



Nano-structured arrays for multiplex analyses and Lab-on-a-Chip applications

Chandra K. Dixit^{a,b}, Ajeet Kaushik^{c,*}

^a National Centre for Sensor Research, Dublin City University, Glasnevin, Dublin 9, Ireland

^b Schools of Biotechnology, Dublin City University, Glasnevin, Dublin 9, Ireland

^c Bio-MEMS and Microsystem Lab, Department of Electrical Engineering, University of South Florida, 4202 E. Fowler Avenue, ENB 118, Tampa, FL 33620, United States

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ABSTRACT

Nanospheres lithographic (NSL) method has been used to fabricate nano-structured arrays (NAs) of hexagonally close-packed gold (Au) using polystyrene beads [PS, diameter ~300 nm] as mask. The developed NA was incorporated with a customized and cheap microfluidics system to demonstrate its applicability as an alternative easy and efficient platform for multiplex analysis and Lab-on-a-Chip applications. The chip functionality was demonstrated with horseradish peroxidase (HRP) and anti-HRP antibody as model for recognition system. The enzyme-linked immunosorbent assay (ELISA) performed on fabricated protein biochip had a detection limit 100 pg/mL for HRP. The antibody chip was also checked for the shelf-life and it was found that these chips could be stored for 50 days when stored at 4 °C without any significant loss of activity. Therefore, NAs based protein biochip with the correct microfluidics could find huge potential application in diagnostics and biosensing technology.

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1. Introduction

An orderly arrangement of nanomaterials on the surface and their size controls the performance of the system depending on the application [1,2]. In this context, the fabrication of periodically arranged nano-structured arrays (NAs) of different materials is crucial and ability of generating desired functionality has been of considerable interest [1–4]. Nanosphere lithography (NSL), among the various techniques such as nano-imprinting lithography [5], hole mask colloidal lithography [6] and electron beam and focused-ion beam lithography [7], has been used to fabricate patterns with excellent size, shape, and spacing control. In addition, NSL is a versatile, high-throughput, and cost-effective technique.

NAs of desired material could be fabricated with NSL technique, wherein primary step is an appropriate choice of deposition mask that governs the shape and order of the developed NAs. Polystyrene (PS) bead-based deposition masks are commonly used for NAs development in NSL [1–3]. Therefore, a deposition mask can be developed by employing a coating of PS beads of appropriate size onto the desired solid support viz. glass and plastics. Further, spatially arranged patterns of the deposited material can be obtained by removing the polystyrene mask [1]. Depending on the nature of the deposited material these NAs may be employed for optical, electrical, magnetic and molecular properties, have got

applications in sensors [8–10], optical and opto-electronic [3,11], photovoltaic devices [12] along with magnetic data storage memory component designing [13,14]. In spite of mentioned significant applications, the application of NSL based NAs as effective electro-active surfaces for biosensor development is not yet well explored. The current generation of biosensor, there is considerable scope for fabricate nano-structured supported platform to achieve high sensitivity, ultra low detection limit, multiplexing capability, low cost, portability and easy operation [15,16]. Among these properties, multiplex application of protein detection is urgently required to detect ultra low concentration of proteins with high sensitivity in clinical diagnosis for human healthcare [17]. Some of these challenges could be achieved using NSL based efficient highly electro-active and sensitive platforms that can either be used as fabricated or in conjunction with microfluidics channel systems for the development of biosensors. In addition, due to the two dimensional electro-actively ordered array formation and ability to oriented immobilization of biomolecules (enzyme, DNA, antibody, microbial cells), these may also be employed for the development of cheap alternative microarray-based immunosensor to perform multiplexed diagnostics along with high sensitivity.

There are several diagnostics approaches such as, microtiter plate-based assays, and microarrays. These approaches have certain associated drawbacks such as cumbersome antibody immobilization procedures and antibody functionality related issues.

In this manuscript we are addressing the problems associated with the pre-existing approaches by employing NAs with a simple model of microfluidics system to demonstrate the use of NAs as an

* Corresponding author.

E-mail addresses: Chandra.dixit2@mail.dcu.ie (C.K. Dixit), ajeet.npl@gmail.com (A. Kaushik).

