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A direct detection of human *papillomavirus* 16 genomic DNA using gold nanoprobes

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ABSTRACT

Nanoparticles have been investigated as flagging tests for the sensitive DNA recognition that can be utilized as a part of field applications to defeat restrictions. Gold nanoparticles (AuNPs) have been widely utilized due to its optical property and capacity to get functionalized with a mixed bag of biomolecules. This study exhibits the utilization of AuNPs functionalized with single-stranded oligonucleotide (AuNP-oligo test) for fast the identification of Human *Papillomavirus* (HPV). This test is displayed on interdigitated electrode sensor and supported by colorimetric assay. DNA conjugated AuNP has optical property that can be controlled for the applications in diagnostics. With its identification abilities, this methodology incorporates minimal effort, strong reagents and basic identification of HPV.

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1. Introduction

Development of highly sensitive and selective DNA detection methods is extremely important in clinical diagnosis because the DNA is usually present at very low concentrations [1]. Conventional DNA amplification using PCR can provide fast results; unfortunately it requires considerable skill and expensive equipment. Nanoparticles have been explored widely as signaling probes for ultrasensitive DNA detection that can be used in field applications. Gold nanoparticles (AuNPs) have been extensively used for biomolecule detection by many research groups mainly because of optical properties and ability to functionalize with a variety of biomolecules [2,3]. AuNPs-probe has been integrated in research as common diagnostics and has shown great potential applications. The colloidal AuNPs are used in the development of several biodetection schemes [4]. Protein-coated gold colloids have been used extensively in lateral flow immunoassay based analytical techniques.

Human *Papillomavirus* (HPV) is the most common sexually transmitted virus that causes cancer [5]. More than 100 types of HPV strains including high and low-risk types have been identified so far. 15 strains of HPV have been identified as placing women at high risk of cervical cancer. Cervical cancer infection was stimulated by high-risk types of Human *Papillomavirus* (HPV) strains 16, 18, 31 and 45 [6]. HPV 16 is the worst for the development of cervical cancer followed by 18, 31 and 45. Cervical cancer infections started when HPV virus in cervix entered cells through micro-abrasions and infected cells. Several weeks later, the virus replicates and infections spread through the cells.

Cervical cancer is the second largest cause of cancer-related death in women worldwide, and it occurs following the persistent infection, with a specific subset of HPV types [7]. Cervical cancer is estimated that globally more than 290 million women have an HPV infection. It can be treated if it detect at the early stage. Infection with the HPV cause cervical cancer have symptoms such as vaginal bleeding, contact bleeding, vaginal mass may indicate the presence of malignancy, moderate pain during sexual intercourse and vaginal discharge [8]. No effective vaccine or specific therapeutic treatment has been reported is available for preventing or curing the disease caused by HPV strain 16. Diagnostic methods are required to identify the types correctly and rapidly, to treat HPV strain virus infection at an early stage. Future developments in early gene specific detection that cause cancer and chronic disease, offer the potential technology for improved capabilities over traditional medical diagnosis, prognosis, and treatment. The identification of genes associated with specific disease states is rising steadily. Furthermore, classification of diseases based on their unique genetic

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identification profiles is increasingly being studied as compared to traditional methods of disease classification [9].

Viral detection using specific biomarker and serological methods with specific anti-HPV antibodies are two types of common detection techniques for the diagnosis of HPV viral infections. Serological tests allow the identification of IgG and IgM antibodies to the HPV strain 16 virus [10]. However, sufficient production of antibodies needs 5 days after the onset of illness. Besides that, the serological test suffered from cross-reaction with another HPV strain due to non-specific antigenic determination by polyclonal antibodies. Currently, virological techniques combined with Polymerase Chain Reaction (PCR) for amplification of specific genes are preferred as they are more selective, low-risk contamination and shorter assay time compared to the serological techniques [11]. The detection includes stained agarose gel electrophoresis for the visualization of PCR product, however it is time consuming and hazardous. With arising of nanotechnology in current technology, the advance of nanobiosensor is replacing such conventional approach.

