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Synthesis of Nano-Graphene Oxide as a Novel Adjuvant for Production of Polyclonal Antibodies in Assessment of Radioimmunoassay System

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ABSTRACT

This study aims to prepare Nano-Graphene oxide (N-GO) for use as a new adjuvant to stimulate immune system against alpha-fetoprotein (AFP) antigen to generate AFP polyclonal antibodies (anti-AFP) to use for measuring AFP in human serum by radioimmunoassay system. The prepared N-GO as a new adjuvant compared with Freund's adjuvant. This study include two groups of New-Zealand rabbits. Group I: immunized with AFP antigen emulsified with N-GO formula and group II: immunized with AFP antigen emulsified with Freund's adjuvant. The produced anti-AFP polyclonal were characterized in terms of titer, immunoresponse and displacement. The data of displacement % of group I immunized with AFP-N-GO formula were (79.5%, 75.4%, and 68.7%) and the data of displacement % of group II immunized with AFP- Freund's adjuvant were (68.7%, 62.8% and 58.7%) at dilution 1/16,000. 125I-AFP tracer was prepared using Chloramine-T oxidation method. 125I-AFP tracer was separated and purified by PD-10 column. The assay's optimization and validation were investigated. The standard curves for produced anti-AFP polyclonal for both of N-GO and Freund's adjuvant were studied, r = 0.996. On conclusion, new adjuvant formula (N-GO) is appropriate to stimulate immune system to produce polyclonal antibodies as a basic component in assessment liquid phase radioimmunoassay to measure hormones or tumor markers.

1. INTRODUCTION

Materials based on carbon Skelton such as carbon nanotubes, fluerne, graphene are ecofriendly and considered as safe and non-toxic materials. Graphene has a unique nanostructure, it consists of SP^2 hyberdization carbon atoms and set in a single layer and every atom of carbon is bounded to three neighboring atoms by covalent bond and arranged in a honeycomb structure [1].

Graphene can be chemically modified with different oxidizing agents to produce graphene oxide (GO) which is enriched with different oxygen function groups such as, CO carbonyl, (-COOH) carboxylic, (-OH) hydroxyl, and epoxy (-O-) [2, 3]. Graphene oxide (GO) is mainly composed of flake graphite natural material which is oxidized to give graphite-bearing oxygen function groups and which can be exfoliated into GO by dispersing into water [4]. Functionalized nano-sized of graphene oxide (N-GO) can be used in different medical field as a drug carrier for different intercellular drugs [5, 6], antibacterial materials [7, 8], gene delivery [9], biosensing [10, 11], cancer treatment [12] and other medical applications [13-15].

Adjuvants are essential parts of vaccine formulations that strengthen the immune system's reaction to an antigen, thereby increasing the vaccination's efficacy [16]. These substances work by mimicking pathogen-associated molecular patterns (PAMPs), which cause innate immune cells to activate and stimulate a more robust adaptive immune response. Adjuvant is work by creating a depot effect; this allows for a slow release of antigen, prolonging its exposure to the immune system [17].

A quintessential example of adjuvants are Freund's adjuvants. It is essential to immunology through strengthening the immune system's reaction to an antigen. Adjuvants are primarily utilized in vaccine research and formulation due to their ability to increase the efficacy of antigens. There are two main types: Complete Freund's Adjuvant (CFA) and Incomplete

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Freund's Adjuvant (IFA). CFA contains heat-killed Mycobacterium tuberculosis, which triggers a robust immune reaction, while IFA lacks this bacterial component but still promotes prolonged antigen exposure through the creation of an emulsion that slowly releases the antigen over time. The use of CFA is associated with a stronger initial immune response due to its inclusion of mycobacterial components that stimulate innate immunity, whereas IFA is preferred for booster doses because it elicits a high level of antibody production without inducing severe inflammation [18, 19].

Alpha-fetoprotein (AFP) is a significant tumor marker utilized primarily in the detection and monitoring of liver cancer and germ cell tumors. AFP is an oncofetal glycoprotein found in a single polypeptide chain with a molecular weight 70 KD [20]. The AFP normal range is 2 to13 ng/ml. Elevated levels of AFP can be indicative of hepatocellular carcinoma, with sensitivities reaching up to 60-70%, making it a vital tool in early diagnosis and prognosis and furthermore, AFP is essential for the evaluation of non-seminomatous testicular cancer, where its levels can help differentiate between various subtypes of germ cell tumors. Also, increased AFP can also be seen in benign liver diseases like cirrhosis and hepatitis [21].

Radioimmunoassay (RIA) is a sensitive and accurate method for determining the concentrations of antigens, like hormones and drugs, within biological samples. In this technique, a high-affinity antibody is competitively bound to radiolabeled and unlabeled antigens, allowing for precise quantification through radioactive emissions detected by gamma counters. Developed in the 1960s by Yalow and Berson [22], RIA revolutionized clinical diagnostics and biomedical research due to its unparalleled sensitivity in detecting very low concentration of substances. The process begins with the introduction of a known amount of radioactively labeled antigen into a sample containing an unknown concentration of the same unlabeled antigen.

Both forms compete for binding sites on the limited amount of antibody molecules. Bound from free antigens are separated using techniques like precipitating with secondary antibodies or adsorption on solid-phase matrices. RIA remains foundational for its extraordinary specificity and capability to measure extremely low analyte concentrations reliably [23].

This work aims to prepare the formulation of Nano-graphene oxide (N-GO) and highlight its role as immune stimulating formula as adjuvant comparison with traditional Freund's adjuvant and evaluation of AFP in human serum using double antibody liquid-phase RIA system.

