Ex vivo Determination of Prostate Specific Antigen (PSA) Using Green Synthesized Magnetic Iron-Oxide Nanoparticles (IONPS)

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Abstract: **- The discovery and introduction of prostate-specific antigen (PSA) testing into clinical medicine enable the diagnosis, treatment and monitoring of prostatic carcinoma, and further revolutionized the management of patients with prostate cancer. Among the current developments in molecular detection of PSA is ELISA. The current research aimed at synthesizing the magnetic Iron-oxide nanoparticles for detection (assay) of prostate specific antigen and comparing the results obtained with that of the ELISA technique. It provides new advancements in molecular detections of PSA using magnetic iron-oxide nanoparticles. The magnetites were green synthesized using blackberry leaves extract, $FeCl_2.4H_2O$ and $FeCl_3.6H_2O$. Serum samples (n=30) were treated and subjected to both ELISA and magnetic iron-oxide nanoparticles immunoassay techniques. Descriptive statistical analysis (α=.05) of T-test, chi-square and ANOVA using SPSS software package, reveals ($p<0.005$) no significance difference between values generated from ELISA and magnetic iron-oxide nanoparticles techniques. Thus, the null hypothesis which asserts that, there is no significant difference between ELISA and magnetic iron-oxide nanoparticles in PSA assay fails to be rejected. It is therefore recommended that magnetic iron-oxide nanoparticles be further exploited with regards to ex vivo determination of PSA and other tumor markers.**

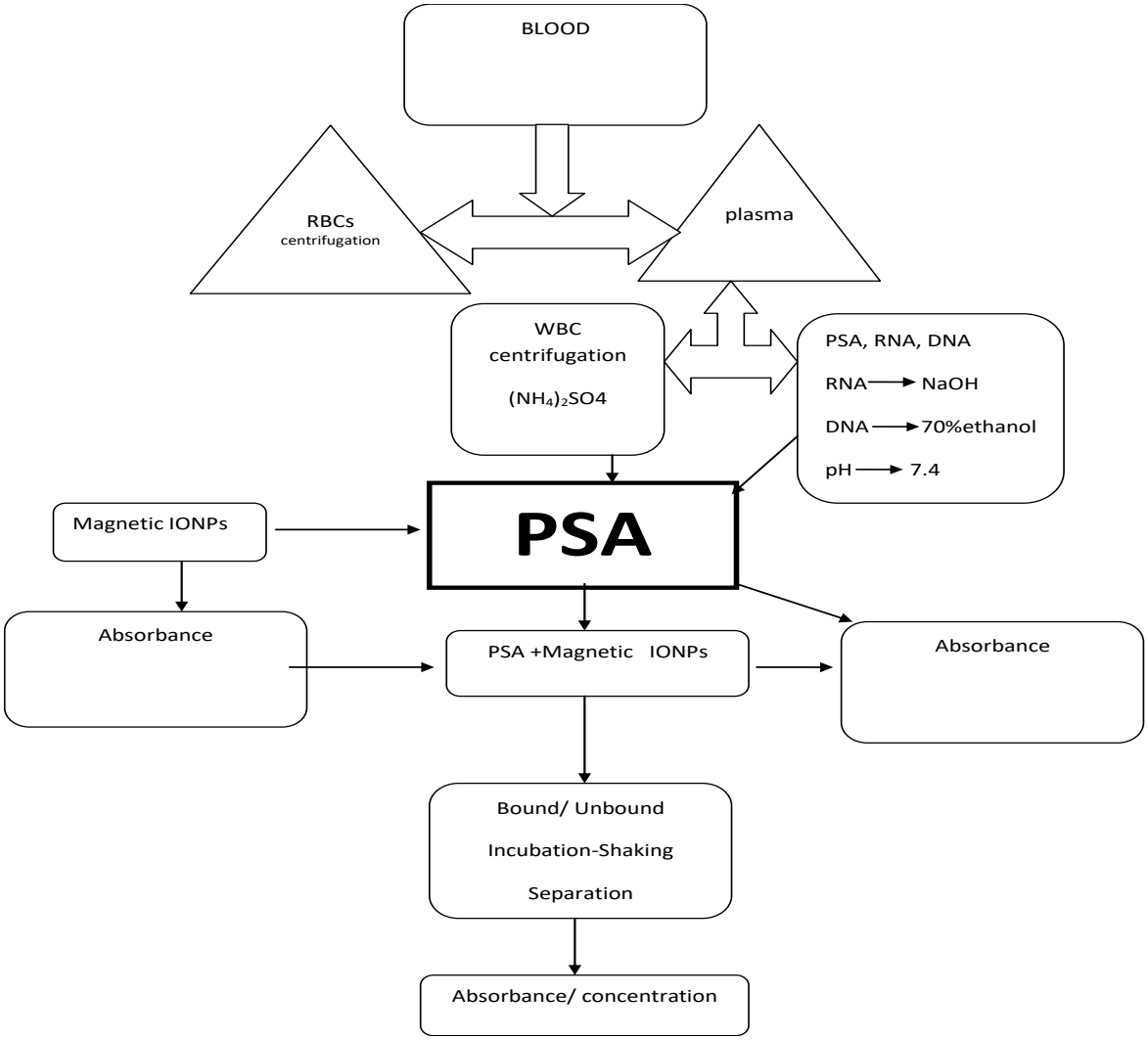
Key words: **Magnetic Iron-Oxide Nanoparticles (IONPS), Prostate specific antigen (PSA), Assay, ELISA.**