Nanobiosensor based nucleic acid hybridization have recently been developed for inexpensive and rapid detection of DNA molecules'[12]. Cancer diagnostic devices which are inexpensive, portable and field ready with high specificity and sensitivity, play significant roles because of cost-effective and compact medical system are needed to diagnose large populations suffering from cancer. Interdigitated electrode (IDE) sensor as microfabricated device is employed to overcome all these limitations faced by serological and virological methods. This electrical based nanobiosensor integrated with biomolecule probes to allow the detection of change in charges when HPV DNA target binds onto the immobilized probe. Due to low fluid volume consumption and high surface to volume ratio, IDE sensor proved to be highly sensitive, capable for direct and specific detection of large species suffering from the cancer. Its fast response time, low detection limit and elimination of labeling method make this type of detection device different from previous detections.

To make real application possible with clinical samples, the target DNA sequence would be longer as genome sequences. Due to limitation of length of clinical target sample, little has been known on detection of long sequence of DNA by nanobiosensor [13]. HPV contains virion that has a double-stranded, circular DNA genome of approximately 7900 bp, and is divided into three regions, viz., the non-coding long control region (LCR, ~1 kb), the protein coding early (E, ~4 kb) and late (L, ~3 kb) regions [14]. The viral genome encodes six early (E1, E2, E4, E5, E6 and E7) and two late (L1 and L2) proteins.

A specific fragment from E6 region (24 bp) was chosen for this study. Functionalization of carefully selected DNA probe sequence on the AuNP based IDE surface enables the hybridization between HPV 16 DNA probe and the target sequence, and thus HPV will be detected. Hybridization with a complementary sequence of DNA would accumulate charges on the surface of the AuNP based IDE contributed by the sequence. Changes in charge upon hybridization induce increasements in resistance and lower the current voltage which constitutes the basis of detection mechanism. This study investigate the feasibility of HPV on AuNP based IDE nanobiosensor offering low fluid volumes consumption, high-throughput analysis, real response time, simple detection and better process control, high surface to volume ratio, ultra high sensitive and selective, reusable, compact system, cost effective device and simple diagnosis [15]. All HPV tests currently in use are rely on the detection of viral nucleic acids because HPV cannot be cultured [11]. In the present study, AuNP-functionalized oligonucleotide probe based IDE nanobiosensor for detection of cervical cancer using deoxyribonucleic acid (DNA) hybridization for highly sensitive and fast response time of HPV was employed for designing a nano diagnostic tool. Further, the obtained results were supported by colorimetric assay.

Table 1
DNA sequences for HPV DNA probe oligonucleotide and complementary 24-mer analyte HPV16A-CMP (AIT Biotech, Singapore).

Name	Sequences
HPV DNA probe oligonucleotide	5′- GGG GTC GGT GGA CCG GTC GAT GTA-3′
Complementary 24-mer target HPV16A-CMP	5′-TAC ATC GAC CGG TCC ACC GAC CCC-3′
HPV16A non-complementary	5′-ATG TAG CTG GCC AGG TGG CTG GGG-3′
HPV16A single mismatch	5′-TAC ATC GAC CGG TGC ACC GAC CCC-3′

2. Materials and methods

2.1. Reagents

Single-stranded 24 mer synthetic oligonucleotide probe designed from Human *Papillomavirus* (HPV) for the identification of target DNA HPV 16 (Accession No: A18875.1). Gold nanoparticle (AuNP) was obtained from Sigma-Aldrich, USA. The sequence of 24-mer probe oligonucleotide, complementary, and non-complementary target oligonucleotides are followed as in Table 1.The 24 mer probe was used as a model system for immobilization, while the complementary 24 mer strand was used as target analyte. Lyophilized DNA samples were reconstituted and diluted in deionized distilled water (ddH_2O) to the desired concentrations prior to use.

2.2. IDE fabrication

The IDE nanosensor was fabricated on a silicon-on-insulator (SOI) wafer with a 145 nm buried oxide layer [16]. Silicon wafer was used as a main substrate in order to form silicon dioxide (SiO_2) as an insulation layer of an electrical device. Schematic diagram of fabrication process flowchart is shown in Fig. 1. The wafer was cleaned using buffered oxide etchant (BOE) to remove native oxide which had been naturally grow on it. Growth of SiO_2 (b) using wet oxidation process provides thicker insulation layer and shorter time consuming compared to dry oxidation process [17]. In order to transfer pattern from a mask on a wafer, photoresist was spin-coated on the growth SiO_2 wafer using spin-coater. Photoresist is a light-sensitive material used to form a patterned coating on a surface. By using deep ultraviolet lithography, a 50 nm silicon layer was patterned and etched when UV-light was exposed on the photoresist (c), pattern from chrome mask was directly transfer onto the photoresist. After development process, aluminum metal was deposited (e) using sputter-coater and acetone was used to strip the unwanted photoresist (f).

