DEVELOPMENT OF NANOPARTICLE BASED IMMUNOSENSOR FOR CANCER ANTIGEN CA125

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DOCTOR OF PHILOSOPHY

By

RAGINI RAGHAV

Department of Biotechnology

JAYPEE INSTITUTE OF INFORMATION TECHNOLOGY

A-10, SECTOR-62, NOIDA, INDIA

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Ovarian cancer has been reported to be the fifth leading cause of mortality with a global burden estimated as 3.6% of worldwide population and 5% in Indian scenario[1-3]. Since 1990-2013, India observed maximum increase in breast cancer (166.4%) closely followed by ovarian cancer (153.7%). Despite observed maximum prevalence of breast cancer, the ovarian cancer situation is alarming with highest mortalities (122%) as opposed to 81.9% and 94.1% in case of breast and lung cancer respectively[4, 5].

High mortality rates could be attributed to the delayed diagnosis of ovarian cancer. Almost 61% of ovarian carcinoma cases are diagnosed when the cancer has already metastasized (stage III), shrinking the 5-year survival rate of the patients to mere 27.4%[5]. While only 15-18% cases are recognized in stage I (when cancer is localized) or stage II (when cancer spreads to regional nodes). The early detection of ovarian carcinoma can improve the 5-year survival rate of the patients-92.3% stage I and 71.7% stage II. Thus, demanding the necessity of screening for early diagnosis of ovarian carcinoma for increasing the survival probability[6].

The clinical diagnosis involves transvaginal ultrasound, computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET), laparoscopy, colonoscopy, biopsy and blood test for biomarkers. Biomarkers such as cancer antigen 125 (CA-125), cancer antigen 19-9 (CA-19-9), alpha fetoprotein (AFP), human chorionic gonadotropin (hCG), cancer antigen 72-4 (CA72-4), CEA (carcinoembryonic antigen), HE4 (Human epididymis protein 4) etc. have been observed to be elevated in ovarian carcinoma[7-10]. However, CA125 is considered as current gold standard with a threshold of 35 U/mL and is the only recommended biomarker by National Cancer Institute for screening, diagnosis and prognosis of ovarian cancer[11, 12]. These biomarkers are quantified using radioimmunoassay (RIA)[13], enzyme linked immunosorbent assay (ELISA)[14], chemiluminescence or

fluorescence based immunoassays (CLIA/FIA) in blood serum[15, 16]. These immunoassays are selective and sensitive, however, are time consuming and require trained personnel for handling sophisticated instruments. Immunosensor have emerged as an alternative, retaining the specificity and sensitivity of immunoassays at the same time being simple and cost effective to be used as a point-of-care device for rapid screening of samples[17-19].

Advances in nanotechnology has revolutionized immunosensors performance with nano-sized materials enabling miniaturization, facilitating high loading capacity of biomolecules, enhancing sensitivity and linear/dynamic range[17-19]. Nanomaterials and conducting polymers based electrochemical immunosensors for quantification of CA125 have attained maximum linear range up to 100 U/mL[20], dynamic range up to 1000 U/mL[21] and LOD as low as 0.0016 U/mL[22]. However, the CA125 levels have been observed to be as high as 17000 U/mL[23, 24] that is much higher than the maximum dynamic range (up to 1000 U/mL) achieved till date[21]. Furthermore, efforts to increase sensitivity for prognostic purpose, compromised on linear/dynamic range of the immunosensor, often failing to encompass even the threshold level in healthy females (35 U/mL)[22]. Hence, the need of the hour is designing an immunosensor strategy optimizing sensitivity and dynamic range suitable for both diagnosis and prognosis.

Objective of the present work is to develop an electrochemical immunosensor for CA125 quantification with following characteristics:

1) High sensitivity
2) Increase Linear range of immunosensor >100 U/mL of CA125 and
3) Increase Dynamic range of immunosensor >1000 U/mL of CA125.

These objectives have been achieved by designing immunosensor fabrication strategy for directed immobilization of antibodies and choice of appropriate nanomaterials and nanostructures for desired immunosensor performance characteristics. The thesis is structured as, Introduction (chapter 1), Review of Literature (chapter 2), Characterization Techniques (chapter 3), Synthesis of Nanomaterials (chapter 4), Directed Immobilization of Antibody for Improved Immunosensor Performance (chapter 5), and Effect of Nanostructures on Performance Characteristics of CA125 Impedimetric Immunosensor (chapter 6).

