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Designing DNA probe from HPV 18 and 58 in the E6 region for sensing element in the development of genosensor-based gold nanoparticles

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Abstract

The E6 region has higher protuberant probability annealing than consensus probe focusing on another region in the human *papillomavirus* (HPV) genome in terms of detection and screening method. Here, we designed the first multiple virus single-stranded deoxyribonucleic acid (ssDNA) for multiple detections in an early phase of screening for cervical cancer in the E6 region and became a fundamental evolution of detection electrochemical HPV biosensor. *Gene* profiling of the virus ssDNA sequences has been carried by high-end bioinformatics tools such as GenBank, Basic Local Alignment Searching Tools (BLAST), and Clustal OMEGA in a row. The output from bioinformatics tools resulted in 100% of similarities between our virus ssDNA probe and HPV complete genome in the databases. The cross-validation between HPV genome and our designed virus ssDNA provided high specificity and selectivity during screening methods compared with Pap smear. The DNA probe for HPV 18, 5′ COOH-GAT CCA GAA GGT ACA GAC GGG GAG GGC ACG 3′, while 5′COOH-GGG CGC TGT GCA GTG TGT TGG AGA CCC CGA3′ as DNA probe for HPV 58 designed with 66.77% guanine (G) and cytosine (C) content for both. Our virus ssDNA probe for the HPV biosensor promises high sensitivity, specificity, selectivity, repeatability, low fluid consumption, and will be useful in mini-size diagnostic devices for cervical cancer detection.

Abbreviations: APTES, 3-aminopropyltriethoxysilane; AuMNPs, gold-coated nanoparticles-coated superparamagnetic nanomaghemite; AuNp, gold-nanoparticles; BLAST, Basic Local Alignment searching Tools; CdTe, Cadmium Telluride; CQD, carbon quantum dots; CRISPR, clustered regularly interspaced short palindromic; E6, early 6 region; *EXO III*, exonuclease III; HPV, human papillomavirus; HR-HPV, high-risk human papillomavirus; HWR, heptapeptide; IDEs, interdigitated electrodes; L1, late 1 region; MWCNTs, multiwalled carbon nanotubes; PANI, positively charge polyaniline; rGO, reduced graphene oxide; SPCE, screen-printed carbon electrode; ssDNA, single-stranded deoxyribonucleic acid; Ti3C2 NSs, two-dimensional MXene Ti3C2 nanosheets.

KEYWORDS
BLAST, Clustal OMEGA, DNA probe, GenBank, Human *Papillomavirus* biosensor, human papillomavirus

1 | INTRODUCTION

Globally, the second leading cause of death for women is cervical cancer caused by human papillomavirus (HPV) infection. HPV strains 16 (50.8%), 18 (17.6%), and 58 (2.6%) became the most leading strains of infection in Malaysia.[1] Recently, a study showed that HPV 58 was rare worldwide but common in Asian countries, including Malaysia. However, the significant detection of HPV-58 in women has not been studied extensively because of the rare case compared to HPV 16 and 18.[2] Sexual interaction between humans came from the oncogenic type of HPV infection initiated cervical cancer. Cervical cancer among married women can be reduced by practicing better cleanliness before, during, and after sexual activities.[3] The main cause of the disease was sexually transmitted infection, as 71% of the total number reported in the United States.[4] The existence of cervical cancer is also because of skin-to-skin contact, cut, and abrasion between humans. Contagious warts spread on the skin affected the immune system of the human body. Most human HR-HPV infections are cleared by the immune system within 1–2 years and are asymptomatic. However, persistent infection with HR-HPV can induce cellular and histological changes leading to cancer.

The screening of cervical cancer at an early stage dominates by a conventional method such as the Pap smear test and colposcopy test for several years. However, due to its limitation, such as false-positive/negative cancer, no specific type of virus was detected. In addition, it requires further testing, insensitive, and more extended time detection. Hence, the conventional method seems unreliable to detect the virus of cervical cancer at an early stage. During the detection, the traditional approach only focuses on the malignancies of the cell. It cannot diagnose the cancerous cell early since HPV cannot be distinguished in cell culture. Other than that, molecular techniques were used to interpret cervical cancer, such as polymerase chain reaction (PCR) and hybrid (II) capture assay. These molecular techniques were time-consuming, required an expert to diagnose the information, and had low sensitivity. In addition, the Cobas HPV test is also one of the biosensing devices to detect the virus of cervical cancer. However, the device has a high probability of producing false-positive results and lower specificity. Most of the current biosensing devices have limitations such as produce cross detection, subjective interpretation, and prevent a further outbreak by early detection (Table 1). Therefore, this diagnosis does not typically replace the direct detection methods as the primary and early tools for screening virus DNA for cervical cancer.

The virus of DNA is likely to be chosen as a biorecognition element in HPV biosensors for early detection of cervical cancer. The advantages of virus DNA are highly optimal coverage density and antifouling properties that prevent the nonspecific binding of nonrelevant molecules.[5] Compared with enzymes and antibodies, virus DNA is gaining popularity as it gets sequence-specific information in faster, easier, and cheaper way. Virus DNA may also be quickly produced and regenerated for various applications.[6] By using virus DNA as a biomarker, the genetic information can be stored significantly compared with RNA. The higher hydroxyl group built in virus DNA resulted in higher stability during the hybridization process.[7,8] Our probe has a higher binding affinity when immobilized on the functionalized surface electrode, especially on AuNPs. Besides AuNPs, carbon quantum dots (CDs) can also be a conductivity enhancer linked with great chemical linkers such as 3-aminopropyl triethoxysilane (APTES) due to their abundant hydroxyl group.[9] The reduced oxide layer on graphene is also vital with linker attachment due to their oxide layer availability.[10] As far as we are concerned, virus DNA is very suitable as a biomarker as it can directly detect the presence of HPV and identify multitudinous types of high-risk HPV.

Virus DNAs from HPV 18 are known to be predominantly caused cervical cancer compared with other strains. The virus DNA from HPV 18 accounts for about 59% and 13% of squamous cell carcinoma and 36% and 37% of adenosquamous carcinoma worldwide.[11] Meanwhile, in Shanghai and Taiwan, 28% and 10% reported high cervical cancer spreading due to virus DNA HPV-58, respectively, while Korea and Japan reported up to 16% and 8%.[11,12] Globally, HPV-58 only contributed3.3 % to cervical cancer but was ranked third pretty much across the board in Asia.[13] Moreover, in Hong Kong, virus DNA from HPV 58 confirmed a 6.9-fold higher risk of developing cervical cancer than other variants on the E6 region.[14] As far as our concern, the virus DNA from HPV 58 is also dominantly can cause mutation on squamous cell carcinoma at an early stage of cervical cancer similar to virus DNA HPV 18.

HPV can be interpreted as a human genome consisting of early (E1–E7) and late (L1–L2) gene expressions

TABLE 1 Comparisons of biosensing devices with limitations and advantages from the previous study

HPV-screening devices	Manufacturing year	Detection technique	Response limit	Sensitivity/ selectivity(%)	Limitation	Advantages	Reference.
Hybrid capture II (instrument)	2004 (Maryland, USA)	Direct genome (Chemiluminescence)	5 h	96.6/89.1	Cannot identify the type of HPV significantly	High sensitivity	63
*Care*HPV (instrument)	2009 (Germany)	Direct genome Chemiluminescence)	3 h	–	Higher chances to cross detection	-	64
Cobas HPV test (instrument)	2014 (Switzerland)	Amplification and genotyping	5 h	91/95	Produced false positive results	Clinically validated in vivo test	65
Abbott real-time high risk (HR) HPV assay (instrument)	2018 (USA)	Amplification with rt-PCR	8 h	78/90	Subjective interpretation	Automated in-vitro test	66
HPVmultidetection genosensor (self-test kit)	2021	SAM	30 min	99/100	NIL	Portable self -test kit Rapid, highly selective and sensitive genosensor Multidetection in a short time	1,67,68

Abbreviations; rt-PCR, reverse-transcriptase-polymerase chain reaction; SAM, self-assembled monolayer.