2.3. Probe design

A 24 mer oligonucleotide (5′-GGG GTC GGT GGA CCG GTC GAT GTA-3′) was designed as the probe using NCBI BLAST nucleotide search tool based on the E6 gene region. The probe sequence was checked for any possible homology and share sequences with any non-HPV species. Moreover, the melting temperature of the probe was ensured to be within a narrow range using oligo software. Then, the probe sequence was checked for potential self-dimer and formation of secondary structures using mfold software (http://mfold.rna.albany.edu).

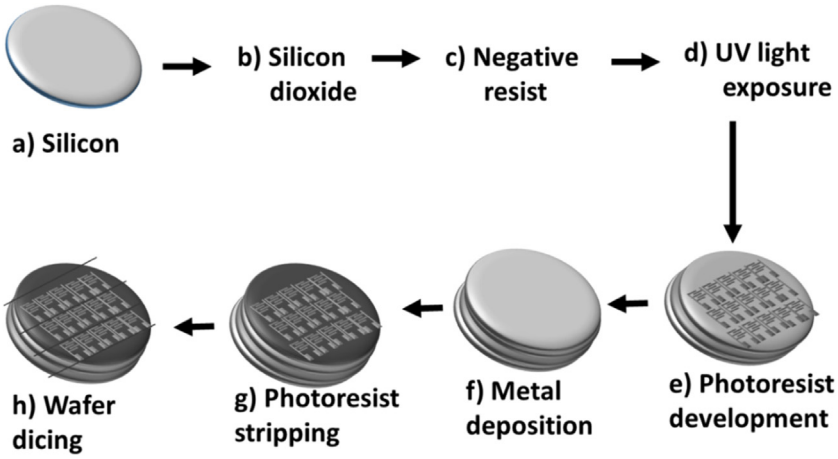

Fig. 1. Schematic diagram of fabrication process.

2.4. AuNP-probe hybridization assay

To optimize the condition of hybridization and the best observation of color changes, various amounts of AuNP-probe and NaCl were mixed to obtain the best color changes. The oligonucleotide target (5′-TAC ATC GAC CGG TCC ACC GAC CCC-3′), that is a part of E6 gene region and complement with HPV probe sequence was designed.

2.5. Genomic DNA extraction

The genomic DNA was isolated from the HPV virus using QIAamp® DNA Mini and Blood Mini Handbook [18]. DNA extraction from clinical samples was performed using a QIAamp® DNA Mini and Blood Mini Handbook and purification kit and stored at −20 °C until use. The amount of genomic DNA was determined using a UV–vis spectrophotometer.

2.6. Analysis of complementation on interdigitated electrode (IDE) sensor

AuNP was deposited on the sensing of the IDE sensor. One micromolar of probe DNA in deionized distilled water was applied in the active area and incubated for 2 h in a moist environment at room temperature. After incubation, excess DNA probe were rinsed free from the surface by washing the device thrice (5 min each) with ddH_2O. IDE device were ready for electrical measurement after immobilization.

After immobilization of the HPV 16 DNA probe, AuNP based IDE current were measured by probing the two terminals, source (S) and drain (D). IDE substrate device was being able to be monitored the current flowing to the drain as well as to the substrate when the source electrode voltage was swept. A single sweep of voltage was supplied to the source of terminal in intervals of 0.05 V with a compliance set at 100 mA. Current was simultaneously measured and displayed in the form of a current-voltage (I–V graph). Hybridization measurements are taken using current-voltage (I–V) characterization (KEITHLEY, 6487) to determine the change in current flow for the AuNP based IDE active area before and after DNA hybridization.

The extracted DNA from HPV strain 16 was first denatured into single stranded DNA by heating in thermoshaker for 2 min and then quench to stop the reaction in ice for 5 min. One microliters of this single-stranded DNA was then applied in active area of IDE and incubated for 15 min. Excess DNA was rinsed free from the surface by washing the chip.

