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**Nano-diagnostics as an emerging platform for oral cancer detection: Current and emerging trends**

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1 **Nano-diagnostics as an emerging platform for oral cancer**  
2 **detection: Current and emerging trends**

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35 **Abstract**

36 Globally, oral cancer kills an estimated 150000 individuals per year, with 300000 new cases being  
37 diagnosed annually. The high incidence rate of oral cancer among the South-Asian and American  
38 populations is majorly due to overuse of tobacco, alcohol, and poor dental hygiene. Additionally, socio-  
39 economic issues and lack of general awareness delay the primary screening of the disease. Availability  
40 of early screening techniques for oral cancer can help in carving out a niche for accurate disease  
41 prognosis and also its prevention. However, conventional diagnostic approaches and therapeutics are  
42 still far from optimal. Thus, enhancing the analytical performance of diagnostic platforms in terms of  
43 specificity and precision can help in understanding the disease progression paradigm. Fabrication of  
44 efficient nanoproboscopes that are sensitive, non-invasive, cost-effective, and less labor-intensive can reduce  
45 the global cancer burden. Recent advances in optical, electrochemical, and spectroscopy-based nano  
46 biosensors that employ noble and superparamagnetic nanoparticles, have been proven to be extremely  
47 efficient. Further, these sensitive nanoproboscopes can also be employed for predicting disease relapse after  
48 chemotherapy, when the majority of the biomarker load is eliminated. Herein, we provide the readers  
49 with a brief summary of conventional and new-age oral cancer detection techniques. A comprehensive  
50 understanding of the inherent challenges associated with conventional oral cancer detection techniques  
51 is discussed. We also elaborate on how nanoparticles have shown tremendous promise and  
52 effectiveness in radically transforming the approach toward oral cancer detection.

53 **Keywords:** Oral cancer detection; Conventional detection; Nanoparticles; Non-invasive; Nano-  
54 biosensors; POCTs

55

## 56 **Introduction**

57 Oral and oropharyngeal cancer is precisely categorized as malignant neoplasm in the lips, oral  
58 cavity, and oropharynx region (ICD 10: C00-C14). Geographic and environmental variables have an  
59 important influence in defining the associated risk factors. Southeast Asian countries account for nearly  
60 40% of the global oral cancer burden, where the causative risk factors include consumption of areca  
61 nuts, tobacco, and smoking. Meanwhile, HPV-16 infection has been linked to oropharyngeal cancer  
62 (OPC) in the younger age group in the US (Gillison et al., 2012; Warnakulasuriya, 2009). Societal  
63 elements and individual lifestyle choices also contribute significantly to the growing cases of oral cancer  
64 (OC). Government screening programs are designed to work at different levels with a defined  
65 framework to prevent the progression of the disease to an invasive stage. Screening sub-mucosal and  
66 mucosal abnormalities often referred to as oral potentially malignant lesions (OPMLs) at an early stage  
67 can effectively improve the quality of life with a 40-50% survival rate (Coletta et al., 2020). However, a  
68 combination of factors such as lack of awareness, unavailability of efficient pharmaceutical adjuncts,  
69 and healthcare practitioners' subjective interpretations can result in late-stage presentation with serious  
70 complications (Mehrotra and Gupta, 2011). Fig. 1 represents a schematic theme of several  
71 determinants involved during the progression of oral oncogenesis.

72 For a definitive diagnosis of oral malignancy, clinicians still rely on the gold-standard biopsy  
73 and histopathological examinations (Sylvie-Louise Avon and Klieb, 2012). This procedure in general is  
74 expensive, invasive, and requires visiting clinics for multiple consultations. It is worth mentioning that  
75 during the COVID-19 pandemic nearly 14 million individuals in England alone missed their oral  
76 examination at desired clinics due to the state-imposed restrictions (Westgarth, 2020). Such  
77 circumstances exacerbate co-morbidities and cause massive backlogs, putting a strain on an already  
78 overburdened healthcare system. Screening aids that provide real-time analysis at the patient's site  
79 with a minimal turnaround time are the need of the hour. With the advent of nanotechnology, engineered  
80 nanoparticles (NPs) have gained substantial attention as biosensors (Bogart et al., 2014). These  
81 engineered NPs act as transducers that are vital in the development of any nanobiosensor. Unique  
82 physicochemical properties of these NPs such as large surface-area to volume ratio enhance their  
83 catalytic and absorbance activity as well as enables easy immobilization of critical biorecognition  
84 elements. The use of nanomaterials may improve the biosensor performance including increased

85 sensitivities and low limit-of-detection of several orders of magnitudes (Malhotra et al, 2018). Further,  
86 metal NPs (gold, palladium, and aluminum) alone and in combination with other classes of  
87 nanostructures have shown excellent biocompatibility (Malekzad et al, 2017). Skillful labeling of gold,  
88 magnetic NPs, and quantum dots have facilitated the development of sensitive optical and  
89 electrochemical immunoassays (Masud et al., 2019a; Perfézou et al., 2012). Additionally, nanoarray-  
90 based platforms that can sense cancer cell surfaces, tissues, and biofluids are being considered for  
91 personalized diagnostic systems (Le et al., 2014). NPs-based Point-of-Care (POC) diagnostic aids such  
92 as LFA-strips have demonstrated significant potential towards detecting viral markers and are most  
93 widely used for the detection of pregnancy at a lower cost and a short period (Butler et al., 2001;  
94 Martiskainen et al., 2021). Needless to say, these facile nanodiagnostic adjuncts can become the  
95 cornerstone in deciding the appropriate treatment strategy and indicating disease prognosis.

96         The aim of the current review is to understand the vital aspects of several diagnostic adjuncts  
97 for the detection of oral malignancy. The review presents the status quo of both conventional screening  
98 aids as well as the upcoming nano-based technologies. Also, discussed briefly are the new age user-  
99 friendly diagnostic innovations and patents for early detection of oral carcinoma. The inherent factors  
100 hindering the clinical translation of nanodiagnostic adjuncts have also been critically analyzed. The  
101 review aims to help the readers gather information about methodological modifications from a diagnostic  
102 point of view. In the future, the knowledge would help the scientific community to better manage the  
103 disease.

104

## 105 **2. Oral malignancy and conventional detection techniques**

106 Presenting a discrete screening algorithm in the case of OC is difficult as the procedures depend on  
107 the patient's history, as well as accessible screening aids. However, at most established clinics and  
108 hospitals a patient's workup starts with analyzing the case history followed by a physical examination  
109 of the oral cavity. Biomarker profiling and locating tumors using imaging techniques remain essential  
110 before proceeding with biopsy. Fig. 2 represents a schematic diagram of the flow chart involved in the  
111 screening of OC.

112 Government-organized sporadic camps that are aimed at screening pre-cancerous lesions often rely  
113 on visual examination and low-cost light-based screening aids. A district-level cancer control program  
114 namely "ASWAS" in Kerala, India was solely based on a visual examination (Philip et al., 2018). Another  
115 study from Uttar Pradesh, India utilized Magnivisualizer for initial screening and cyto-brush analysis for  
116 further validation of OC (Parashari et al., 2014). Commercial screening aids that use the principle of  
117 chemiluminescence (ViziLite), tissue autofluorescence (Velscope), and/or multispectral fluorescence  
118 (IDENTAFI 3000) for visual discrimination between dysplasia and healthy mucosa are frequently used  
119 in different clinical settings (Cicciù et al., 2019; Macey et al., 2015). Several of these index tests have  
120 shown to have limited applicability as they often present false negative or false positive results (Macey  
121 et al., 2015). To rule out such 'risk of Bias" judgments, additional biomarker analyses are performed.  
122 Bottom-up proteomics and top-down proteomics strategies, as well as powerful mass spectrometry, are  
123 used to detect proteins that serve as biomarkers in OC. Currently, cancer antigen 125 (CA 125), tissue  
124 polypeptide antigen (TPA), interleukin-6 (IL-6), Cytokeratin fragment 21-1 (CYFRA 21-1), tumor  
125 necrosis factor-alpha (TNF-alpha), Mac- 2, and telomerase are considered as promising protein  
126 biomarkers in the case of early detection of oral cancer (Zhang et al., 2009). According to (Huang et  
127 al., 1999) P53 mutations were found in salivary DNA in 62.5% of patients with oral cancer. Identification  
128 of epigenetic modifications such as hypermethylated promoters of tumor suppressor gene (TSG) in oral  
129 rinses has been shown to assist in monitoring and diagnosis of OSCC (Liyanage et al., 2019). For the  
130 fabrication of more practical detection systems, a clear idea about the relative occurrence of possible  
131 prognostic, diagnostic and post-operative markers in the different biological fluids can play a crucial role  
132 in understanding the progression of the disease. Whole saliva can act as an excellent auxiliary tool in  
133 such cases as the mode of sampling is non-invasive, and cost-effective and further saliva can be easily  
134 transported and stored. Concomitant increase (400%) in the levels of CA-125 and CYFRA 21-1 protein  
135 markers in saliva of patients with OSCC has been reported previously(Nagler et al., 2006). It was  
136 observed that the values of CYFRA 21-1 were ~3 folds higher in pre-malignant cases as compared to  
137 the healthy individuals(Rajkumar et al., 2015). Though the CA-125 level increases significantly in  
138 patients with OSCC, It has lower levels of expression at the early stages of oral cancer(Geng et al.,  
139 2013). From these findings, it can be suggested that CYFRA-21-1 can be demarcated as a good  
140 diagnostic marker for early detection of OSCC as compared to CA-125. Another combinatorial salivary  
141 marker analysis study considered matrix metalloprotease 9 (MMP-9) and 8- hydroxy-2'-

