Exosomes and Paper-Based Biosensors for Early Oral Cancer Screening

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Abstract

Oral cancer is a severe hazard across the world due to its poor overall survival and potential to metastasize to different sites of the body. Despite recent advances in diagnostics, detecting Oral Potentially Malignant Disorders (OPMDs) remains difficult. Conventional clinical diagnostics with assisted vital staging and optical approaches like velscope to identify OPMDs are both complex and ineffective. Rapid, easier, non-invasive and non-expensive methods with high sensitivity and specificity for precise diagnosis of OPMDs and Oral Cancer is the need of the hour to improve patient survival.

Exosomes has recently become an appealing cancer biomarker in non-invasive early detection. Exosomes have unique physiology and pathology characteristics that reflect the cancer microenvironment and play a critical role in the incidence and development of cancer. Paper based biosensors have generated a lot of interest because of their excellent qualities like reduced technique sensitivity and real time output implying prospective role in cancer detection.

In this review, we discuss in detail about paper based biosensor tools for early detection of various biomarkers, especially molecular identification and exosomes in oral cancer.

Keywords: biosensors, paper based, oral cancer, early detection, exosomes

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INTRODUCTION

Oral cavity cancers are among the most common cancers in developing countries. Every year, around 300,000 new incidences of oral cancer are recorded, with over 140,000 fatalities occurring globally. This is mostly because of the Oral Potentially Malignant Disorders (OPMD) which are usually asymptomatic, are often disregarded in the early stages, resulting in a high death rate, particularly among male cigarette and alcohol users (1,2).

Cancer detection and treatment is a worldwide issue. Initial diagnosis is usually confirmed with a tissue biopsy. Subsequent research utilising image approaches such as X-rays, magnetic resonance imaging, Positron emission tomography - computed tomography (PET-CT), ultrasound imaging and biomarker identification could be performed. Genes, nucleic acid sequences (DNA, RNA microRNA), proteins/enzymes, lipids, secondary metabolites, extracellular vesicles/exosomes, and circulating tumour cells are examples of biomarkers that are expressed differentially in normal and disease phases (3).

Traditional methods for detecting biomarkers include Polymerase chain reaction (PCR), ELISA, immunohistochemistry, immunofluorescence, biochemical tests etc. But, recent trends are liquid biopsy, strip based and point of care based diagnostics. The most sensitive and specific methods for identifying biomarkers in clinical diagnostics are immunoassays and PCR. The identification of biomarkers is crucial for the early diagnosis and prognosis of illnesses like cancer as well as for the prompt and effective therapy that follows to enhance patient quality of life and boost survival rates. Additionally, biomarkers can be employed to track treatment efficacy and illness recurrence (4).

Some biomarkers are present at extremely low quantities, and some biomarkers are linked with only one form of cancer. But oral cancer is heterogeneous, and several biomarkers are connected with mouth cancer. Because of these factors, detecting the levels or expression of biomarkers in complicated samples can be difficult. Few confounding factors like age, medications and

other systemic illnesses need to be eliminated. Detection of a panel of biomarkers is frequently necessary for diagnosis over a single biomarker (5).

Advanced clinical tests are not available in some areas, especially in developing countries, due to a lack of financial resources and trained personnel, which has an impact on accurate diagnosis and subsequent treatment. These clinical tests, however, require complex and expensive protocols involving multiple equipment and multiple level assays, such as characterization, high reagent and sample consumption (6).

Biosensors are analytical instruments with a bioreceptor which recognises the target, and a transducer, which converts the recognition event into a measurable signal. Even at low concentrations, biosensors have a high sensitivity for detecting a single biomarker or a collection of biomarkers. In order to diagnose diseases like cancer, a point-of-care (PoC) format that can be used outside the laboratory, researchers have been focused on creating biosensor-based techniques. Although PoC may not replace clinical examination but can be an useful adjunct in the diagnosis, particularly for diagnostic applications due to their ability to give a simple and speedy preliminary screening. Several cancer biomarker diagnostic tests are already available allowing rapid and non-invasive diagnosis (7).