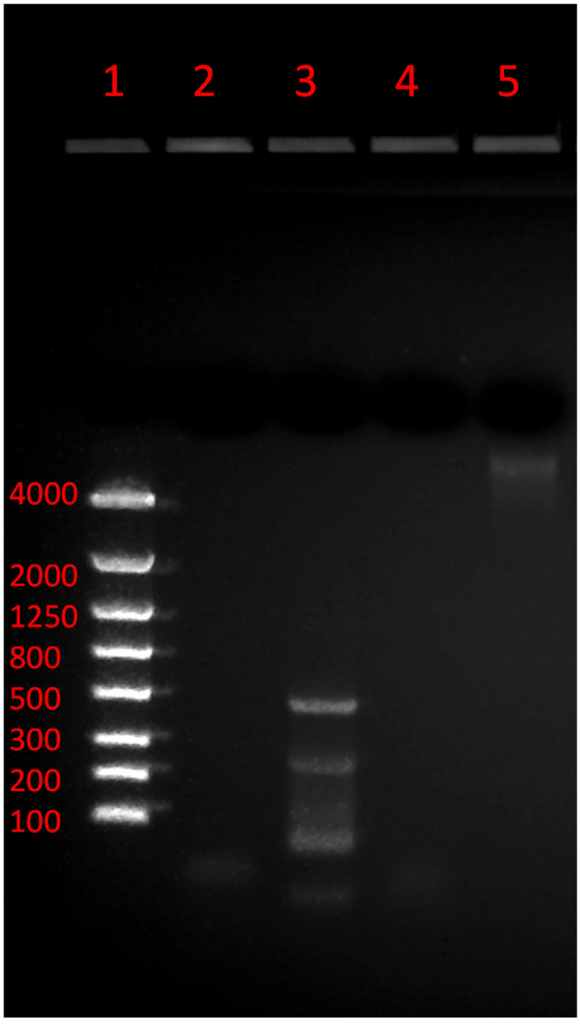

Fig. 2. Agarose gel showing comparison of genomic DNA of HPV extraction by using QIAamp® DNA Mini and Blood Mini Handbook [18] with PCR product from multiple sequence of HPV and single band of PCR product. Negative control without template from HPV DNA genomic was used for confirmation of successful amplification by using PCR. Lane 1: FlashGel DNA Marker 100 bp- 4 bp. Lane 2: Multiple sequence HPV PCR product (H00892). Lane 3: HPV PCR product. Lane 4: Negative control PCR reaction (without template). Lane 5: Genomic DNA extraction from Human *Papillomavirus* (HPV).

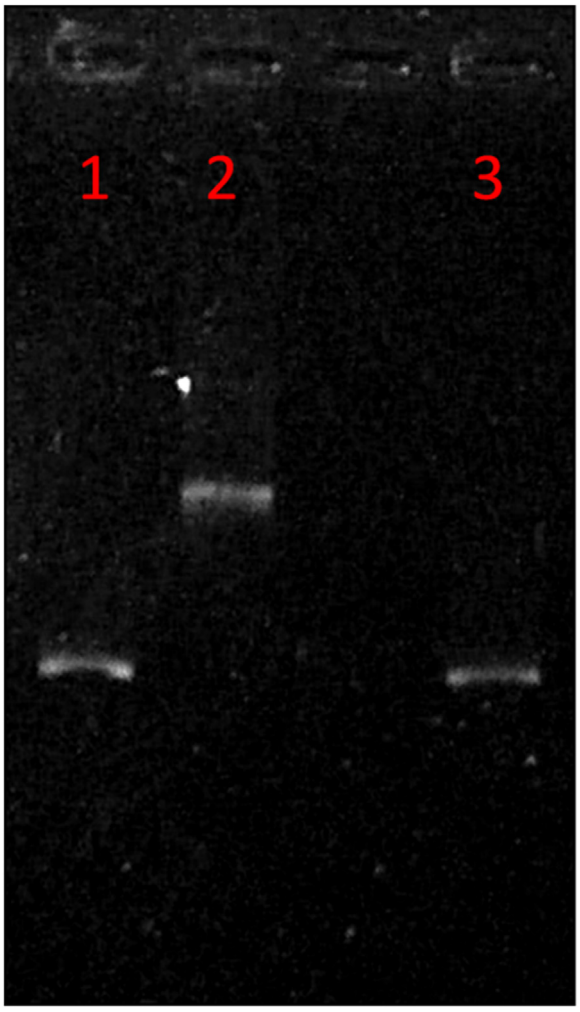

Fig. 3. Agarose gel showing probe hybridization. Hybridization of probe with target DNA. Lane 1: probe. Lane 2: Hybridization with DNA. Lane 3: Hybridization with non-complementary DNA.