142 deoxyguanosine (8-OHdG) in patients with OSCC. The representative biomarkers analysis in  
143 unstimulated whole saliva indicated that MMP-9 could be employed as both a universal OSCC  
144 screening marker as well as a prognostic marker (over a period of 9 months). However, the levels of 8-  
145 OHdG, a pivotal marker for measuring the effect of endogenous oxidative DNA damage did not show  
146 positive correlation and authors suggest further research to be done to clarify the contradicting  
147 differences (Shin et al., 2021). Liquid biopsy based detection of circulating tumor cells (CTCs) and  
148 circulating tumor DNA (ctDNA) in biological fluids has gained enormous attention in the recent years  
149 (Soda et al., 2019). ctDNA being majorly released into the blood stream may reach saliva via passive  
150 diffusion or active transport. Recent studies have reported the presence of ctDNA in 95% of the patients  
151 enrolled at late stages of OSCC. Patients with clinical reoccurrence of OSCC post-surgery also showed  
152 elevated levels of ctDNA suggesting that salivary ctDNA can be a valuable biomarker for oral cancer  
153 follow-up and surveillance(Cristaldi et al., 2019). .

154 To properly analyze the depth and loco-regional invasion of the tumor, cross-sectional imaging is  
155 required. Scanning techniques like computed tomography (CT), and positron emission tomography  
156 (PET), and magnetic resonance imaging (MRI) provide information about resectability and also help a  
157 clinician plan a proper treatment strategy (Waech et al., 2021) (Arya et al., 2012). Clinical staging at  
158 this point is possible. However, for a definitive tumor nodes metastasis (TNM) staging biopsy is a  
159 mandate. Visual inspection of the gross tissue and tissue staining lets a skilled technician asses the  
160 deformities in the cellular architecture of the patient. A detailed analysis of the frequently used medical  
161 adjuncts in clinical settings is summarized in initial screening, biomarker assay, advanced optical  
162 imaging followed by biopsy and histopathological assay table 1.

163 Conventional screening methods such as MRI, CT, ultrasound, biopsy, and other techniques as listed  
164 in table 1 have inherent shortcomings which limit their usability. While the low-cost technologies  
165 compromise the sensitivity, the scanning techniques are relatively costly. On the other hand, the biopsy  
166 is invasive, requires skilled labor and significant time. In developing countries, where available  
167 resources are scarce, portable adjuncts that facilitate self-examination can be highly effective for  
168 detecting oral malignancies at an initial stage. Additionally, a good understanding of different biomarkers  
169 may help in the development of LFA-based detection formats. Such diagnostic kits can be used  
170 throughout the continuum of care in oncology.

171

172 **Table 1.** Conventional Screening aids for the detection of oral carcinoma

<b>Screening Adjunct</b>	<b>Principle</b>	<b>Biomarker</b>	<b>Pros</b>	<b>Cons</b>	<b>Ref.</b>
VELscope (400-430 nm)	Loss in tissue autofluorescence due to disruption of intact collagen, elastin, and increased vascularity	Oral mucosa	Non-invasive, cost-effective	Low-sensitivity, false-positive reporting	(Cicciù et al., 2019)
ViziLite (light 430, 540,580 nm)	Illumination with Chemiluminescent light for assessing tissue reflectance	Oral mucosa	Non-invasive, cost-effective, convenient	Low-sensitivity, false-positive reporting	(Mehrotra and Gupta, 2011)
IDENTAFI (405 and 575 nm)	Illumination with green-amber light for capturing both tissue autofluorescence and reflectance, for observing vascularity	Oral mucosa	Non-invasive, relatively high sensitivity (82-87%)	The risk of bias judgment needs further validation	(Messadi, 2013)
Vital staining	Toluidine blue staining of the lesion and microscopic examination	Oral tissue	Inexpensive, non-invasive	False-positive report, low sensitivity	(Missmann et al., 2006)
ELISA	The presence and quantity of antigens binding is detected by	Saliva, serum, blood	Sensitive, can detect low levels of the biomarker	Laborious requires skilled expertise	(Prasad and McCullough, 2013)



Specific antigen-  
antibodies reaction

HPLC	Column chromatography based identification of specific markers	Biofluids containing serum proteins	Quantitative measurement, sensitive	Expensive requires technician for result interpretation	(Reddy et al., 2012)
PCR and PCR-RFLP	DNA amplification, genetic mapping using restriction enzyme sites	Serum and saliva (can indicate HPV infection and genetic polymorphism)	Reproducible, sensitive	Tedious sample preparation, expensive, and requires expertise	(Herrero et al., 2003; Sreelekh a et al., 2001)
DNA microarray	Measurement of multiple tissue-specific gene expression based on complementary sequence binding	Oral tissue	Sensitive, moderately costly	Tedious instrument handling	(Ziober et al., 2008b)
Mass-Spectroscopy	Measurement of the mass-to-charge ratio of ions based on the intensity	Saliva based metabolic proteins	Details about molecular profiling, sensitive	Expensive instrumentation, not easily accessible	(Hu et al., 2007)

MRI	Use of magnetic fields, and radio waves for imaging the suspected lesion	Tumours located in soft tissue	Can accurately detect the status of invasion, and depth of tumor	Expensive, complicated instrumentation, cannot image patients with metal implants	(Ziober et al., 2008b)
PET	Imaging-based on the usage of a radioactive isotope	Tumors located in head and neck	Can facilitate early detection by a non-invasive mode	Lowered accuracy due to short decay time of the radioactive substance, expensive	(Civantos et al., 2003)
CT	Ionizing radiation, X-ray coupled with an electronic detector for imaging of tissues	Tumors located in soft tissue	Moderately less expensive gives an idea about the spread of carcinoma	Exposure to radiation and contrast agents	(Handschel et al., 2012)
Ultrasound	Usage of high-frequency sound waves	Head and neck	Moderately expensive, non-invasive	Biased interpretation, cannot report vascularization	(Lodder et al., 2011)

Brush biopsy	Use of cytobrush for collection of oral cells followed by microscopic examination	Oral cells	Non-invasive, easy clinical procedure, cost-effective	Not highly sensitive, sampling error and contamination	(Gupta et al., 2014)
Incisional biopsy	Microscopic examination following removal of a small section of the lesion using a scalpel	Oral tissue	Sensitive, accurate analysis can be performed	Invasive, patient discomfort, requires a skilled clinician	(Kusuka wa et al., 2000)
Fine-needle biopsy	Fine-needle aspiration followed by microscopic examination	Oral cells	Low cost	Chances of bleeding and infection, false-negative reporting	(Flach et al., 2013)
Immunohistochemistry	Immunochemical staining for identification of antigen-antibody interaction	Oral tissue	Widely used method for validation	Chances of human error, tedious sample preparation	(Lenouvel et al., 2021)

173

174 **3. New-age products and Patents for oral cancer diagnosis**

175 Integration of technology from several trans-disciplinary fields has introduced several simplified  
 176 diagnostic aids and kits for early detection of OC. For instance, artificial intelligence (AI)-based  
 177 technologies that analyze enormous datasets have shown considerable improvement in predicting the

178 occurrence of OC. Reports suggest that the accuracy of prediction analysis is better as compared to  
179 the conventional cox- and logistic regression analysis methods (Khanagar et al., 2021). Other products  
180 include visualizing aids, salivary diagnostic kits, and real-time imaging tools. A few of these new-age  
181 commercial products and patents are discussed below.

