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Development of an indirect ELISA to detect antigens using DNA origami nanoantenna labeled specific monoclonal antibodies

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ABSTRACT

This research examines the development of an indirect ELISA to detect antigens using DNA origami nanoantenna-labeled specific monoclonal antibodies. Hepatocellular carcinoma (HCC) is one of the five most common cancers and the second leading cause of cancer-related death worldwide. More than 60% of cases are detected in the late stage, with the absence of specific symptoms in the early stage of the disease. Adding measurements of AFP (Alpha-Fetoprotein), TXN (Thioredoxin), and DCP (Des-γ-Carboxy Prothrombin) has the potential to improve the accuracy of diagnosing hepatocellular carcinoma. DNA origami technology is revolutionizing the forefront of cancer diagnostics, particularly for HCC. In this study, an indirect enzyme-linked immunosorbent assay (ELISA) method targeting the assessment of the effectiveness of specific monoclonal antibody-conjugated DNA origami particles was developed. The results of DNA origami folding into complex nanostructures and labeled biomarkers like AFP, DCP, and TXN have explored the high specificity and sensitivity for targeting HCC. We found the cut-off values for AFP, DCP, and TXN to be 0.132, 0.156, and 0.150, respectively. The coefficients of variation for samples remained consistently under 10%, indicating the method's high degree of stability and reproducibility. This innovation, combined with biomarkers, promised in early HCC detection, holds the potential to significantly enhance patient outcomes in the fight against liver cancer. The platform also has enormous potential for advancing personalized medicine.

Contribution/ Originality: The originality study of both the DNA origami-conjugated monoclonal antibodies (e.g., Alpha-Fetoprotein, Thioredoxin, and Des-γ-Carboxy-Prothrombin) and indirect ELISA methods has enhanced sensitivity and specificity in hepatocellular carcinoma antigen detection. This innovative platform holds enormous potential for advancing personalized medicine and improving the clinical outcomes of HCC patients in Vietnam.

1. INTRODUCTION

Hepatocellular carcinoma is one of the leading causes of cancer-related deaths worldwide. It is also a prevalent and aggressive form of liver cancer with limited treatment options. Early detection and accurate diagnosis are crucial for successful treatment and improving patient outcomes. Recent years have witnessed significant progress in identifying biomarkers that aid in the diagnosis and prognosis of HCC [1]. Among the most promising biomarkers, such as AFP, DCP, and TXN [2] AFP is a well-known and widely used biomarker for HCC detection. The fetal liver primarily produces this glycoprotein during embryogenesis, which significantly decreases after birth [3]. However, in cases of HCC, the tumor cells can produce AFP, leading to its elevated levels in the blood [4]. And the elevated AFP levels are particularly useful for the diagnosis of early-stage HCC and monitoring the response to treatment [4]. The second biomarker protein, DCP also known as prothrombin induced by vitamin K absence-II (PIVKA-II), is another crucial biomarker for HCC [2]. HCC cells aberrantly produce DCP, a prothrombin precursor, due to their reduced vitamin K-dependent carboxylation [5]. The levels of DCP increase in the blood during HCC development, especially in the presence of AFP-negative HCC cases. Therefore, DCP serves as an important complementary marker to AFP, enhancing the overall sensitivity of HCC diagnosis [5]. The third biomarker, TXN, is a redox-active protein, plays a significant role in protecting cells from oxidative stress. Various cancers, including HCC, over express TXN, making it a potential biomarker for the disease. Its upregulation in HCC patients has been associated with tumor progression, invasion, and metastasis. Consequently, monitoring TXN levels may provide valuable prognostic information and help in identifying high-risk HCC patients who require more aggressive treatment strategies [6]. In conclusion, AFP, DCP, and TXN are valuable biomarkers for the early detection, diagnosis, and prognosis of HCC [7]. When used individually or in combination, they provide critical information for clinical decision-making.

Currently, clinical diagnostic methods such as liver biopsy, computed tomography (CT) scans, ultrasound imaging, and biomarker determination such as α-fetoprotein (AFP) are considered the "gold standards" for diagnosing liver cancer [8]. However, these tests have limitations as they often detect the disease at later stages, reducing the survival chances of liver cancer patients. Early detection of HCC could increase patient survival rates by enabling effective early treatments such as surgical resection or localized treatment methods [9]. The need arises to research new biomarkers for diagnosing and detecting liver cancer at an early stage.