2. MATERIAL AND METHODS

2.1. Chemicals and reagents

Graphite (extremely pure fine powder) was obtained from Merck Co. Germany. Potassium permanganate ($KMnO_4$) was bought from BDH chemicals ltd, England. $NaNO_3$ and sulfuric acid (H_2SO_4, 95-97%) were obtained from Sigma- Aldrich, Germany. Hydrogen peroxide (30 % H_2O_2, Alpha, India). Pure human alpha-fetoprotein (AFP) antigen, International Reference Preparation (IRP), goat anti-rabbit, normal rabbit serum (NRS) were obtained from Scottich Antibody Production Units (SAPU), Scotland, polyethylene glycol (PEG-8000), Complete and incomplete Freund's adjuvant, Chloramin-T, Mineral oil, Sigma Co., USA. Sodium Iodid-125 ($Na^{125}I$), Izotop. Each ingredient utilized was pure enough for analytical usage.

2.2. Animals

Six adult male New Zealand white rabbits, weighing 2-3 kg at 4 months age, served as the host animals for the production of the polyclonal antibodies against AFP.

2.3. Methods

i. Synthesis of Nano - Graphene Oxide:

The modified Hummers method was used to synthesis Nano-Graphene oxide (N-GO) from pure natural graphite powder [24, 25]. This process involved mixing 0.5 g of graphite and 0.5 g of NaNO3, then adding 23 ml of 98% concentrated sulphuric acid with stirring at least three hours until a homogenous heavy gelatinous is formed. After that, 3 g of $KMnO_4$ was gently mixed to the gelatinous solution at temperature under 20 °C. After two hours of stirring the mixture at room temperature (30 °C), the resulting soluting was diluted with 30 ml of distilled water and 10 ml of 30% H_2O_2 was added to the suspension for converting the unreacted manganese dioxide and permanganate into soluble sulphate. To obtain graphene oxide sheets, the resultant mixture was repeatedly rinsed with distilled water, sonicated for 1 hour using ultra-sonication, and the residual solid material was dried for 24 hours at 80 °C. The N-GO prepared was characterized in terms of X-ray diffraction, IR- spectrum and SEM- micrographs.

ii. Production of anti-AFP polyclonal

This study included 6 adult healthy male White New-Zealand rabbits, weighing 2-3 kg. They were divided into two groups, each with three rabbits. In this study the production of anti-AFP polyclonal was done by two methods.

The first method was the production of anti-AFP polyclonal against AFP antigen which prepared by using nano-graphene oxide (N-GO) as a novel adjuvant formula to stimulate immune system of rabbits.

The second method was the production of anti-AFP polyclonal which done by using Freund's adjuvant as a traditional method to compare the immunoresponse with a novel adjuvant formula (N-GO) in the first method.

iii. Schedule of immunization

Group I (first method): In order to create a novel adjuvant formula (100 µg N-GO/ 1 ml mineral oil) at a ratio of 1:3, 0.3 mg of AFP/750 µl H2O emulsified with 2.25 ml of N-GO was used to immunize rabbits. Each rabbit received 0.1 mg of AFP antigen subcutaneously and intramuscularly in 1 ml of emulsion (100 µg N-GO/ 1 ml mineral oil) at 2-week intervals as part of the immunization program.

Group II (second method): The rabbits in this group received an immunization of 0.3 mg AFP diluted in 750 µl distilled water and emulsified with 2.25 ml Freund's adjuvant complete (FAC) at a ratio of 1:3 According to Chapman [26]. The basic vaccination schedule, which included initial immunization and three booster doses were given every two weeks. Subcutaneous and intramuscular injections of 0.1 mg AFP antigen in 1 ml emulsion were given to each rabbit. The boosters were administered in the same method, but with Freund's adjuvant incomplete instead of complete.

Blood samples were drawn from the rabbits via the marginal ear vein two weeks after the primary immunization and blood samples were also collected, two weeks after each booster for the immunization schedule [27]. Antiserum (anti-AFP) was separated from the blood samples and stored at – 20 ^{o}C until using.

iv. Characterization of the obtained antibodies for RIA system

Anti-AFP was characterized and tested for both dilution titer and displacement factors, as well as immunoresponse.

In order to determine the titer and maximum displacement percent between the maximum binding percent (Bo%, 0.0 ng/ml) and minimum binding percent (highest standard B%, 550 IU/ml) for each bleeding harvested antisera using the RIA system, various antibody dilution curves were constructed with each of the AFP standards zero and high levels (0.0 and 550 IU/ml).

***Assay design*:** Anti-AFP (100 ml) was put into the assay tubes at the following serial dilutions: 1/500, 1/1000, 1/2,000, 1/4,000, 1/8,000, 1/16,000, 1/32,000, 1/64,000, and 1/128,000, then100 µl of AFP standards (0.0 or 550 IU/ml) and 100 µl of ^{125} I- AFP were added to each tube, respectively. After three-hour incubation period at 37 °C, the following order of separating reagents were added to assay tubes: 100 µl of non-immune rabbit serum at a dilution of 1:200, 100 µl of goat anti-rabbit serum (the second antibody) at a dilution of 1:50 and 500 µl of 4% polyethylene glycol (PEG-8000). The assay tubes were incubated at room temperature for 30 minutes and were centrifuged for 15 minutes at 4000 rpm at 4 ^{o}C. After that, the tubes were accurately decanted, and a gamma-counter was used to count the sediment that contain the precipitated antibody.

For all bleeding of each rabbit, the percentage binding for both (Bo /TA) and (Bs /TA) were determined. Dilution curve for each AFP (0.0 or 550 IU/ml) were calculated as % binding of labeled AFP antigen (^{125} I- AFP) against antiserum of AFP at serial dilutions. In the optimized assay, the antiserum dilutions that provide a highest displacement percentage between zero and high standards were selected as the optimum antibody titer [28, 29]. Titer and displacement for each rabbit were plotted as displacement % against serial dilutions of the anti-AFP polyclonal.