To detect a wide range of analytes, optical and electrochemical transduction techniques are typically utilised in combination with paper-based analytical devices (PADs). Colorimetry (CM) is a viable optical detection approach for PADs, giving various advantages such as rapid, cheap and visible in naked eye. Furthermore, the utilisation of cellphones, operating systems, and wifi allows for real-time analysis (8).

Exosomes derived from cancer cells have also been found using Paper Based Biosensors (PBB) (9). An innovative paper-based aptasensor for the detection of exosomes was developed by Chen et al (10). Multi-walled carbon nanotubes (MWCNTs) were coated on micro-pore filter paper to form the basis of Ji et al immunosensor in 2018 for the detection of early-stage prostate cancer (11). Multi-walled carbon nanotubes (MWCNTs) were coated on micro-pore filter paper to form the basis of Ji, Lee, and Kim's (2018) immunosensor for the detection of early-stage prostate cancer. An innovative paper-based aptasensor for the detection of exosomes was developed by Chen et al (4-6).

Biosensors

"A biosensor is a device that employs specialised isolated enzyme-mediated biological activities to detect chemical substances either by thermal, electrical, or optical signals," according to the International Union of Pure and Applied Chemistry (IUPAC) (12).

Clark LC (1918-2005), commonly known as the "Father of the Biosensor," fabricated biosensors in the 1960s using "enzyme electrodes" to measure glucose content using Glucose Oxidase. Technological advances developed more advanced and compact biosensors for measuring glucose, lactate, and urea from biological fluids. Chromatography and capillary electrophoresis are few other developments of new diagnostic instruments. Recently multiplex immunosensor gadgets are introduced which can have several specific electrodes per square centimetre (13).

Components of Biosensor(14)

Two elements

1. Biological component - the analyte (blood, stools, serum, saliva, and urine), Bioelements produced by biological engineering (enzyme, antibody, nucleic acid, cells, tissue, etc)

2. Physical Constituent

a. A detector that detects the biomolecule and generates a stimulus

b. The transducer converts the signal produced by the analyte's interaction with the biological element into another signal that can be measured and quantified more readily (Electrochemical, Piezoelectric, Optical, etc)

c. The signal processors or electrical components responsible for displaying the results in a user-friendly manner are referred to as amplifiers or display units.

Working Principle of Biosensors

A biosensor is a bioanalytical device that includes a biosensitive layer that is connected to the device framework and assists in

signal detection. The biosensitive layer keeps the biological receptor component on the biosensor membrane stable. In most cases, the targeted biological material takes the form of an enzyme. Using an electro-enzymatic approach, enzymes are transformed into corresponding electrical signals. The oxidation of the enzyme is a frequent physiologic reaction. Oxidation causes the pH shift which will further have an immediate effect on the current. The pH shift will have an immediate effect on the enzyme's present carrying capacity that is proportional to the enzyme being tested (15).

Saliva based biosensors

Analysis of biomarkers from blood is a standard clinical diagnostic approach. Saliva has grown in popularity as a diagnostic fluid, with increasing assays and technological developments for detecting salivary analytes. Comparatively, Saliva samples are easier to obtain, less technique sensitive and can identify a variety of normal and diseased conditions. Recently several salivary biomarkers have been developed to aid in diagnosis, disease monitoring and prognosis of a variety of cancers (16).

Salivary-based sensors are classified into three major types depending on their biosensing targets: Biosensors for deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein (17).

i) DNA biosensor: DNA from body fluids can be isolated by a series of steps like centrifugation and purification. DNA biosensors can be used to detect target DNA primer sequence binding with the complementary sequences. Carbon electrodes and other metallic electrodes like gold, Indium Tin Oxide can be used. Ratiometric Electrometric DNA sensor can also be used to directly analyse the target DNA in collected samples using exonuclease III mediated amplification with dual signal ratiometric output. Biomarkers even in lower quantities can be identified (18).