Nanotechnology is commonly defined as a field of research involving materials and devices with dimensions smaller than 100 nanometers, which is equivalent to the size of biological macromolecules [10]. In nano-DNA technology (DNA- deoxyribonucleic), the building blocks are nucleic acid strands like DNA; these strands are often synthesized and used outside living cell environments [11]. DNA is well-suited for constructing nanostructures because of the base pairing rules that govern the interactions between two nucleic acid strands, which form precise nanostructures through helical structures [12]. These properties simplify the process of synthesizing nucleic acid structures by controlling their design. For biological applications, nanoparticles need to be surface-functionalized to enable binding with biological entities such as DNA, antibodies, and enzymes. Common functional groups include amino groups, biotin, streptavidin, carboxyl, thiol, silica, or charged surfaces [13].

Scientist are developing a novel structural design known as DNA origami technology, which creates nanostructures from a single long strand of DNA [14]. This long strand can fold back onto itself through self-interactions or fold back using shorter strands known as "staples." This can create two- or three-dimensional nanostructures, allowing them to carry more cargo simultaneously when reaching their target [15]. DNA nanorobots exemplify this uniqueness by carrying multiple antibodies simultaneously, thereby enhancing the targeting of cancer antigens. This proves more effective compared to other nanoproducts that only attach a single type of antibody. Recently, there have been encouraging achievements in the application of DNA nanoparticles in biomedical diagnostics [16]. This research direction aligns with the modern trends of the world, addressing health-related issues in human health and harnessing the interdisciplinary strength of science and application. In the

future, there should be a greater emphasis on highly applicable biological products, specifically diagnostic kits and pharmaceutical processes using nanomaterials [17].

2. MATERIAL AND METHODS

2.1. Recombination Antigen and Monoclonal Antibody

The Institute of Genome Research (IGR), Vietnam Academy of Science and Technology, provided the recombinant antigens (rAgs) and monoclonal antibodies (mAbs). The concentrations of purified rAgs were AFP (0.589 ng/ml), DCP (0.784 ng/ml), and TXN (0.662 ng/ml). Besides, the purified mAb concentrations were 1.012 mg/ml, 1.078 mg/ml, and 1.054 mg/ml of AFP, DCP, and TXN, respectively.

2.2. DNA Origami

The single-stranded antibody-conjugated DNA origami particles were designed and optimized in collaboration with the Gattaquant DNA Nanotechnologies (München, Germany). We create DNA origami particles loaded with antibodies are created using genetic material from the M13mp18 virus. The particles take shape through a computer-designed framework and scaffold, which are pre-programmed into a box-like form. The 3D software meticulously plans the structure. This involves defining sequences for staple and scaffold strands that assemble autonomously into the intended shape. The strategically designed staple strands bind to specific scaffold regions, guiding the structure's formation.

2.3. Colleting Serum Samples

A total of 80 serum samples from patients diagnosed with HCC, hepatitis B virus infection, and cirrhosis were collected from 103 Military Hospital in Hanoi, Vietnam. We stored the serum sample at 4°C before subjecting them to analysis and assessment. A total of 30 serum samples from healthy individuals who participated in a voluntary blood donation program were also collected and used as control samples.

2.4. Indirect ELISA Method

Microtiter plate wells (Costar, Corning Inc., USA) were coated with 50 µL of diluted serum (from 1:2 to 1:50 ratio) in coating buffer (0.5 M carbonate buffer, pH 9.6) and incubated at 4°C overnight. After discarding unbound serum, the wells were washed three times with Phosphate-Buffered Saline (PBS) containing 0.05% Tween-20 detergent (PBST). Next, all nonspecific binding was blocked with 200 µL of Bovine Serum Albumin (BSA)-containing PBS (from 0.5% to 2%) for 1 h. Continuously, the washing was done three times with PBST, and then 50 µL of detectable antibody (an IGR's production, dilution from 1:20 to 1:500 ratio) was added and incubated at 37°C for 1 h. After washing with PBST, a total of 50 µL of Goat anti-mouse IgG (H + L) secondary antibody Horseradish Peroxidase (HRP) (Invitrogen, USA) (diluted from 1:10,000 to 1:25,000 in PBS buffer containing 1.5% BSA) was added and incubated at 37°C for 1 h. Repeat washing steps, then add 50 µL of 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate (Life Science Technology, USA) and incubate at room temperature for 15 min. The reaction was terminated with 50 µL of 0.5M HCl. We recorded the optical density at 450 nm (OD_{450}) using a Biotek ELX808 ELISA plate reader (Biotek, USA). The working conditions were optimized by determining the highest ratio of OD_{450} positive (P) to negative (N). Each experiment and all samples were evaluated in triplicate.

2.5. Data analysis

The data were analyzed by nonparametric one-way analysis of variance using the student's t-test (for normally distributed data) or the Mann-Whitney test (for normally undistributed data). We performed all statistical analyses were performed using SPSS for Windows (SPSS, Chicago, USA), the results using Pearson's $\chi 2$ test and Fisher's exact test. In all cases, $P < 0.05$ indicates a statistically significant difference.