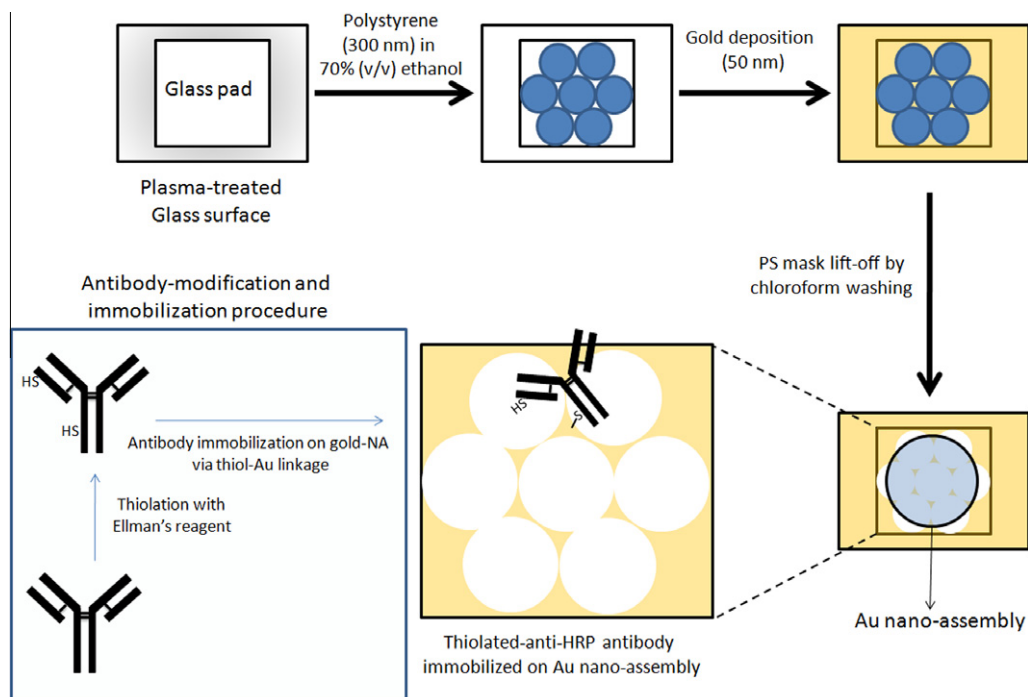


Fig. 1A. Schematic representation of the methodology for nano array (NA) development using nano sphere lithography (NSL). Antibody activation by thiolating them using Ellman's reagent and subsequently their immobilization on the NAs is also depicted.

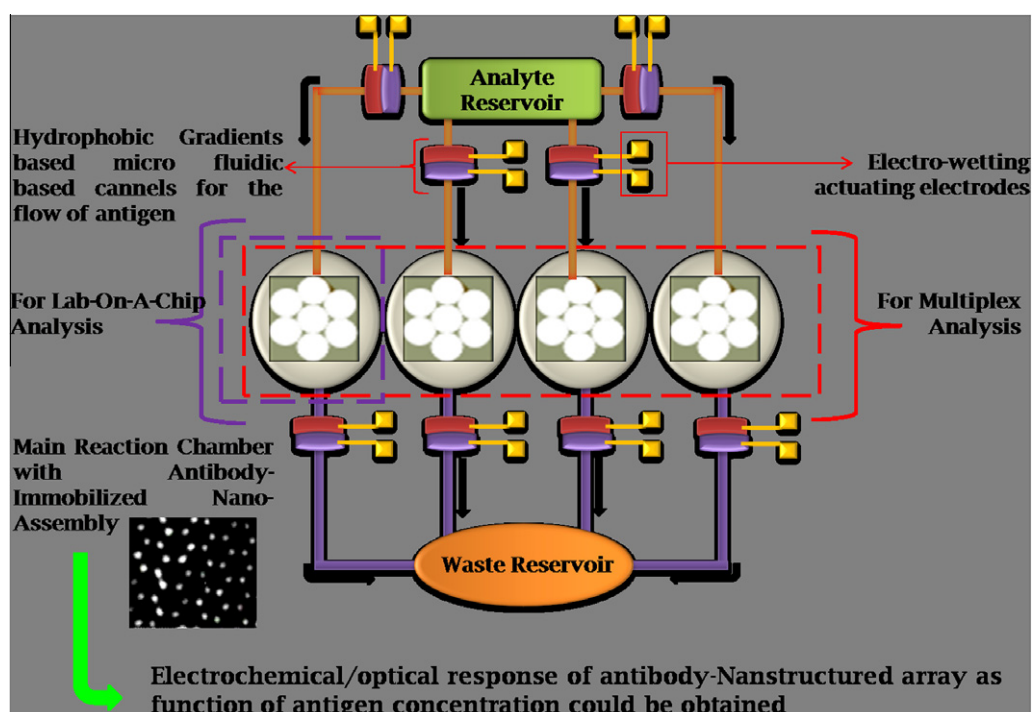


Fig. 1B. The set-up of micro-injection-based microfluidics system employed in conjunction with the developed NA. The developed system could be employed for Lab-on-a-Chip and multiplex analyses.

alternative platform for the development of a Lab-on-a-Chip prototype. We have employed NSL technique to fabricate hexagonally packed nano-structured arrays of Au by using polystyrene bead as a mask. The immunoassay application was demonstrated on the developed NAs by immobilizing anti-horseradish peroxidase

(HRP) antibody and performing a concentration dependent colorimetric immunoassay for the recognition of HRP. However, further improvements in the design of microfluidics system must be performed accordingly, for incorporating this platform to develop immunoassays.

