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CARBON NANOTUBES MODIFIED ELISA SYSTEM DEVELOPMENT FOR IMPROVED DETECTION OF BIOMARKERS

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Abstract

This study developed and evaluated a nanomaterial-modified ELISA system to analyze a peptide biomarker (Human VEGF). Multiwalled carbon nanotubes (MWCNTs) were used as nanomaterial-based modifiers for the capture/detection antibodies and HRP enzyme. CNTs modifications of the ELISA method at these three different binding steps were tested and compared. The most efficient modification step was concluded depending on these works' sensitivities, linearities, and repeatabilities. We proved that CNTs could be used successfully to enhance the performances compared to the traditional ELISA method by achieving higher sensitivities and, therefore, more confident results, especially with a 0.5 pg/ml the limit of detection value. When comparing the three modification steps of CNTs binding, we observed the most sensitive results with the CNTs/detection antibody complex usage. Improved results (sensitivity is doubled compared to the test when no CNTs were used), improved reaction with serum samples, and good stability compared to the unmodified conventional ELISA system were obtained when CNTs were utilized with the detection antibodies as signal enhancers.

Keywords: ELISA, Carbon nanotube, Bioanalysis, Biotechnology

BİYOMARKERLERİN İYİLEŞTİRİLMİŞ TESPİTİ İÇİN KARBON NANOTÜPLERLE MODİFİYE EDİLMİŞ ELISA SİSTEMİNİN GELİŞTİRİLMESİ

Özet

Bu çalışmada, bir peptit biyobelirtecini (İnsan VEGF) analiz etmek için nanomateryalle modifiye edilmiş bir ELISA sistemi geliştirdi ve değerlendirdi. Çok duvarlı karbon nanotüpler (MWCNT), yakalama/tespit antikorları ve HRP enzimi için nanomateryal bazlı modifiye malzemesi olarak kullanıldı. ELISA yönteminin bu üç farklı bağlanma aşamasındaki CNT modifikasyonları test edildi ve karşılaştırıldı. Bu çalışmaların hassasiyet, doğrusallık ve tekrarlanabilirliklerine bağlı olarak en verimli modifikasyon adımı belirlendi. CNT'lerin kullanıldığı ELISA metodunda, özellikle 0,5 pg/ml tespit limit değeri ile daha yüksek hassasiyetler ve dolayısıyla daha güvenilir sonuçlar elde edildiği ve geleneksel ELISA yönteminede performansı artırmak için başarılı bir şekilde kullanılabileceğini kanıtlandı. Ayrıca ticari serum örneklerinin analizinde farklı modifikasyon adımlarının performansları ve tüm bu bağlanma adımlarının stabilitesi değerlendirildi ve geleneksel ELISA yöntemi ile karşılaştırıldı.

Anahtar Kelimeler: ELISA, Karbon nanotüp, Biyoanaliz, Biyoteknoloji

Cite

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1. Introduction

The ELISA method is a high-sensitivity laboratory diagnostic technique used to detect the presence of antigens or antibodies in a wide variety of biological samples by serial attachment to a solid surface, usually a polystyrene multi-well plate, to obtain quantitative results [1, 2]. This method can measure the immunological reaction due to an enzymatic activity influenced by the possible application areas, including health, food, and environmental analysis [3, 4]. ELISA systems are performed on 96-well microplates that are made of polystyrene. Each well can contain a different molecule or samples of the sample molecule [5]. This property can make the system suitable for performing multiple analyses simultaneously. Depending on the binding sensitivity of the target molecule or antibody, the

sensitivity and test duration of the ELISA test may vary [6].

Sandwich type ELISA system is the most commonly used system among other indirect and competitor ELISA systems [7, 8]. In the sandwich ELISA mechanism, the antibody is immobilized at the bottom of the microplate wells. The antigen sample is added to the antibody, and the complex is expected to form. Excessive unbound molecules are removed by washing. A biotin-labeled secondary antibody, a detection antibody, binds to the antigen bound to a different epitope capture antibody. After removing the streptavidin-labeled enzyme is added to the medium and by binding biotin and streptavidin, the enzyme is and attached to the antibody. By adding the enzyme's substrate (TMB) to the surface, the substrate reacts with the enzyme and turns into a blue-colored molecule. A stop buffer is added to the wells, which stops the TMB colour change and creates a more stable colour, and the absorbance in each well is measured by spectrometers suitable for microplates. The formation of the coloured product is directly proportional to the concentration of the target molecule [9].

