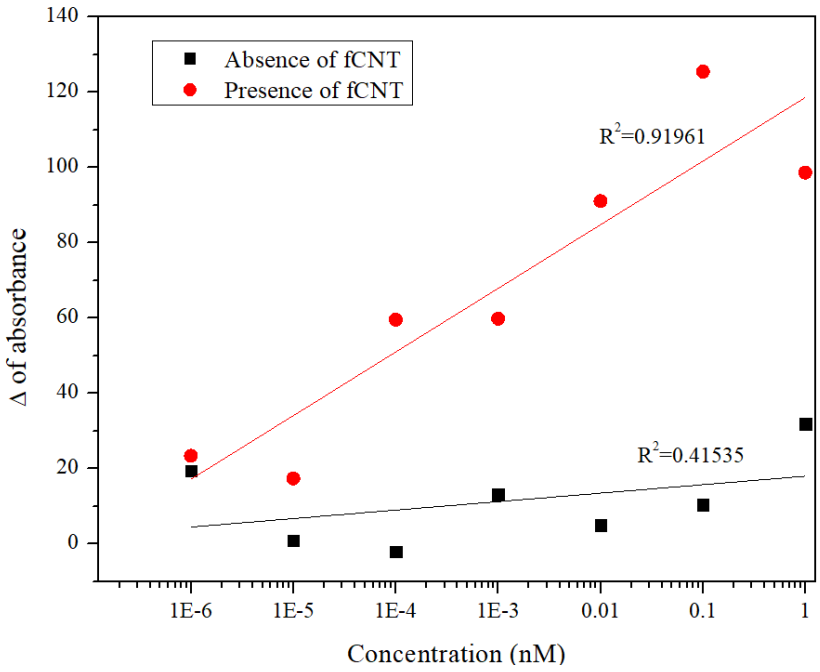

Figure 6. Comparison of the change in absorbance with or without fCNT.

From Figure. 5, a more pronounced color change was observed, where the detection limit after fCNT was introduced went down to 10 pM in contrast to the typical ELISA method (100pM), as shown in Figure 4. Also, a more consistent result can be observed after adding fCNT. From Figure. 6, the data shows that the absorbance only increases to 31.90% at 1 nM with linear regression of 0.41535 for the typical ELISA method. Upon incorporating with fCNT, the absorbance increased to 98.61% and 125.44% at 1 nM and 100 pM, respectively, with its linear regression of 0.91961. The improvement results increased by approximately 3-fold in detection limit compared to the typical ELISA for the detection of cTnI. Nevertheless, modifying ELISA by employing fCNT shows noticeable benefits in early detection of heart attack risks.

5. CONCLUSION

The ELISA technique is used to detect cTnI, a cardiac biomarker that causes AMI. To bolster the performance of ELISA, fCNT is utilized as a signal amplifier. This study involves a comparative analysis between conventional ELISA and its modified version. The functionalization of APTES and GA on fCNT is characterized by using FTIR and FESEM techniques. The functional groups on the fCNT demonstrate enhanced performance in capturing targeted biomarkers. While the standard ELISA exhibits a detection limit at 100 pM, the incorporation of fCNT significantly reduces it to an impressive 10 pM. This translates to a three-fold improvement in detection limits compared to traditional methods. These results underscore how the integration of fCNT into ELISA enhances the sensitivity of biomarker detection.

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