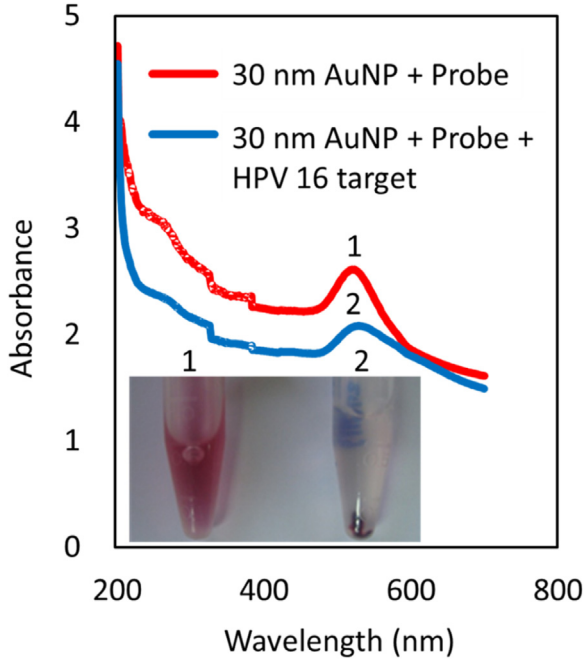

Fig. 4. UV–vis spectra of the respective samples of 30 nm AuNP with probe in red line and combination of AuNP + probe + HPV16 DNA probe in blue line. Figure inset displays the colour changes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

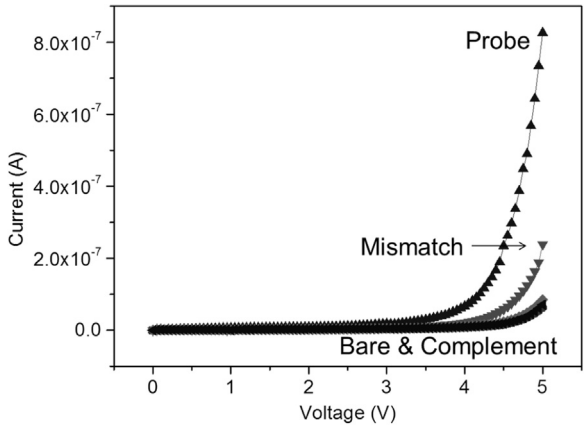

Fig. 5. Complementation on IDE sensor. After complementation with the target, the probe DNA will be removed from the AuNP and the spectrum back to the original position.

3. Results and discussions

3.1. AuNP-HPV probe characterization assay

Total DNA from virus can be purified from whole blood, plasma, serum, buffy coat, bone marrow, body fluids, lymphocytes, cultured cells, tissue, and forensic specimens [19,20]. In this study, total DNA from HPV 16 was isolated from serum plasma. AuNP-HPV oligo probe assay was optimized for visual detection using 24 mer complementary sequences.

Agarose gel showing comparison of genomic DNA of HPV extraction by using QIAamp® DNA Mini and Blood Mini Handbook [18] with PCR product from multiple sequence of HPV and single band of PCR product (Fig. 2). Negative control without template from HPV DNA genomic was used for confirmation of successful amplification by using PCR. The expected size of the genomic DNA from HPV is 7.9 kb [21].

Complementary DNA after hybridization with the probe was separated by gel electrophoresis (Fig. 3). The decrease in migration of the complex was observed with increase by hybridization of the complementary DNA in the sample (Lane 2) when compared to the migration of probe (Lane 1) and the negative control (Lane 3) which is hybridization of HPV probe with non-complementary DNA.

Hybridization was revealed by shift in band with lower electrophoretic mobility. This in fact acts as an evidence for duplex formation of the oligonucleotide to the probe. In turn the hybridization was evident from the mobility shift proportionate to the concentration of the complementary DNA.

Color change from red to purple was observed upon adding complementary DNA. Unhybridized AuNP-oligo probe has stability and tends to form dispersion even after addition of NaCl. Addition of non-complementary DNA also exhibited dispersion upon addition of NaCl. Visual change in color of the solution from red to purple was noticed with target DNA. This preliminary test infact to confirm that the AuNP-oligo probe hybridization with the target DNA present in test sample.

Hybridization of AuNP-HPV oligo probe with the HPV 16 target DNA formed double stranded DNA that destabilizes the AuNPs with NaCl and shows the purple colour (Fig. 4). However, in the absence

of complementary DNA strand, the AuNP- oligo probes does not and the solution remains red.