182 Modernized visualizing screening aid that uses the principle of autofluorescence has been developed  
183 to overcome the shortcomings associated with light-based detection systems (LBDS). GOCCLES®  
184 (Glasses for Oral Cancer – Curing Light Exposed – Screening) fabricated with filters are reported to be  
185 a cost-effective alternative for VELscope that can aid in visualizing oral lesions via dental curing light  
186 (Moro et al., 2015). However, the limitations of the study include a small cohort study (sample size-61)  
187 non-randomized multicenter trial on patients at high risk for OSCC. Additionally, inter-observer  
188 variability leads to the subjective interpretation of the data. Further stages of clinical trial need to be  
189 conducted to accurately define the diagnostic performance of the system.

190 OralScan, a handheld optical imaging multimodal device developed by Sascan Meditech, Kerala, India  
191 is reported to be highly efficient in detecting early lesions and preventing unwarranted biopsies (DST,  
192 2020). Another, Bengaluru-based startup in collaboration with AIIMS Delhi and Bhubaneswar  
193 developed a mobile app that analyses oral lesions with the help of Artificial intelligence, making the  
194 overall diagnosis easily accessible and affordable (BerryCare, 2020). It is interesting to note that few of  
195 these innovations involve the direct usage of different components of artificial intelligence for presenting  
196 their data to the user. Though there is a booming growth of AI, especially in terms of its association with  
197 a cancer diagnosis and precision medicine, certain drawbacks such as ambiguous AI algorithms can  
198 lead to erroneous data generation. Other concerns regarding patient data privacy and security breaches  
199 limit the clinical implementations of AI on large scale (Sunarti et al., 2021)

200 Multiple large trial studies have proved SalliMark™ OSCC an efficient salivary biomarker diagnostic kit.  
201 The non-invasive kit uses bio-chemistry analytics for the early detection of OSCC. Analysis of specific  
202 biomarkers expressed during the early stages of OSCC is used for better risk stratification  
203 (SalliMark™ OSCC, 2021). The procedures for saliva collection and shipping of the sample via pre-paid  
204 services may not be suitable for individuals with socio-economic challenges. Further, unfortunate  
205 logistical constraints may render the sample unusable.

206 Cook, Watson, and Festy from King's College London, have patented an imaging apparatus that uses  
207 only a green wavelength of light for imaging vascular tissue with better contrast. Angiogenesis and/or  
208 neovascularization commonly occurring during oral lesion formation could be monitored with higher  
209 accuracy due to the light absorption characteristics of hemoglobin (Hb) (US8364222B2, 2013).  
210 However, the technique involves performing surgery on the patient making the overall process invasive  
211 and causing inconvenience to the patient. The procedure also would require a highly-skilled technician  
212 and advanced medical setups.

213 Another research group patented a technique using a complex compound consisting of a fluorescent  
214 and radioactive marker that is selectively taken up by pre-malignant and malignant cells. In the case of  
215 oral malignancy cyto-brush is the suggested method of cell collection. The exfoliated cells with the  
216 compound can be examined further via fluorescence microscope or flow cytometry and also by PET  
217 and single-photon emission computerized tomography SPECT (US6750037B2, 2004). The work  
218 patented by the research group represented a minimally invasive detection system that limited the  
219 patient trauma and is also inexpensive. However, the use of cytobrush for the collection of cells may  
220 induce sampling error issues and the use of PET-based screening may suffer from lowered accuracy  
221 due to the short decay time of the radioactive substance

222

223

224 **Table 2.** Products and patents for early screening of oral cancer

Name	Company/patents	Collaborators/Inventors	Working Principle	Ref.
GOCCLÉS®	Pierrel S.p.A., (owner of the rights on the GOCCLÉS medical device)	NA	The device is equipped with dental curing lights: Elipar S10 3M ESPE, Led. B Carlo de Giorgi and Optilux 501 Kerr Corporation for illumination of the oral mucosa	(Moro et al., 2015)
OralScan (handheld device)	Sascan Meditech	Sree Chitra Tirunal Institute for Medical Sciences & Technology, Thiruvananthapuram	Diffuse Reflectance, Tissue autofluorescence, cloud-based machine learning, and application of oxygenated hemoglobin (HbO <sub>2</sub> ) absorption maps for biopsy guidance	(DST, 2020)
BerryCare (mobile app)	Atom360	AIIMS Delhi and Bhubaneswar	Screening and segmenting oral cancer via AI-based analysis of images of the cancerous lesion	(BerryCare, 2020)
SalliMark™ OSCC (Salivary kit)	PeriRX, Broomal, Pennsylvania	NA	Salivary biomarker analysis for risk assessment of OSCC	(SalliMark™ OSCC, 2021)

Imaging apparatus	US8364222 B2 (Patent)	Richard James Cook Timothy Frederick Watson Frederic Festy	Selective green wavelength based illumination endoscopic imaging apparatus to image vascular tissues	(US8364222B2, 2013)
The fluorescent and radioactive Detection technique	US6750037 B2 (Patent)	Edwin L. Adair Jeffrey L. Adair	Screening method based on uptake of a complexed compound containing fluorescent molecule (5-ALA, protoporphyrin IX, tetrakis carboxy-phenyl porphine (TCPP), hematoporphyrine derivative, photofrin, and photofrin II) and radioisotope <sup>64</sup> Cu or <sup>67</sup> Cu	(US6750037B2, 2004)

225

226

#### 227 **4. Nano-biosensors: A step towards advanced detection platforms**

228 Nanotechnology is redefining health care strategies and is predicted to have a significant impact on  
229 both the therapeutic and diagnostic sectors in the coming years. Nano biosensors are nano-scale  
230 analytical frameworks that incorporate nano-conjugated biological entities as a transducer for the  
231 identification of specific bio-chemical, or physical analytes in minuscule quantities (Huang et al., 2021).  
232 Nanodiagnosics is being developed as potentially transformative tools for the rapid, convenient, and  
233 cost-effective detection of multiple forms of cancer (colorectal, liver, lung, breast, cervical, prostate,  
234 Leukaemia, and oral) (Ali et al., 2021). Over the last 15 years, researchers have shown tremendous  
235 interest in nano biosensors for the early detection of oral cancer. This is evidenced by the substantial  
236 rise in the number of publications as shown in Pubmed (Fig. 3). The details of different nanotechnology-  
237 based techniques used for the detection of oral carcinoma are summarized in Table 3. The following

238 section will address the details of these relevant nano-based oral cancer screening methods that aid in  
239 the early diagnosis and help clinicians monitor the different phases of oral malignancy.

#### 240 **4.1 Surface-enhanced Raman scattering (SERS)**

241 SERS is a well-known spectroscopic technique with an ultrasensitive spatial resolution that trumps  
242 other traditional techniques. Raman spectroscopy works on the principle of inelastic scattering, in which  
243 molecules in tissues interact with the incident photons and scatter at a different wavelength (Moore et  
244 al., 2018). The Raman spectrum is a 'biochemical fingerprint' of the molecules present in the sample  
245 tissue, which are depicted as molecule-specific bands. For efficient SERS-based detection, free-  
246 electron metals such as Ag, Au, and Cu are taken into consideration. A two-step process generates a  
247 Raman signal. The first step involves incident local field enhancement (by the excited surface plasmon  
248 resonance (SPR) of the plasmonic nanoparticle) leading to the polarization of the adsorbed analyte  
249 molecule and transformation of far-field to near field frequency. The second subsequent step involves  
250 the plasmonic nanostructures working as a transmitting optical antenna wherein Raman shifted  
251 frequencies are transmitted from near field to far-field where the detector is located. The overall  
252 phenomenon of plasmon-enhanced Raman scattering (PERS) demonstrated by Ag and Au  
253 nanomaterials with nano—gaps, tips, holes, voids, grooves, bumps, and ridges can be considered as  
254 excellent SERS-active substrates. Apart from using noble metal nanoparticles as SERS-substrate for  
255 the cancer diagnosis, a new class of active SERS-substrate composed of metal oxides has been  
256 emerging recently wherein, the selective SERS enhancement of metal oxides originates from photon-  
257 induced charge transfer (PICT) process based on Herzberg–Teller selection rule. Apart from the  
258 electromagnetic enhancement mechanism for noble nanoparticles, the remarkable SERS activity, in  
259 these metal oxide NPs is due to the chemical enhancement mechanism. The underlying principle for  
260 efficient electron transition involves the development of a stable charge-transfer complex due to  
261 metastable electronic states and high electrostatic potential, (Lin et al., 2021). (Fig. 4i) Analytical  
262 techniques based on SERS can be classified as label-free or label-mediated. Direct label-free detection  
263 of analytes, requires that the analyte be SERS-active and that its environment be devoid of interfering  
264 molecules to maintain high specificity (Blanco-Formoso and Alvarez-Puebla, 2020). The latter involves  
265 indirectly detecting the analyte by functionalizing the SERS active molecule as a label or a reporter  
266 molecule on the capturing moiety and detecting the reporter's SERS signal as a surrogate measure of  
267 the analyte (Zhang et al., 2019) (Fig. 4ii).