3. FINDINGS

3.1. DNA Nanomaterials Creation

Nano-DNA technology has demonstrated a promising application in the field of nanotechnology. Here, we harness the physical and chemical properties of DNA, not its genetic characteristics. Through the foundation of supplementary Watson-Crick base pairing, single-stranded DNA sequences can be folded and formed into a complete 3D structure. The process begins with a scaffold strand, a single-stranded DNA fragment sourced from bacteria and shapes it by incorporating over 200 additional short oligonucleotide strands referred to as staple strands. The resulting DNA molecules can be folded into various 2D shapes, such as squares, circles, or five-pointed stars, at the nanoscale, and can be stacked together to create 3D structures. To construct complex structures, developed computational programs can analyze connections, simulate factors influencing the folding and formation process into the eventual stable state, and devise a computer algorithm to calculate the folding path of scaffold DNA strands to achieve any desired shape. The moldability of computational software enables precise placement of binding molecules, like protein molecules, onto the surface of DNA origami structures.

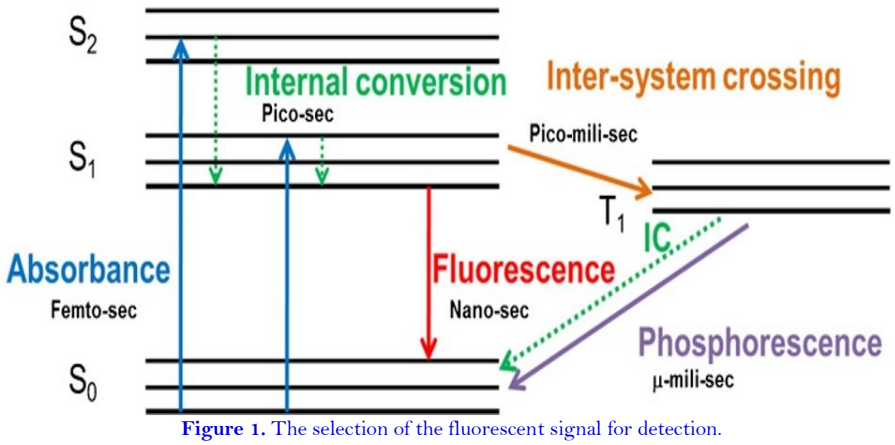

Figure 1. The selection of the fluorescent signal for detection.

In order to find the most suitable fluorescent signal, it is crucial to be able to compare candidate fluorophores. Certain properties exhibit variations, such as the excitation and emission spectra of fluorescent molecules. Generally, when a fluorescent molecule absorbs a photon, it becomes excited to a higher energy state (S1 or S2). The fluorescent molecule can only emit light in the form of fluorescence at the lowest energy level of S1. Therefore, the molecule must undergo an energy relaxation process to reach the lowest S1 energy level. We referred to this purpose as as nonradiative internal conversion. The molecule can also transition from the excited state to a third state (T1) through intersystem crossing. The energy decrease from T1 back to the initial state occurs through nonfluorescent or phosphorescent processes. These states and processes are crucial parameters for selecting appropriate fluorescent-emitting materials (Figure 1).

A monoclonal antibody-labeled nanofluorescent DNA structure for early diagnosis of liver cancer has been designed according to the principle above. We stain the original DNA origami particles green, the monoclonal antibodies (mAb) are stained yellow, and the target recombinant antigens (rAg) are stained red. The gold-stained antibodies are attached to the DNA origami particles. It is impossible to find the target antigens carried by the DNA origami particles in areas that fluorescence both yellow and red (Figure 2).

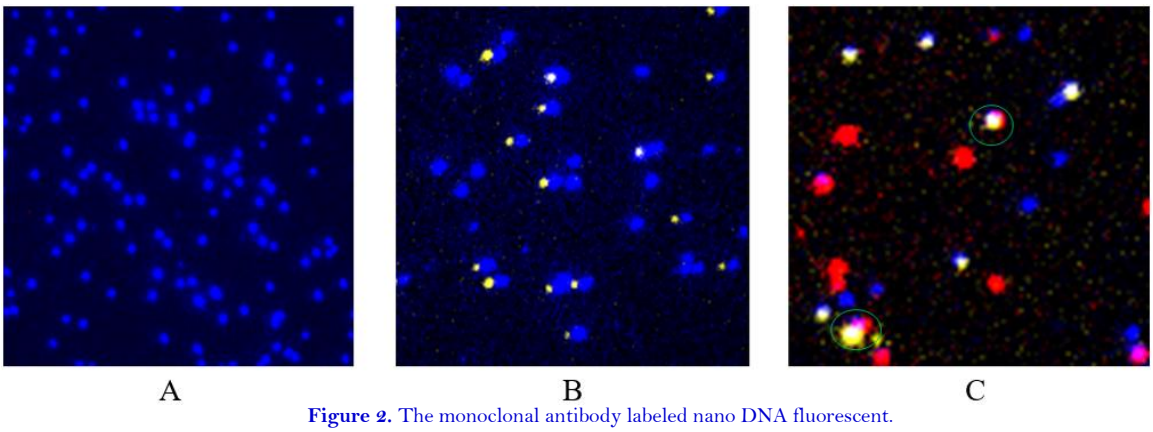

Figure 2. The monoclonal antibody labeled nano DNA fluorescent.