ii) RNA biosensor: The RNA biosensor operates on the recognition of sequence by employing sequence specific forward and reverse primer sequence hybridisation between nucleic acids. A probe of complementary sequence of the target site is added for the detection. The biological sample and complementary strand of target mRNA is added and the probe is hybridised with it. Electrochemical and optical methods could be used to detect hybridisation. MicroRNAs are endogenous RNAs that range in length from 21 to 25 nucleotides. They have a vital regulatory function in animals and plants by directing the degradation or suppression of particular mRNAs(19).

iii) Protein biosensor: Electrochemical biosensors could be used for detecting cancer specific protein levels. These protein based biosensors are sensitive, rapid, and cheap PoC devices. Antibiotics and aptamers are coated to electrodes for the detection of analytes using biosensors. Aptamer biosensors have high sensitivity for detection when compared to traditional biosensors(19).

Paper based biosensors

Paper is made of cellulose fibre (unbranched glucose) which is helpful for fabrication of biosensors because of its flexibility, weight, cost, ease of storage and transportation, combustibility, biocompatibility, and biosafety management. Conducting paper has lately been investigated for the development of novel approaches for printed basic electronics components and low-cost sensor kits. The use of flexible paper as a substrate for PoC PAD development as an alternative to traditional biomarker detection is growing. (20-22)

A linear polymer consisting of D-glucose units connected by -(1,4) glycosidic links, cellulose is known as. Cellulose paper is also easy to manufacture, store, and move. This polymer possesses distinctive physical and physicochemical properties, such as high porosity, hydrophobicity, strength, thermal stability, and biocompatibility. It also has a high sorption capacity. Because of its vast number of holes, cellulose paper permits reagent immobilisation and drying, making it appropriate in construction of a variety of devices. (20-22)

PADs are very valuable for localities with limited resources and they meet the requirements of WHO's ASSURED (Affordable, Sensitive, Specific, User-friendly, Rapid and Robust and Deliverable) concept, making them good PoC devices. Furthermore, they may be connected to electronic displays. To achieve great sensitivity, nanoparticles (NPs) produced from various materials have been employed in PADs. (20-22)

Furthermore, to ensure specificity, these particles may be simply functionalized with biorecognition components. PADs are user-friendly since they do not need intrusive collections and produce simple-to-understand findings without the need for experienced staff or expensive equipment. Because of cellulose features such as porosity and capillary forces, they give a quick reaction and relatively fast results. (20-22)

PAD constituents make them durable gadgets. High surface-to-volume ratios, adsorption abilities, biological samples compatibility, chemical functional groups for protein and antibody immobilisation, and ease of sterilising are few advantages

of paper. Furthermore, the capacity of the paper matrix to store and transfer reagents reduces the requirement of reagents. (20-22)

In order to analyse glucose in urine samples semi-quantitatively, the first paper device was created in 1956. Since then, paper has been utilised in a wide range of applications, including forensics, environmental monitoring, food and water quality management, diagnostics, and treatments. Paper based electrode for printing silver paste which electrochemically attracts and binds anti-sIl2Rα for the detection of cancer by Kumar et. al (20). Ge et al. developed an electrochemiluminescent immunodevice for the multiplexed detection of cancer biomarkers using chitosan grafted on screen-printed conducting carbon paste on a paper substrate (21). Numerous polymers, PEDOT: PSS, were discovered to have excellent heat and cold resistant ease of film production, and were superior over old electrodes (22).

Classification of Paper-based biosensors (PBB)

PBB are majorly classified into three types, the dipsticks test, LFA, and μ-PADs.

i) Dipstick test: The dipstick paper-based biosensor is a diagnostic tool that uses an optical detection approach to identify immobilised chemicals and is used for qualitative examination. Dipstick assays, such as uric acid and glucose strips, pathogen detection strips, and so on, are now accessible. Dipstick testing is simple to create, which bodes well for commercial enterprises. The fundamental disadvantage of the dipstick is that it cannot be used for quantitative analysis. Colorimetric dipstick changes colour and is used to detect colour visually. As a result, this kind is ideal for obtaining quick and reliable results. A few benefits include quick standardisation, simple design, and constraints such as only optical mode is available and only one step analysis is supported. (23,24)

ii) Lateral flow assays (LFA): The LFA paper-based biosensor is a more advanced variant of a dip-stick test that uses optical and electrochemical sensing techniques to deliver a controlled flow of sample. These strip test advances are suitable for designing multistep assay. It is made up of 4 major parts: a sample pad, absorbent pad, a conjugation pad and a detection pad. The advantages include electrochemical mode and flexible sample movement, while the disadvantages include relatively low sample volume, which requires expert personnel and takes a long time to optimise, and the manufacturing process (25,26).