I. INTRODUCTION

The introduction of prostate specific antigen (PSA) testing into clinical medicine in 1986 revolutionized the management of patients with prostate cancer. The major limitation of this tumor marker stems from its in ability to provide a clear distinction between benign prostate disease and prostate cancer, especially in patients with upper limit of normal or slightly increased PSA values (Vashi and Oesterling 1997). Prostate specific antigen is a 28,400 Da glyco-protein 40 comprising 237 amino acid residues (Lundwall and Lilja, 1987), with five inter-chain disulphide bonds and approximately 8% carbohydrate in the form of a N-linked oligosaccharide side chain. In seminal plasma, PSA can be shown to exist in five isoforms, two biologically active and differing in the degree of glycosylation, and three biologically inactive or `nicked' forms (Zhang, *et al.*, 1995). This inherent heterogeneity has proved problematic in the various purifications, and some procedures have not yielded a product in which all isoforms are represented. PSA exhibits serine protease activity (EC3.4.21.77) similar to chymotrypsin (Watt, *et al* 1986). Over the past few decades, nanomedicine has emerged as a promising means to deliver anticancer therapeutics to tumours as a result of its preferential and selective accumulation at tumor sites via the enhanced permeability and retention (EPR) effect (Davis, *et al* 2008; Torchilin, 2014). It is known that physical properties of nanoparticles such as size, shape, and surface charge have profound effects on systemic transport of the nanoparticles in solid tumors (Decuzzi, *et al* 2010). Nanoparticles and their biosensing dynamism have recently received considerable attention. Magnetic signals experience little interference from native biological background as most biological molecules have negligible magnetic susceptibilities and thus appear transparent to external magnetic fields (Mahmoudi, 2015). Because of this unique property, magnetic sensing can be applied to both *in vivo* as well as *ex vivo* point-of-care molecular diagnostics (Mahmoudi, 2015). Despite years of research and hundreds of reports on tumor markers in oncology, the number of markers that have emerged as clinically useful is pitifully small (Mahmoudi, 2015). It is imperative that we attempt to understand the reasons that multiple studies of the same marker lead to differing conclusions. A variety of methodological problems have been

cited to explain these discrepancies. Unfortunately, many tumor marker studies, in nanomedicine, have not been reported in a rigorous fashion, and published articles often lack sufficient information to allow adequate assessment of the quality of the study or the generality of their results findings (Burton and Altman, 2004). Therefore, the main drive of this research project is to further explore the capabilities of newer synthetic nanoparticles (IONPs) in the diagnosis of cancers with specific reference to prostate. Iron oxide nanoparticles (IONPs) have been extensively used during the last two decades, either as effective bio-imaging contrast agents or as carriers of biomolecules such as drugs, nucleic acids and peptides for controlled delivery to specific organs and tissues. There are several important criteria (e.g. size and size-distribution, charge, coating molecules, and plasma protein adsorption) that can be effectively tuned to control the in vivo pharmacokinetics and bio-distribution of the IONPs (Hamed, *et al* 2015). Super paramagnetic iron oxide (γ-Fe_2O_3 and Fe_3O_4) nanoparticles (IONPs) are biocompatible, bio degradable and non-toxic and have been used for a wide range of biomedical applications such as tumors or vascular imaging (Rosen, *et al*., 2012). drug delivery (Arami, *et al*, 2011), in vivo tracking of labeled cells (Berman, *et al*., 2011), magnetic separation of cells or molecules (Xu, *et al.*, 2011), or as an iron supplement for patients with anaemia (Lu, *et al*., 2010). Immediately after their administration *in vivo*, a host of innate immunological mechanisms start to recognize and collect these foreign particles and direct them to the major elimination pathways of the body (Khlebtsov, *et al*., 2011). It is well known that whilst the size of the iron oxide crystals determines the magnetic properties of IONPs, the additional molecules on their surface act as the main interface between the IONPs and the body's immune system (Krishnan, 2010). The result of this research finding will add meaning to general understanding of nanoparticles applications in the field of nanomedicine. Also similar approaches can be designed for all other tumor-markers.