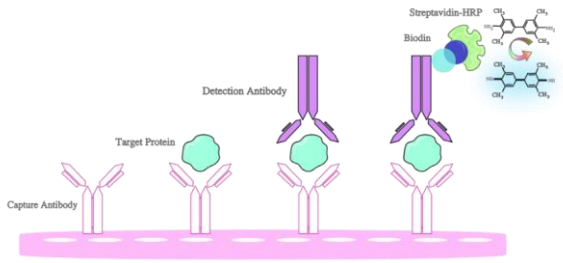

Figure 1. Schematically shown entire step-by-step working principle of traditional sandwich ELISA system from left to right.

ELISA systems are simple, easy to access, and can give fast results. Especially in the healthcare field, ELISA systems play an important role. It is one of the methods considered reliable among laboratory techniques today. However, ELISA systems are not infallible. Low detection limit and high sample volume are just some of the main shortcomings of traditional ELISA systems. The low presence amounts of biological target molecules, which require sensitive analysis, limit the use of the system [10]. The sensitivity of the ELISA may often need to reach the desired concentration level to meet the demand for biomarker analysis in biological samples, especially in the early diagnosis of diseases. Increasing the sensitivity of ELISA systems will be an essential step in healthcare [11, 12].

To develop a system that involves nanotechnology and materials science, as one of the emerging technologies, can provide a beneficial touch to the ELISA system. Current research articles in the literature used different nanomaterials with ELISA systems. Nanostructures such as nanoparticles or graphene nanostructures were used in some steps carried out within the scope of these published studies, and effective signal improvement was observed [12, 13]. However, there is no example of using nanomaterials in every ELISA experimental step and determining the most efficient method for enhancing the signal amplification, system stability, and real sample usage [10]. Carbon nanotubes can also be used in developing highly sensitive sensor/biosensor systems, as they have great chemical and mechanical stability. Many studies show that biosensors modified with carbon nanotubes can show better reproducibility and sensitivity [14]. In this current work, nanotubes were applied at each experimental step in the sandwich-type ELISA method, and their effectiveness was compared.

2. Materials and Methods

2.1. Reagents

Multiwalled carbon nanotubes (MWCNTs) containing 8% carboxylic acid were obtained from Thermo Fisher (USA). N-(3-dimethylaminopropyl)-N0-ethylcarbodiimide hydrochloride (EDC), N-Hydroxysulfosuccinimidesodium salt (NHS), 4-Morpholineethanesulfonicacid (MES), 3,3', 5,5' tetramethylbenzidine (TMB) substrate, and Tween-20 were obtained from Sigma Aldrich (GERMANY). Human VEGF ELISA Matched Antibody Pair Kit (ready-to-use detection/capture antibodies, HRP enzyme, and target molecule ELISA kit) was obtained from Tonbo Biosciences (USA). 96 "F" base microplates and sterile covers were obtained from Şahinler Kimya (TR).

2.2. Instruments

Thermo Scientific™ Varioskan™ LUX device was used for the analysis of ELISA systems, Thermo Scientific™ NanoDrop Spectrophotometers device was used for absorbance measurements of supernatants, and HITACHI SU5000 FIELD EMISSION SCANNING ELECTRON MICROSCOPE (FE-SEM) was used for SEM imaging. AYBU Central Research Laboratory facilities were used in the analyses performed with all these devices.

2.3. ELISA Method

In order to coat the 96-well plate, 100 µL of the capture antibody (10 g/mL) was added to each well. The well was blocked with 400 µL of PBS buffer (0.01 M PBS containing 1% BSA) following a 12-hour incubation period at 4 °C and four rounds of washing with PBST buffer (0.01 M PBS containing 0.05% Tween 20). At 37 °C, the blocking response was maintained for 40 minutes. Standard antigen (Human VEGF) solutions (200 µL) produced in PBS buffer at concentrations of 0, 0.5, 2, 5, 50, 200, 400, and 800 pg/ml were added to the corresponding wells of the microtiter plate and incubated for 1 h at 37 °C. The wells were cleaned with PBST buffer six times after incubation. Following eight washing sessions with PBST buffer, 200 µL of the biotin-modified detection antibody (10 g/mL in PBS buffer) was applied to each well and incubated at 37 °C for 1 hour. Streptavidin modified HRP probe (100 L) was added to each well and then each well was washed three times with washing buffer before the

reaction continued for another 60 minutes at 37 °C. TMB substrate (100 L) was added to each well and then swabbed three more times before being incubated for 30 minutes at 37 °C. Finally, H_2SO_4 (50 L, 2 M) was used to terminate the catalytic process. Thermo ScientificTM VarioskanTM LUX microplate reader was used to measure the absorbance at 450 nm. Three times each of the measurements were taken. Figure 1 depicts all of the classic ELISA method's phases.