iii) μ-PAD: Microfluidics technology applications have enabled the development of several PBB that use optical, electrochemical, chemiluminescence/ Electro-chemiluminescence and have the potential to overcome the major shortcomings of the LFA type. It not only improved sample flow but also enabled additional multiplexing and quantitative analysis. Numerous μ-PAD variants have been created, with detecting methods including electrochemical, optical, chemiluminescence, and piezo-resistive micro-electromechanical systems (MEMS). Several μ-PAD are created for the measurement of glucose and uric acid utilising bodily fluids. Benefits include allowing for many detections for quantitative analysis and variable sample flow; downsides include the necessity for expertise, a lengthy optimization and manufacturing process, and incompatibility with mass production (27–32).

Extracellular Vesicles

Extracellular vesicles (ECVs) are membrane-bound structures that are categorised according to their size, density, and origin (33)(34). ECVs are composed of proteins (>92,897), lipids (584) and genetic material miRNAs (4934) and mRNAs (27,642) (35).

Exosomes have gotten a lot of attention among the three types of ECVs, since they have potential diagnostic and therapeutic use. Exosomes are bilipid layer ECVs of size 30-120 nm in diameter which are formed by multivesicular bodies (endosomes) with the plasma membrane. They are found abundant in liquid biopsy with concentrations of ≥109 vesicles/ml (36) by means of exocytosis they reach the extracellular matrix (ECM); thus, they act as natural nanocarriers and intercellular messengers.

Exosomes were once thought to be a straightforward way to get rid of undesired cellular waste. However, these "trash bags" and their critical functions in intercellular communication. Exosomes engage in both physiological and pathological activities by transferring cargo molecules into the target cells thereby influencing their characteristics and can have positive or negative effects. Dysregulation nucleic acids and proteins in pathological conditions could be used for molecular diagnosis and targeted therapy (37).

Exosomes

Exosomes are one of the most significant contributors to tumor growth and metastasis, delivering a wide range of molecules that could be implicated in cancer pathogenesis (38).

Their abundance in liquid biopsy and molecular components in exosomes could be used as early diagnostic markers in Oral Potentially Malignant Disorders (OPMDs) and Oral Squamous Cell Carcinoma (OSCC). Hurvitz et al analysed the quantification of salivary exosomes from healthy and OSCC using Nano Tracking Analysis and significantly higher concentration of Exosomes were evident in OSCC when compared to healthy individuals (39), similarly quantification study on plasma exosomes by Heravi et al showed higher quantitative levels of circulating exosomes in OSCC when compared to healthy and benign lesion. There are no quantification studies on OPMDs (40).

Sharma et al analysed morphological alterations of salivary exosomes under Atomic Force Microscopy from OSCC that showed increased vesicle size, irregular morphology, increased intravascular aggregation (41).

The components of liquid biopsy include circulating tumor cells, cell-free DNA and circulating tumor DNA, exosomes, and circulating cell-free nucleic acids (42). Real-time monitoring of malignant transformation, cancer growth and emerging genetic mutations within the tumour is possible using liquid biopsy (43). Molecular testing is becoming more widely used, with over 80% of medical oncologists are moving towards biomarker analysis at first biopsy (44). ExoDx® Prostate (IntelliScore) and ExoDx®Lung, cancer diagnostic products based on exosome profiling, were introduced in the United States in 2016 (45).

Role of oral fluid-based biosensors in Oral Cancer

Various biomarkers have been studied for the use of diagnosis of oral potentially malignant disorders and malignant transformation of oral cancer have been established. Salivary proteins such as IL-8, TNF-alpha, Epidermal Growth Factor Receptor (EGFR), and miRNAs can be used to diagnose oral cancer. IL-8, an anti-inflammatory chemokine, has a crucial function in tumour angiogenesis and metastasis (46).