Conceptual framework



II. MATERIALS AND METHODS

Reagents: All chemicals and reagents used were of analytical grade purchased from Sigma company USA**,** this includes Ferrous Chlorides ($FeCl_2.4H_2O$ and $FeCl_3.6H_2O$), Double distilled water, Ammonium sulphate $(NH_4)_2SO4$, Ethanol and Aqueous sodium hydroxide.

Apparatus/equipment: Spectrophotometer, Incubator-shaker, Cuvettes, Conical flask, Magnetic stirrer, pH meter, 250mls beaker, Micropipette, Pasteur pipette, Gloves, Facemask, 40 sterile 5mls syringes, Whatman No 1 filter paper, sterile 20mls Syringe, and Weighing balance.

Study design: The current research is a pilot experimental study; therefore 30 samples were utilized.

Specimen collection: Blood specimens were collected from volunteers; both apparently healthy and individuals and prostate cancer patients who wished to participate in the research.

Synthesis of Magnetic iron-oxide

Fresh leaves of blackberry were removed from trees and were authenticated by specialist at the department of botany in Federal University Dutse. The leaves were washed, sun dried and powdered using kitchen blender. 100g of powdered blackberry leaf extract were dissolved in 100ml of double distilled water and allowed to stay overnight in the refrigerator at 4^oC and then filtered with Whatman No 1 filter paper, $FeCl_3.6H_2O$ and $FeCl_2.4H_2O$ (1:2 molar ratios) were dissolved in 100ml of double distilled water in a 250ml beaker and heated at 80^oc with mild stirring using magnetic stirrer, under atmospheric pressure. After 10mins, 20mls of the aqueous solution of the extract was added to the mixture, immediately, the light green colour of the extract changes to dark brownish colour. After 10mins, 20mls aqueous solution of NaOH was added to the mixtures at the rate of 3mls per min to allow the iron oxide precipitate uniformly, the mixture was then allowed to cool down and the IONPs were obtained by decantation. The magnetite formed were washed 3 times using distilled water and ethanol and later air dried at room temperature. The absorbance was determined at different wavelength (300-700nm).

Samples preparation

$(NH_4)_2SO_4$ (4.3M) was prepared 24hrs before use. Centrifugation of serum at 1000g was done to remove any remaining lipid that would compromise the salt precipitation, the total sample volume was determined and transferred to a beaker containing a stirrer bar. Stirring gently, the ammonium sulphate was added drop by drop slowly and continuously to produce 50% final saturation i.e. 1:2. Measuring and adjustment of pH to 7.4 was done. The beaker was kept in the stirring place at 4^oc overnight to ensure complete precipitation; the Supernatant was taken for the assay. The absorbance of PSA in the fluid was determined at different wavelength (380-780nm).

Assay

Absorbance of equal volumes of PSA in serum and magnetic IONPS, was noted independently and then mixed in a Cuvette. Equal volume of 70% ethanol was added to wash unbound magnetite and DNA contaminants (in case there is any). Incubation at 37^0c for 10mins in a shaking incubator was carried out, unbound are removed using Pasteur pipette. Absorbance of the mixture was taken. Concentration of PSA was determine using beers law.