In Figure 2, the original DNA origami particles are stained green, the monoclonal antibodies (mAb) are stained yellow, and the target recombinant antigens (rAg) are stained red. The DNA origami particles attach the gold-stained antibodies to them. Regions displaying both yellow and red fluorescence demonstrate the capability to detect the target antigens carried by the DNA origami particles.

3.2. Optimization of ELISA Indirect Conditions

The formation of chemical bonds with functional groups of amino acids easily links antibodies and DNA origami. Among these, Lysine is one of the most commonly utilized amino acids for connecting DNA and antibodies, as it is often located on the antibody's surface, making it easily accessible. An antibody can contain up to 80 lysine molecules [18] which can lead to varying degrees of binding heterogeneity between the antibody and DNA, such as differing amounts of DNA attached to the antibody and antibodies bound to DNA at different positions [19, 20]. However, we can control and limit the heterogeneity associated with this chemical equilibrium can be controlled and limited to a certain extent by adjusting the concentration of antibodies and DNA, and fine-tuning specificity using chemical methods [21].

Bridging molecules commonly link the lysine of antibodies with DNA molecules. These bridging molecules typically carry two functional groups. One end of this molecule will bear one of the functional groups such as N-hydroxysuccinimide (NHS) esters, sulfonyl chlorides, isocyanates, or isothiocyanates, which will form a bond with the remaining free amino group on lysine. The other end of the bridging molecule might have maleimide, which will then bond with DNA strands that have been modified with thiol-modified or azides in the next reaction (Figure 3).

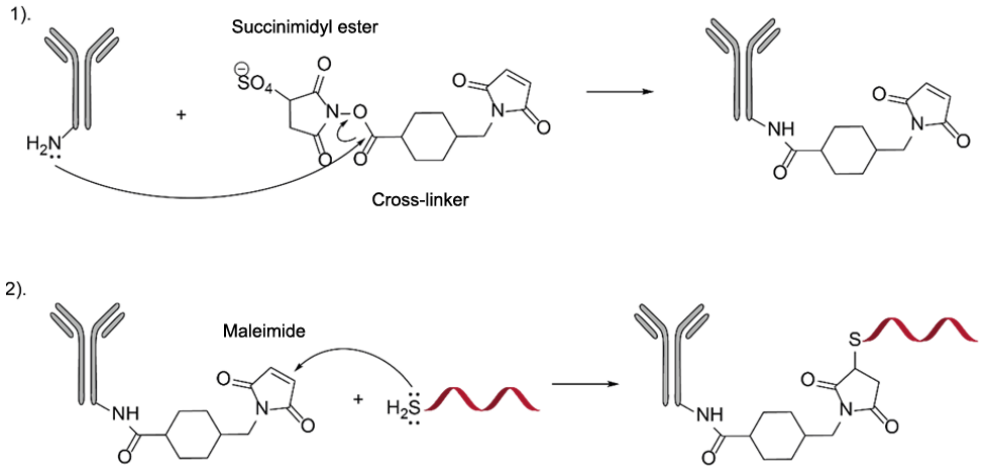

Figure 3. The reaction for forming a bond between antibodies and DNA nanostructures.

In Figure 3, the linkage between the lysine of antibodies and DNA molecules is commonly achieved through bridging molecules. These bridging molecules typically carry two functional groups. One end of this molecule will bear one of the functional groups such as N-hydroxysuccinimide (NHS) esters, sulfonyl chlorides, isocyanates, or isothiocyanates, which will form a bond with the remaining free amino group on lysine. On the other end, the bridging molecule, there may be maleimide, which will then bond with DNA strand that have been modified with thiol or azides.