The absorbance of AuNP was showed at wavelength around 550 nm, and it was relying upon their absorbance [22]. UV–vis spectra of the respective samples of 30 nm AuNP with probe is shown in red line and combination of AuNP-probe and HPV16 DNA target is shown in blue line. This concept with colour changes has been described before and matches here [23,24].

The above obtained result from DNA complementation was further confirmed on IDE is shown in Fig. 5. The bare IDE substrate gave the basic spectrum and there was a drastic change upon adding the probe and it was back to the original position upon complementation. This is due to detachment of probe DNA from GNP. The control mismatch DNA sequence shows the reduction with the signal but not to the level of target DNA.

In this paper, a sensitive and fast DNA detection technique based on the single stranded oligonucleotide test was demonstrated on IDE. This AuNP-oligo test system was also shown with the identification of HPV from its genomic DNA. UV–vis spectroscopic examinations offer a proof for the development of AuNP-probe oligo test. To exhibit the importance of the technique for clinical diagnostics, extensive validation needs to be performed. With the current methods, the recognition of presence of HPV could be performed with good sensitivity and specificity within the turnaround time of 30 min. A complementation to the currently available methods for HPV detection [25].

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References

[1] W. Lian, S.A. Litherland, H. Badrane, W. Tan, D. Wu, H.V. Baker, P.A. Gulig, D.V. Lim, S. Jin, Ultrasensitive detection of biomolecules with fluorescent dye-doped nanoparticles, Anal. Biochem. 334 (2004) 135–144, http://dx.doi.org/10.1016/j.ab.2004.08.005.
[2] P.C. Chen, S.C. Mwakwari, A.K. Oyelere, Gold nanoparticles: from nanomedicine to nanosensing, Nanotechnol. Sci. Appl. 1 (2008) 45–65, http://dx.doi.org/10.2147/NSA.S3707.
[3] D. Philip, Synthesis and spectroscopic characterization of gold nanoparticles, Spectrochim. Acta Part A Mol. Biomol. Spectrosc. 71 (2008) 80–85, http://dx.doi.org/10.1016/j.saa.2007.11.012.
[4] Z. Liang, J. Zhang, L. Wang, S. Song, C. Fan, G. Li, A centrifugation-based method for preparation of gold nanoparticles and its application in biodetection, Int. J. Mol. Sci. 8 (2007) 526–532, http://dx.doi.org/10.3390/i8060526.
[5] F.T. Cutts, S. Franceschi, S. Goldie, X. Castellsague, S. De Sanjose, G. Garnett, W.J. Edmunds, P. Claeys, K.L. Goldenthal, D.M. Harperi, L. Markowitz, Human papillomavirus and HPV vaccines: a review, Bull. World Health Organ. 85 (2007) 719–726, http://dx.doi.org/10.2471/BLT.06.038414.
[6] J. Doorbar, Molecular biology of human papillomavirus infection and cervical cancer, Clin. Sci. (Lond) 110 (2006) 525–541, http://dx.doi.org/10.1042/CS20050369.
[7] K. Kawana, K. Adachi, S. Kojima, S. Kozuma, T. Fujii, Therapeutic human papillomavirus (HPV) vaccines: a novel approach, Open Virol. J. 6 (2012) 264–269, http://dx.doi.org/10.2174/1874357901206010264.
[8] A.R. Cirilli, S.J. Cipot, Emergency evaluation and management of vaginal bleeding in the nonpregnant patient, Emerg. Med. Clin. North Am. 30 (2012) 991–1006, http://dx.doi.org/10.1016/j.emc.2012.08.010.
[9] J. Su, B.J. Yoon, E.R. Dougherty, Accurate and reliable cancer classification based on probabilistic inference of pathway activity, PLoS One 4 (2009) e8161, http://dx.doi.org/10.1371/journal.pone.0008161.