III. RESULTS

Absorbance of the synthesized magnetic iron-oxide nanoparticles

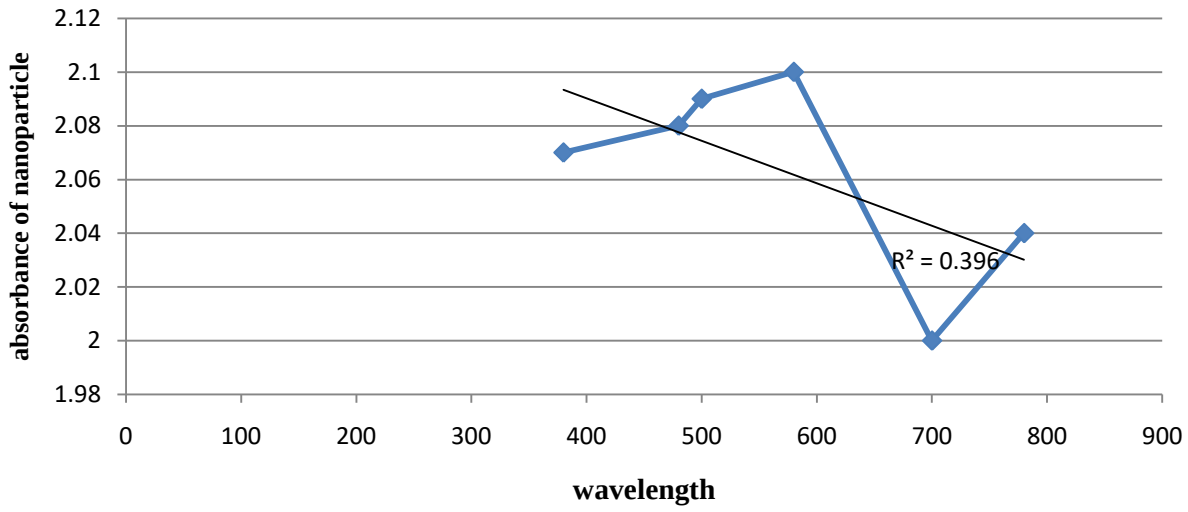


Fig. 1 Graph of absorbance VS wavelength

The table above shows absorbance of the synthesized magnetic iron-oxide nanoparticles at different wavelengths. Peak absorbance was found to be at wavelength 580nm, thus it chosen for the assay. This was in line with two others findings of Hamed, *et al*, (2015) and Rosen, *et al*, (2012), in which the former found peak absorbance at 560nm and the latter at 580nm.

Samples assay

Absorbance for each of the 30 treated samples was first taken at 580nm independently, then equal volume of the magnetic IONPs was added to each, and absorbance of mixture taken after 5mins of incubation at 37^{o}C in a shaking incubator. It was found that absorbance of the mixture is greater than each of the sample and magnetic IONPs singly. This is also in line with the Beers' law, which asserts the higher the concentration the higher the absorbance of light.

Magnetic IONPS concentration

Amutha and Sridhar, (2018), technique was adopted with modification of extract to blackberry leaves and thus 100nm size documented on their literature was taken to be the size of the synthesized magnetite, and thus the concentration is 100nm/ml. This is owing to lack of state-art-equipment to characterize and determine the size of the synthesized magnetic iron-oxide nanoparticles.

PSA Concentration

The concentration of analyte (PSA) was determined thus;