Cancer biomarkers can be evaluated and discovered using oral exfoliated cells and a saliva-based biosensor. When compared to traditional biopsy techniques, patients experience far less worry and discomfort. An unique biosensor was fabricated by Weigum et al. Nano-Bio-chip Cellular (NBC) analysis for the categorization of malignant and premalignant lesions and was also utilised for the evaluation of EGFR and cytomorphometry in exfoliative cytology models. The NBC sensor test was used to quantify and identify morphological changes in the nucleus and EGFR expression in cancer tissues(47).

Exosomal microRNA in Oral cancer

Oral Squamous Cell Carcinoma (OSCC) derived Exosomes are involved in tumor growth that are mediated by miR-21-5p, miR-342-3p, miR1246 by activation of Nuclear Factor kappa B inflammatory pathway (40)(48). miR-142-3p regulates angiogenesis by elevated expression of Type I TGFβ receptor (TβRI) in the donor cancer cells and increase of TβRI action in recipient endothelial cells(49). Metastatic potential of OSCC are regulated by expression of miR-21, miR-342-3p, miR-1246 which further downregulates Snail,Vimentin and E-cadherin pathways (50). Drug resistance via miR21 by activation of phosphatidylinositol 3 kinase (PTEN) and Pyruvate Dehydrogenase Deficiency (PDCD4)(51,52). Inflammatory modifications by miR-24-3p by downregulation growth factors further inhibits signal transducer and activator of transcription (STAT) and extracellular signal-regulated kinases (ERK) protein of T cells(53) .

Exosomal microRNA in Oral Potentially Malignant Disorders

Studies have been done on serum, plasma and salivary exosomes on OSCC and OPMD patients to be used as diagnostic biomarkers (54). A cell line study on plasma derived exosomes from patients with erosive and non-erosive lichen planus co-incubated with T lymphocytes (Jurkat cells) suggested a significant internalisation of OLP-Exosomes by T cells within 48 hrs which lead to T cell proliferation but T cell activation was not evident (55).

Exosome-derived miRNAs have been found to be freeze–thaw resistant and stable for 5 years at an ultra-low temperature of 208°C (56). Exosomal miRNAs have a high level of stability, making them a suitable biomarker for illness monitoring (57). Qiao peng et al in 2018 reported in a study among the Chinese population that miR34a-5p, miR130b-3p, miR29c-3p derived from circulating exosomes were upregulated and miR301b-3p and miR144-3p were downregulated in Oral Lichen Planus when compared to healthy individuals (58). J-S Byun et al in 2015 reported in a study among the South Korean population that miR-4484 derived from salivary exosomes are associated with Oral Lichen Planus when compared to healthy (59).

Conclusion

Novel innovative biosensors using cellulose and exosomes are PBBs and microfluidic PADs in OPMDs screening and cancer diagnostics. Chairside diagnostic tests have recently gained importance over traditional lab tests, because of its rapid and ease in procedure to do without the need for qualified professors. Furthermore, biosensors refer to POC devices designed to aid in the early diagnosis, periodic monitoring, and treatment of diseases. These devices rely on natural reactions to differentiate and estimate specific biomarkers. Conventional diagnostics aided with adjuvant methods have saliva as an oral fluid that has benefits over serum and tissues for several biomedical diagnostics, including noninvasive, smaller sample, convenient storage and transit, and more affectability.

Besides the complex nature of cancer cells and tumor derived exosomes. As a result, several studies on identification of cancer specific paper based biosensors are warranted. Collaborative data of epidemiology, molecular data bank, genomics and proteomics data might aid in gaining a better knowledge of cancer etiologies and pathogenesis, as well as selecting effective exosome indicators.

The future of diagnostic biosensors is mainly focused toward a bigger population. As a result, gadgets must be portable, user-friendly, and affordable. With the rapid advancement of biosensor-based test technologies, early cancer screening may enter a new age, significantly improving therapy outcomes and patient survival.

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Conflict of Interest

All the authors declare no conflict of interest.

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