268 (Girish et al., 2014) developed hierarchical TiO<sub>2</sub> nanostructures (needular, bipyramidal, and leaf-like)  
269 and further incorporated Raman active 30 nm silver nanoparticles (AgNPs). The fabricated SERS  
270 catheter could distinguish between normal and malignant tissues in oral cancer patients, SERS spectra  
271 were obtained by the quick absorption of cryosection onto the SERS substrate. It was observed that  
272 the nano-leaf-like structure with numerous 20-150 nm adjacent parallel surfaces could potentially  
273 accommodate a higher number of AgNPs as compared to nano-needular and bipyramidal structures.  
274 The AgNPs in the TiO<sub>2</sub> nano-leaf acted as efficient inter-structure hot-spots and the overall  
275 nanostructure demonstrated the highest enhancement factor of  $\sim 10^6$ . Intense spectral peaks obtained  
276 at 645, 680, 794, 825, 1189, 1326, 1585, and 1618 cm<sup>-1</sup> corresponded to the tumour spectra whereas,  
277 normal tissues with intact collagen from connective tissue displayed peaks at 870 and 950 cm<sup>-1</sup>.  
278 Principal component analysis (PCA) was performed to classify normal and malignant tissues with 97.24  
279 % accuracy within 25-30 minutes for each patient.

280 (Connolly et al., 2016) used silicon-coated silver nanopillar substrates (SERStrates™) procured from  
281 SILMECO (Denmark) for recording SERS signal of salivary and oral cells extracted from patients with  
282 OPC. The manufacturer used a patented technique for the fabrication of the SERS substrate that did  
283 not involve lithography and complex chemistry. SERS spectra were recorded upon the addition of 20 ul  
284 thawed saliva onto the substrate. Distinct spectral signatures (1224, 1275, 1409, and 1417 cm<sup>-1</sup>) were  
285 obtained which reflected molecular and cellular changes associated with oral malignancy. An additional  
286 pilot study was conducted wherein desquamated oral cells from OSCC patients were used. Patients  
287 with cancer demonstrated intense peaks at 1347 and 1543 cm<sup>-1</sup>. The diagnostic accuracy of saliva and  
288 oral cells was reported to be 73% and 60%, respectively, using principal component analysis (PCA)  
289 and linear discriminant analysis (LDA). The study established the potential of SERS as a non-invasive,  
290 label-free technique for detecting OC.

291 In a recent study, (Liu et al., 2021) developed a plasmonic Ag nanocube (AgNC)-based SERS detection  
292 platform that used the principle of nicking endonuclease assisted signal amplification (NESA). AgNCs  
293 with sharp edges and a strong electromagnetic field generated superior-quality SERS hot spots,  
294 enhancing the SERS signal by a factor of  $10^8$ . NESA provides the advantage of recycling the target  
295 analyte such as DNA sequences and micro-RNA which improves the assay sensitivity via signal  
296 amplification. In this study, the authors, combined heated Au electrodes with NESA for the identification  
297 of OC-specific target DNA (tDNA) sequences. The SERS signal was generated by hybridizing the SERS

298 tag (AgNCs/sDNA/4-MBA) on the heated Au electrode which contained the capture DNA (cDNA). In  
299 presence of tDNA, a complex structure containing the SERS nanotag and cDNA/tDNA duplex was  
300 formed which could be cleaved by Nt.BstNBI enzyme. This resulted in a decrease in the SERS signal  
301 which was proportionally related to the tDNA concentration. The nano biosensor demonstrated  
302 excellent sensitivity over a broad linear range of 10 fM –1 nM and a 1 h cleavage time. High selectivity  
303 of the proposed sensor in terms of single- double and complete base mismatch at 1586 cm<sup>-1</sup> were  
304 10.2%, 7.1%. and 2.8% respectively. This shows the application potential of the sensor for early clinical  
305 detection of oral cancer (Fig. 5).

306 (Fălămaş et al., 2020) investigated the amplification of salivary distinctive Raman bands utilizing label-  
307 free, gold nanoparticles mixed with saliva from smoking and non-smoking volunteers and OC patients.  
308 SERS hotspots were formed as a result of the clustering of AuNPs during the air-drying of saliva  
309 samples on glass slides. Using PCA, oral cancer saliva could be distinguished from healthy patients  
310 based on multiple SERS signals ascribed primarily to amino acids and proteins. The study revealed  
311 that the individuals with cancer had a greater overall level of the 2126 cm<sup>-1</sup> band area allocated to  
312 thiocyanate C–N stretching vibrations.

313 A few key points that need to be addressed while utilizing SERS-based sensors for cancer diagnostics  
314 include the surface geometry of the nanoparticle and intimate contact between detecting analyte and  
315 plasmonic SERS substrate. Though reshaping of NP enhances the SERS activity, reliable reproduction  
316 of such particles at a large scale is practically challenging. Another major problem frequently  
317 encountered is the fouling of the SERS substrate in presence of undiluted biological fluids which leads  
318 to lower signal intensity and reduces the reusability of the substrate. Extensive sample pre-treatment is  
319 thus required which makes the detection process cumbersome and less economical. Introduction of  
320 stealth-modification by utilizing zwitterionic molecule and/or nanoshearing mechanisms can enhance  
321 the anti-fouling properties. The use of L-cysteine has been reported to decrease the BSA-mediated  
322 fouling of hybrid SERS substrate (graphene-oxide supported star-like gold nanoparticles) by  
323 80.53%(Panikar et al., 2018). Another report suggests the use of tri-methyl amine N-oxide (TMAO)  
324 derived zwitterionic polymers that aim at improving the hydration and stability of gold chips (Li et al.,  
325 2019). For SERS-based microfluidic systems, another approach for the generation of anti-fouling  
326 SERS-substrate can be nanoshearing that essentially incorporates the tunable alternating current  
327 electrohydrodynamic force (ac-EHD). The developed method enhances the diffusion of biomarkers and

328 SERS-nanoprobes resulting in increased fluid flux and minimized non-specific attachment (Panikar et  
329 al., 2021). However, both these techniques have their limitations and need further optimization to be  
330 implemented as a standard procedure in the development of active SERS substrate.

#### 331 **4.2 Colorimetric nanobiosensor**

332 Colorimetric detection is one of the most widely used optical detection techniques because of its easy  
333 operation, visible radiation, and rapid reading. Free electrons are naturally oscillating in metallic  
334 nanoparticles due to their confinement in a finite region. Absorption happens when metallic NPs are  
335 irradiated with a frequency that equals the inherent natural frequency of the oscillating electrons. It  
336 results in non-propagating oscillations known as the localized surface plasmon resonance (LSPR)  
337 phenomenon (Iarossi et al., 2018). The principle of the colorimetric assays depend on LSPR effect  
338 manifested by several metallic nanoparticles. Colorimetric assays typically proceed via a two-step  
339 method wherein, the first step includes specific recognition of the target analyte with the plasmonic  
340 metal NPs followed by the conversion of these specific recognition events into measurable optical  
341 signals in the visible range. Such interactions can either promote aggregation of the metal NPs or induce  
342 morphological alterations leading to dramatic spectral shifts (blue shift/red shift)(Kailasa et al., 2018).  
343 Typically, metal NPs (Ag, Au, Cu, and Pt) exhibit SPR behavior. Easily-tunable physicochemical  
344 properties and biocompatibility make AuNPs excellent nano sensing probes (Fig. 6i) (Akshaya et al.,  
345 2020). Over the years, this technique has been used to detect several cancers such as leukemia, oral  
346 cancer, lung cancer, exosomes from ovarian cancer, and tumor-specific CD8+ T Cells (Im et al., 2014;  
347 Ribaut et al., 2016; Soler et al., 2018; Valsecchi et al., 2016).