(Figure 1).[15] Referring to Figure 1, the long control region (LCR) helps in regulating viral DNA transcription and replication in black and green indicators for L1 and L2. Three HPV oncogenes (E5–E7) are indicated by the purple, dark blue, and red color, while E1 and E2 are shown in orange and pastel blue, respectively. The virus's life cycle proceeded throughout the epithelium and is directly connected to differentiation, ending with the production and release of the progeny of virus from the terminally differentiated cells.[15] The most exciting feature is that transcripts of E6 and E7 can be recognized in cells. The combination sites indicate the open reading frame (ORF) of E1, E2, E4, and E5 were adversely affected by flanking the host cell DNA.[16] For the entire E6 and E7 ORF and a minor part of the E1 ORF, HPV mRNAs present hybridized probes, implying that these were the central portion of the HPV genome present.[17,18]

Most aggressive cervical cancer develops by the viral oncogenes in the E6 and E7 region of the HR-HPV genome.[19] The viral sequences are integrated into the host chromosome after the squamous cells are invaded by high-risk HPV, which inhibits the E2 ORF and contributes to the expression of E6/E7 in the cells.[20,21] The viral proteins in the E6 region bind to the ligase E6AP and forms a complex that diminishes p53 protein—thereby the function is deactivated. The cell becomes unstable and has uncontrollable growth. RB was produced after the tumor suppressor binds with viral proteins in the E7 region, this discrete RB protein through ubiquitination. Retinoblastoma (Rb) protein is a tumor suppressor, which plays a pivotal role in the negative control of the cell cycle and in tumor progression. Since HPV strains consist of almost 8000 bp, it is highly recommended that only 30-mer oligonucleotides have to be used.[22] The effectiveness of virus single-stranded deoxyribonucleic acid (ssDNA) probes in the sensing implementations with a nanosensor has higher sensitivity and specificity. They require only a medium probe length with faster hybridization and immobilization kinetic strength compared with ionic strength. Matching the sequence database has consistently proven to be a helpful way to discover the function of an elusive gene whose sequence is accessible in the research facility.[23]

Tools for bioinformatics have significant relevance and are used in various fields of life sciences.[24] A number of tools are available on multiple constructive sequences of DNA probes for biosensing application. Many tools are available to carry out primary DNA and protein analysis, and most of them are compatible with various platforms, and many remain undetected.[25] However, the different bioinformatics tools preserved a complicated sequence of uses; thus, the designation of biomarker could not be handled more efficiently. Hence, we constructed the identification of virus ssDNA probe started with analysis with

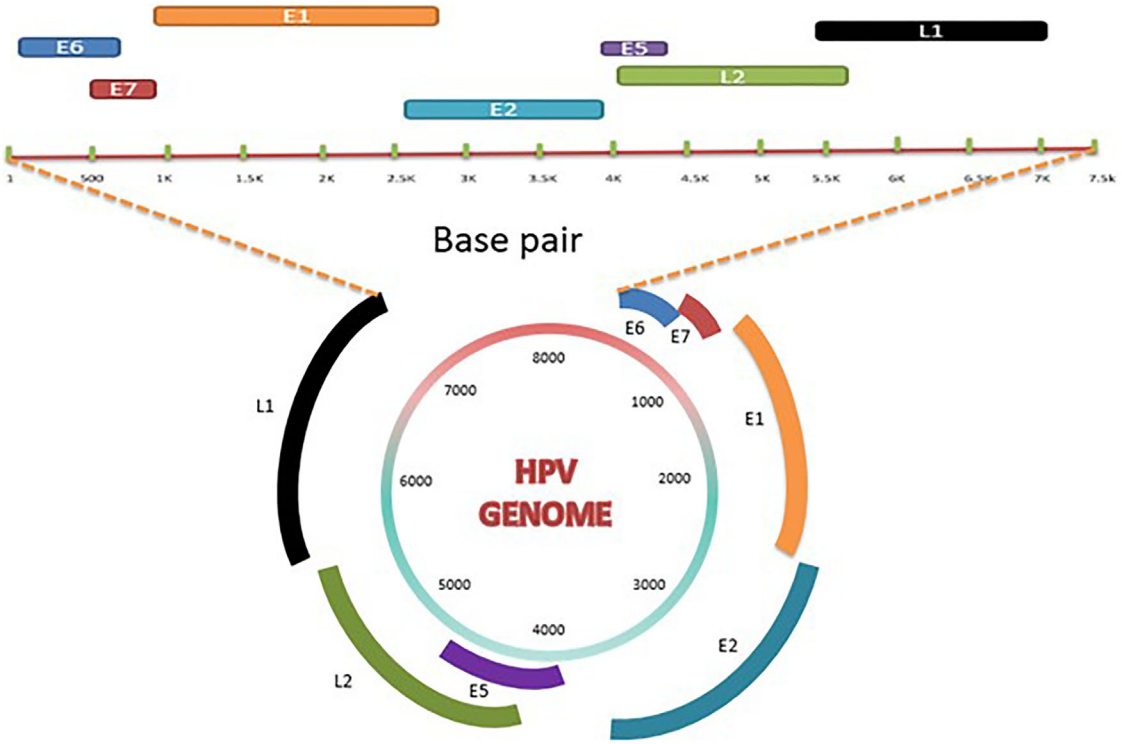

FIGURE 1 The map of high-risk HPV genome shown in the circular and linear form stranded ssDNA: (E1–E7) Early gene, participate in the virus DNA reproduction, transcription, translation regulation, and transformation from the three functional areas in the HPV genome; (L1–L2) Late encoded region which prominent to virus infection

GenBank, finding similarities by Basic Local Alignment Searching Tools (BLAST), and differentiating virus ssDNA with the database by Clustal OMEGA.

Tools for bioinformatics have been of significant relevance and were used in various fields of life sciences.[24] There were vast wide opportunities on multiple constructive sequences of DNA probes for biosensing application. Many tools were available to carry out primary DNA and protein analysis, and most of them were compatible with various platforms, and many remain undetected.[25] However, the different bioinformatics tools preserved a complicated sequence of uses; thus, the designation of biomarker could not be handled more efficiently. Hence, we constructed the identification of virus ssDNA probe started with analysis with GenBank, similarities finding by BLAST, and differentiate virus ssDNA with the database by Clustal OMEGA.

In bioinformatics, position of DNA, RNA, and protein sequence is made to determine the structural, functional, or epigenetic relationship. Biomarkers were dependent on predicting the structure or composition interaction of such biomolecules for drug discovery, cancer treatment, cancer classification, and so on. Thus, the bioinformatics tools were very beneficial for such new and emerging research studies as an accessory or alternative tool. Furthermore, experimental technologies include time and cost implications, so that bioinformatics tools as an alternative methodology complement biologists in their research studies to predict protein structures and their functions.[25]

The short length of virus ssDNA-designed results in higher stability during the hybridization process. The hybridization process is responsible for detecting the target sequences of the virus caused by HPV 18 and 58. The length of the virus ssDNA nucleotide sequence applied in this study was 30-mer the only specificity usually is dependent on the length and annealing temperature. The shorter the virus ssDNA, the more efficiently they will bind or anneal to the target.[26–28] The percentage of GC content in each virus the ssDNA sequence was recorded up to 67.77%. GC contains a number of guanine (G) and cytosine (C) codons in a nucleotide. The effectiveness of a higher percentage of GC content is proved as it consists of three hydrogen bonds that create higher stability binding affinity than thymine (T) and adenosine (A) base pair. The advantage of using virus ssDNA as sensing molecules rather than other molecules is that DNA can be reused and is highly stable, which helps in keeping genetic information safe.[29]