2.4. Immobilization of Enzymes and Antibodies to Nanotube Surfaces

Carboxylic acid-modified MWCNTs at 50 µg/ml concentration were washed three times in 10mM MES pH 5. They were incubated for 30 minutes at 37°C in a solution containing 15 mM EDC and 22.5 mM NHS prepared in 10mM MES buffer pH 5. At this stage, the surfaces of the CNTs were activated, suitable for binding for enzymes and antibodies containing amino groups (figure 2). Immediately after the activation step, MWCNTs were incubated for 6, 12, and 18 hours with gentle shaking at 37°C with a solution of 1 mg/mL enzyme or antibody prepared in 10mM sodium bicarbonate buffer, pH 8 containing 2% Tween20. The suspension was centrifuged, the precipitate was collected and washed well with PBS (pH 7.4), and the supernatant absorbance was measured at A280 by UV-vis spectroscopy to observe the enzyme and antibody binding efficiency to the nanotubes. Finally, the obtained enzyme/antibody-CNTs conjugate was diluted in the desired ratio in PBS buffer (pH 7.4) and stored at 4 °C. It was sonicated for at least 5 minutes before each use.

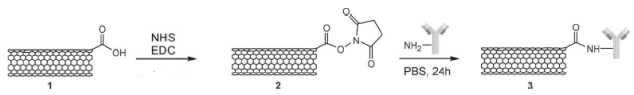

Figure 2. Antibody (or enzyme) binding onto the carboxylic acid-modified carbon nanotube surfaces with EDC/NHS activation.

2.5. ELISA Method with Microplate Surfaces Modification by Carbon Nanotubes

This work package includes attaching the CNTs to the surface of the ELISA microplates. Since the system's surface material has been produced with polystyrene, it has provided the system adsorption properties, and the adhesion of the carbon nanotubes to the surfaces was achieved without any problems with the carbon nanotubes purchased from Thermo Fisher containing 8% carboxylic acid. Carboxylic acid modifications increasing the surface area of these nanotubes provided very stable immobilization of capture antibodies containing amino groups. The performances of the modified ELISA systems and the traditional ELISA method were compared over the parameters of sensitivity, reproducibility, stability, and reliability.

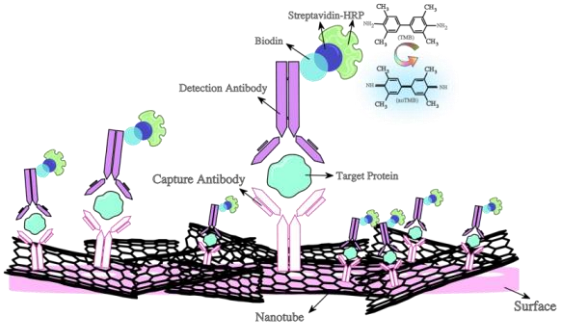

Figure 3. ELISA method overview with the microplate surface modification with carbon nanotubes.

2.6. ELISA Method with Binding of Detection Antibody to Carbon Nanotubes

In this work package, the detection antibody was immobilized to carboxylic acid-modified CNTs surface, and this complex was used in the relevant step in the ELISA system. With the CNTs included in the system, the amount of detection antibody that binds to a single target molecule was increased with multiple antibodies attached to the surfaces of carbon nanotubes, as shown in Figure 4. In the next step, the bound HRP enzyme amount also increased, and thus, the reacting TMB substrate also multiplied. By this way, more sensitive ELISA analysis results could be obtained by providing signal amplification. The obtained data were also compared with a conventional ELISA method that not modified with nanotubes.