Chapter one presents the worldwide status of ovarian cancer and its diagnosis, presently adopted diagnostic methods and their drawbacks and need for an early diagnosis for increasing the survival rates. Review of Literature of clinical diagnostic techniques for ovarian cancer and technological developments in immunosensing technology for CA125 quantification has been presented in detail in **chapter two**. **Chapter three** describes the characterization techniques employed in the study. The physicochemical characterization of nanomaterials was done using scanning electron microscope (SEM), transmission electron microscopy (TEM), ultraviolet-visible spectroscopy (UV-vis), energy dispersive X-ray spectroscopy (EDX), X-ray diffraction (XRD), and fourier transform infrared spectroscopy (FTIR). The fabrication of immunosensors was characterized employing contact angle measurements and electrochemical analysis employing electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV). **Chapter four** presents synthesis of gold nanostructures (nanoparticles and nanochains) using amino acids, core shell gold-silver nanoparticles and copper oxide nanostructures (cuprous and cupric oxide nanoparticles).

Immobilization of antibodies in correct orientation i.e. orthogonal orientation onto the electrode surface is of paramount importance in improving the performance characteristics such

as sensitivity, linear and dynamic range, and limit of detection of the immunosensor. In **Chapter five**, we have evaluated different covalent strategies for antibody immobilization with CA125 immunosensor as model system.

After designing a suitable fabrication strategy for antibody immobilization in **Chapter six**, we have studied the effect of different nanomaterials and their morphological variations on immunosensor performance characteristics - sensitivity, linear/dynamic range of label free impedimetric immunosensor. The hypothesis/probable mechanism responsible for extending the dynamic range of the immunosensor has also been proposed.

SUMMARY

- We report the synthesis of well dispersed spherical gold nanoparticles, gold nanochains, core-shell gold-silver nanoparticles, cuprous and cupric oxide nanostructures in pure phase.
- We present a simple strategy for maximizing the immunosensor performance by directed immobilization of antibody using amino coated nanostructures and antibody in coating buffer of pH 9.0.
- AuNPs, Cu_2O and CuO NPs based impedimetric immunosensors displayed sensitivity of 363.05, 383.83, and 397.92 $\Omega/(U/mL)/cm^2$ that was almost ~1.3 times that of Au-Ag NPs based immunosensor (283.83 $\Omega/(U/mL)/cm^2$) and twice that of AuNCs based immunosensor (190.84$\Omega/(U/mL)/cm^2$).
- AuNCs and Au-Ag NPs based impedimetric immunosensor exhibited extended dynamic range of 1250 U/mL and 1000 U/mL with increased Linear range as well upto 150 U/mL.
- AuNCs, Au-Ag NPs as well as AuNPs based impedimetric immunosensor retained >80% stability up to 30 days while others were stable up to 20 days.

FUTURE SCOPE

- The effect of different shapes and sizes of nanoparticles on performance of the immunosensor can be studied.
- Proposed fabrication strategy can be extended for quantification of other biomarkers.
- A multianalyte immunosensor can be fabricated for a panel of biomarkers CA125, carcinoembriyonic antigen (CEA), alpha fetoprotein (AFP), cancer antigen 19-9 (CA 19-9), human epididymis 4 (HE4), cancer antigen 72-4 (CA 72-4) etc., for ovarian cancer screening with improved sensitivity and specificity.