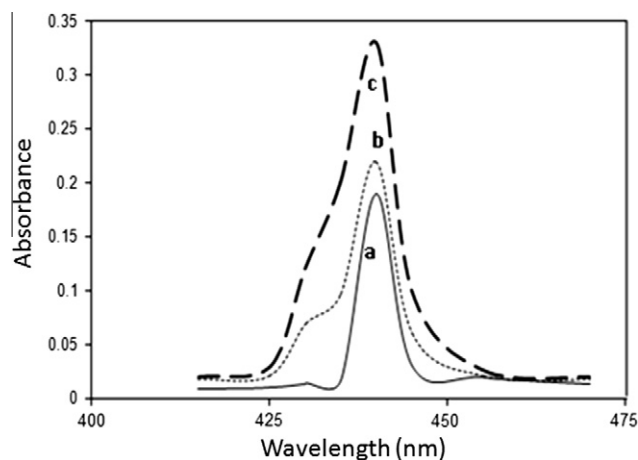


Fig. 2. Spectrophotometric analyses of the color developed after horseradish peroxidase (HRP) capture by the immobilized anti-HRP antibody. Antibodies capture on the NA were thiolated and captured via sulfhydryl-gold linkage. The HRP capture was repeated thrice on the same surface immobilized with antibody by repeated regeneration of the antibody using 10 mM NaOH. A decrease in the amount of captured HRP was detected over three repeats.

2. Materials and methods

2.1. Materials required

Polystyrene (PS) beads (diameter 300 nm), anti-HRP antibody and HRP were purchased from Sigma–Aldrich, Ireland. Rhodamine (Rh)-conjugated HRP were procured from Sigma Chemical Co., St. Louis, MO. Bovine serum albumin (BSA; 10 $\times$), 0.15 M phosphate buffered saline (PBS), tetramethyl benzidine (TMB), pH 7.4, thiolation kit (Traut's reagent, Ellman's reagent and ethylenediaminetetraacetic acid (EDTA)) and bicinchoninic acid (BCA) was obtained from Thermo Scientific, Ireland.

2.2. Fabrication of NSL based nano-structured arrays

Glass substrate was plasma-treated for 3 min at 29 W power in an oxygen-plasma chamber (Harick Plasma, NY, USA). The hydrophilicity of the surface was monitored by contact angle measurement using G2 2 contact angle goniometer (Kruss GmbH, Germany). PS solution (2.5% (v/v) in 70% (v/v) ethanol) was drop-coated on the treated glass and was allowed to dry in ambient conditions. Au film (50 nm) was deposited onto PS-coated glass using

PECVD reactor Europlasma, model CD300 (Oudenaarde, Ghent, Belgium). Subsequently, the polystyrene beads were removed by dissolving in chloroform with the aid of sonication for 3 min. Au nanostructures were then characterized with scanning electron microscope (SEM Jeol Instruments (JSM 840)). The fabrication procedure is depicted in schematic Fig. 1A.

2.3. Thiolation of the anti-HRP antibodies

Anti-HRP antibodies (10 mg/mL) were dissolved in PBS, pH 8.0, with 5 mM EDTA. Traut's reagent (2-iminethiolane-HCl) at 10-fold molar excess (46 μ L of 14 mM Traut's reagent) was added to the anti-HRP antibody solution and incubated at room temperature for 90 min. the unreacted Traut's reagent was removed by passing the sample through a desalting column. The amount of thiolation was confirmed as described in the guidelines supplied; whereas, antibody was quantitated with bicinchoninic acid (BCA) assay (Supplementary Fig. 1).

2.4. Immobilization of anti-HRP antibody onto NAs and assay development

Thiolated anti-HRP antibodies were further diluted (50 μ g/mL) in 0.15 M phosphate buffer saline (PBS, pH 7.4). Anti-HRP antibody solution was incubated on the NA assembly for 1 h at 37 $^{\circ}$ C followed by washing using PBS. A glass surface, activated as mentioned previously, was used as a control surface to assess the non-specific binding of the antibody under same conditions. The developed color was measured with UV spectrophotometer at 450 nm wavelength (Fig. 2). The amount of immobilized antibody was characterized using bicinchoninic acid (BCA) assay. The amount of antibody immobilized on the bare glass surface equivalent to the void space of one hexagonal arrangement of the Au islands was calculated and deducted from the total amount of antibody bound on the NA. This correction allows the approximation of the amount of antibody bound on the Au islands of the NA. The immobilized antibody was characterized using atomic force microscopy (AFM; Veeco Scientific). After antibody quantification, the surface was blocked with BSA (3% (v/v)) in PBS.