The challenge for developing a biosensor device depends on the credibility of the biomarker and target DNA and the detection activities with the help of a transducer for

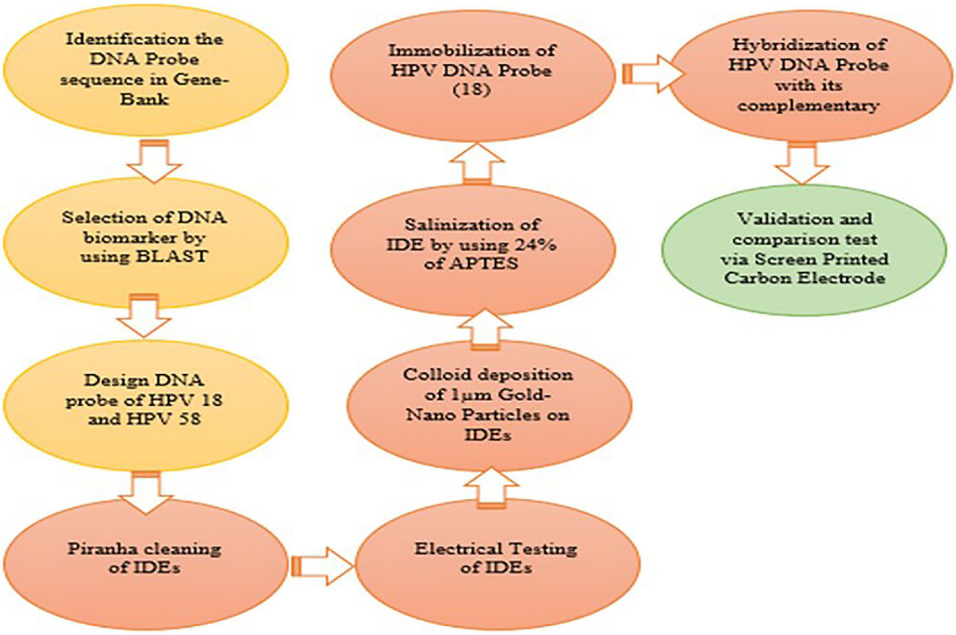

FIGURE 2 Research overview in development of high selectivity and sensitivity nanoparticles-based biosensor for early detection of cervical cancer

electrical measurement. Figure 2 illustrates the flow of the research starting from designing a DNA probe of HPV up to application in a biosensor. In this study, the designation of virus ssDNA from HPV 18 and 58 maximizes its efficiency as a biorecognition element. Along with target sequences of virus ssDNA, the hybridization process helps in increasing the sensitivity and specificity of advanced biosensors. The lab-on-chip developed a combination of DNA extraction, biosensor, and portable voltammetry measurement. In the current study, most of the biosensing devices detect only one type of high-risk HPV toward virus ssDNA. The detection also focused on the L1 region that resulted in more disadvantages compared with the E6 region. To date, no research describes multidetection of various types of high-risk HPV in the E6 region. Therefore, this paper elucidates the first design of multiple virus ssDNA as a probe in the E6 region for screening of HPV 18 and 58 at an early stage of cervical cancer.

2 | MATERIALS AND METHODS

2.1 | Analysis of HPV sequences from high-risk HPVs such as HPV 18 and 58 via GenBank

The GenBank was used to obtain the information of 7000 base pair and 150 HPV types worldwide. Encyclopedic databases are attainable over the interlacing and can be openly obtained (http://www.ncbi.nlm.nih.gov). The similitude between the DNA probes with the databases must be within 80–100% to maintain the quality of the sequences. The alignment of new sequences with previously established genes or proteins contains essential information on general and phylogenetic properties.[30] Efficient DNA sequencing methods consider making the amino acid sequence of proteins accessible more conveniently than some of its structures or functions.[31]

2.2 | Identification of viral DNA as a probe for HPV 18 and 58 via BLAST

A grouping database via homology search cross-examined using the BLAST-programming on HPV 18 and HPV 58 oligonucleotide reference sequences. Primer-BLAST programming facilitates fast arrangement finding in a constantly updated coding sequence. In addition, various BLAST programs have been used by researchers to construct lines of DNA probe. When developers submit a query, either as a FASTA sequence or as a sequence identification identifier, it sends a search to the BLAST operating system. The question and results will be stored up to 24 h after the RID is issued in a structured format. The RID identifies the query and allows for multiple settings, including the familiar report BLAST, a simplified "hittable," XML, and ASN1.[32] The BLAST webpage promotes an ideal parameter setting, providing several links,

or specific purposes. When the primary goal is to identify the sequence or the comparison of an intraorganism, the best way is to search quickly and substantially by using the BLAST database program.

The goal of BLAST was to provide users a quality control method, which guarantees accurate results, superior runtime posts, and authenticates many types of the analysis process while remaining convenient and straightforward to use.[33] For instance, in DNA comparisons, there is noise in each codon from the rapidly mutated third-base position and comparisons of noncoding frames.[34] Consequently, amino acids have chemical characteristics that allow the analysis of degrees of similarity rather than simple identity or nonidentity recognition.

2.3 | Differentiation virus ssDNA for HPV 18 and 58 via Clustal OMEGA

Clustal represents a set of several homology-modeling computer algorithms used in biotechnology and informatics software. Clustal serves as an assistant for phylogenetic analysis. In their respective markets, the study of each method and its code are also comprehensive.[35] Clustal OMEGA has the widest variety of operating systems out of all the Clustal tools. Multiple sequence alignment via Clustal OMEGA conducted for various alignment sequences of the HPV 18 and 58 amino acids in the E6 viral protein among HPV genotypes. The method's purpose was to give factual parameters such as similarity and identity of DNA probe with other HPV 18 and 58 sequences in the database. Using multiple sequence alignment can validate the location of ssDNA probes in HPV 18 and 58 synthetically.[36] The most critical part of this method was the region of viral protein that exists, such as the E6 region. The different areas also gave additional factual parameters.

2.4 | Predicted virus ssDNA as a target for the development of biosensors

The prediction on virus ssDNA target sequences obtained from GenBank (https://www.ncbi.nlm.nih.gov/genbank/sequenceids/). The target sequence must be in similar length with the virus ssDNA of the probe designed. The similarities of 30-mer oligonucleotides with the databases were acknowledged and selected based on the content of GC codons. Along with the virus ssDNA probe, target sequences for ssDNA were also profiled and designed in the E6 region to maximize the capability during the hybridization process.

3 | RESULTS AND DISCUSSION

3.1 | The importance of bioinformatics tools for analyzing HPV sequences

Computer science based instruments have opened up unique information regarding the depiction and defense of biomolecular genomes within individual genomic DNA. The current research aims to conduct a comprehensive analysis of E6 protein parts among all the high probability and reliability of E6 region compared with other region on HPV genome. Bioinformatics tools are potent tools developed to perform primary analysis of DNA. A DNA probe is the essential material applied in the research as it is used for detecting the high-risk HPV genome (18 and 58). The length of nucleic acids obtained before designing any virus ssDNA sequences, making the hybridization process more reliable. The length of virus ssDNA must be in 24–35 base pairs for their effectiveness in detecting the target virus ssDNA.[6,37,38]

3.2 | Identification virus ssDNA for HPV 18 and 58 by using GenBank

The DNA sequence for HPV 18 and 58 has been identified using the GenBank database assigned to accession number: (A) LC509004.1, (B) MH348928.1 known as HPV type 18 K1872 DNA, complete genome, and HPV type 58 isolate 58CNTZ11 E6 protein (E6) and E7 protein (E7) genes, complete CDs. The ssDNA sequence is illustrated in detail in Figure 3. GenBank database proved that the highlighted DNA sequences were identified and analyzed from the genome of HPV 18 and HPV 58. Both virus ssDNAs were identified with 100% matched with ssDNA in the E6 region. Hence, as far as our concern, both virus ssDNAs for each HPV genome effectively discover their similarities in BLAST and differentiate it by using Clustal OMEGA.