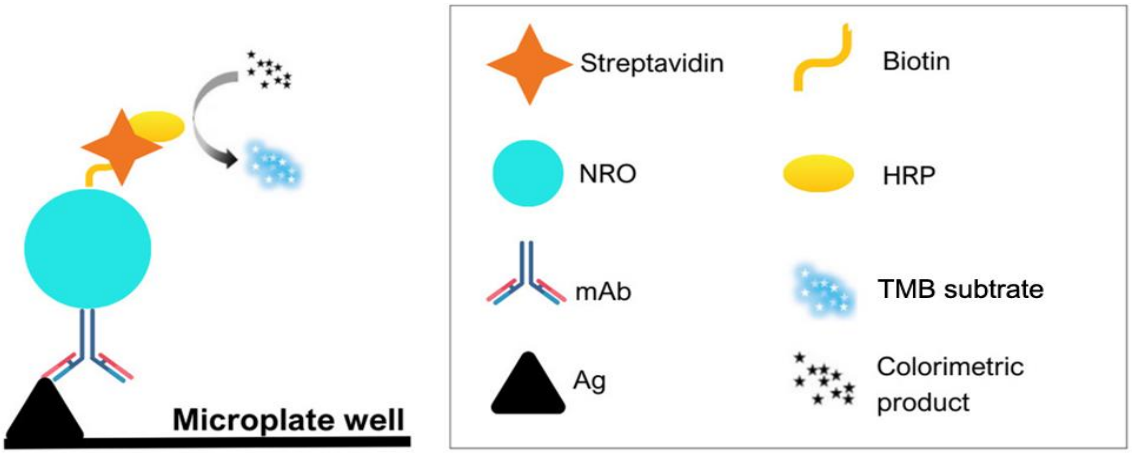

Figure 4. Schematic model of the indirect ELISA protocol. The four steps are: Coating of microplate wells with antigen; adding NanoDNA Origami (NRO) with biotin-conjugated antibodies to the microplate well; detection of biotin by streptavidin-hrp; and finally enzymatic activity monitored by addition of TMB chromogenic substrate.

The assay procedure immobilizes samples containing the target antigens on a solid surface. The DNA origami-conjugated monoclonal antibodies are then introduced and allowed to bind to the target antigens. We add an enzyme-linked secondary antibody after thorough washing to remove an unbound component. This secondary antibody recognizes and binds to the monoclonal antibodies conjugated to the DNA origami structures. We then add a substrate solution to initiate an enzymatic reaction, which leads to the development of a colored product. The intensity of this color is proportional to the presence of the target antigen (Figure 4).

The ELISA reaction conditions corresponding to the highest positive/negative (P/N) values are generally considered to be the optimal conditions. The optimal reaction conditions of the study have been performed in a square matrix titration test Table 1.

Table 1. P/N values of antigen- antibody indirect ELISA square matrix titration test.

Antibody dilution	Antigen dilution											
	AFP				DCP				TXN			
	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16
1:2	3.134	2.749	2.496	1.911	2.940	2.967	2.501	2.368	2.765	2.808	3.087	2.785
1:4	3.087	2.456	2.098	1.644	2.987	3.015	2.762	2.509	3.144	2.997	2.974	2.862
1:8	2.987	2.509	1.905	1.753	3.077	3.149	3.067	3.396	3.399	3.054	3.178	2.890
1:16	2.701	2.765	2.501	1.966	3.295	3.503	2.883	2.630	2.793	3.194	3.019	2.984
1:32	2.353	2.808	2.894	1.696	3.450	*3.578*	3.091	2.765	3.167	3.280	2.966	2.483
1:64	1.678	2.546	*3.598*	2.034	2.900	3.439	2.634	1.931	2.307	*3.423*	2.305	1.956
1:128	0.854	1.983	2.408	2.173	2.865	2.995	2.014	1.705	2.206	2.787	1.839	1.600

As shown in Table 1, the optimal coating antigen dilution was determined to be 1:8 for AFP, 1:4 for both DCP and TXN. The optimal working dilution of specific monoclonal antibody binding nano DNA origami is 1:64 for AFP and TXN, and 1:32 for DCP. We optimize the other reaction conditions by varying a single parameter at a time.

The other results indicated that the optimum dilution of HRP-conjugated Streptavidin was 1:15,000 for AFP (Figure 5A), and 1:20,000 for DCP and TXN (Figures 5D and 5G). The best blocking solution was determined to be a carbonate buffer containing 1.5% BSA (Figures 5B, 5E, and 5H). Both the mAbs and the HRP-conjugated Streptavidin were incubated for 60 minutes, respectively (Figure 5C, 5F and 5I).