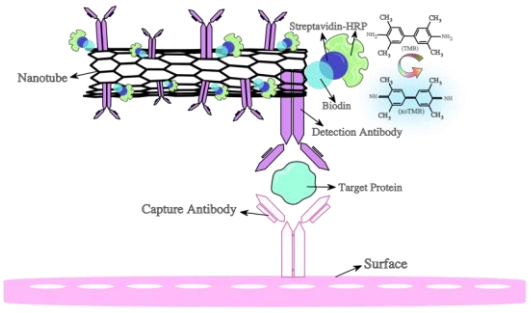

Figure 4. ELISA method overview with the CNTs modified detection antibodies.

2.7. ELISA Method with Binding of HRP Enzyme to Carbon Nanotubes

As seen in Figure 5, the binding of streptavidin-linked HRP enzyme to carboxylic acid-modified CNTs was performed. In the last step of the analysis, which was started with the same procedures as traditional ELISA method, the study continued by examining the effect of modified carbon nanotubes on the signal-producing HRP enzyme. The effect of the CNTs/HRP complex on the performance of the ELISA system was investigated, and its advantages compared to the traditional ELISA method were determined.

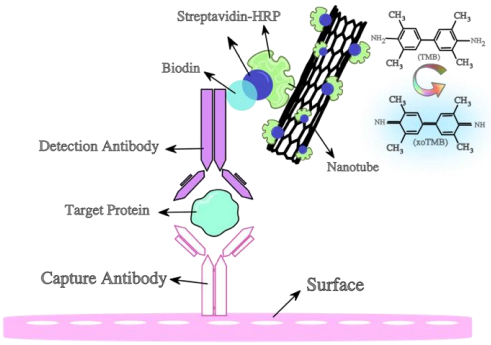

Figure 5. ELISA method overview with the CNTs modified HRP enzyme.

3. Results and Discussion

3.1. Binding of Enzymes, Antibodies to the CNTs Surfaces

After the enzyme/antibody immobilization step with the EDC/NHS covalent binding protocol, the binding efficiency was checked by measuring the absorbance values of the obtained supernatants in A280. When the absorbance values of the supernatants (unbound enzyme/antibody residues) were obtained as a result of the binding achieved by incubation at different periods that were examined, it was seen that the most effective binding was obtained after 12 hours of incubation (Figure 6). Incubation time longer than 12 hours shows no significant decrease in the A280 absorbance value. Therefore, it was decided that the optimum incubation time was 12 hours.

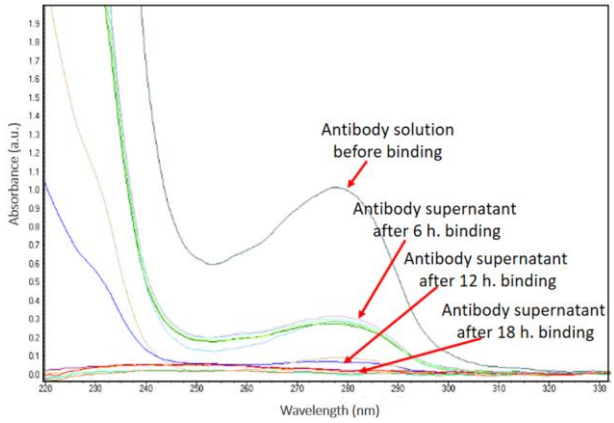

Figure 6. Spectra were measured for the supernatants before and after binding to the CNTs at A280 absorbance.

The solutions obtained by CNTs and antibody binding were diluted at different ratios and used in ELISA systems in the next steps. Before use, SEM images of these solutions were taken, and their morphologies were examined. As seen in Figure 7, there is a difference between plain MWCNTs and antibody-coupled nanotubes in terms of both nanotube thickness and surface density. The SEM images and UV-Vis spectroscopy measurements concluded that the enzymes and antibodies were successfully bound to the MWCNTs surfaces.

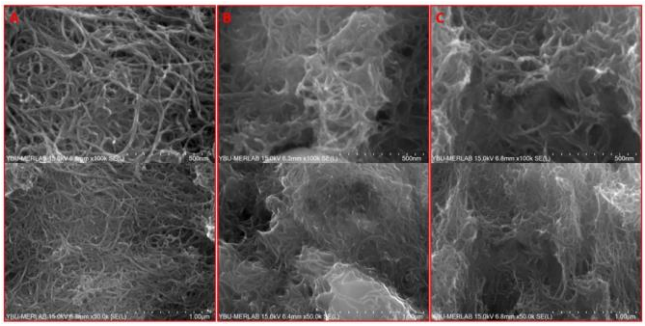

Figure 7. SEM images for the unmodified (A) and enzyme (B) and antibody (C) modified CNTs with 500 nm and 1 µm scale, and 6.8mmx100k and 6.8mmx50k pixels.