348 (El-Sayed et al., 2005) conjugated AuNPs (35 nm) with monoclonal anti-epidermal growth factor  
349 receptor (anti-EGFR) for the diagnosis of malignant oral epithelial cell lines (HOC 313 clone 8 and HSC  
350 3). The homogenous and specific binding of the EGFR expressed by the cancer cell lines to the Ab-  
351 coated AuNPs increased the  $\lambda_{max}$  at 545 nm. The absorption maximum for normal HaCaT (normal  
352 keratinocyte cell line) which was at 0.01 increased to 0.06 and 0.07 in the case of HOC 313 clone 8  
353 and HSC 3 suggesting an increase in binding efficiency by 600 and 700% respectively. The overall  
354 study illustrates the potential application of plasmonic AuNPs for efficient differentiation between  
355 cancerous and non-cancerous cells by deploying a simple student microscope for SPR scattering-  
356 based imaging as well as with SPR-absorption spectroscopy.

357 (Wang et al., 2013) demonstrated a label-free OC detection method using rose bengal (RB)  
358 conjugated- gold nanorods (GNRs). The RB dye in the nanoprobe with specificity for oral cancer cells  
359 (CAL-27 and Tca8113) induces end-to-end assembly of the GNRs leading to aggregation and  
360 significant shift in the LSPR of the GNRs. The quantitative sensing range for the cancer cells based on  
361 the LSPR shift was  $2.2 \times 10^3$  to  $30.3 \times 10^3$  cells mL<sup>-1</sup> with a detection limit of 2000 cells ml<sup>-1</sup>. Further,  
362 the NIR-absorption of the GNRs probe facilitates optical absorption imaging. The enhancement in NIR-  
363 absorption intensity with an increase in the number of cancer cells can be visualized following irradiation  
364 with a LED light source. A linear correlation was obtained between the absorption intensity of the  
365 nanoprobe and the concentration of cancer cells in the range of 10 and  $50 \times 10^3$  cells/mL. The study  
366 highlights the potential application of the conjugated probe as a sensitive NIR- optical biodiagnostic  
367 tool.

368 It has been previously reported that the enzyme PKC  $\alpha$  which initiates differentiation and proliferation  
369 of malignant cells is hyperactivated in several cases of oral cancer (ZHANG et al., 2005). (Kang et al.,  
370 2010) devised a colorimetric technique for cancer detection employing the interaction between anionic  
371 citrate-functionalized AuNPs (~20 nm) and cationic protein kinase C (PKC)  $\alpha$  - specific peptide with +5  
372 charge resulting in blue color aggregate formation via a non-crosslinking pathway (Fig. 6ii). However,  
373 the presence of the enzyme PKC  $\alpha$  (highly activated in cancer cells) induced phosphorylation of PKC  $\alpha$   
374 -specific peptide and reduces its cationic charge to +3 and declined the aggregation propensity of  
375 AuNPs resulting in the formation of red coloured dispersed AuNPs. The visual colour changes of the  
376 GNPs dispersion following the addition of cell lysates (A546, B16 melanoma, HeLa and CHO) indicated  
377 the concentration of the enzyme PKC  $\alpha$  in the range 0 - 0.2  $\mu$ g/mL. The resultant phosphorylation ratio  
378 mediated by the enzyme was further validated using Matrix-Assisted Laser Desorption - Ionisation-Time  
379 of Flight Mass (MALDI-TOF-MS) spectrometry and western blotting experiments.

380 Though colorimetric-based disease detection is considered most simple, fast, and effective, few  
381 technical drawbacks limit their application for the sensitive determination of disease biomarkers.  
382 Quantitative determination of analyte concentration via visual color change can be subjective and thus  
383 reduces the overall accuracy of the detection system. Apart from this, uncontrollable and self-  
384 aggregation of the nanomaterial in a complex sample matrix may generate false-positive data along  
385 with high background signals (Krishnan, 2022). Recent developments include the incorporation of 2D  
386 materials such as graphene, transition metal oxides, and MXenes along with nanoparticles. These 2D

387 materials can be used as templates for efficient conjugation for the fabrication of hybrid nanomaterials.  
388 Further, they also possess a higher amount of analyte binding sites and enzyme-mimicking activity(Zhu  
389 et al., 2021). These modifications can enhance the sensitivity and selectivity of the analytical system  
390 and can be employed for the detection of several OC markers in the future.

### 391 **4.3 Fluorescent Nanobiosensor**

392 Fluorescence is an optical phenomenon that occurs when photons are absorbed at one wavelength  
393 and emitted at another. Vibrational relaxation and solvent reorganization result in Stokes shift. Following  
394 the absorption of light, the excited electron can be subjected to several photophysical events (radiative  
395 emission, nonradiative emission, vibrational relaxation, intersystem crossing, and internal conversion)  
396 finally, returning to the ground state, while releasing energy in the form of photons at higher  
397 wavelengths. (Fig. 7) Quantum dots (QDs), polymer dots (PDs), and upconversion nanoparticles  
398 (UCNPs) are the most frequently used fluorescent nanoprobe for cancer diagnostic applications  
399 (Berezin and Achilefu, 2010). Conventional fluorophores underperformed for biosensing applications  
400 due to their shorter fluorescence lifetimes, low coefficients of molar absorption, limited absorption  
401 spectra, and sensitivity to photodegradation, resulting in a substantially low signal-to-noise ratio during  
402 bioassays. Fluorescent nano-bio sensors have proven to be a highly sensitive and selective optical  
403 sensing aid for the on-site recognition of biomolecules in a dynamically active living cell (Bhardwaj et  
404 al., 2017).

405 Currently, quantum dots (QD) are being used for *In vitro* and *In vivo* molecular and cellular imaging of  
406 OSCC. QDs have been shown to exhibit a low nonspecific binding, high fluorescence intensity, and  
407 resistance to photobleaching when used for *In vitro* imaging. In this regard, water-soluble thioglycolic  
408 acid stabilized CdTe QDs and peptide-conjugated QD800 have been used for immunofluorescent  
409 labeling of different human OC cells such as Tca8113 and BcaCD885 (Li et al., 2006; Yang et al., 2010;  
410 Zhao et al., 2011). These fluorescent nanoprobe with higher tissue penetration ability can promote  
411 early determination of OC as compared to conventional immunoassays using organic fluorescent dyes.  
412 Further (Zhu et al., 2017) developed a nanoconjugated probe consisting of Ag<sub>2</sub>Se QDs coupled with  
413 cetuximab for targeted imaging and therapy of orthotopic tongue cancer. The developed nanomaterial  
414 with NIR-emission at 900 nm significantly decreased tumour growth and enhanced the survival rate of

415 the nude mice (57.1%). This work suggests the potential applicability of QDs-based nanotheranostics  
416 for OC.

417 Carbon-based nanomaterials such as CQDs have gained notable attention over the years for their  
418 exceptional non-blinking photoluminescence and biocompatible property. Recently(Sri et al., 2018)  
419 used novel zwitterionic CQDs (2-5 nm) for imaging CAL-27 cells with a high quantum yield of ~80%.  
420 The zwitterionic property provides a better advantage over negative and positive CQDs which are  
421 known to have lower fluorescent quantum yield and biocompatibility respectively. Interestingly the  
422 research group reported the low toxicity even at exceptionally high concentrations of (1600  $\mu\text{g mL}^{-1}$ )  
423 CQDs used in the experimental procedure. Another study demonstrated the use of UCNPs for Förster  
424 resonance energy transfer (FRET)- based detection of MMP-2 (matrix metalloprotease-2) in OC cells  
425 (CAL-27). The UCNP@p-QD nanoprobe consisted of  $\text{SiO}_2$ -coated UCNPs and were further conjugated  
426 with  $\text{CuInS}_2/\text{ZnS}$  core/ shell QDs via MMP-2 sensitive peptide. The mechanism of detection was based  
427 on MMP-2 mediated digestion of the peptide which released the bound QDs decreasing the  
428 fluorescence emission intensity at 600 nm and simultaneously increasing the fluorescence emission  
429 intensity from the UCNPs (475 nm). The detection of MMP-2 could be performed on a broad range of  
430  $10^6$ -1  $\text{pg mL}^{-1}$  (Chan et al., 2018).