The anti-AFP that had the highest titer and displacement was selected and subjected to purification using the sodium sulphate / ammonium sulphate precipitation technique in accordance with Parham et al. [30].

The purification procedure was carried out as a follow: A neat rabbit antiserum of AFP (5 ml) and 900 mg of sodium sulphate powder were added little by little during a period of one hour at 4 ^{o}C while stirring continuously. Centrifugation was used for 30 minutes at 10,000 rpm at 4 ^{o}C to pellet the precipitate that had produced. After that, 5 ml of saturated ammonium sulphate (SAS) solution were added drop by drop while stirring continuously for 1 hour at 4^{o}C. The mixture was then centrifuged for 30 minutes at 10,000 rpm at 4^{o}C. Supernatant was discarded, the precipitate of IgG-AFP was re-dissolved in a minimum volume (5 ml) of phosphate buffer (0.05 M, pH 7.4) and dialyzed against phosphate buffer (0.002 M, pH 7.4) at 4 ^{o}C for 48 hour with 4 changes of dialysis buffer.

v. Preparation of AFP standards

Standards of AFP were prepared and purified from human cord blood [31]. The AFP antigen was purified from cord serum using ammonium sulphate precipitation method according to Stanely and Randy [32]. NaCl (0.9 %) was used to dilute the cord serum after purification and the concentration of AFP antigen was evaluated by spectrophotometer at 280 nm [33]. The working standards of AFP (ranged from 5-550 IU/ml) were performed and compared with International Reference Preparation (IRP).

vi. Radioiodination of AFP

The radioiodination of AFP antigen with radioactive ^{125}I was prepared using Chloramine-T as oxidizing agent according to Hunter and Greenwood [34]. The following components were added to the Eppendorf tube as follows, 20 µg AFP antigen / 10 µl phosphate buffer (0.5 M, pH 7.4) and 5 µl of Na^{125}I (500 µCi, 18.5 MBq). To start the reaction, 10 µl of phosphate buffer (0.05 M, pH 7.4) containing 10 µg Chloramine –T was used. The iodination reaction mixture was mixed by vortex for 60 second at room temperature and the reaction was stopped by addition 10 µg of sodium metabisulphite in 10 µl phosphate buffer (0.05 M, pH 7.4) and 20 µg of potassium iodide in 100 µl phosphate buffer (0.05 M, pH 7.4) as a carrier. Gel chromatography on PD-10 column (sephadex G-25) was used to separate and purify the reaction mixture of labeled ^{125}I-AFP tracer from free radioactive iodide (^{125}I). Following the injection of the reaction mixture onto the PD-10 column, the elution buffer (0.05M phosphate buffer containing 0.01% BSA and 0.1% sodium azide) was added. The fractions of ^{125}I-AFP tracer in pure form were separated and counted by using the Radioisotope Dose Calibrator (CRC-15 R Captunic Ionization Chamber, Mark, International Equipment Co., USA).

The elution pattern was created by plotting radioactive counts versus fraction numbers. ^{125}I-AFP peak was used to calculate the radioactivity percentage. The fractions of ^{125}I-AFP tracer were collected together as stock and kept at – 20 ^{o}C till use. In order to use the stock solution of ^{125}I-AFP tracer as a working tracer in the RIA system, it was diluted with assay buffer to give about 20,000 cpm using a gamma counter. The specific activity of the prepared ^{125}I-AFP tracer, radiochemical yield, maximum binding, and non-specific binding were calculated

vii. Optimization of AFP-RIA system

The optimization processes of the assay reaction including some parameters such as sample volume, incubation time were carried out using double antibody liquid phase RIA technique.

The assay was set up as follows: assay tubes were arranged in pairs then, 100 µl of the obtained anti-AFP with suitable dilution, standards or unknown samples (50, 100 or 200 µl) and 100 µl of ^{125}I-AFP tracer counted ~ 20,000 cpm were added and mixed. The mixture was then incubated with different incubation time (1, 3, 24 h) at 37 ^{o}C. The process for separating the bound from free fractions was performed by adding 100 µl of second antibody (goat anti-rabbit-IgG) at dilution (1:50), 100 µl of normal rabbit serum (NRS) at dilution (1:200) and 500 µl of polyethylene glycol (PEG-8000) at concentration (4%) to all assay tubes. After standing for 30 minutes at room temperature, the tubes were centrifuged for 15 minutes at 4000 rpm at 4 ^{o}C. After that, the supernatant was decanted and the bound fraction was counted by gamma counter. Two sets of AFP standard curves were created based on the outcomes of optimization studies performed using the liquid-phase RIA.

viii. Validation tests of AFP-RIA system

Validation tests applying the optimized conditions of the liquied phase AFP-RIA system such as sensitivity, intra and inter assay precision, accuracy (recovery and dilution tests) and method comparison were studied.

3. RESULT AND DISCUSSION

X-Ray Diffraction

X-ray diffraction (XRD) is analytical method used to identify the phase of solid materials (crystalline or amorphous).

Also, it is used to determine the interlayer atomic space of this material. XRD depends on interaction of monochromatic radiation with the sample to produce constructed and diffracted rays. By applying Bragg equation: $n\lambda = 2d \sin \theta$

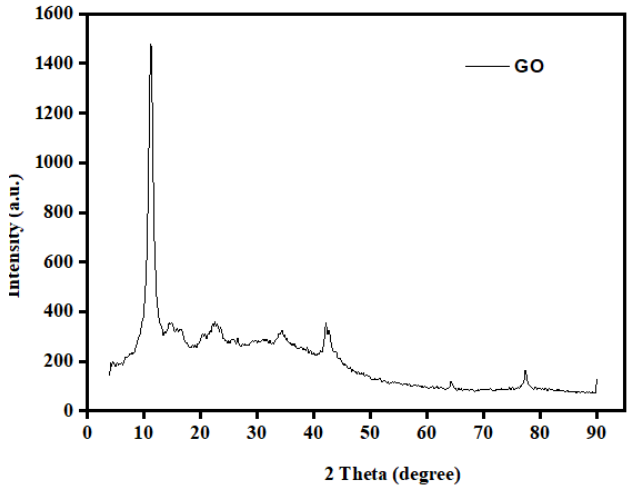
Where

λ is wave length of the electromagnetic radiation

θ is diffraction angle

d is inter atomic spacing of sample

Scanning the sample through 2θ range, all possible diffraction can be obtained and by applying Bragg equation, d spacing is calculated. Figure (1) shows a strong and sharp diffraction signal at about 2θ = 10.6° indicating a highly crystal material and interlayer spacing is 0.78 nm.