3.2. ELISA system obtained by modifying microplate surfaces with CNT-bound capture antibodies

Carbon nanotubes are modified to the surface of polystyrene microplates in this work package. During the experiment, different carbon nanotube concentrations (1 µg/ml, 5 µg/ml and 10 µg/ml) were tried and in response, different target molecule concentrations (0, 0.5, 2, 5, 50, 200, 400, 800 pg/ml) were used for observing logarithmic calibration curves.

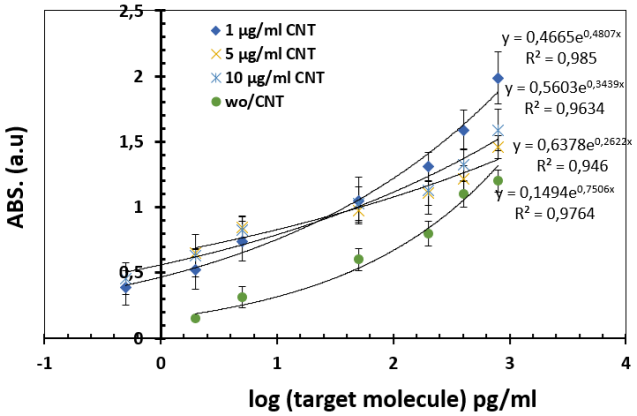

Figure 8. Logarithmic calibration curves for ELISA methods with unmodified and different concentrations of CNTs modified microplate surfaces.

As seen in the calibration graphs in Figure 8, although a logarithmic calibration curve is obtained for the target molecule of the carbon nanotube unmodified ELISA system, the absorbance values are lower than the carbon nanotube-modified ELISA systems. Higher absorbance values and more sensitive (lower detection limit) values were obtained for the CNTs-modified system at all three different concentrations than the unmodified system. While the lowest analysis value for the unmodified system was 5 pg/ml, significant results were obtained for the 1 µg/ml and 5 µg/ml CNTs modified systems for the 0.5 pg/ml value.

When 3 different CNTs amounts are compared with each other, it is seen that at the concentration level of 1 µg/ml, the absorbance value increased significantly and the most linear curve was observed. As a result, it was decided that 1 µg/ml CNTs concentration is more favourable than other concentrations, with an increase in

both linearity and signal amplification. In addition, since the carbon nanotube concentration to be modified on the surface of the ELISA system is kept low, it can be said that it is an economically appropriate concentration level.

3.3. ELISA system obtained by modifying CNTs with detection antibody

In this part of the work, the detection antibody is immobilized on carboxylic acid-modified CNTs surfaces. The unmodified ELISA system was compared with detection antibodies coupled with different carbon nanotube concentrations (1 µg/ml, 5 µg/ml, and 10 µg/ml) and the unmodified ELISA system. Different target molecule concentrations (0, 0.5, 2, 5, 50, 200, 400, 800 pg/ml) were tested for each system, and calibration curves were drawn.

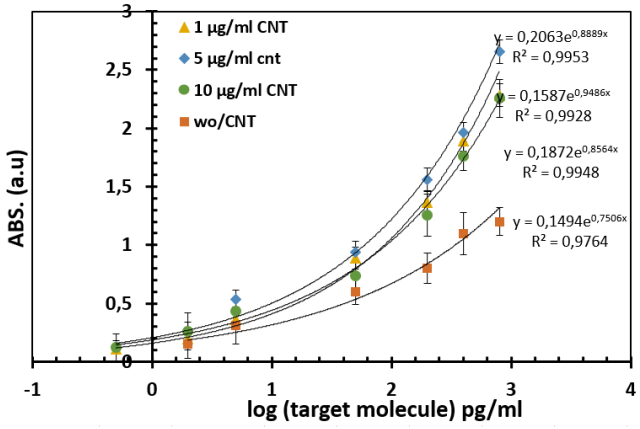

Figure 9. Logarithmic calibration curves for ELISA methods with CNTs modified detection antibodies and unmodified ELISA methods.