431 Song and colleagues, developed a 3D network of carbon nanotubes (3DN-CNTs) on a Silicon pillar  
432 substrate to detect the oral cancer biomarker cytokeratin-19 antigen (CYFRA 21-1) in clinical samples  
433 (Fig. 8). To ensure structural stability, the template was coated with aluminum oxide ( $\text{Al}_2\text{O}_3$ ) and then  
434 modified with an amino silane reagent to generate a SAM for antibody immobilization. After incubation  
435 of 3DN-CNTs with the target analyte, the enhanced fluorescence signal generated from Alexa 558-  
436 tagged detector Ab was used for the quantitative detection of CYFRA 21- 1 in the concentration range  
437 of 0.1-  $10^3 \text{ ng mL}^{-1}$  with a detection limit of  $0.5 \text{ ng mL}^{-1}$ . The fabricated sensor system had 20 times  
438 higher sensitivity as compared to the conventional sandwich-type ELISA. It was suggested that 3DN-  
439 CNTs provided a larger surface area and higher number of binding sites for capture-Ab binding as well  
440 as the hierarchical architecture of 3DN-CNTs eased the accessibility of biomolecules through the  
441 ordered pathways of 3DN-CNTs templates. (Song et al., 2018).

442 (Li et al., 2020) recently developed non-invasive nano-graphene oxide (NGO)-based  
443 immunofluorescent probe (NGO-BBN-AF750) consisting of graphene oxide nanoclusters functionalized

444 with AF750-6Ahx-Sta-BBN GRPR-specific peptides via hydrogen and  $\pi$ - $\pi$  bonds (Fig. 6c). Taking the  
445 advantage of the fact that oral cancer cells overexpress gastrin-releasing peptide receptor (GRPR),  
446 they reported ~ 96% cellular uptake of the nanocluster in HSC-3 cells within 60 min. When compared  
447 to normal oral mucous tissues, the malignant tissues exhibited a high-intensity fluorescent signal (2.5-  
448 fold increase), indicating a high affinity and specificity for HSC-3 cells. These findings indicate that nano-  
449 GO with a higher amount of functional group (carboxyl, hydroxyl, and oxygen), and enhanced  
450 permeability and retention (EPR) effect can be fine-tuned for suitable biosensing applications.

451 (Wu et al., 2021) recently published a study focusing on the detection of exosomes in saliva as a  
452 potential biomarker associated with OC, among other malignancies. They fabricated a fluorescent nano  
453 biosensor that incorporates magnetic and fluorescence bio-probes (MFBPs) coated with QDs for signal  
454 amplification. Aptamer functionalized magnetic microspheres were designed for the specific binding of  
455 CD63 proteins on exosomes. Under experimentally optimized conditions, 0.25 mg mL<sup>-1</sup> of the  
456 synthesized nanoprobe interacted with the target sample for 30 min and promoted the reshaping of  
457 aptamers and release of QDs-conjugated DNA concatemers as a fluorescent signal. The one-step  
458 signal amplification procedure was based on the concept of "one exosome numerous QDs". The  
459 analytical attributes of the sensor included an exosome detection range of 500 - 10<sup>5</sup> particles/ $\mu$ L and a  
460 detection limit of 500 particles/ $\mu$ L. The intra-and inter-assay variability was reported to be 3.09% and  
461 5.43% respectively.

462 Over the years, molecular imprinting technology has gained enormous attention. The development of  
463 molecular imprinted fluorescent nanoparticle (MIFN) sensors has shown promising results in the  
464 detection of cancer protein markers such as Interleukin-2 (IL-2) in serum (Piloto et al., 2020). The  
465 process involves the incorporation of fluorescent nanoparticles such as QDs, CQDs, and/or UCNPs as  
466 a fluorescent source into MIP specific recognition unit. The hybrid system fabricated via the sol-gel  
467 method, free-radical polymerization, and reverse microemulsion are reported to have high sensitivity,  
468 selectivity, and stability. However, several parameters such as the amount of monomer/crosslinker that  
469 can affect the MIP layer thickness and subsequently the luminescence efficiency of the fluorescent  
470 probe needs to be carefully optimized to successfully implement these techniques for OC detection.  
471 (Wang et al., 2020)

472

#### 473 4.4 Electrochemical nano-biosensor

474 Electrochemical nano biosensors are deemed promising due to their simplicity, rapidity, and cost-  
475 effectiveness, as well as their potential to be easily coupled with electronics for mass production (Lin et  
476 al., 2020). Three integrated constituents are required to design an electrochemical biosensor system:  
477 (i) a recognition element that interacts with the analyte; (ii) a signal transducer that converts the analyte-  
478 biomolecular layer interaction into a measurable signal; and (iii) an electronic data management system  
479 (Fig. 9) (Topkaya et al., 2016). Various NPs such as CNTs, graphene oxide, zinc oxide, AuNPs,  
480 AgNPs, zirconium oxide, etc. are incorporated in electrochemical nano biosensors to improve electron  
481 transfer efficiency between the electrodes and analyte. It improves the immunosensor's function by  
482 increasing its sensitivity and stability, enabling ultrasensitive detection of multiple OC biomarkers  
483 (Hasanzadeh et al., 2017). Numerous electro-analytical techniques are used to quantify these signals,  
484 including voltammetry, amperometry, field-effect transistors, impedimetric methods,  
485 electrochemiluminescent assays, and electrical approaches based on nanochannel/nanopore  
486 structures (Naresh and Lee, 2021).

487 For the label-free identification of salivary IL-8, an electrochemical immunosensor was devised using  
488 an Indium tin oxide (ITO) electrode modified with AuNPs/r-GO nanocomposite. The AuNPs here work  
489 as a conduction pocket for smooth charge diffusion between the reaction mixtures to the surface of the  
490 electrode. The  $\pi$ -conjugated r-GO facilitates better channeling of electrons as compared to GO. The  
491 AuNPs/r-GO electrode has the highest redox current due to the synergistic role of AuNPs and r-GO in  
492 the electron transfer process. 1 ng/mL of the analyte was incubated with Anti-IL8/AuNPs-rGO/ITO  
493 nanoprobe and quantified using Differential Pulse Voltammetry (DPV). The fabricated nanosensor  
494 demonstrated a high degree of specificity, sensitivity, and rapid detection within 9 minutes (Verma et  
495 al., 2017). The analytical performance of the fabricated sensor in terms of recovery was 94.15% for the  
496 IL-8 spiked saliva sample. Remarkable is the data concerning the long-term stability, with a 94.3%  
497 performance retainment after three months and a 91.8% stability after four months of dry storage.

498 Ding and colleagues reported the first use of vertically aligned carbon nanotube arrays (VANTA)  
499 arranged in 2D interdigitated electrodes (IDEs) for the electrochemical sensing of CIP2A- an oral  
500 cancer-related oncoprotein. The high height: width aspect ratio (3:1) VANTA incorporated IDEs utilized  
501 here, possessed unique light absorption, chemical inertness, and electrical conductive properties along



502 with high surface area. Ab was immobilized on VANTAs, and samples were analyzed using  
503 electrochemical impedance spectroscopy (EIS). The fabricated VANTA-IDE sensor had lower sensing  
504 ranges (5-400 pg ml<sup>-1</sup> in PBS and 1-100 pg ml<sup>-1</sup> in saliva) as compared to the commercial ELISA Kit.  
505 and also minimal interference in the presence of a high concentration of BSA. It must be noted that the  
506 nature of the interaction between the antigen and the immobilized Ab on the VANTA varied across  
507 different reaction matrices. Lower dissociation constant ( $K_D$ ) values 13 pg ml<sup>-1</sup> obtained from Hill  
508 equation in case of saliva as compared to PBS indicated a positive cooperative mode of Ag-Ab binding  
509 on the sensing platform. Overall a detection limit of 0.24 pg/mL was obtained in saliva specimen within  
510 a short response time of 35 minutes (Ding et al., 2018).