Also, from XR diffraction pattern, it can calculate the crystallite size of graphene oxide applying the Scherrer equation: $D = Ky/B \cos \theta$

Where

D is crystallite size nm

K is shape factor equals 0.94

y is wave length of X-ray 0.15406 nm

B is full width half Maxima in radians

θ is diffraction angle.

By applying this equation, it was found that the crystallite size of graphene oxide is 29 nm.

IR – Spectrum

As indicated in Figure (2), the FTIR spectra of N-GO show a very broad peak at 3427.84 cm^{-1}, which is corresponding to –OH group stretches. The peaks in aliphatic –CH that were observed at 2923.91 and 2853.91 cm^{-1} correspond to symmetric and anti-symmetric stretching vibrations [35]. The stretching vibration mode of the C=O, C=C and =C–O groups may be related to the peaks located at 1741, 1622, and 1461 cm^{-1}, respectively. The stretching of alcoholic C-OH and carboxylic COOH is represented by the bands that appear at 1383 and 1120 cm^{-1}, respectively. The C-O-C stretching is apparent by the peak at 1268.1 cm^{-1}, while the C-O group's vibration mode is indicated by the peak at 1036 cm^{-1} [36]. These distinct oxygen function groups on the surface provide confirmation of graphite's oxidation and provide information on graphene oxide's hydrophilic nature.

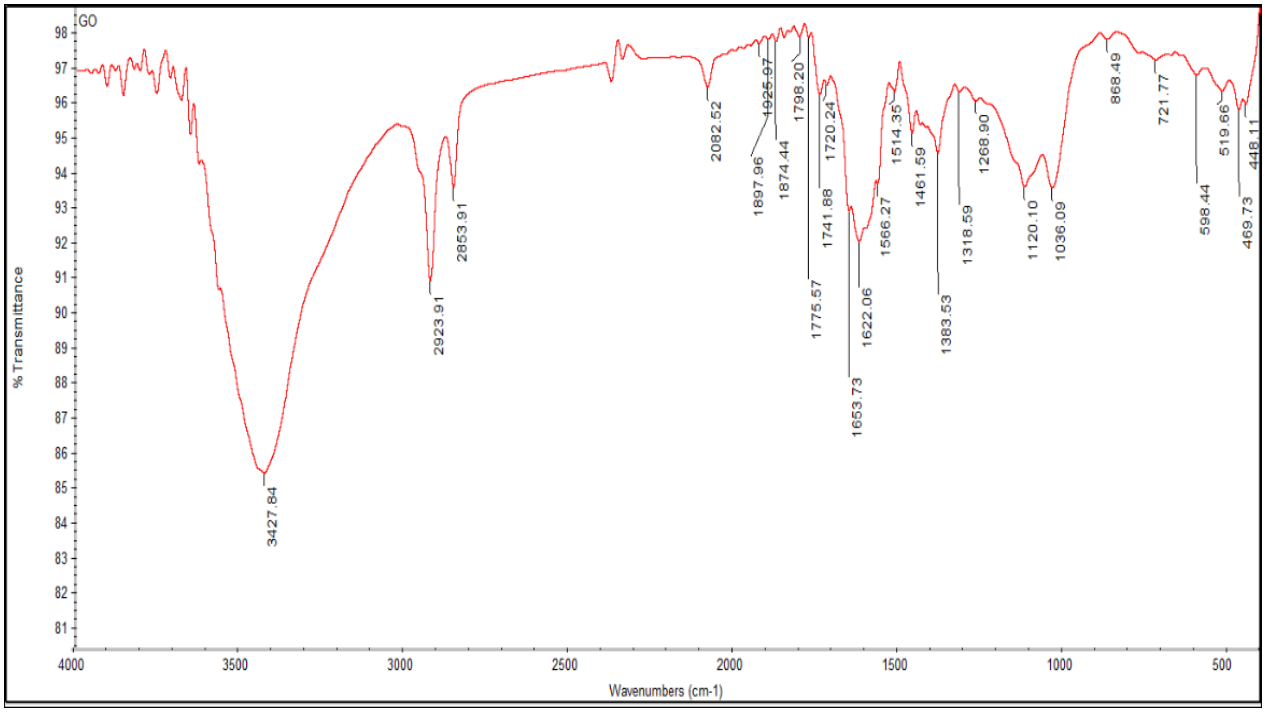


Fig. (2): IR spectra of the synthesized N-GO.

SEM - micrographs

Figure (3) showed that N-GO has layered structure which are very thin and nearly homogeneous in its shape.