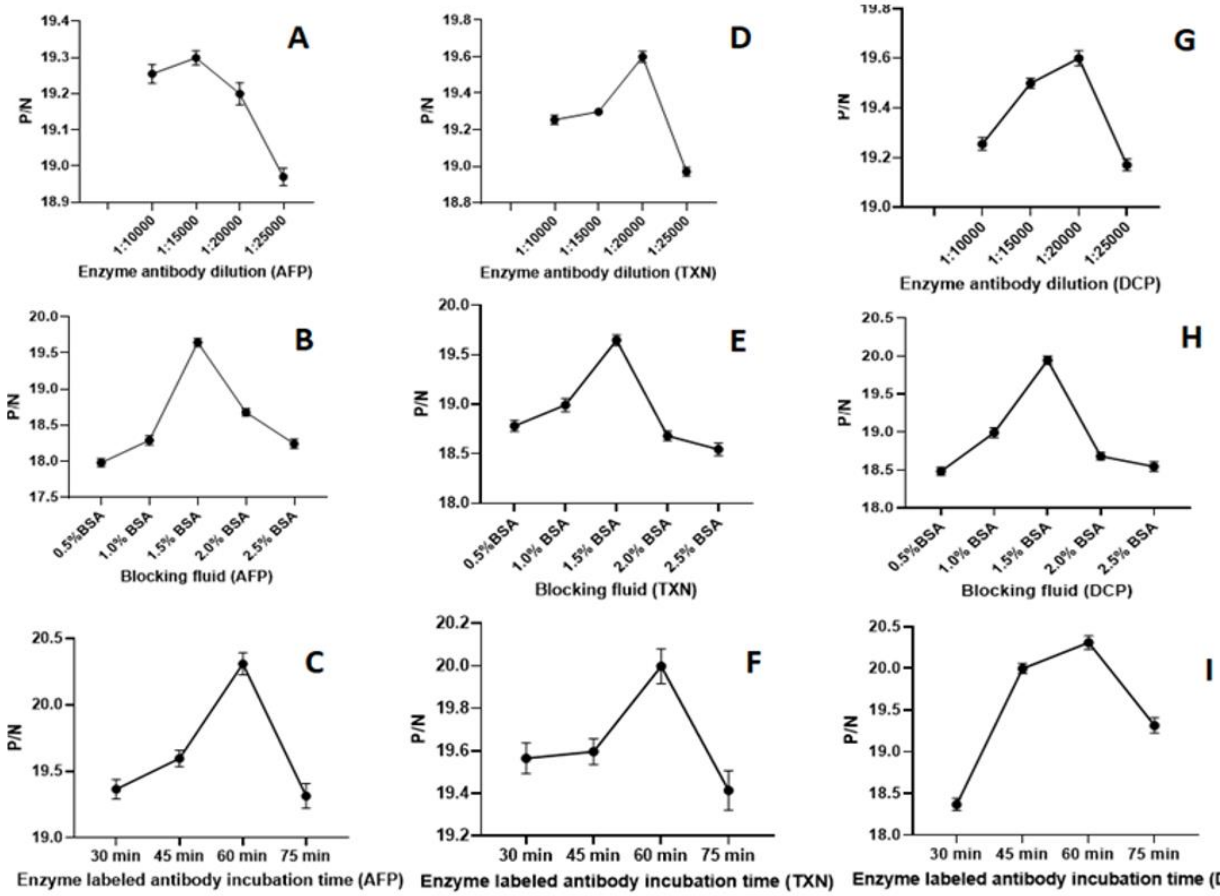

Figure 5. Optimization of ELISA indirect conditions. The optimal dilution of the HRP-conjugated streptavidin was selected according to the P/N value for AFP (A), TXN (D), and DCP (G). The optimal blocking buffer was selected according to the P/N value for AFP (B), TXN (E), and DCP (H). The optimal enzyme labeled antibody incubation time was selected according to the P/N value for AFP (C), TXN (F), and DCP (I).

3.3. Determination of Cut-Off Value in the Indirect ELISA

To determine the cut-off value of positive and negative samples, 30 human negative serum samples without AFP, DCP, and TXN antibodies were used to detect them by the indirect ELISA method. In the samples containing AFP antigen, the results showed that the average of OD_{450} values was 0.202, standard deviation was 0.069 and cut-off value was determined to be 0.132 (Figure 6A). Additionally, the samples containing DCP antigen also indicated that the mean values of OD_{450}, standard deviation, and cut-off value were 0.185, 0.037, and 0.156, respectively (Figure 6B). Similarly, the samples containing TXN antigen have been shown to have a mean OD_{450} value of 0.185, a standard deviation of 0.05, and a determined cut-off value of 0.150 (Figure 6C). Therefore, they could determine that the human serum samples are positive if the OD_{450} value is on the cut-offline; otherwise, the samples could be determined to be negative.