3.3 | Genotyping virus ssDNA by using BLAST

Multiple configurations are essential for recognizing critical residues, predicting conformational changes, and scrutinizing evolution.[39] DNA biomarkers have been selected from the gene profiling and involved two strains of HPV that cause cervical cancer HPV 18 (LC509006.1) and HPV 58 (MH348928.1). BLAST has been applied in the maximal way to identify, summarize, and construct the sequence of DNA probe from HPV 18 (Table 2) and HPV 58 (Table 3) complete genome, respectively. The virus ssDNA

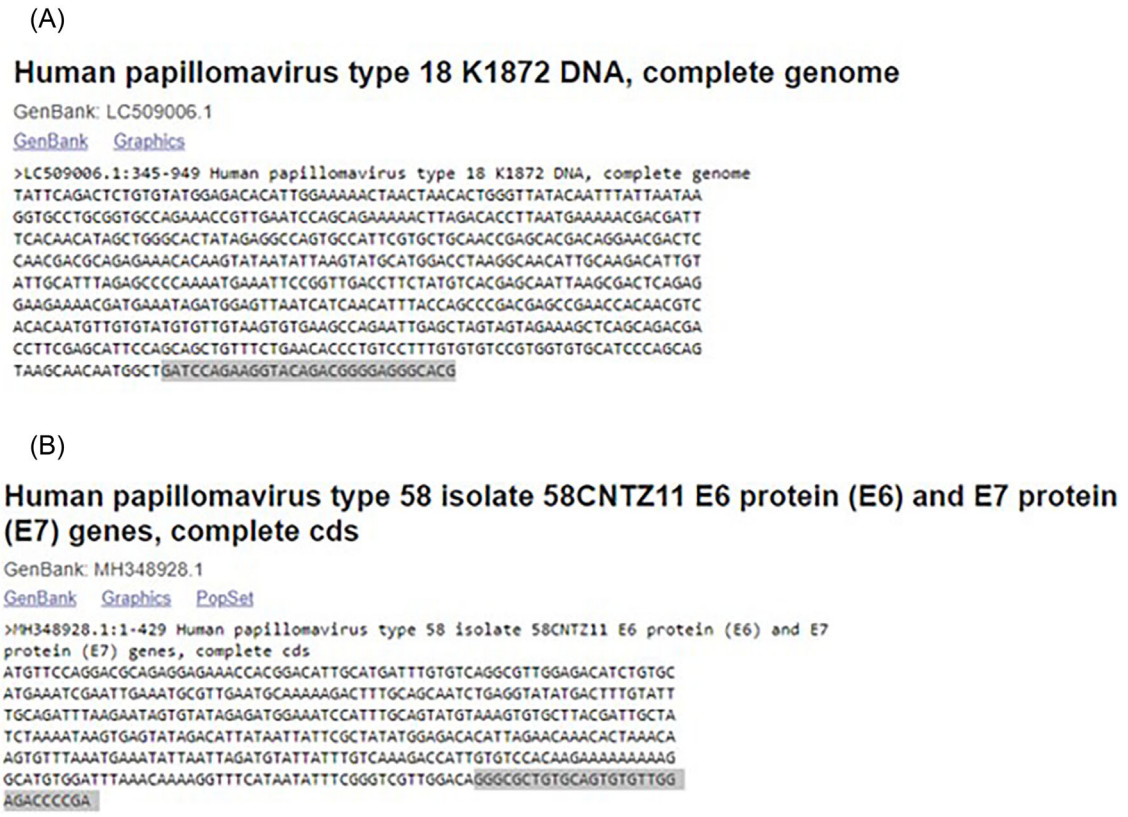

FIGURE 3 Highlights of virus ssDNA analyzed in GenBank databases. (A) HPV 18; (B) HPV 58

TABLE 2 Basic Local Alignment Sequence Tools (BLAST) results for identifying viral ssDNA in HPV 18

Description	Common name	Max score	Total Score	Query cover	E-Value	Per identity	Accession
Human papillomavirus type 18 K1872 DNA, complete genome	HPV	60.0	60.0	100%	3e-06	100%	LC509006.1
Human papillomavirus type 18 K0619 DNA, complete genome	HPV	60.0	60.0	100%	3e-06	100%	LC509005.1
Human papillomavirus type 18 K2087 DNA, complete genome	HPV	60.0	60.0	100%	3e-06	100%	LC509004.1
Human papillomavirus type 18 K1835 DNA, complete genome	HPV	60.0	60.0	100%	3e-06	100%	LC509003.1
Human papillomavirus type 18 TK032 DNA, complete genome	HPV	60.0	60.0	100%	3e-06	100%	LC509001.1
Human papillomavirus type 18 K1929 DNA, complete genome	HPV	60.0	60.0	100%	3e-06	100%	LC509009.1
Human papillomavirus type 18 K1506 DNA, complete genome	HPV	60.0	60.0	100%	3e-06	100%	LC509008.1
Human papillomavirus type 18 K1456 DNA, complete genome	HPV	60.0	60.0	100%	3e-06	100%	LC509007.1

identified by BLAST for HPV 18 and 58 resulted in 100% similarities with the whole genome in the databases. The E-value obtained indicates homologous identity with the database. For both HPV types, 3e-06 is the expected value (E), which was recorded with high confidence that the virus ssDNA results from a homologous relationship. BLAST shows the classification of nucleic acids requests from libraries, with genes encoding also being translated reasonably before any techniques and procedures are conducted.

TABLE 3 BLAST results for the designing of viral ssDNA in HPV 58

Description	Common name	Maximum score	Total score	Query cover	E-Value	Per identity	Acc Len	Accession
Human papillomavirus type 58 isolate 58CNTZ25 E6 protein (E6) and E7 protein (E7) genes, complete cds	HPV	60.0	60.0	100%	3e-06	100%	761	MH348942.1
Human papillomavirus type 58 isolate 58CNTZ24 E6 protein (E6) and E7 protein (E7) genes, complete cds	HPV	60.0	60.0	100%	3e-06	100%	761	MH348941.1
Human papillomavirus type 58 isolate 58CNTZ20 E6 protein (E6) and E7 protein (E7) genes, complete cds	HPV	60.0	60.0	100%	3e-06	100%	761	MH348937.1
Human papillomavirus type 58 isolate 58CNTZ14 E6 protein (E6) and E7 protein (E7) genes, complete cds	HPV	60.0	60.0	100%	3e-06	100%	761	MH348931.1
Human papillomavirus type 58 isolate 58CNTZ12 E6 protein (E6) and E7 protein (E7) genes, complete cds	HPV	60.0	60.0	100%	3e-06	100%	761	MH348929.1
Human papillomavirus type 58 isolate 58CNTZ11 E6 protein (E6) and E7 protein (E7) genes, complete cds	HPV	60.0	60.0	100%	3e-06	100%	761	MH348928.1
Human papillomavirus type 58 isolate 58CNTZ06 E6 protein (E6) and E7 protein (E7) genes, complete cds	HPV	60.0	60.0	100%	3e-06	100%	761	MH348923.1
Human papillomavirus type 58 isolate 58CNTZ03 E6 protein (E6) and E7 protein (E7) genes, complete cds	HPV	60.0	60.0	100%	3e-06	100%	761	MH348920.1