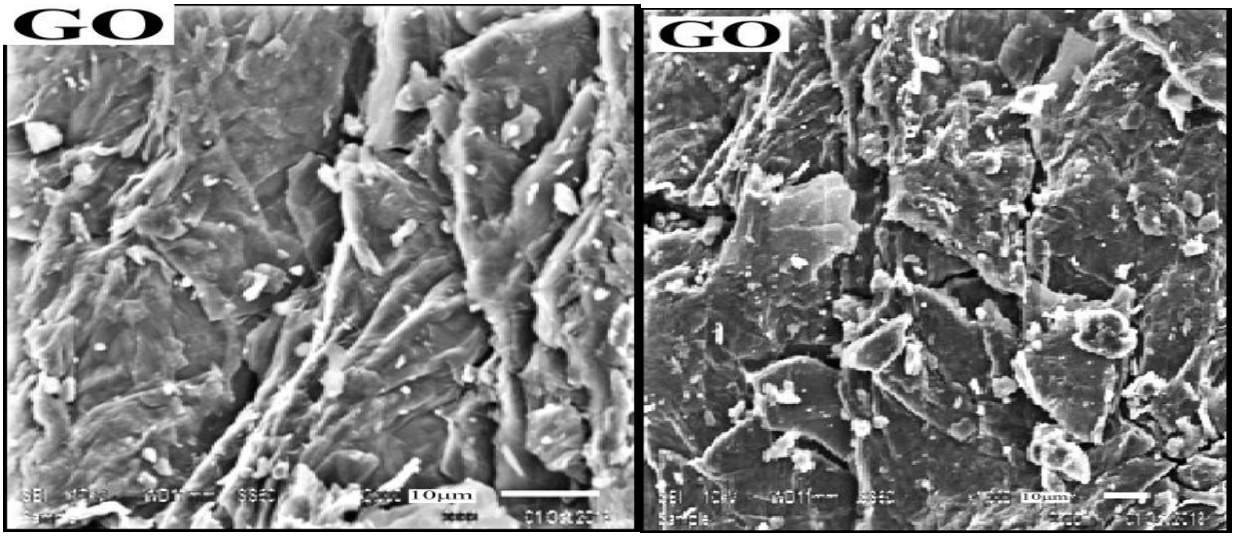


Fig. (3): SEM micrographs of graphene oxide in different magnifying scale.

Polyclonal antibody production

In order to produce polyclonal anti-AFP, six mature male white New Zealand rabbits were immunized with highly purified AFP antigen. The rabbits were divided into two groups

In the first group (R_1, R_2 and R_3), each rabbit was given 1 ml emulsion from a new formula of the modified adjuvant which consisted of (0.1 mg AFP in 250 µl distilled water emulsified with 75 µg N-GO in 750 µl mineral oil). In the second group (R_4, R_5 and R_6), each rabbit was given 1 ml emulsion which consisted of (0.1 mg AFP in 250 µl distilled water and 750 µl of Freund's adjuvant complete) as a traditional adjuvant. Three booster doses in addition to the initial vaccination were used to produce polyclonal antibodies against AFP. The first booster dose was administered after two weeks from the initial dose and the remaining three boosters were administered in the same method.

For characterization of the harvested polyclonal antisera for AFP, some tests were carried out prior to routine application in RIA system. The criteria for selecting antisera for RIA system are mainly dilution titre, displacement percentage and immunoresponse profile**.**

The anti-AFP dilution titer and displacement percentage are determined by calculating the percentage of anti-AFP binding at various dilutions for each individual bleeding antisera with each of the AFP standards zero and high levels (0.0 and 550 IU/ml) using a fixed quantity of tracer. To determine the highest displacement percentage between the highest binding percentage (Bo%, 0.0 IU/ml) and lowest binding percentage (Bs %, 550 IU/ml), the binding percentages of each of the two variables (Bo/TA and Bs/TA) were estimated for each bleeding (Bl_1, Bl_2, Bl_3, Bl_4) for each rabbit.

Group I showed the highest displacement percentage and a successful immunoresponse profile when immunized with AFP with N-GO as a modified adjuvant formula. The results showed that, R_1, R_2 and R_3 gave highest displacement, (79.5%, 75.4%, 68.7%) respectively, with dilution titre 1/16,000 after two weeks from the third booster.

Additionally, the outcomes of group II, which immunized by AFP-Freund's adjuvant emulsion, the findings gave a high displacement and good immunoresponse profile. The results showed the following, R_4, R_5 and R_6 gave highest displacement (68.7%, 62.8% and 58.7%) respectively, with dilution titre 1/16,000 after two weeks from the third booster.

The optimum titre and displacement percentage for immunized rabbits with AFP immunogen by two methods were presented in Figures (4 and 5).

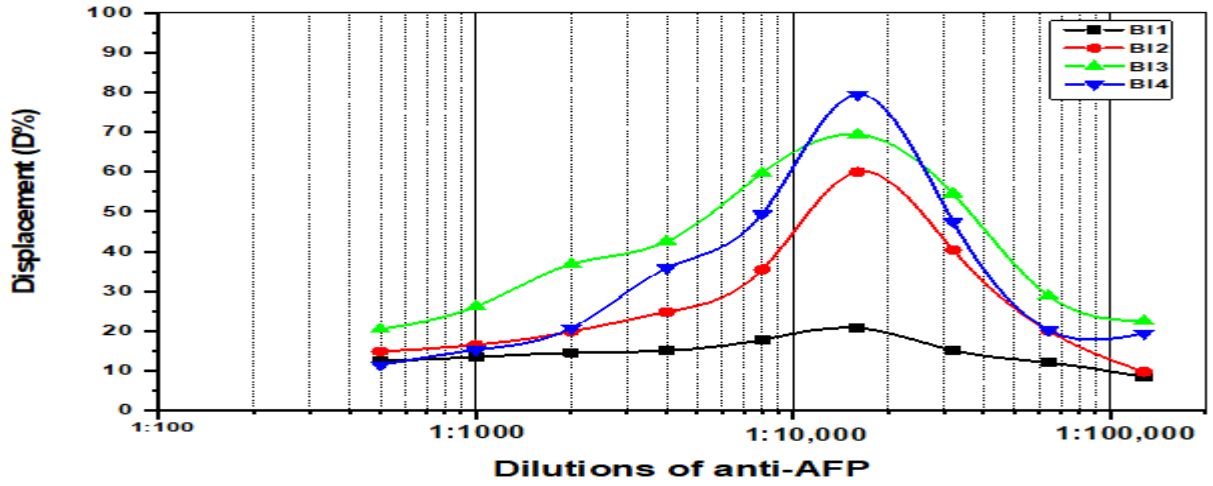


Fig. (4): Dilution titer and displacement of anti-AFP polyclonal for rabbit (1) Group (I) which immunized with AFP-N-GO formula.