511 Silver molybdate ( $\beta$ -Ag<sub>2</sub>MoO<sub>4</sub>) nanoparticles on ITO-coated glass substrate have recently been utilized  
512 for the label-free detection of salivary IL-8 (Fig. 10). Ab was covalently attached to the immunoelectrode  
513 surface via EDC-NHS bioconjugation chemistry. The Ag<sub>2</sub>MoO<sub>4</sub>/ITO electrode provided an optimal  
514 microenvironment for different biomolecular interactions required for the sensing of IL-8. The higher Ag  
515 valences and greater O-Ag-O bond length provided a biocompatible environment for the immobilization  
516 of IL-8 Ab on the AgO sites. DPV method was used to assess the IL-8 antigen's sensing response at a  
517 wide range of concentrations (1 fg mL<sup>-1</sup> to 40 ng mL<sup>-1</sup>) within a response time of 10 minutes, the  
518 BSA/anti-IL-8-/Ag<sub>2</sub>MoO<sub>4</sub>/ITO immunosensor was able to detect IL-8 with a detection limit of 90 pg/mL.  
519 Additionally, the fabricated electrodes demonstrated the same DPV measurements over a period of 4  
520 weeks when stored at 4 °C. (Pachauri et al., 2020).

521 (Zhang et al., 2015) used a top-down lithographic approach to fabricate a monocrystalline silicon  
522 nanowire (SiNW) field-effect transistor (FET) sensor device for the label-free, multiplexed detection of  
523 two common oral cancer biomarkers, IL-8 and TNF- $\alpha$  (Fig. 7c). The high surface-to-volume ratio of the  
524 SiNW enables efficient biomolecular interaction with high affinity, this, in turn, enhances the sensitivity  
525 of the analytical system. The Ab of both the biomarkers were immobilized on the SiNW array surfaces  
526 and allowed to interact with a specific Ag Considering the different isoelectric points of the detecting  
527 analytes, utilization of low ionic strength 1x PBS (pH-8.5) as the measuring buffer took into  
528 consideration the Debye-screening length and resulted in accumulation of carrier on positively charged  
529 n-doped SiNW in case of IL-8 binding and depletion of SiNW bulk for TNF- $\alpha$  binding. The alterations in  
530 the resistance of the FET biosensor resulting in a signal generation were recorded both before and after

531 target binding. The LOD for both biomarkers in was reported to 10 fg ml<sup>-1</sup> in buffer and 100 fg ml<sup>-1</sup> in  
532 artificial saliva

533 (Tiwari et al., 2017) reported easy synthesis of lanthanum oxide La(OH)<sub>3</sub> NPs by co-precipitation  
534 method. The enhanced electron-transfer ability and availability of a large number of binding sites for  
535 biomolecules exhibited by La(OH)<sub>3</sub> make them suitable for electrochemical sensing applications. The  
536 study suggests the use of non-toxic L-cysteine as the capping agent instead of the most frequently used  
537 3-aminopropyl triethoxysilane (APTES) or olylamine (Fig. 11). The detection of CYFRA 21-1 in the  
538 range of (0.001-10 ng mL<sup>-1</sup>) was performed at an optimized pH of 7.0. Higher and lower pH denatured  
539 the bound antibody due to their interaction with OH<sup>-</sup> and H<sup>+</sup> ions. The decrease in the peak current upon  
540 binding of the target analyte with increasing concentration was due to the formation of an electrically  
541 insulating Ag-ab complex that hindered the electron transfer between the BSA/anti-Cyfra-21-1/Cys-  
542 La(OH)<sub>3</sub>/ITO immuno electrode and the electrolyte species [Fe(CN)<sub>6</sub>]<sup>3-/4-</sup> present in the reaction buffer.  
543 The fabricated sensor exhibited a low detection of 0.001 ng mL<sup>-1</sup> and the response time was 5 min.  
544 Additionally, the immune electrodes had a shelf-life of 13 weeks when stored at 4 °C.

545 Salivary CYFRA 21-1 has been frequently reported to be expressed at high levels in the case of OC.  
546 Extensive research has been conducted over the past few years for the sensing of CYFRA-21-1 via  
547 electrochemical methods by Kumar and his colleagues. The architectural framework has been slightly  
548 modified across the different studies. Different NPs (ZrO<sub>2</sub>, nHfO<sub>2</sub>, and TiO<sub>2</sub>) have been  
549 electrophoretically deposited onto APTES treated ITO coated glass electrode followed by conjugation  
550 with mAb -anti-CYFRA-21-1 and BSA as the blocking agent. (Kumar et al., 2020; Kumar et al., 2018b;  
551 Kumar et al., 2016a; Kumar et al., 2015). The measurements were performed in PBS buffer at pH-7  
552 containing [Fe(CN)<sub>6</sub>]<sup>3-/4-</sup> as the electrolyte species. The linear calibration curve for an increase in anodic  
553 peak current/ charge transfer resistance vs. different concentrations of CYFRA-21-1 was plotted to  
554 further analyze the analytical performance of the respective electrochemical sensors. The authors  
555 suggest that the increase in the peak current was due to tyrosine amino acid residues in the CYFRA-  
556 21-1 molecules that undergo oxidation and channel a higher number of released electrons. Additionally,  
557 conformational alterations following Ag-Ab binding can facilitate the easy transfer of electrons to the  
558 electrode. ZrO<sub>2</sub> nanodots were highly efficient as they had a broad CYFRA 21-1 detection range of 0.5-  
559 50 ng mL<sup>-1</sup> with a detection limit of 0.5 ng mL<sup>-1</sup> as compared to nHfO<sub>2</sub> (2-18 ng mL<sup>-1</sup>; LOD- 0.21 ng mL<sup>-1</sup>

560 <sup>1</sup>) and TiO<sub>2</sub> (0 -12 ng mL<sup>-1</sup>; LOD-0.24 ng mL<sup>-1</sup>). However, immuno electrode .with nHfO<sub>2</sub> showed the  
561 highest shelf-life of 8 weeks when stored at 4 °C.

562 In a separate study, (Malhotra et al., 2010) developed an ultrasensitive Single-Walled Nanotube  
563 (SWNT) forest-based immunosensor for the detection of secreted IL-6 from different *in vitro* OC cell  
564 preparations. The working mechanism of the fabricated sensor was based on the binding of capture  
565 Ab-coated SWNT forests with IL-6 and further interaction with a multi-labeled conjugate system. The  
566 conjugated complex consisted of secondary Ab that was attached with 106 HRP per 100 nm of multi-  
567 Walled Nanotube (MWNT). The signal generated was measured using rotating disc amperometry at  
568 3000 rpm using 1mM hydroquinone as a mediator. The authors suggest that this particular multi-labeling  
569 strategy exhibits a 60-fold lower detection limit as compared to treating with Ab2biotin-streptavidin-HRP,  
570 providing 14 to 16 HRPs on one Ab. However, the conjugated system had a short shelf life of 10 days  
571 and also required 1h of incubation with the target analyte for the generation of a readable signal.

572 From the above studies, it can be understood that modified nanomaterials on the electrochemical  
573 sensor surface enhance the interfacial adsorption of the detecting analytes as well as improves the  
574 overall electron-transfer kinetics. Modifying the nanoarchitecture in electrochemical sensing platforms  
575 can significantly improve the detection of crucial biomarkers such as miRNA for OC diagnosis (Masud  
576 et al., 2019b). Different nanoforms of graphene such as nanoribbons, and nanoflower with excellent  
577 conductivity and electron-transfer rates have shown significant potential in the development of  
578 graphene-based electrochemical biosensors (Ismail et al., 2017). However, the requirement of intricate  
579 modifications of the nanoparticles compromises the reproducibility of the sensing system. Nanomaterial  
580 aggregation and flaking hamper the stability and shelf-life of the sensors. Considering the fluctuating  
581 sensor-to-sensor batch reproducibility, mass production of these sensors is still not a feasible option.  
582 These issues have recently been circumvented by the incorporation of sol-gel and ceramics along with  
583 nanoparticles(Ferrag and Kerman, 2020). Another major challenge that needs to be addressed for real  
584 sample analysis is the use of raw samples which increases the possibilities of non-specific adsorption  
585 of unwanted molecules. Extensive sample dilution, to minimize the matrix effect has been a major  
586 roadblock against deploying these electrochemical sensors as POC devices.