HRP (1–10,000 pg/mL) was reconstituted in 0.15 M PBS, pH 7.4 and incubated on anti-HRP immobilized NA such that one concentration is provided on one chip. The incubation was performed for 1 h at 37 $^{\circ}$ C. Chips were extensively washed with PBS. These HRP-captured chips were incubated in TMB solution for 30 min and the color was allowed to develop. The enzyme reaction was stopped using 1 N H₂SO₄ and allowed to settle for 5 min (Fig. 3).

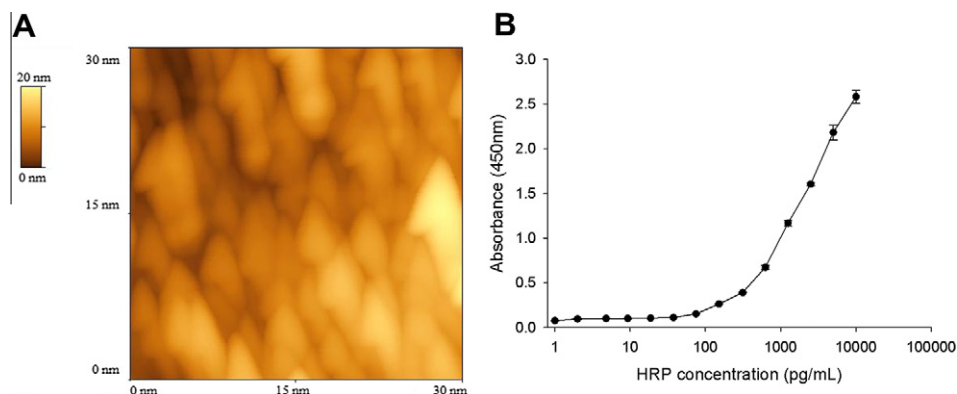


Fig. 3. Analysis of the developed surface with immobilized anti-HRP system. (A) The atomic force microscopy image of captured antibody on the NA surface. A scan was performed in a 30 $\times$ 30 nm² area corresponding to the gold island of 30 nm arm-length. A densely packed surface with antibodies was obtained corresponding to an efficient antibody thiolation and immobilization. (B) An immunoassay was performed to obtain a minimum detection limit of the HRP detection with the developed platform. HRP was detected corresponding to a concentration as low as 10 pg/mL; whereas the detection limit was found to be 100 pg/mL (blank + 3SD).

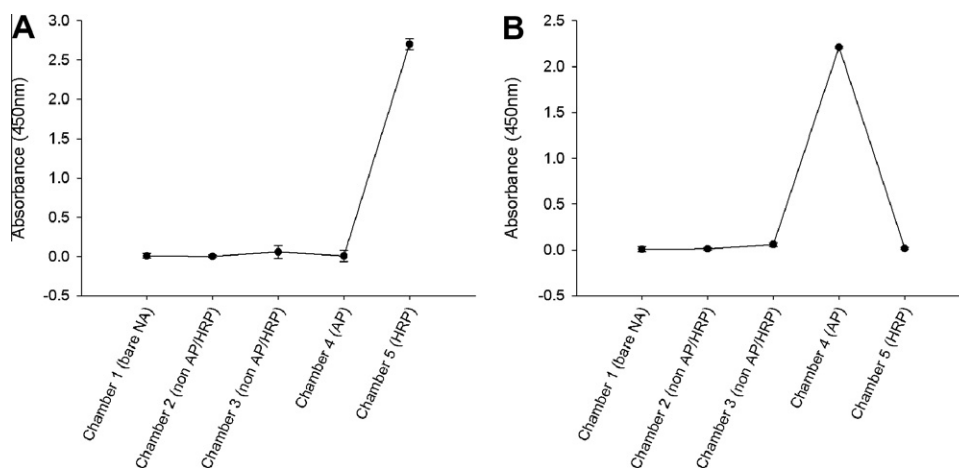


Fig. 4. Functionality analysis of the developed hybrid microfluidics-NA system using a spiked alkaline phosphatase (AP) and HRP enzymes. The first three chambers were system controls such that first chamber was bare Au NA, second chamber was BSA-blocked Au NA; whereas, third chamber was immobilized with an antibody not specific to HRP/AP. All these process controls were employed to analyze the non-specificity of biological molecules toward the developed NA. The fourth chamber was immobilized with anti-HRP antibody and fifth chamber contains anti-AP antibody. In this experiment (A) the specific capture of HRP and (B) AP was demonstrated.