Calibration graphs drawn in Figure 9 show an increase in absorbance values in the modified ELISA system with three different CNTs concentrations compared to the unmodified system. Even when the target molecule ratio in the system is kept to a minimum, an increase in the signal of the CNTs modified systems can be observed. In the calibration curves, depending on the amount of target molecule, it is seen that the most linear value is obtained with 5 µg/ml (R2=0.9953) and 1 µg/ml (R2=0.9928) CNTs concentrations. Considering the signal amplification values, it was concluded that the complex prepared with 5 µg/ml CNTs concentration, which achieved successful results in terms of linearity, sensitivity and higher signal generation, had the most effective use in the ELISA system.

3.4. ELISA system obtained by modifying CNTs with HRP enzyme

In this part of the study, the HRP enzyme, which is in the last binding step of the ELISA method, was immobilized to CNTs and used in the relevant step. HRP enzyme complexes with different CNTs concentrations (1 µg/ml, 5 µg/ml, and 10 µg/ml) were tested with different concentrations of the target molecule (0, 0.5, 2, 5, 50, 200, 400, 800 pg/ml). Moreover, the calibration curves were observed and compared with the unmodified ELISA system.

At this stage of the experiment, while the amount of target molecule is kept at 0,5 pg/m, the data of the conventional unmodified ELISA system cannot be obtained. However, the data of the ELISA system modified with carbon nanotubes at the minimum concentration level can be obtained. The most important finding from the graph is that the system prepared with 5 µg/ml concentration of CNTs makes a significant difference compared to other concentrations. When the target molecule is increased to 800 pg/ml, the concentration level of 5 µg/ml CNT also reaches 2,5 a.u. in the absorbance value and is far ahead of the others. Not only as signal amplification, but the R2 value of 5 µg/ml concentration level also increased to 0.9989 and gave a good result in terms of linearity.

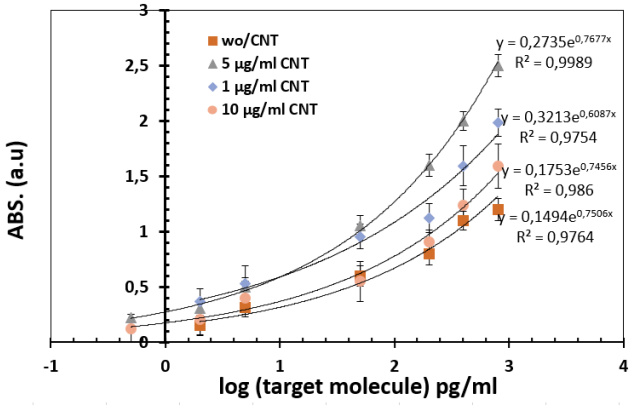

Figure 10. Logarithmic calibration curves for ELISA methods with CNTs modified HRP enzyme and unmodified ELISA methods.

3.5. Testing of CNTs modified ELISA systems with a commercial serum sample

After examining the results of independent modifications one by one, carbon nanotube-modified ELISA systems, in which the most appropriate CNT amount was determined, were tested with a different concentration of target molecule prepared in commercial serum diluted 1:5 ratio in PBS buffer solution. Calibration curves of ELISA systems with and without CNTs modifications are given in Figure 11. When the results are examined, the calibration curves for the target molecule analyzed in serum do not differ in sensitivity and linearity compared to the analysis performed in buffer solution.

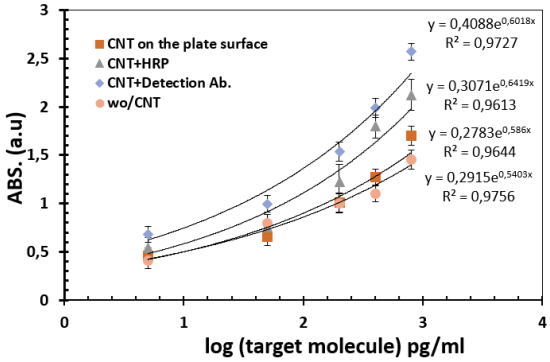

Figure 11. CNTs modified and unmodified ELISA methods' testing with serum samples.

Even when the amount of target molecule in the system is kept to a minimum (5 pg/ml), it is observed that the system with CNTs modified with detection antibody makes a difference when compared to other systems in terms of the absorbance value examined. When the linearity is examined, it is seen that the R2 value of the CNTs modification in the detection antibody is 0.9727, which is a much better result for linearity compared to other systems.