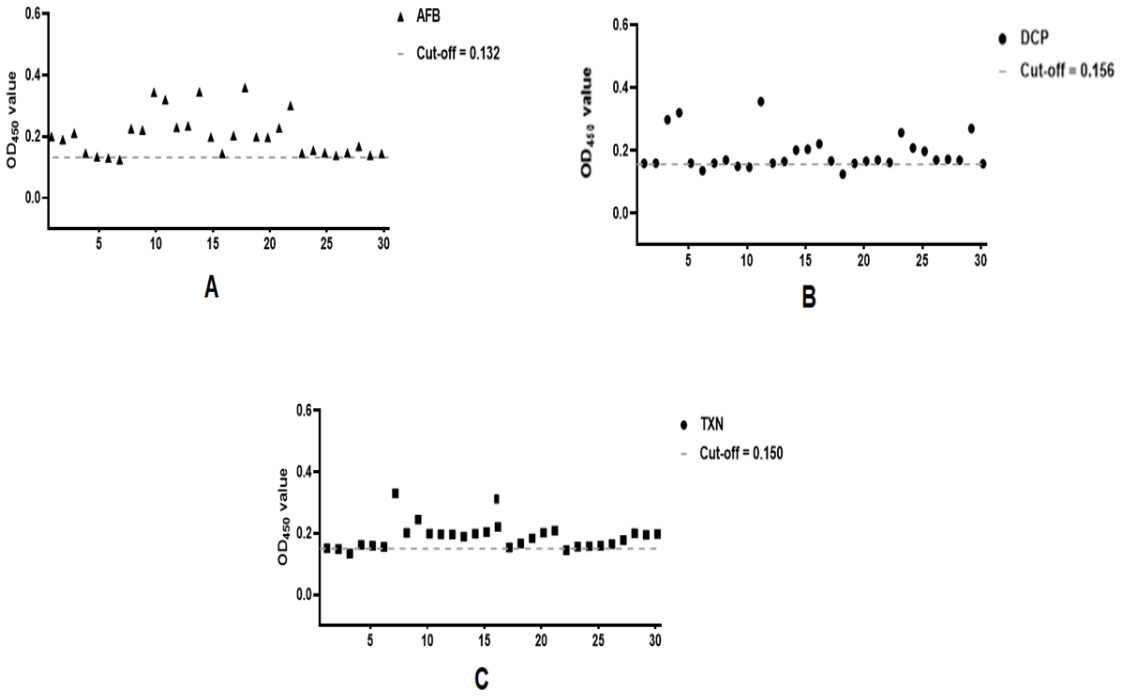

Figure 6. Determination of the cut-off value for the indirect ELISA. The average value plus the standard deviation of three times was used as the cut-off value, which was found to be 0.132 for AFP, 0.156 for DCP, and 0.150 for TXN. Meaning that if the serum OD value of the test is above the cut-off value, it is considered positive for the corresponding antibody. OD values below the cut-off value are evaluated as negative.

3.4. Assessing Sensitivity, Specificity and Reproducibility in Indirect ELISA

To gauge the sensitivity and specificity of mAb-carrying NROs, commercial kits used in the experiment include Human Trx (Thioredoxin) ELISA Kit (Finetest, Wuhan, China), Human DCP (Des-gamma carboxyprothrombin) ELISA Kit (Finetest, Wuhan, China), and Human AFP (Alpha Fetoprotein) ELISA Kit (Finetest, Wuhan, China). We subjected recombinant antigens and monoclonal antibodies produced by the self-laboratory (Register intellectual property of IGR in Vietnam) to ELISA, following the protocol of the commercial kits above. In which commercial antigens are diluted 1:64, recombinant antigens and monoclonal antibodies produced by the laboratory are diluted 1:2. The conjugated antibody is goat anti-mouse IgG (H + L) secondary antibody HRP (H + L) (Invitrogen, USA).

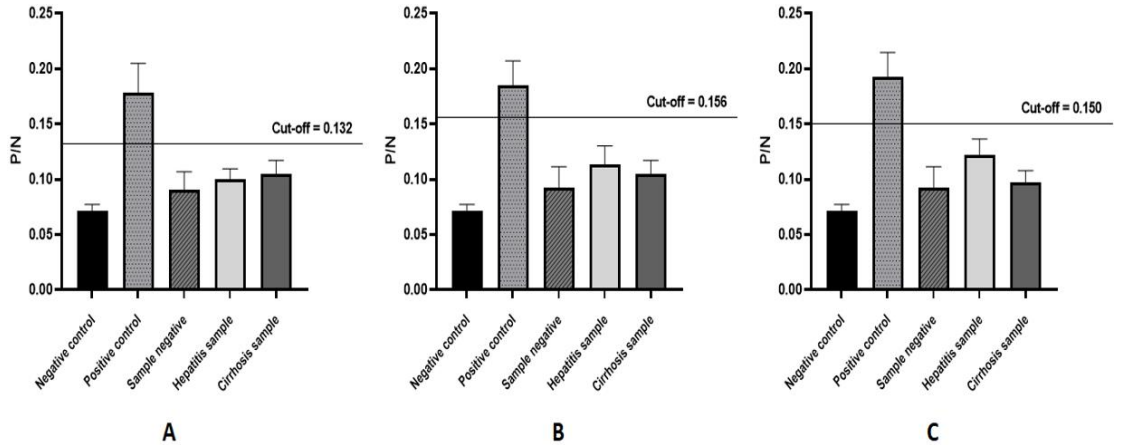

Figure 7. Specificity analysis of the indirect ELISA. NRO-mAb for the negative control, positive control, sample negative, Hepatitis sample, and Cirrhosis sample were detected by the indirect ELISA, and the P/N values were recorded. The P/N values of the other four anti-serums were below the cut-off value except that of Positive control.