[10] D.J. Marais, C.C. Sampson, M.I. Urban, F. Sitas, A.L. Wiliamson, The seroprevalence of IgG antibodies to human papillomavirus (HPV) types HPV-16, HPV-18, and HPV-11 capsid-antigens in mothers and their children, J. Med. Virol. 79 (2007) 1370–1374, http://dx.doi.org/10.1002/jmv.20874.
[11] A.A.T.P. Brink, P.J.F. Snijders, C.J.L.M. Meijer, HPV detection methods, Dis. Markers 23 (2007) 273–281.
[12] K. Xu, J. Huang, Z. Ye, Y. Ying, Y. Li, Recent development of nano-materials used in DNA biosensors, Sensors 9 (2009) 5534–5557, http://dx.doi.org/10.3390/s90705534.
[13] M.E. Ali, U. Hashim, S. Mustafa, Y.B. Che Man, M.H.M. Yusop, M. Kashif, T.S. Dhahi, M.F. Bari, M.A. Hakim, M.A. Latif, Nanobiosensor for detection and quantification of DNA sequences in degraded mixed meats, J. Nanomater. 2011 (2011), http://dx.doi.org/10.1155/2011/781098.
[14] N. Ganguly, S.P. Parihar, Human papillomavirus E6 and E7 oncoproteins as risk factors for tumorigenesis, J. Biosci. 34 (2009) 113–123, http://dx.doi.org/10.1007/s12038-009-0013-7.
[15] N. Azizah, U. Hashim, S.C.B. Gopinath, S. Nadzirah, Gold nanoparticle mediated method for spatially resolved deposition of DNA on nano-gapped interdigitated electrodes, and its application to the detection of the human Papillomavirus, (2016). 10.1007/s00604-016-1954-9.
[16] S. Nadzirah, S. Ahmad, U. Hashim, Titanium dioxide nanowires-Based interdigitated electrodes for biomedical application, IEEE-EMBS Conf. Biomed. Eng. Sci. (2012) 126–128.
[17] T. Adam, U. Hashim, T.S. Sutikno, T.Nazwaa Dhahi, Material engineering for nano structure formation: fabrication and characterization, Procedia Eng. (2012) 361–368, http://dx.doi.org/10.1016/j.proeng.2012.10.041.
[18] A. Technologies, June 2012 Blood Mini Handbook Sample & Assay Technologies QIAGEN Sample and Assay Technologies, (2012).
[19] Y.Y.N. Lui, K.W. Chik, R.W.K. Chiu, C.Y. Ho, C.W.K. Lam, Y.M.D. Lo, Predominant hematopoietic origin of cell-free dna in plasma and serum after sex-mismatched bone marrow transplantation, Clin. Chem. 48 (2002) 421–427.
[20] Qiagen, QIAamp DNA Mini Kit – QIAGEN, Qiagene Protoc. (2012) 72.
[21] J.C. Prado, I.E. Calleja-Macias, H.U. Bernard, M. Kalantari, S.A. Macay, B. Allan, A.L. Williamson, L.P. Chung, R.J. Collins, R.E. Zuna, S.T. Dunn, R. Ortiz-Lopez, H.A. Barrera-Saldaña, H.A. Cubie, K. Cuschieri, M. Von Knebel-Doeberitz, G.I. Sanchez, F.X. Bosch, L.L. Villa, Worldwide genomic diversity of the human papillomaviruses-53, 56, and 66, a group of high-risk HPVs unrelated to HPV-16 and HPV-18, Virology 340 (2005) 95–104, http://dx.doi.org/10.1016/j.virol.2005.06.024.
[22] M. Apte, G. Girme, A. Bankar, A. Ravikumar, S. Zinjarde, 3, 4-dihydroxy-L-phenylalanine-derived melanin from Yarrowia lipolytica mediates the synthesis of silver and gold nanostructures, J. Nanobiotechnol. 11 (2013) 2, http://dx.doi.org/10.1186/1477-3155-11-2.
[23] N.Z. Jin, S. Anniebell, S.C.B. Gopinath, Y. Chen, Variations in spontaneous assembly and disassembly of molecules on unmodified gold nanoparticle, Nanoscale Res. Lett. 11 (2016) 399.
[24] S.C.B. Gopinath, T. Lakshmipriya, K. Awazu, Colorimetric detection of controlled assembly and disassembly of aptamers on unmodified gold nanoparticles, Biosens. Bioelectron. 51 (2014) 115–123.
[25] J.P. Kerishnan, S.C.B. Gopinath, S.B. Kai, T.H. Tang, H.L. Ng, Z.A.A. Rahman, U. Hashim, Y. Chen, Detection of human papillomavirus 16-Specific IgG and IgM antibodies in patient sera: A potential indicator of oral squamous cell carcinoma risk factor, Int. J. Med. Sci. (2016) 13.