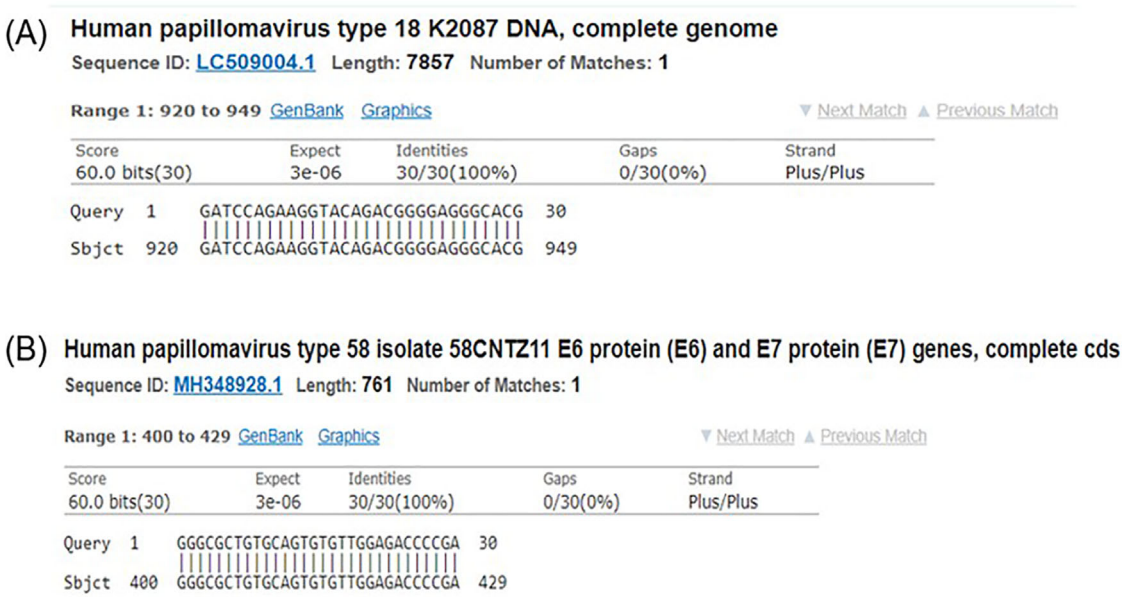

FIGURE 4 Position of DNA probe in GenBank databases. (A) HPV 18; (B) HPV 58

Users can apply BLAST software to show the location and relation on the databases. The allocated number indicates the location of the DNA probe in the complete genome. That is why the similarities between DNA probe sequences and the whole genome databases reach 100%. Figure 4 shows the position of a 30-mer oligonucleotide of DNA probes detected with a complete genome of HPV 18 (A) and 58 (B) on 920th until 949th and 420th until 429th,

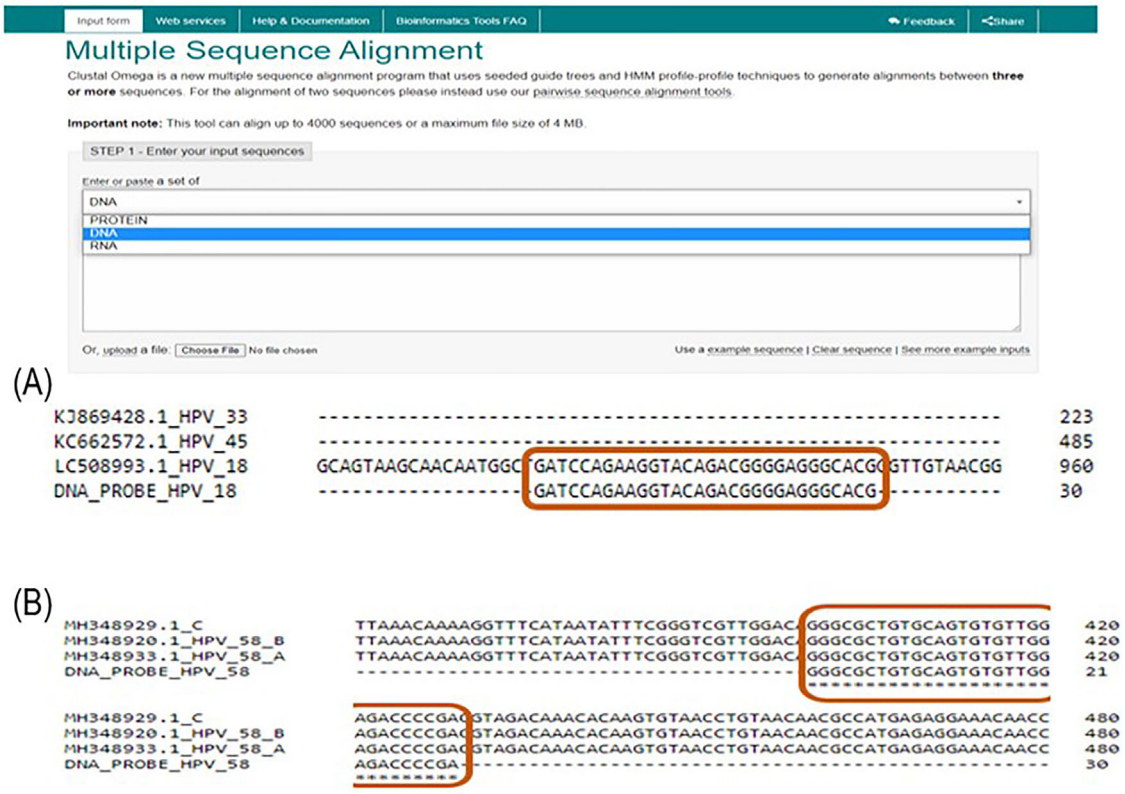

FIGURE 5 DNA selection via CLUSTAL OMEGA for multiple sequence alignment. (A) Result of multiple sequences for DNA probe HPV 18 with complete genome of HPV 18, 33, and 45 for specificity and selectivity; (B) Result of multiple sequence alignment for DNA probe HPV 58 with complete genomes of HPV 58

respectively. As a result, the similarities between the DNA probes are up to 100% as it is perfectly matched.

This research aimed to analyze the importance and use of the residue of samples in the E6 genome region of the HPV 18 and HPV 58 for sensing employing bioinformatics analysis. BLAST was meant to separate proteins or DNA queries from libraries, with genomic DNA mostly adequately translated before any experimental setup. The E-value close to zero suggests the alignment of the amino acid deposit reserves of the E6 protein in the high-risk HPV and reveals complementary sequences in the findings of this analysis and the results of the short sequences of the related E6 protein.

3.4 | Multiple sequence alignment by using Clustal OMEGA

The first step of Clustal OMEGA started with selecting input either protein, ssDNA, or RNA. For sure, ssDNA has been chosen; it must be compared with ssDNA of HPV 18 and 58 that existed in the database. By using Clustal OMEGA, the comparison between each HPV strain was obtained.[40] A sequence ssDNA probe designed in HPV 18 compared with HPV 33,45 and 18 is shown in Figure 5A, meanwhile the same type of HPV genome complete sequences can be applied onto the designed DNA probe as shown in Figure 5B for HPV 58.[41] The purpose is to highlight the similarities between the designated DNA probe of HPV 58 with the preserved DNA sequences in the database. In addition, columns that indicate identical amino acids in the same position in all types were analyzed and marked with an asterisk (*), which showed fully conserved residues. Symbol (−) showed no consensus of the groups.[42]

Multiple sequence alignment in Clustal OMEGA also preserved many features. One of the informative features is the phylogenetic tree for the designed DNA probe of HPV 18 and 58 with the different and same type of HPV genomes. A Phylogenetic diagram shown an evolutionary connection between the organisms that are connected to the same species.[43] The evolutionary connection is the connection between family called *Papillomaviridae* with its species called 'types'. In a phylogenetic tree, the branching sequence represents how organisms or other groups evolved from a set of similar ancestors.[44] If two or more related trees have a more recent common ancestor and a less recent common ancestor, they are less connected.[45]