$$PSAConc = \frac{ABStest}{ABSstd} XConc.\ ofSTD$$

Normality test

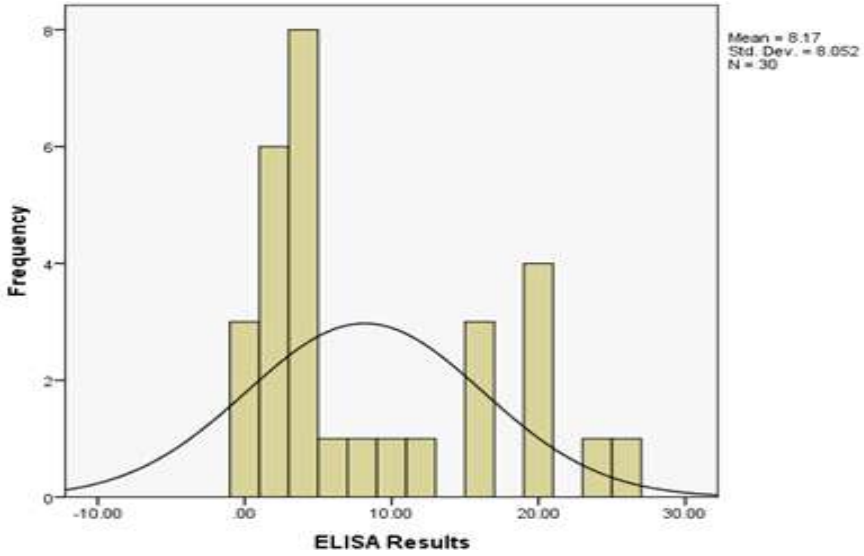


Fig. 2: ELISA normality distribution Curve

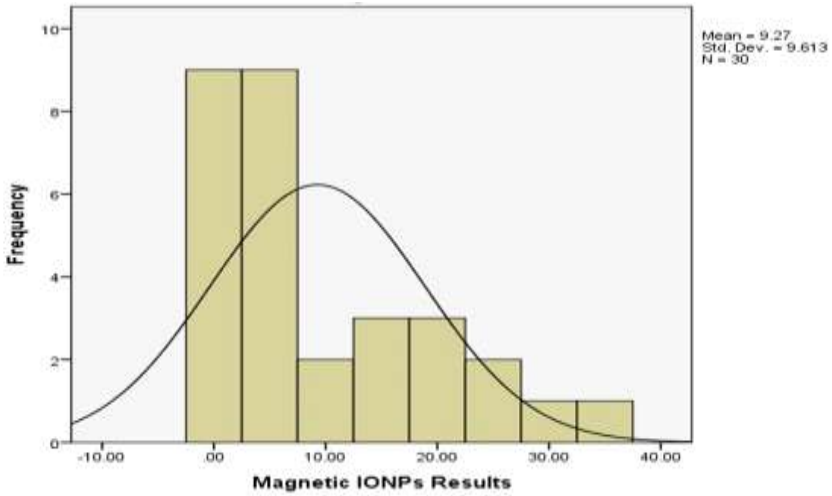


Fig. 3: Magnetic ion-oxide normality distribution Curve

Table 1.0. Concentration of PSA results using and Magnetic IONPS techniques

Lab No.	ELISA RESULTS(ng/ml)	Magnetic IONPS RESULTS(ng/ml)	Difference
1.	3.0	3.0	0.0
2.	3.0	3.0	0.0
3.	20.0	34.0	14.0
4.	6.0	7.0	1.0
5.	2.0	2.0	0.0
6.	4.0	4.0	0.0
7.	0.0	1.0	1.0
8.	20.0	21.0	1.0
9.	19.0	18.0	1.0
10.	15.0	13.0	2.0
11.	9.0	9.0	0.0
12.	8.0	9.0	1.0
13.	15.0	19.0	4.0
14.	23.0	24.0	1.0
15.	4.0	4.0	0.0
16.	12.0	13.0	1.0
17.	15.0	17.0	2.0
18.	0.0	0.0	0.0
19.	1.0	2.0	1.0
20.	3.0	3.0	0.0
21.	4.0	5.0	1.0
22.	26.0	29.0	3.0
23.	3.0	4.0	1.0
24.	1.0	0.0	1.0
25.	4.0	5.0	1.0
26.	2.0	2.0	0.0
27.	2.0	2.0	0.0
28.	20.0	24.0	4.0
29.	0.0	0.0	0.0
30.	1.0	1.0	0.0
Total	245.0	278.0	

Table 2.0. ELISA and Magnetic IONPs Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	Df	Sig.	Statistic	df	Sig.
ELISA RESULT	.264	30	.000	.843	30	.000
Magnetic IONPS	.238	30	.000	.841	30	.000

Significance values =.000 at 95% confidence level in both the KS and Shapiro-wilk tests. It then follows that, the data is normally distributed, as there is no significant difference statistically. So, on the basis of normality tests, the null hypothesis which says there is no difference between ELISA and Magnetic Ion-oxide nanoparticles techniques in PSA assay cannot be failed. Meaning that, they both are efficiently the same.

Table 3.0. Pearson Correlation test

		ELISA	Magnetic IONPs
ELISA	Pearson Correlation	1	.967**
	Sig. (2-tailed)		.000
	N	30	30
Magnetic IONPs	Pearson Correlation	.967**	1
	Sig. (2-tailed)	.000	
	N	30	30

Pearson correlation test, shows there is no significant difference between ELISA and Magnetic IONPs assay techniques at 95% confidence level. significant difference between results.