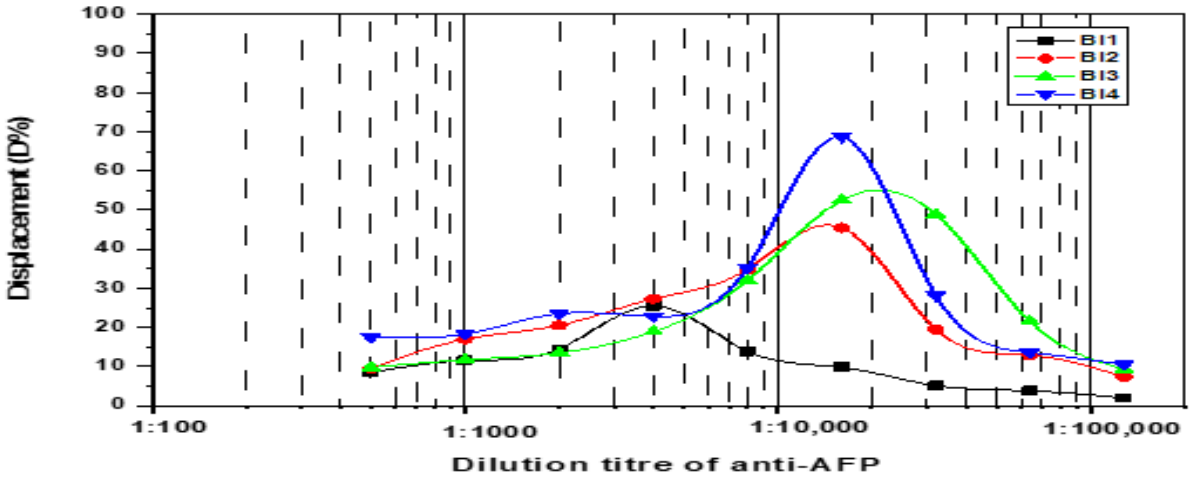


Fig. (5): Dilution titer and displacement of anti-AFP polyclonal for rabbit (4) Group (II) which immunized with AFP- Freund's adjuvant.

Based on the acquired results, it can be stated that rabbits immunized with the AFP-N-GO formula (R1 group I) and AFP-Freund's adjuvant formula (R4 group II) gave the high displacement between the zero and high standard, respectively. The immunoresponse and displacement % results are consistent with previous studies [37, 38]. As stated by the authors, active immunization is occurring when an antigen is introduced into a host animal. This is identified by an initially slow antibody synthesis. Synthesis of antibodies becomes faster and produced in larger quantities after receiving the same antigen another time. Moreover, antibodies synthesized in response to stimulation of antigens by plasma cells and lymphocytes.

The immunoresponse profile was created by plotting the maximum displacement percent and titer for each individual rabbits bleeding against the immunization time. Figure (6) shows that the immunized rabbits gave successful immunoresponse to the AFP immunogen.

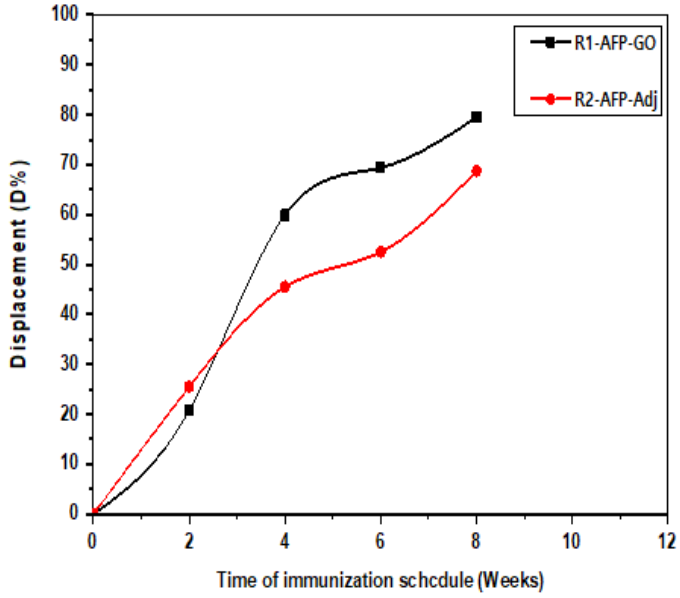


Fig. (6): Immunoresponse profiles for each of rabbit (1) which immunized with AFP-N-GO formula and rabbit (4) which immunized with AFP-Freund's adjuvant.

The antisera from each R_1 and R_4 were purified separately with sodium sulphate/ ammonium sulfate method. The concentrations of IgG-AFP antibodies were estimated by UV spectrophotometer at 280 nm. The following equation can be used to calculate the IgG-AFP concentration:

$$^{0.1\%}E_{280} = 1.4$$

The concentration of the purified IgG-AFP antibodies for R_1 and R_4 obtained were 8.2 and 7.9 mg/ml respectively. The results obtained are in good agreement with previous report [39] that stated the concentration of IgG ranged from 1-20 mg/ml

The set of AFP working standards were prepared from purified cord blood with the following concentrations (5, 10, 20, 50, 100, 200, 550 IU/ml). The prepared standard were validated and compared with International Reference Preparation (IRP) standards. The results of the local prepared standards gave a good correlation against IRP reference and the range of the recovery was 97.6 % to 102.5 %. The findings corresponding with previous studies stating that the recovery percentage has to be 100 ± 15% [29].