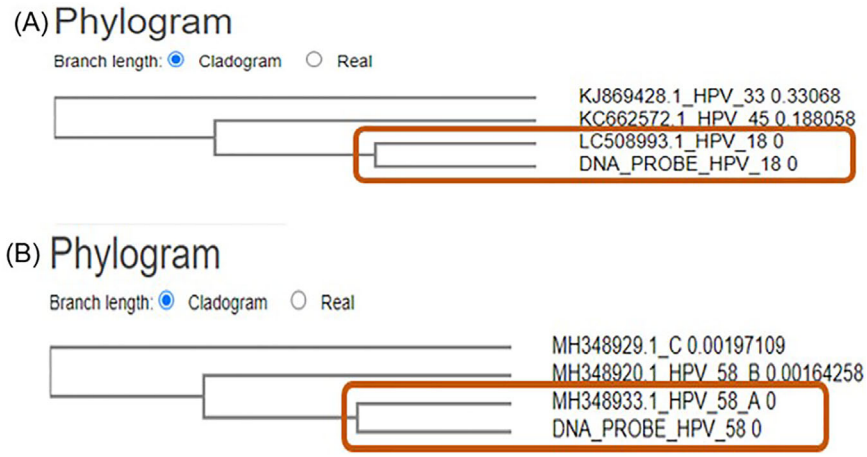

FIGURE 6 Phylogram of DNA probe. (A) HPV 18; (B) HPV 58

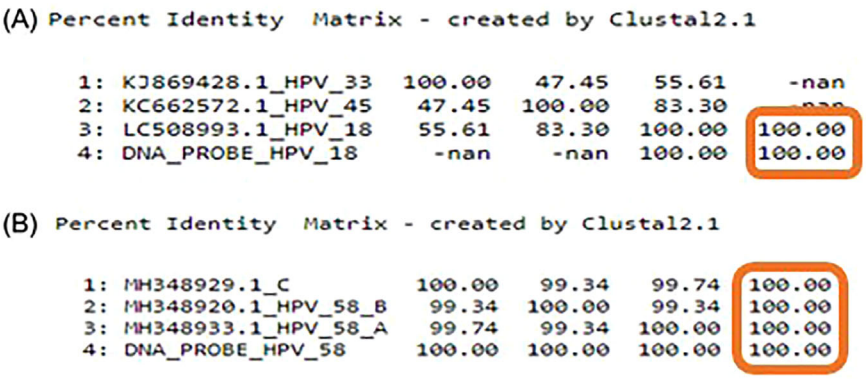

FIGURE 7 Percentage matrix of DNA probe. (A) HPV 18 with HR-HPV (33 and 45); (B) HPV 58 with similar type of complete HPV genome (HPV 58)

It is possible to draw phylogenetic trees of different equivalent types. Rotating a tree does not modify the information it holds regarding its branch points. Hence, if the branch point connecting to the branches means there were connected.[46] The phylogenetic diagrams of HPV 18 (A) and 58 (B) are shown in Figure 6, respectively. In Figure 6, HPV 18 and 58 branches relate to "zero" values. The interpretation of how the tree's organisms developed from a set of similar ancestors reflects the sequence in which the branches bind. Each branch point (also referred to as an internal node) represents a divergence event or divided into two descendant groups from a single group.[47]

Multiple types of high-risk HPV can cause cervical cancer or not due to its phylogenetic classification.[46] A phylogeny classification is evolution of a group related to their ancestor. A phylogenetic classification is related to the high and low risks of HPV type described to two kinds of ancestors for HPV genotyping. Meanwhile, an epidemiologic classification refers to the number of HPV types caused by the phylogenetic classification. HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, and 82 are listed as high risk, while 6, 11, 40, 42, 43, 44, 54, 61, 72, and 81 are defined as low risk. High-risk types of HPV have a high probability of causing cervical cancer and vice versa. Hence, a phylogenetic and epidemiologic classification analysis can also be applied to the identification of HPV genotyping.

Finally, the percentage matrix of the virus ssDNA can also be identified by using multiple sequence alignment. The percentage matrix is related to the capacity and rigidity of designed DNA probes with the existing DNA database for the same HPV genome. Figure 7 illustrates the percentage matrix for the DNA probe of HPV 18 with HPV 33 and 45 (A), while HPV 58 with the same type of HPV genome (B) significantly. In (A), virus ssDNA from HPV 18 designed were differentiated into different types of HPV. The difference sequence alignment was aimed to study the inaccurate percentage when hybridized with HPV types. On contrary, data in (B) resulted 100% with the same HPV types (HPV 58) which indicates similarities with complete genome in the database. Hence, Clustal OMEGA is proved highly sensitive, specific, and selective to identify and analyze the virus ssDNA for HPV 18 and 58, respectively.

3.5 | ssDNA target sequences predicted from virus ssDNA for HPV 18 and 58

The similitude of target sequences depends on the length of the ssDNA probes. Target sequences designed are aimed

to provide higher sensitivity, specificity, repeatability, and selectivity during the hybridization process. Virus ssDNA nucleotide sequences of the HPV 18 and 58 in the E6 region were identified as 5′ CGT GCC CTC CCC GTC TGT ACC TTC TGG ATC 3′ and 5′ TCG GGG TCT CCA ACA CAC TGC ACA GCG CCC 3′. Target sequences and virus ssDNA probe designed and have been achieved for HPV 18 and 58. Thus, the development of HPV biosensors will be wholly established by the target of nucleotide sequences.

3.6 | The efficiency of the E6 region in the HPV genome

The current biosensor evolution focuses on the designation of viral DNA in the L1 region, which states various limitations of detecting cervical cancer in the early phase. The cellular reaction is related to the reconstitution of the p53 and pRb antiproliferative pathways and the pro-senescent mTOR signaling.[48] In addition, the L1 expression might be lost during cell integration, leading to the false negative during detection. In addition, the L1 expression is the late stage, which is very speculative to detect cervical cancer at early stage compared with the E6 region. The L1 expression also has lower chances of annealing, resulting in lower specificity for detecting high-risk HPV types. Viral protein in the E2 region has a lower chance to design viral DNA as a probe as it can block viral transcription oncogene when binding to E6 viral protein. During the transcription and integration process, viral DNA in the E6 region will disrupt the E2 ORF, and E2-mediated transcriptional is eliminated at the early region, resulting in the viral protein in the E2 region being unutilized.[34,49–51] Furthermore, due to the higher sequence succession races in the HPV region, the fragment probe used for distinguishing L1 sequences is not 100% reliable. In contrast to E6, L1 may have a greater probability of disappointment in hybridizing and can lead to mispriming instantaneously.[52] All the comparisons between the genome region are listed in Table 4 prominently.[53]

Recent interactome analyses led to the identification of hundreds of novel cellular proteins that could interact with E6 or E7.[54] E6 can mark Bak for degradation, an essential proapoptotic protein, and bind to p300, thus inhibiting its p53 activation mechanism compared with the L1.E6 region of viral DNA having higher efficiency compared with another area in the HPV genome. In terms of maintaining the progression of HPV-positive, the E6 region is involved in transformation and immortalization of cellular in HPV genome compared with L1 region and acts as a target for intervention of therapeutic method. Viral DNA in the E6 area was designed with multitypes of target DNA, leading to a multidetection ability of the HPV biosensor.