Table 4.0. Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	271.667[a]	195	.000
Likelihood Ratio	125.706	195	1.000
Linear-by-Linear Association	27.094	1	.000
N of Valid Cases	30		

The chi-square test shows no significant difference between both ELISA and Magnetic IONPs, thus the null hypothesis fails to be rejected, basis of chi-square test.

IV. DISCUSSION

The optical activity of iron-oxide nanoparticles and their magnetic property made them a novel target for various applications in the field of nanomedicine, specific to immunoassay. Most biological molecules have magnetic susceptibilities owing to a cocktail of differently charged amino acids forming the peptide backbone of these biomolecules. For PSA, the amino acids sequences forming the five (5) different chains are accordingly polar, hydrophobic, neutral, positively and negatively charged. Over roll, PSA has a net positive charge and some magnetic property. To show the mode of magnetic detection by nanoparticles, new advancements in both magnetic material syntheses and sensing technologies have been made by this research study, and it shows great promises in PSA detection and quantification. Green synthesis of magnetic ion-oxide nanoparticles was made using blackberry leaves extract, coupled with 1:2 molar concentrations of $FeCl_3.6H_2O$ and $FeCl_2.4H_2O$ in a 100mls of double distilled water. Heating was done at 80oc with gentle stirring using magnetic stirrer. NaOH (20mls) was added at the rate of 3mls per minute so as to prevent agglutination and allow for uniform precipitation of the magnetite. Then the mixture was allowed to cool to room temperature and magnetite decanted. Accordingly, a total of 30 samples were used for the assay. And, equal volumes each of the samples and 4.3M of $(NH_4)_2SO_4$ were mixed and centrifuged at 4000g for 30minutes to pellet albumin, immunoglobulins and clotting profile. Then, the supernatant was taken and divided into two; one was used to assay PSA using ELISA technique and the other using magnetic ion-oxide nanoparticles. Absorbance each of samples and magnetic IONPs was taken at different wavelengths and maximum value was observed at wavelength 580nm, after blanking with distilled water. Again, equal volumes each of samples, Magnetite and 70% ethanol were mixed and incubated for 10 minutes in an incubator-shaker at 37oc after adjusting the pH to 7.4 with 1 drop NaOH. Then, unbound magnetites/contaminants (DNA if any) were removed using pastuer pipette. Further, concentration of the PSA was determined from the mixture using spectrophotometry. Still more, results were compared with that of ELISA kit. Data and research hypothesis were respectively analyzed and tested using SPSS software package, version 20. Arithmetic average of both measurements (mean) is 8.72. Mid-point data (mode) distribution of ELISA and magnetic IONPs are 4.00 and 4.5 respectively. The degree of dispersion (standard deviation) in both data sets stands at 8 and 9. This shows exquisite homogeneity among the data groups. Skewness and Kurtosis tests for the measure of both the central tendency and dispersion for the ELISA and magnetic Ion-oxide nanoparticles show a difference of 0.263, which means data are homogenous. Again, normality curves were symmetrical, continuous and bell-shaped. Similarly, T-tests, Chi-square, and ANOVA tests all show .000 significant differences among the data sets. It then follows that, the data is normally distributed and there is no significant difference statistically. So, on the basis of these descriptive analytical tests, the null hypothesis which says there is no difference between ELISA and Magnetic Ion-oxide nanoparticles techniques in PSA assay failed to be rejected. Meaning that, they both are efficiently the same. Still more, it has to be reported that magnetic IONPs technique of PSA assay is cumbersome and labour intensive.

V. CONCLUSION

Although, the number of tumor markers reported in oncology is pitifully small, the study of a particular marker of interest (in which case PSA) will improve point-of care molecular diagnostics and care of patients living the disease in question. Nanoparticles and their bio-sensing dynamism gave them an excellent characteristic as well as use in field of molecular medicine, specific to diagnostics. Magnetic ion-oxide nanoparticles, when greenly synthesized at 100nm, are found to be as effective as ELISA kit used in PSA assay. There is need to expand the scope of this work to treat a larger samples size.

VI. RECOMMENDATIONS

Magnetic iron-oxide nanoparticles can be used for PSA assay. It is recommended that this research should be repeated using larger samples size, to allow for generalization of findings. Other tumor-markers should be studied and thus a nanostructure(s) designed for their assay. Awareness for the medical community as well as biotechnology entrepreneurs of the novelty of this approach towards diagnosis and management of cancers.

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