2.5. Microfluidics incorporation with the NA developed platform

To demonstrate the capability of devising Lab-on-a-Chip system with the developed NA platform a microfluidic system with five reaction chambers was designed using one millimeter thick Teflon plastic (Polypenco plc, Farnell, Leeds, UK). Teflon was chosen because of its non-sticky and inert nature that minimizes the non-specific protein adsorption. The design and assembly of the microfluidics system is described in Fig. 1B. Two types of antibodies (anti-HRP and anti-alkaline phosphatase (AP) antibody) were employed to demonstrate the functionality of the developed prototype. Antibodies were immobilized manually by incubating antibody samples in respective chambers followed by washing. Antibody immobilization was performed for capturing each type of antibody in the designated chambers; whereas, in the other two chambers mouse antibodies were immobilized, which were not raised against either HRP or AP. Subsequently, the system was completed by mounting a sheet on the top of the channel system in order to make it a closed-system. Spiked sample containing two different enzymes were injected into the system as described. The samples were injected at flow rate of 10 $\mu\text{L}/\text{min}$ whereas; washing was performed at 200 $\mu\text{L}/\text{min}$ flow rate.

3. Results and discussion

The self assembly of PS beads onto hydrolyzed surface via drop casting was studied using microscopy (Supplementary Fig. 2). Various experimental conditions, such as, (a) high concentration, low evaporation rates, (b) high concentration of polystyrene bead in matrix at high evaporation rates, (c) low concentration of polystyrene bead in matrix at high evaporation rates and, (d) low concentration of polystyrene bead in matrix at low evaporation rates, were optimized for analyzing uniformity of distribution of the PS beads on the surface. Slow rates of evaporation of the solvent system were found to generate high levels of uniformity over a given number of sets of experiments (Supplementary Fig. 2A–D). In the post lift off array only gold triangles of the height of 100 nm were detected and were confirmed by SEM. Arm-length of each gold triangle was found to be 0.03 μm . The probable justification for this difference is that the coinciding regions of the beads which were considered in the calculations never covered with gold after liftoff.

Immobilization of desired biomolecule on a conducting surface is crucial step in designing a biosensor. The high antibody immobi-

lization density is desirable to make a diagnostic device. In addition, preserving the functional conformation of the immobilized antibody is also critical. Immobilization of the thiolated antibodies on Au islands improves the site-directed capture with preserved conformation. The amount of immobilized antibodies was calculated by employing BCA assay for protein estimation (Supplementary Fig. 1). Total amount of antibody captured on the array after correction of the 'non-specificity factor', was 5 $\mu\text{g}/\text{mL}$, which corresponds to an immobilization efficiency of 10%. In addition, AFM (Fig. 3A) was also used to confirm the antibody immobilization. Primarily, the functionality of the immobilized antibody was assessed using a colorimetric approach. HRP was captured on the anti-HRP antibody functionalized NA. TMB-mediated colorimetric immunoassay (Fig. 2) was performed to examine the immunoassay performance on the developed NAs. The captured antibody was found functional after immobilization. The negative and positive controls were also analyzed. There was significantly negligible non-specificity in the HRP capture. In addition, the control values for colorimetric and fluorescence imaging was deducted from their respective experimental values.

After confirming the functionality of antibody-immobilized Au array prototype, an enzyme immunoassay (EIA) was performed with HRP (1–10,000 pg/mL). The detection limit of this assay was 100 pg/mL of HRP (Fig. 3B). The control for this study was HRP incubated on the blocked surface without antibody. The control values were subtracted from the experimental values.

Similarly, the integrated microfluidic system-based concept for Lab-on-a-Chip was also critically assessed. The five-chambered system was mounted on an oxygen-plasma-treated glass surface. Subsequently the NAs were developed in the spaces of each of the five chambers. Later, thiolated anti-HRP antibodies were immobilized on the developed NAs. The spiked sample containing HRP and AP was then injected for 15 min at described flow rates, followed by washing with PBS. TMB was then injected through the system and collected from the over-flow waste containers. The collected TMB from each chamber was then mixed with 50 μL of 1 N H_2SO_4 and spectrophotometrically analyzed at 450 nm. A yellow color was obtained with the samples corresponding to the chambers where HRP was captured by anti-HRP antibody (Fig. 4). Similarly, *p*-nitrophenyl phosphate disodium salt (pNPP) solution was passed through the system. The sample was collected as done for TMB substrate analysis. The collected solution was stopped by mixing with 2 N NaOH, which was measured spectrophotometrically at 405 nm. A clear signal for each type of

enzyme-substrate reaction was obtained confirming the performance of the prototype assembly of NSL-based NAs integrated with the five-chambered microfluidic system.