TABLE 4 Comparisons between recognition sites in viral DNA for HPV 18 and 58 with its limitation and advantages

Recognition site	Type of biomarker	HPV-type	Sample collection method	Limitation	Advantages	Reference
L1 region	ssDNA	HPV-16	Cervical brush	During integration L1 expression will be lost	Form icosahedral capsids around the viral genome	52
L1 region	DNA Aptamer	HPV-16	Cervical brush	Higher chances in mispriming	Rapid diagnostic	69
L1 region	ssDNA	HPV-16	Cervical brush	Lower chances in annealing	Conserved amino acids	70
L2 region	DNA Aptamer	HPV16,18	Cervical brush	Risks for false-positive results		
E6 region	ssDNA	HPV-18 and HPV 58	Saliva/cervical brush	NIL	E6 expression remains during integration Higher in priming More probability in annealing expression E6 is more pivotal in detection at an early stage of cancer	1,68

Abbreviations: DNA, deoxyribose nucleic acids; *ssDNA*, single-stranded deoxyribose nucleic acid; HPV, human papillomavirus.

Other than that, the E6 region proved more reliable as it produced a higher percentage of DNA virus on early detection.[55] Precise sample coordinates in E6 have reported a particularly prominent probability renewal rather than a consensus sample focusing on L1.[56] Apart from that, few studies have shown a conspicuous part of the E6 protein in the cell's activation and preservation of cell transition. They function effectively as an oncoprotein by strengthening the decimation of various core administrative proteins of the host genome, coming back to their roots as the side effects of cervical cancer.[14] Sequence oligonucleotides must be developed to create virus ssDNA biomarkers.[57] This study has proven the stability, sensitivity, and high specificity of viral DNA encoded in the E6 region.

3.7 | Future works on developing nanosensor-based gold nanoparticles

Globally, more than 500,000 women were affected by cervical cancer and 274,000 among them died. It is also reported that cervical cancer is the second most common cancer after breast cancer in Malaysia and worldwide. The results show that early detection at the early stage of the cancer is much necessary, as cervical cancer does not show any signs during its cancerous growth. The conventional screening tests such as Pap smear and colposcopy test indicated many disadvantages, such as resulting false negative and false positive. They require more time. In addition, previous researchers have limited knowledge of DNA detection in the lab on chips due to the expensive equipment and not well-trained technicians.[58] Hence, a sensitive nanosensor-based AuNPs is much reliable and label-free as a screening test kit for early detection of cervical cancer.

This research aims to detect the presence of HR-HPV directly from the patient's DNA via a rapid, simple, mini reusable system and low fluid volume consumption with high conductivity and selectivity nanosensor-based AuNPs. During the study, the base for the nanosensor must be stable and provide a more significant surface area for the sensing mechanism with sufficient sensitivity. To read the biological changes, they should be in the minimal size of biomolecules (micromolar) and lower consumption energy. According to Ref.[59] the point of care (POC) highlighted that the method applied for early detection of cervical cancer must be autonomous, faster detection, and sensitive to straightforward virus detection. To corroborate method, AuNPs can enhance the conductivity of the genosensor. Gold-nanoparticles (AuNPs) are biocompatible, have stable structure, efficient electron transportation and cost-effective compared with other nanomaterials such as diamond.[60]

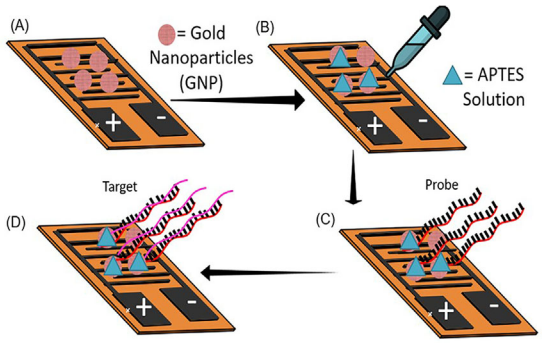

FIGURE 8 The diagram of the development of genosensor-based GNPs chemically treated with APTEs solution. Five steps applied: (A) Deposition of colloid GNPs on IDEs. (B) Silanization treat by using APTES treatment. (C) Immobilization of designed DNA probe on IDE. (D) Hybridization of target DNA with DNA probe

As referred in Figure 8, the different steps to develop nanosensor-based AuNPs are crystal clearly explained and illustrated. The process starts with colloid deposition of AuNPs on the bare IDE and ends with target hybridization on the nanosensor. IDEs is interdigitated electrodes fabricated with two connections tracks on glass substrates. Here, the study applied on Silicon Oxide (SiO2) based IDEs to provide abundance of hydroxyl groups during surface functionalization with AuNPs. As shown in (A), 10 M AuNPs were deposited on the plain IDE to enhance the conductivity as its very stable and efficient electron transportation. Next in (B), solution of APTES is dropped on the colloid AuNP by using micropette as it enhances the binding during ssDNA probe immobilization in (C) and target ssDNA hybridization in (D). Immobilization process of self-assembled monolayer virus ssDNA probe on the active sites of IDE illustrated in (C). The hybridization process between virus ssDNA probe and its target ssDNA illustrated in (D). At the end of the APTES's chemical structures, the amine acts as a linker during conjugation of AuNPs and hybridization of the ssDNA probe with the target on the IDE substrates. Before the immobilization process, the binding of AuNPs and APTES takes place by dehydration synthesis.

The dehydration synthesis process by removing the hydroxyl group indicates the attachment of APTES with AuNPs to enhance the conductivity. Next, AuNPs-APTES acts as a linker during immobilization by a binding amine group (–NH2) with carboxylic acid-modified on the virus ssDNA probe. The condensation process successfully helped in binding the AuNPs-APTES and virus ssDNA.[61] The covalent bonds formed with the linker from the APTES solution are called amide bonds. The covalent binding of dehydration synthesis for AuNPs-APTES and amide bond is shown in Figure 9: (A) dehydration

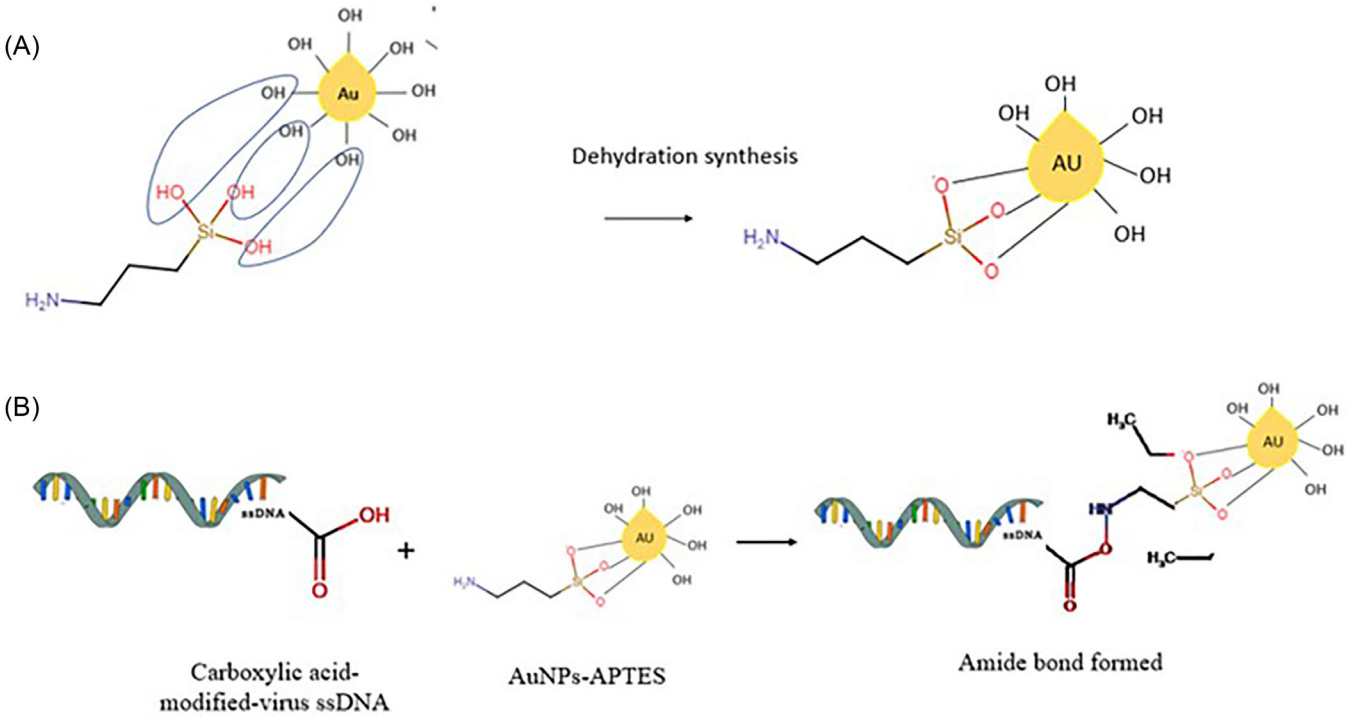

FIGURE 9 The diagram of covalent binding between AuNPs, APTES, and virus ssDNA for HPV 18 and 58: (A) dehydration synthesis of AuNPs with APTES and (B) formation of the amide bond