Using gel chromatography on a PD-10 Sephadex column, the elution pattern of ^{125}I-AFP was created by plotting activity (µCi) versus fractional number, as shown in Figure (7). In the figure, there are two peaks that indicate the radiochemical yield of ^{125}I-AFP (69.3%), free ^{125}I (15.5%), with specific activity equal 34.7 µCi/µg. The maximum binding (B_o %) is 68.9% and nonspecific binding (NSB %) is 2.4% using the local liquid-phase AFP-RIA system. These findings agree well with the findings reported by Pillai and Bhandarkar [29].

Optimization of the assay:

Evaluation of the optimal conditions for the locally prepared AFP-RIA system were undertaken via investigating the effect of both sample volume and incubation time.

Sample volume

Four different standard concentrations of AFP (0.0, 5, 50 and 550 IU/ml) were assayed with different volumes (50, 100 and 200 µl) for each standard in assay tubes. The assay procedures were performed for each volume in separated assay. The data in Table (1) showed that the binding percentage increased with the increased the sample volume of AFP to 100 µl. Also, the results demonstrate that applying 100 µl of AFP standards results in in the highest differences in displacement percentage (D %) between the different levels of AFP (zero, low, medium, and high). Therefore, 100 µl of sample volume of AFP was optimum

The addition of 200 µl to the higher concentration standard gave low counts, which could be wrongly interpreted as a low concentration standard. This is because of the high dose hook effect [29, 40].

Different incubation times in the range of 1, 3 and 24 hours for the local liquid phase AFP-RIA were studied, at room temperature. Four different standard concentrations of AFP (0.0, 5, 50 and 550 IU/ml) were assayed. As shown from the data presented in Table (2), 3-hour incubation time was optimum for the assay. Also, the results showed that the displacement percent between (zero and high) concentrations of AFP standard increases as incubation duration increases and reaches its highest optimum value within three hours and gave approximately the same results with 24-hours incubation time. From the results obtained, it can be concluded that, three hours is suitable as incubation time to set the antigen-antibody complex. The results are good agreement with results obtained by previous study [41].

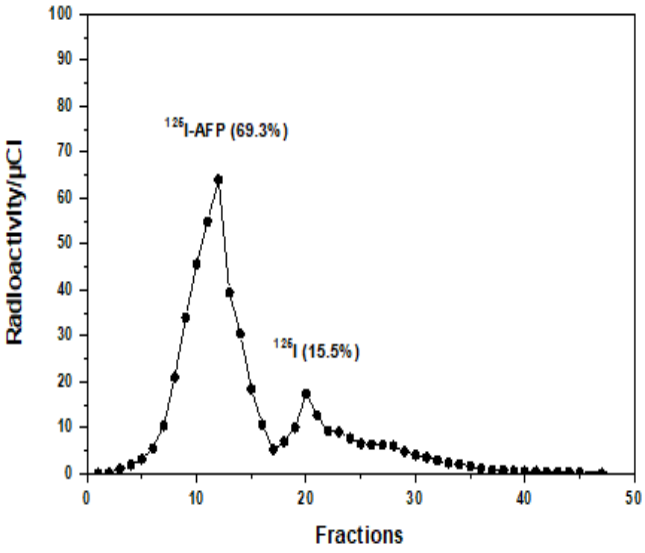


Fig. (7): Elution pattern of radioiodination mixture of AFP and purification of ^{125}I-AFP tracer using PD-10 sephadex G-25 column.

Table (1): Effect of sample volume on the liquid phase AFP-RIA.

Standard volume / AFP standard	50 µl		100 µl		200 µl	
	Group I	Group II	Group I	Group II	Group I	Group II
Maximum binding (B_o)[a]	56.4	61.5	77.2	72.9	69.1	67.0
Low (5 IU/ml)[b]	77.1	73.6	84.5	83.2	75.3	68.7
Medium (50 IU/ml)[b]	28.5	26.4	39.2	37.1	32.9	31.5
High (550 IU/ml)[b]	10.5	11.2	10.0	10.1	9.8	11.2
Displacement (D %)[C]	81.4	81.9	87.0	86.1	85.8	83.3

a: the data are presented as percent bound (B/TA).
b: the data are presented as percent bound (B/B_o).
c: D%: displacement between zero and high standard ($D\ \% = B_o - B_H/B_o$).

Incubation time

Table (2): Effect of incubation time on the liquid phase AFP-RIA.

Standard volume / AFP standard	1 hr		3 hr		24 hr	
	Group I	Group II	Group I	Group II	Group I	Group II
Maximum binding (B_o)[a]	71.5	67.9	79.0	74.8	79.5	75.4
Low (5 IU/ml)[b]	79.4	78.1	85.2	83.9	85.7	84.5
Medium (50 IU/ml)[b]	33.9	36.5	40.7	38.5	41.8	39.6
High (550 IU/ml)[b]	11.8	10.9	11.2	10.7	11.0	10.5
Displacement (D%)[C]	83.5	83.9	85.8	85.7	86.2	86.1

a: the data are presented as percent bound (B/TA).
b: the data are presented as percent bound (B/Bo).
c: D%: displacement between zero and high standard ($D\ \% = B_o - B_H/B_o$).