The obtained results suggest that the NSL approach, even being extensively studied, could be used widely for developing customized surfaces. In addition, these customized surfaces can be employed for developing immunoassay-based biosensors for diagnostics and other analytical application. On such study that shows the use of nanoparticles-based localized plasmon can also be employed in conjunction with enzyme immunoassays for improving the sensitivity of the developed assay. Therefore, we propose that silver or gold nanoarrays can also be used to perform high sensitivity immunoassays along with their potential applicability in the development of microfluidics-based platforms. In addition, these NAs could be employed as potential alternates for conventional solid supports used for Lab-on-a-Chip applications because development of NAs is less laborious and can be customized according to the requirements.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.bbrc.2012.02.018](https://doi.org/10.1016/j.bbrc.2012.02.018).

References

- [1] J-F. Masson, M-P. Murray, M. Ethota, L.S. Livea, Nanohole arrays in chemical analysis: manufacturing methods and applications, *Analyst* 135 (2010) 1483–1489.
- [2] B.J.Y. Tan, C.H. Sow, T.S. Koh, K.C. Chin, A.T.S. Wee, C.K. Ong, Fabrication of size-tunable gold nanoparticles array with nanosphere lithography, reactive ion etching, and thermal annealing, *J. Phys. Chem. B* 109 (2005) 1100–11109.
- [3] C.L. Haynes, R.P. Van Duyne, Nanosphere lithography: a versatile nanofabrication tool for studies of size-dependent nanoparticle optics, *J. Phys. Chem. B* 105 (2001) 5599–5611.
- [4] S. Chen, M. Sveghendahl, R.P.V. Duyne, M. Kall, Plasmon-enhanced colorimetric ELISA with single molecule sensitivity, *Nano. Lett.* 11 (2011) 1826–1830.
- [5] S.-W. Lee, K.-S. Lee, J. Ahn, J.-J. Lee, M.-G. Kim, Y.-B. Shin, Highly sensitive biosensing using arrays of plasmonic Au nanodisks realized by nanoimprint lithography, *ACS Nano* 5 (2) (2011) 897–904.
- [6] H. Fredriksson, Y. Alaverdyan, A. Dmitriev, C. Langhammer, D.S. Sutherland, M. Zach, B. Kasemo, Hole-mask colloidal lithography, *Adv. Mater.* 19 (2007) 4297–4302.
- [7] Z.-P. Tian, K.L.B. Chen, Nanotechnology, unique nanopore pattern formation by focused ion beam guided anodization, *Nanotechnology* 21 (2010) 405301.
- [8] A.J. Haes, R.P. Van Duyne, A nanoscale optical biosensor: sensitivity and selectivity of an approach based on the localized surface plasmon resonance spectroscopy of triangular silver nanoparticles, *J. Am. Chem. Soc.* 124 (2002) 10596–10604.
- [9] J.C. Riboh, A.J. Haes, A.D. McFarland, C.R. Yonzon, R.P. Van Duyne, A nanoscale optical biosensor: real-time immunoassay in physiological buffer enabled by improved nanoparticle adhesion, *J. Phys. Chem. B* 107 (2003) 1772–1780.
- [10] A.J. Haes, L. Chang, W.L. Klein, R.P. Van Duyne, Detection of a biomarker for Alzheimer's disease from synthetic and clinical samples using a nanoscale optical biosensor, *J. Am. Chem. Soc.* 127 (2005) 2264–2271.
- [11] K. Kempa, B. Kimball, J. Rybczynski, Z.P. Huang, P.F. Wu, D. Steeves, M. Sennett, M. Giersig, D.V.G.L.N. Rao, D.L. Carnahan, D.Z. Wang, J.Y. Lao, W.Z. Li, Z.F. Ren, Photonic crystals based on periodic arrays of aligned carbon nanotubes, *Nano Lett.* 3 (2003) 13–18.
- [12] S.S. Williams, M.J. Hampton, V. Gowrishankar, I.-K. Ding, J.L. Templeton, E.T. Samulski, J.M. DeSimone, M.D. McGehee, Nanostructured titania-polymer photovoltaic devices made using PFPE-based nanomolding techniques, *Chem. Mater.* 20 (2008) 5229–5234.
- [13] S.M. Weekes, F.Y. Ogrin, W.A. Murray, Fabrication of large-area ferromagnetic arrays using etched nanosphere lithography, *Langmuir* 20 (2004) 11208–11212.
- [14] M. Albrecht, G. Hu, I.L. Guhr, T.C. Ulbrich, J. Boneberg, P. Leiderer, G. Schatz, Magnetic multilayers on nanospheres, *Nat. Mater.* 4 (2005) 203–206.
- [15] P.R. Solanki, A. Kaushik, V. Varun, B.D. Malhotra, Recent advances in nanostructured metal oxides based biosensors, *Nat. Asia Mater.* 3 (2011) 17–24.
- [16] A. Kaushik, P.R. Solanki, K. Kaneto, C.G. Kim, S. Ahmad, B.D. Malhotra, Nanostructured iron oxide platform for impedimetric cholesterol detection, *Electroanalysis* 22 (2010) 1045–1055.
- [17] D.A. Giljohann, C.A. Mirkin, Drivers of biodiagnostic development, *Nature* 462 (2009) 461–464.