synthesis of AuNPs with APTES and (B) formation of an amide bond. Hence, the binding between the AuNPs-APTES and virus ssDNA probe will bond perfectly.[62]

Next, electrical measurement must be conducted on each step to identify and record the different levels of current–voltage measurements (I–V) as a negative charge existed on the ssDNA of the probe. Thus, the designed DNA probe for HPV 18 and 58 effectively detected the complementary target as the binding sites were the same. The designation of the DNA probe proved the selectivity and sensitivity of the nanosensor.

3.8 | Comparison between current and previous biosensing elements with limitations and advantages

Early detection of cervical cancer relies excessively on the sensitivity and selectivity of biosensing elements. Currently, detection of cervical cancer highly depends on DNA amplification, PCR, and HPV genotyping, which results in higher chances of false positive/negative cases. The speculative detection results due to the lower specificity biosensing elements. There were multitudinous molecular techniques applied for cervical detection; however, the detection can only be diagnosed at the late stage of cancer, triggering cross-contamination results.

Most of the current biosensing devices have preferred label-based assays to detect the virus of cervical cancer. Label assay is performed with high reliability during the immobilization and hybridization processes. However, label assays also performed end-point results and not allowing real-time kinetic measurements. In addition, mis-critical information and weak binding of compound-sare noted. It also resulted in lower conductivity during amplification from the transducer into measurement of signal such as voltammetry. These technologies have produced lower sensitivity and assays robustness when applied to measure the sensitivity of biosensors. The challenge of ligand/molecule fluorescence labeling, antibodies coupling, and radiolabeling can be only overcome by using label-free assays. In nanoscale molecules characterization, label-free assays play a significant role in validating the direct binding of small molecules and fragments to target sequences. The label-free technologies were designed and modified with a high ability to measure the specific ssDNA hybridization signals without affecting the condition of the nanobiomaterials. The development of highly sensitive, selective, and specific HPV biosensors allowing label-free assays technique with AuNPs as conductive electron transmission. Surface modification via AuNPs is efficient and has high compatibility, superior photostability, and excellent water solubility, resulting in remarkable biomedical applications for long-term imaging, high-sensitivity detection,

TABLE 5 Comparisons with other biosensing systems that were used for HPV 18 and 58 for reported HPV DNA

Type of HPV	Detection techniques	Biomarker sensing element(electrode/ linker/ probe/label)	Type of assay	Limitation	Advantages	Reference
HPV 18(malignant cells)	Electrical enhanced	AuNPs-SA complex/ CRISPR-Cas12a/ nitrocellulose/DNA	Label based	Produced unanticipated interaction and complicated protocols	High reliable	[71]
HPV 18(Malignant cells)	Differential Pulse Voltammetry(DPV)	SPCE/rGO-MWCNTs/L-Cysteine/DNA	Label-based	High cost	Sensitive and rapid diagnostic	[31]
HPV 18(malignant cells)	Fluorescence	Ti3C2 NSs/ /EXO III/DNA probe	Dye-label based	Crowded attachment led to unspecific result	–	[72]
HPV 18 and 58(antibodies)	Gold-thiol interaction	AuMNPs/ anti-dsDNA antibodies labeled with HWR-peptide-modified-CdTe quantum dots	Label based	Not adequate to detect cancer at an early stage	–	[57]
HPV 58	Electrochemical Impedance Spectroscopy(EIS)	AuNp/PANI hybrid nanocomposite mRNA/CDNA	–	Complicated protocols	Able to discriminate complementary and mismatching sequences	[73]
HPV18 and 58	Voltammetry	IDEs modified AuNPs/APTES/oligo-modified-carboxylic acid (DNA)/Target DNA	Label-free	NIL	Portability and automation Rapid, highly selective, and sensitive nano sensor Multidetection in a short timeSimple instrumentation	[1,68]

Abbreviations: CRISPR, clustered regularly interspaced short palindromic; rGO, reduced graphene oxide; MWCNTs, multiwalled carbon nanotubes; SPCE, screen-printed carbon electrode; Ti3C2 NSs, two-dimensional MXene Ti3C2 nanosheets; EXO III, exonuclease III; AuMNPs, gold-coated nanoparticles-coated superparamagnetic nano maghemite; *HWR*, Heptapeptide; *CdTe*, Cadmium Telluride; *IDEs*, Interdigitated Electrodes; APTES, 3-aminopropyltriethoxysilane; AuNp, gold-nanoparticles; PANI, positively charge polyaniline.

and target-specific treatment. Higher abundance of the hydroxyl group allows the hydrolysis and condensation process of APTES on AuNPs, resulting in a higher electrical current passed through it. Most of the current biosensing element designed virus DNA as a biomarker in the L1 region, which showed a higher chance of mispriming and performed false positive/negative results.

Future HPV biosensors consisting of promising virus ssDNA as a biomarker must have qualities of high sensitivity, selectivity, repeatability, and specific detection. Currently, biosensing element provided complicated label assays with longer consumption of time. Apart of that, antibodies as a probe for biosensing element resulted higher chances on cross contamination on end users. The limitation of the current biosensing element draws higher inadequate consenqueces while practicing in the real sample of HPV. Hence, Table 5 lists comparisons with other biosensing systems used for HPV 18 and 58 for reported HPV DNA.

The E6 region has higher ability in detecting virus ssDNA for all high-risk HPV types, including HPV 18 and 58. The E6 region presents promising higher chances in multidetection of high-risk HPV types because it is only the viral gene that is constantly maintained and retained in the HPV genome during cell integration. The performance of virus ssDNA for HPV can also diagnose any asymptomatic regression and helps in preventing further outbreaks. It can also detect the target ssDNA target from HPV 18 and 58 simultaneously. To the best of our knowledge, our biosensor is the first sensing system with multiple detection types of high-risk HPV strains (HPV 18 and 58) due to the regeneration of virus ssDNA and able to detect multiples strains on ssDNA target sequences.

4 | CONCLUSION AND FUTURE PERSPECTIVES

Gene analysis, disease diagnostic, and monitoring of the environment become a conceivable application for recent research trends. In this study, the designation of DNA probe for sensing element was highlighted as the primary point to make a high conductivity nanosensor. The specific region has been studied and analyzed, which indicates the efficiency of the designed DNA probe. The DNA probe also resulted in high specificity and selectivity hybridized with its specific target DNA. The GC content calculated in each DNA probe was upgraded up to 66.77%, which resulted in 100% similarities in BLAST software. Thus, by using the virus ssDNA in the E6 region expected for the configuration of the probe, the sensitive detection of HPV by the nanosensor tremendously exceeds the degree of cervical malignancy in patients with low-quality cytological discrepancies from the standard.

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