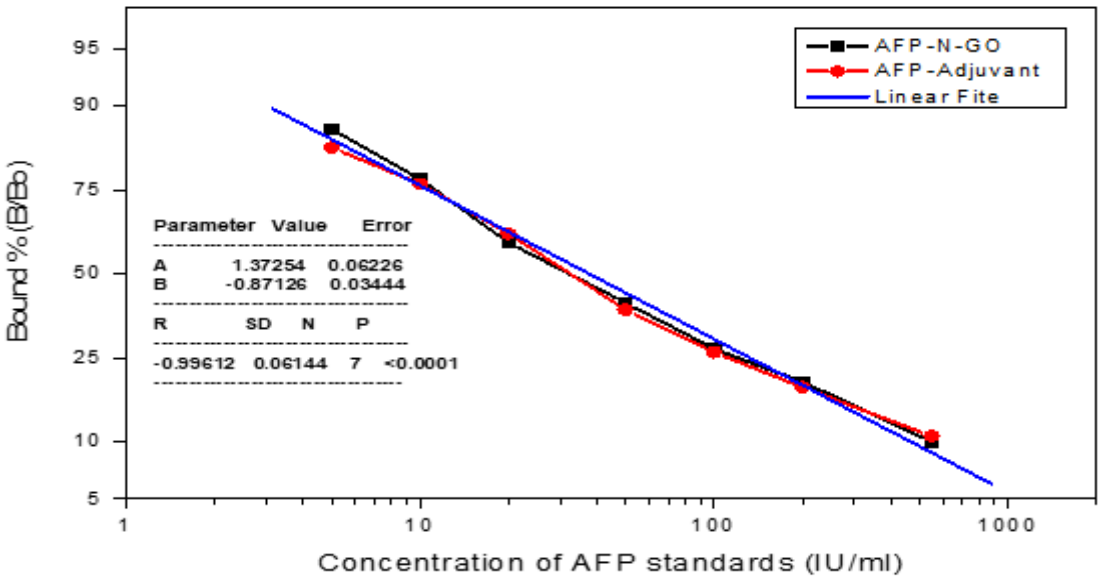


Fig. (8): Dose response curves of liquid phase AFP-RIA for new adjuvant N-GO compared with Freund's adjuvant.

From the obtained results, it was found that the optimum conditions for AFP-RIA technique as follow: 100 µl of AFP standard or sample, 100 µl of ^{125}I-AFP tracer approximately 20,000 cpm were added to 100 µl of anti-AFP at dilution (1/160,000). The content of the tubes were mixed and incubated at 37 ^{o}C time for 3 hrs. At the end of the incubation time, separating agent were added as a following sequence, 100 µl of second antibody (goat anti-rabbit IgG) at dilution (1:50), 100 µl normal rabbit serum (NRS) at dilution (1:200) and 500 µl of polyethylene glycol (PEG-8000, 4%). After vortex mixing, assay tubes were incubated for 30 min at room temperature and then centrifuged at 4000 rpm for 15 min at 4^{o}C. The assay tubes were decanted and the sediment containing the precipitate antibody-antigen complex was counted by gamma counter.

Optimized standard curves for the liquid-phase AFP-RIA with new adjuvant N-GO compared with Freund's adjuvant were demonstrated in Figure (7), the data showed that good linearity and the correlation coefficient "r" equal (0.996).

Validation tests for estimation of AFP-RIA:

Sensitivity is the smallest or minimum detectable concentration (MDC) of the ligand. The sensitivity or minimum detection limit of RIA techniques were calculated by the interpolation of the mean minus two standard deviations of 20 replicates of the zero IU/ml AFP standard. Sensitivity of AFP-RIA were 0.84 and 0.91 for group I and group II, respectively. Therefore, local liquid phase RIA technique for measurement of AFP is very high sensitivity [29, 40].

Precision is a statistical index of the ability of an assay to yield the same result when the assay is repeated on the same sample. Intra-assay (within-assay) precision were assessed using 10 replicate of sample, while inter assay (between-assay) precision were measured by assaying samples in duplicate of 10 separate assays. The obtained results showed that the coefficient variation CV% of intra assay were found to be not more than 6.9 % and 8.4 % for group I and group II respectively. While CV% of inter assay were found to be not more than 9.7 % and 10.5 % for group I and group II respectively. The results are in a good agreement with previous studies [40, 42]. They reported that the permissible limit for CV% of the intra-assay and the inter-assay is ≤ 10 % and ≤ 15 %, respectively.

The assay accuracy was evaluated by recovery and dilution tests. Dilution test was carried out using patient serum samples diluted serially from 1:2 to 1:16 using assay buffer and AFP concentration of the diluted samples were measured along with undiluted serum samples to assess the linearity of the assay. The obtained results show that the dilution test for AFP-RIA ranged from 95 % to 108 % for group I and 92 % to 102 % for group II. The recovery tests measured the concentration in human serum samples before and after adding known amount of AFP standard. The recovery of group I ranged from 96 to 105% and group II ranged from 93 to 107%. The obtained results showed that recovery and dilution tests indicated accurate calibration and appropriate matrix that in good agreement with other studies [29, 41].

Method comparison: AFP concentrations of 20 clinical samples in different value (low, medium and high) measured using the developed two system for each of group I and group II were compared with commercial kit AFP-RIA (Dia-Source). The obtained results showed highly positive correlation between the results obtained from the present system and commercial kit where r= 0.9761 for group I and r=0.9689 for group II.

CONCLUSION

The present study indicated that, Nano-graphene oxide (N-GO) was prepared as a new adjuvant formula for stimulating the immune system to produce polyclonal antibodies. N-GO was characterized as a follow: N-GO has been prepared with a strong and sharp diffraction signal at about 10.6°, highly crystal material and interlayer spacing was equal 0.78 nm and sharp diffraction at size 29 nm. AFP-N-GO formulation adjuvant raised polyclonal antibody with highest displacement percentage more than AFP- Freund's adjuvant in immunized rabbits. The labeling of AFP with^{125}I gave radiochemical yield 69.3 %, with specific activity 34.7 µCi/µg.

On conclusion, the locally prepared N-GO could possibly be utilized as a novel adjuvant formula to enhance the host animal immune response for generation of polyclonal antibodies to evaluate hormones and tumor markers such as AFP using radioimmunoassay technique.

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