Nano-structured Arrays Revisited As A Platform for Multiplex Analyses and Lab-On-A-Chip Applications

Chandra K. Dixit^{1,2}, Ajeet Kaushik^{*3}

¹ National Centre for Sensor Research, Dublin City University, Glasnevin, Dublin 9, Ireland,

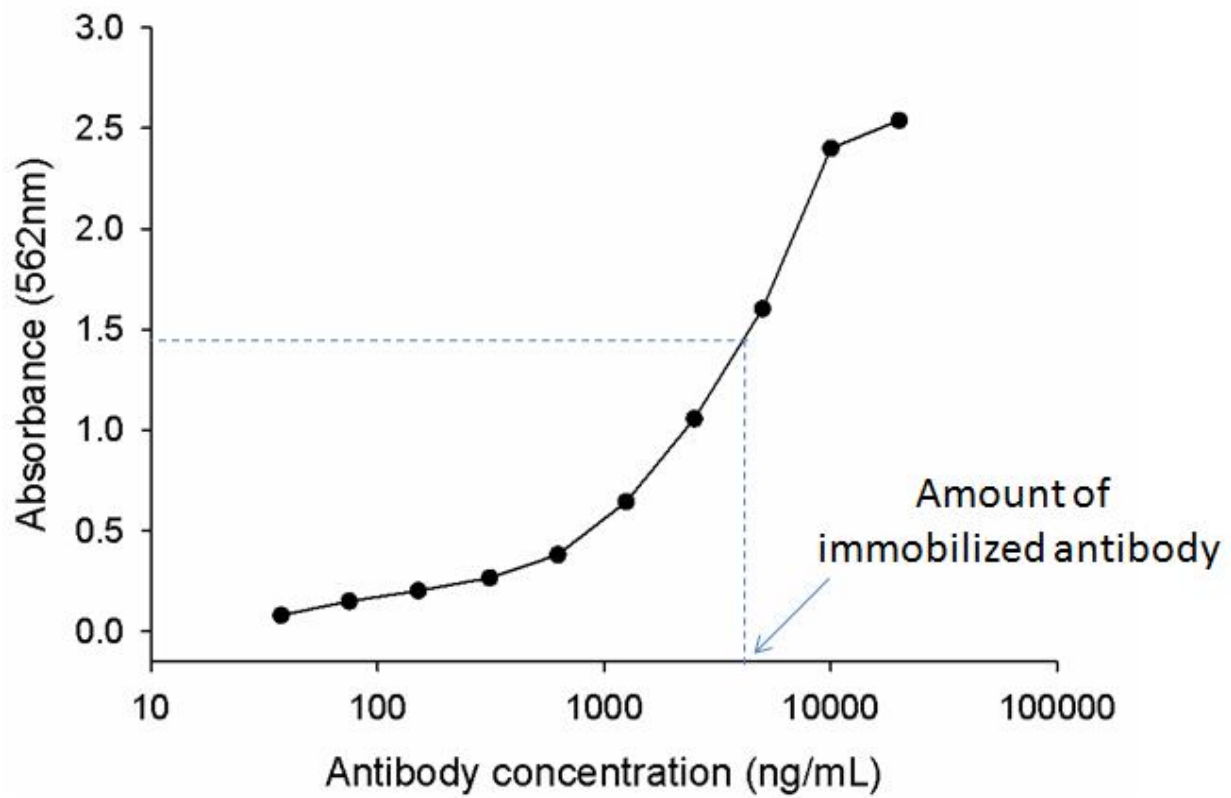
² Schools of Biotechnology, Dublin City University, Glasnevin, Dublin 9, Ireland,