Table 1: Comparison of performance characteristics of fabricated immunosensors

Nanostructures	Type of Immunosensor	Dynamic Range (U/mL)	Linear Range (U/mL)	Sensitivity (Ω/(U/mL)/cm^2) /%Change in response per unit antigen	>80% Stability upto (in days)	RSD
Asn-AuNPs	Impedimetric	1-750	1-100	363.05 (20.8%)	30	6.92%
Au-Ag NPs	Impedimetric	1-1000	1-150	283.83(25.2%)	30	8.28%
AuNCs	Impedimetric	1-1250	1-150	190.84(22.4%)	30	7.36%
Cu_2O NPs	Impedimetric	1-500	1-100	383.83(13.2%)	20	9.42%
CuO NPs	Impedimetric	1-500	1-100	397.92(16.4%)	25	8.69%

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LIST OF PUBLICATIONS DURING Ph.D. THESIS WORK

International Journals

- Ragini Raghav and Sudha Srivastava, *Immobilization Strategy for Enhancing Sensitivity of Immunosensors: L-Asparagine –AuNPs as a promising alternative of EDC-NHS activated citrate-AuNPs for Antibody Immobilization,* Biosensors and Bioelectronics, 2016, 78,396-403 (Indexed in Scopus, Impact Factor 6.4).

- Ragini Raghav, Priyanka Aggarwal and Sudha Srivastava, *Tailoring Oxides of Copper - Cu_2O and CuO Nanoparticles and evaluation of organic dyes degradation,* AIP Conference Proceedings, 1724, 020078, 2016 (Indexed in Scopus).

- Ragini Raghav and Sudha Srivastava, *Core-shell Gold-Silver nanoparticles based impedimetric immunosensor for cancer antigen CA125*, Sensors and Actuators B: Chemical, 2015, 220, 557-564 (Indexed in Scopus, Impact Factor: 4.09).

- Ragini Raghav and Sudha Srivastava, *Copper (II) oxide Nanoflakes based Impedimetric Immunosensor for Label Free Determination of Cancer Antigen-125,* Sensor Letters, 2016, 14(1), 97-101 (Indexed in Scopus, Impact Factor: 0.56).

- Ragini Raghav and Sudha Srivastava, *Direct ELISA based Reagentless Amperometric Immunosensor for Cancer Antigen 125*, NanoTrends 2014, 16(2), 1-6 (Indexed in Google Scholar)

Other Publications

- Ragini Raghav and Sudha Srivastava, *Gold nanoparticles based colorimetric detection of Cobalt (II) ion*, Sensor Letters, 2015, 13(3), 254-258 (Indexed in Scopus, Impact Factor: 0.56).
- Conference Proceedings: *Heavy Metal Ion Detection Exploiting Surface Plasmon Resonance of Gold Nanoparticles.* 17th National Seminar on Physics and Technology of Sensors, Jamia Millia Islamia, New Delhi.
- Conference Proceedings: *Impedimetric Immunosensor for Detection of Cancer Antigen 125 using amine functionalized cupric oxide nanoparticles*. 9th National Conference on Solid State Chemistry and Allied Areas (ICAS-2015), Bhaskaracharya College of Applied Sciences, University of Delhi, Delhi.

International Conferences

- Colorimetric Assay for Cobalt detection using label free Gold Nanoparticles in Aqueous Solution. ALIGARH NANO-IV, International Conference on Nanoscience and Nanotechnology, Aligarh Muslim University, Aligarh, Uttar Pradesh.
- Heavy Metal Ion Detection Exploiting Surface Plasmon Resonance of Gold Nanoparticles. 17th National Seminar on Physics and Technology of Sensors, Jamia Millia Islamia, New Delhi.
- Citrate Stabilized Silver Nanoparticles Based Colorimetric detection of Mercury (II) ions in Aqueous Solution. Conference on "Frontiers in Nanoscience, Nanotechnology and their Applications (NanoSciTech – 2012)" at Punjab University, Chandigarh, Punjab.
- Optimization of immobilization of antibodies onto gold electrode surface for efficient sensing. ALIGARH NANO-II, National Conference on Nanoscience and Nanotechnology at Aligarh Muslim University, Aligarh, Uttar Pradesh.
- Nanobiosensor: An Edge over Conventional Heavy metal Ion Sensing Tools. National Conference on Recent Trends in Material Science, JUIT, Himachal Pradesh.

ACADEMIC ACHIEVEMENTS

- Selected for Travel Support from University of Luxembourg (2013) for attending Cycle 9 of "European Ph.D. School on Nanoanalysis using finely focused ion and electron beams" Belvaux, Luxembourg.
- Invited for lecture/ key speaker at The National work shop on Bioinformatics-Discovery in Biology-2011 at Department of Microbiology, Bundelkhand University, Jhansi, Uttar Pradesh.