According to these performance characteristics of our CNTs modified ELISA systems, we can conclude that they are comparable with the novel works in the literature. It has been shown to functionalize nanoparticles with antibodies and enzymes using a variety of conjugation chemistries. Liu et al. created the ZnFe2O4@MWNT)-ELISA technique for performance enhancement, which is comparable to our idea [15]. This technique, which has similar advancements to our system, can detect CEA in human serum in a linear range of 0.005 to 30 ng/mL with a LOD of 2.6 pg/Ml. Another innovative study used the AuNP-dendrimer-ELISA system to detect human chorionic gonadotropin (hCG) at a linear range of 0.1–6.4 IU/L with a LOD of 0.03 IU/L, 20 times lower than a single AuNP-ELISA system and 27 times lower than the conventional ELISA [16]. Additionally, three distinct diagnostic biomarkers at pg/ml level were discovered using an ELISA approach based on carboxyl graphene oxide (cGO). This approach yielded results that were many orders of magnitude lower than those of classical ELISA because of the high loading capacity of cGO [17].

The more precise analytical process should be devised to prevent errors when significant medical choices are based on the results of the biomarker study. It is possible to functionalize CNTs, which makes them an intriguing improvement for ELISA assays.

3.6. Testing the storage stability of CNTs modified ELISA systems

The systems prepared using the most appropriate amount of CNTs for each modification step were tested with a fixed concentration of target molecule (200 pg/ml) on certain days for long-term stability analysis. The results of the experiments performed with 200 pg/ml target molecule were compared with the ELISA systems' first day of preparation, and the systems' stability percentages were determined. Materials prepared for each system (CNTs-modified antibodies, enzymes, and prepared ELISA plate) were stored at 4 °C and tested on different days.

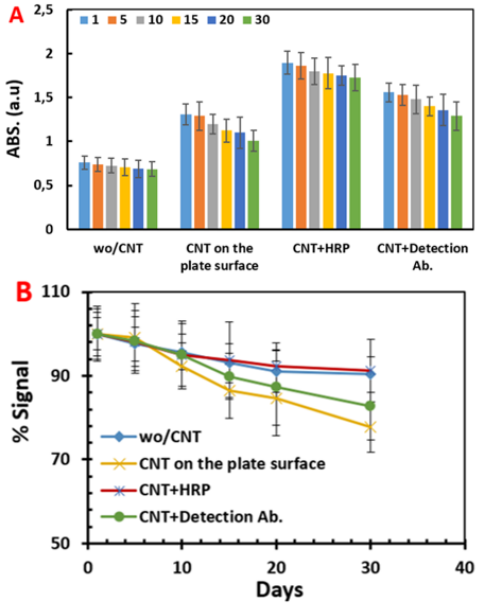

Figure 22. Storage stability results for the CNTs modified and unmodified ELISA system. A) measured absorbance results for each system by continuous tested days, B) the percentage of signals by tested days depends on the first-day absorbance result.

As seen in Figure 12, according to the results repeated for 30 days, the stability of the unmodified ELISA system is 90% compared to the first day's results. On the other hand, the stability of the ELISA system in which the HRP enzyme binds to CNTs was 91%, and the system in which the detection antibodies were attached to the CNTs was 83%. The system in which the capture antibodies were bound on the CNTs and modified onto the ELISA plate was 78%. According to these results, it can be said that the system that maintains its stability most effectively is the unmodified system and the ELISA system in which the HRP enzyme binds to CNTs. At the same time, it is seen that the system in which ELISA plates are prepared with CNT-bound capture antibodies quickly loses its stability even though it is stored at 4 °C.

4. Conclusion

In this work, we have developed ELISA methods where MWCNTs were used as carriers of the capture/detection antibodies and HRP enzyme for the analysis of peptide biomarker (Human VEGF) as a sample target molecule. CNTs modifications of the ELISA method at the three different binding steps were tested and compared. The most successful and useful one was concluded in this work for the first time in the literature. In this study, we established the theory that CNTs might be utilized effectively to improve the outcomes of conventional ELISA tests, producing better sensitivities and, thus, more reliable findings, particularly in the region around the limit value of 0,5 pg/ml. When comparing the three modification steps of CNTs binding onto the detection/capture antibodies and HRP enzyme, we observed the most sensitive results with the

CNTs/detection antibody complex usage. improved results (sensitivity is doubled compared to the test when no CNTs were used), improved reaction with serum samples, and acceptable stability compared to the unmodified conventional ELISA system were obtained when CNTs were utilized with the detection antibodies as signal enhancers.

5. Acknowledgment

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