⁴Bio-MEMS and Microsystem Lab, Department of Electrical Engineering, University of South Florida, 4202 E. Fowler Avenue, ENB 118, Tampa, FL 33620, United States

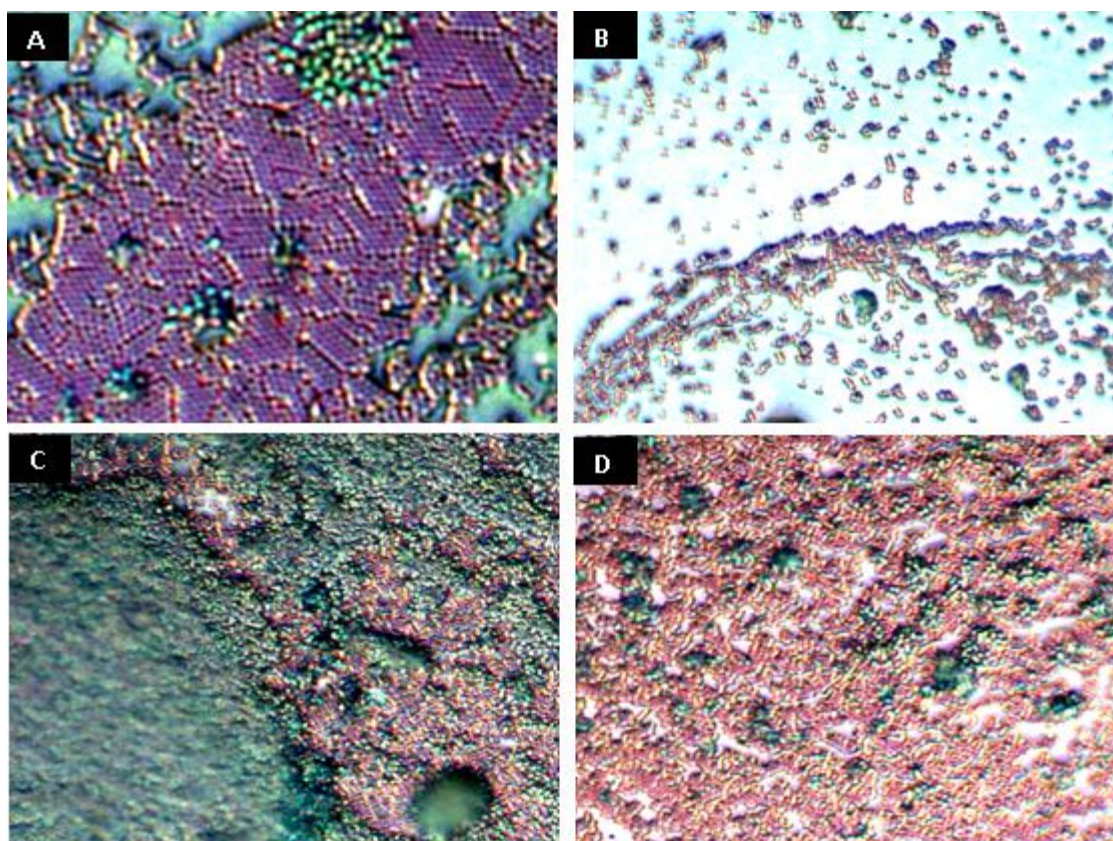
* Corresponding Author: ajeet.npl@gmail.com (Ajeet Kaushik), Chandra.dixit2@mail.dcu.ie (Chandra K Dixit),
Tel. +1-[813-974-7942](tel:+18139747942)

ABSTRACT

Nanospheres lithographic (NSL) method has been used to fabricate nano-structured array (NA) of hexagonally close-packed gold (Au) using polystyrene beads [PS, diameter ~ 300 nm] as mask. The developed NA was incorporated with a customized and cheap microfluidics system to demonstrate its applicability as an alternative easy and efficient platform multiplex analysis for Lab-on-a-Chip applications. The chip functionality was demonstrated with horseradish peroxidase (HRP) and anti-HRP antibody as model for recognition system. The enzyme-linked immunosorbent assay (ELISA) performed on fabricated protein biochip had a detection limit 100 pg/mL for HRP. The antibody chip was also checked for the shelf-life and it was found that these chips could be stored for 50 days when stored at 4°C without any significant loss of activity. Therefore, NA-based protein biochip with the correct microfluidics could find huge potential application in diagnostics and biosensing technology.



Supplementary Figure 1. Quantitation of the antibody immobilized on NA using bicinchoninic acid (BCA) assay. The dotted line on X-axis corresponds to the antibody concentration bound on the surface.



Supplementary Figure 2. Analysis of the effect of various physical conditions on the NA formation.