Another gauge of the specificity of mAb-carrying NROs and cross-reactions was performed with diagnostic samples for both hepatitis B virus infection and Cirrhosis. As depicted in Figure 7, the positive control sample

(featuring recombinant antigen) displayed P/N values exceeding the designated cut-offline, whereas negative reactions registered values below this threshold. Additionally, the results showed that the mAb-carrying NROs didn't react with any other serums, which supports the strong specificity of the standard indirect ELISA protocol. To evaluate the reproducibility of the procedure, the average intra-assay and inter-assay coefficients of variation (CVs) were determined. The CVs for all samples remained consistently under 10%, indicating the method's high degree of stability and reproducibility.

4. DISCUSSION

Researchers have proven the effectiveness of screening and early cancer detection in curing and enhancing the survival rates of cancer patients. However, statistical results indicate that most Vietnamese cancer patients diagnosed with cancer are already in advanced stages. Nevertheless, due to issues related to selecting appropriate screening techniques, as well as the demand for significant equipment and financial resources, these programs have been conducted only within specific population groups and for certain types of cancer. One of the primary reasons for the high cost of early cancer screening tests is the use of imported testing kits, which local medical facilities have not been proactive in producing these kits. As a result, individuals at risk of common cancers in Vietnam who are facing economic difficulties have limited opportunities to access early diagnostic testing for early-stage treatment.

Vietnam is currently developing and using cancer screening kits that stand out for their use of inorganic nanoparticles. However, this method exhibits drawbacks associated with inorganic structures, such as difficulties in altering the nanostructure's shape or substituting parts during optimization. This could decrease the effectiveness of the products. Furthermore, previous research has raised concerns about potential toxicity to normal tissues when using inorganic nanomaterials [22]. The utilization of nanomaterials derived from DNA enhances biocompatibility and reduces cell toxicity [23]. Additionally, the flexible structure allows for the formation of various nanostructures and precise assembly of different sensing elements onto DNA nanostructures down to the nanometer level. This enables the creation of a programmable sensing system with high efficiency for cancer detection, even during the initial stages when only a small number of cancer cells are present in the body [24].

The anti-body antigen principle-based early cancer detection technology attaches single-stranded antibodies to fluorescent DNA nanostructures, which can be performed both inside and outside the body [25]. This is a notable advantage of the type of kit. As of now, DNA nanotechnology is entirely new in Vietnam, with no research publications on the application of DNA nanomaterials in medicine, particularly for early diagnosis of common cancer types in Vietnam such as liver cancer, lung cancer, and colorectal cancer. By utilizing DNA origami-conjugated monoclonal antibodies, this indirect ELISA method offers enhanced sensitivity and specificity in antigen detection in serum human samples [26]. The incorporation of DNA origami structures into the assay provides a versatile platform for immobilizing antibodies and increasing the efficiency of antigen-antibody interactions [27]. This approach holds promise for various applications, including diagnostic testing and biomarker detection.

The interaction between biotin and streptavidin, which combines DNA origami with specificity of single-domain antibodies, is a big step forward in nanobiotechnology [28]. This strategy empowers researchers and scientists to engineer versatile nanoscale structures with programmable molecular recognition capabilities. The applications of this technology extend across diverse fields, from targeted drug delivery to ultra-sensitive biosensing, holding the potential to revolutionize medicine, diagnostics, and beyond.

5. CONCLUSION

In summary, the extra method to detect antigens using DNA origami nanoantenna labeled specific monoclonal antibodies was developed successfully on a laboratory scale. The combination of nano DNA fluorescent allows the binding of multiple antibodies such as AFP, DCP, and TXN at the same time or labeling alone, increasing the efficiency of HCC detection. From the test results, it has been proven the sensitivity, specificity, stability, and

reproducibility of the method are relatively high. Researchers should continue to refine and expand upon this conjugation strategy. This study anticipates witnessing the emergence of innovative solutions to some of the most pressing challenges in science and healthcare.

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Transparency: The authors state that the manuscript is honest, truthful, and transparent, that no key aspects of the investigation have been omitted, and that any differences from the study as planned have been clarified. This study followed all writing ethics.
Competing Interests: The authors declare that they have no competing interests.
Authors' Contributions: All authors contributed equally to the conception and design of the study. All authors have read and agreed to the published version of the manuscript.

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