#### 587 **4.5 Other Nanomaterials based approaches for oral cancer diagnosis**

##### 588 **Optical coherence tomography (OCT)**

589 OCT uses infrared light to provide cross-sectional architectural images of epithelial layers and basement  
590 membranes, making it ideal for early detection of OSCC (Green et al., 2016). It has a resolution of  
591 approximately 10  $\mu\text{m}$ , which is better than existing MRI, CT, and ultrasound techniques (Pande et al.,  
592 2016; Troutman et al., 2007). AuNPs are effective OCT contrast agents as they are biocompatible and  
593 capable of generating LSPR at near-infrared wavelengths. For instance, AuNPs (71 nm) conjugated  
594 with EGFR mAb was used as an effective OCT-contrast agent for imaging oral dysplasia in a hamster  
595 model. The reflected light from the immobilized mirror and each scattered particle from the specimen  
596 generated a signal that was detected by the spectrometer. Microneedles and ultrasound were used to  
597 deliver AuNPs which improved OCT penetration and increased the contrast level by approximately  
598 150% (Kim et al., 2009). It must be noted that in the case of OCT, NPs with different anisotropic  
599 geometries have been used to increase the imaging contrast. However, specific NPs geometries  
600 wherein the plasmon band are close to the central wavelength of the OCT source lead to very strong  
601 interaction with the plasmon resonance and enhance the backscattered light. Recently it was observed  
602 that branched-AuNPs with three distinct plasmonic peaks are highly efficient as compared to nanorods  
603 matching only one central wavelength, or nanospheres that scatter light only if their size is big (bulk  
604 effect) (Ponce de León et al., 2012).

### 605 **Magnetic resonance imaging**

606 MRI has been effective for assessing primary tumors, bone invasion, and delineating tumor margins  
607 during surgery. Contrary to conventional MRI contrast agents, Nano-contrast agents can identify unique  
608 biomarkers with increased blood circulation half-life, displaying excellent MRI contrast features (Fig 12i).  
609 Oral cancers have also been examined using a nano-contrast agent containing a combination of folate-  
610 conjugated chitosan and magnetic poly (lactide-coglycolide) (PLGA) NPs.

611 Asifkhan and colleagues synthesized an MRI contrast agent by combining folate-conjugated chitosan  
612 and magnetic poly (lactide-coglycolide) (PLGA) NPs having a magnetization of 35 emu/g. Oral cancer  
613 cells with folate receptors showed enhanced nanoparticle uptake by receptor-mediated endocytosis  
614 and were measured via mean fluorescence intensity (MFI). The T2 relaxation time was decreased, with  
615 an increase in the NPs relaxivity ( $232.7 \text{ mM}^{-1} \text{ S}^{-1}$ ), resulting in high contrast cancer images as compared  
616 to commercially available contrast agent ferumoxytol which has a relaxivity of  $85 \text{ mM}^{-1} \text{ S}^{-1}$  (Shanavas  
617 et al., 2017).

### 619 **Photoacoustic imaging**

620 Photoacoustic imaging technique generates ultrasonic transients from tissues by employing short laser  
621 pulses resulting in transient thermoelastic expansions. The ultrasonic transducer captures  
622 photoacoustic waves and converts them into photoacoustic images based on their arrival times.  
623 Photoacoustic imaging, in comparison to traditional optical imaging, provides a greater imaging depth  
624 and a higher spatial resolution resulting in enhanced imaging (Fig 12ii). Several exogenous contrast  
625 agents have been used to enhance the performance of the imaging modality, however, AuNPs are  
626 preferred because of their ability to conjugate proteins and generate higher photoacoustic imaging  
627 signals (Bayer et al., 2017; Zhang et al., 2016).

628 (Luke et al., 2014) pioneered the use of ultrasound-guided spectroscopic photoacoustic imaging (sPA)  
629 to detect lymph node micrometastases in a metastatic OSCC murine model. The study demonstrated  
630 that 40 nm anti-EGFR antibody conjugated, molecularly activated plasmonic nanosensors (MAPS)  
631 shifted their  $\lambda_{\max}$  towards the NIR region. Due to the molecular interactions between EGFR-targeted  
632 MAPS and tumor cells, its injection into the lymph nodes resulted in an increased spectroscopic signal.  
633 Furthermore, a single peritumoral injection of MAPS allowed for the rapid detection of micrometastases  
634 as tiny as 50  $\mu\text{m}$ , within 30 minutes. The sensitivity and specificity of the developed system were  
635 reported to be 100% and 87% respectively. the sPA is considered to be better compared to  
636 commercially available radiographic techniques such as PET which has nearly 50% sensitivity.  
637 However, the work faces major challenge toward clinical acceptance due to the use of large 40 nm  
638 AuNPs that reduces systemic clearance and induces long term physiological toxicity.

### 639 **Diffusion reflection imaging**

640 In this technique, the white light that enters the tissue is either absorbed or transmitted, the remaining  
641 undergoes repeated elastic scattering and becomes diffusely reflected. The reflected light is strongly  
642 influenced by cytological and morphological deformities that occur during cancer formation, such as  
643 nuclear size and collagen content variation, changes in ECM structure, epithelial thickness, and blood  
644 flow (Stephen et al., 2013). It aids in determining surgical margins and can be used to distinguish  
645 between normal mucosa, OPMD, and oral cancer (Chen et al., 2015).

646 (Ankri et al., 2016) attached GNRs (25 x 11 nm) to EGFR mAb and used diffusion reflection imaging to  
647 examine the margins of human OSCC specimens. The uptake of the Ab-conjugated GNRs was nearly

648 21.8  $\mu\text{g}$  for cancer whereas for negative control cells the cellular uptake obtained was 0.2  $\mu\text{g}$ . After 15  
649 minutes of incubation with 50  $\mu\text{l}$  GNR, tissue sections were imaged using the hyperspectral imaging  
650 system. Air scanning electron microscopy (air-SEM) visualized that the GNRs-EGFR propagated 1mm  
651 between the tumor and the healthy tissue, indicating tumor margins of 1mm. The sensitivity and  
652 specificity of the diagnostic mode in terms of differentiating *ex-vivo* positive and negative resected  
653 tumour margins as tested on mice-model were observed to be 82 and 75%.

#### 654 **Modified immunoassay**

655 In our previous work, we have developed nano-ELISA for salivary osteopontin (OPN) detection,  
656 wherein, multiple antibodies attached to single gold nanorods (GNRs) generated an amplified response  
657 in the presence of a low concentration of OPN as compared to classical ELISA. The GNRs-in the nano-  
658 ELISA acted as a concentrator of signaling Ab and amplified the signals upon antigen binding (Fig. 13i).  
659 The GNR-based nano-ELISA had increased sensitivity and had a detection limit of 0.02  $\text{ng mL}^{-1}$ ,  
660 whereas, for conventional ELISA, it was 0.14  $\text{ng mL}^{-1}$ . The same study was also validated using gold  
661 nanospheres (AuNS) wherein the detection limit for OPN was 0.03  $\text{ng mL}^{-1}$ . The analytical performance  
662 of the modified nano-ELISA system validated on OPN-spiked whole and simulated saliva showed good  
663 recovery rates of 96.25-98.7%. The study emphasizes the critical necessity of employing different  
664 anisotropic nanoparticles with a large surface area that not only enhances the sensitivity but also  
665 reduces the wastage of excess unreacted Ab making the sensing system more economical.  
666 (Chakraborty et al., 2018).

#### 667 **Microfluidics**

668 Microfluidic systems frequently referred to as "Lab-on-a-chip," can serve as a miniature automated  
669 version of integrated experimental operations on a single device (Ziober et al., 2008a). Currently, this  
670 technique is used in a variety of biomedical applications, including disease detection, drug delivery, and  
671 drug discovery. Processing minuscule biopsy samples or identifying tumor biomarkers in biological  
672 fluids promptly while maintaining high repeatability and consistency boosts its therapeutic potential for  
673 cancer management (Fig.13ii). In a recent report, polythiophene was used as bulk heterojunctions for  
674 spin-coating of organic photodetectors (OPDs) and incorporation onto microfluidic chips made of  
675 poly(methyl methacrylate) (PMMA). The immunogold-silver assay (IGSA) was used to detect various  
676 salivary analytes, including MMP-8, IL-1, and IL-8, using the immunoreaction compartment of the

677 microfluidic chip. The chip's immuno-reaction compartment was oriented with the OPDs to measure the  
 678 photocurrents from the light absorbed ( $\lambda_{650}$ ) by the IGSA. This diagnostic platform was cost-effective  
 679 and provided multiplexity in the early diagnosis of OC (Dong and Pires, 2017).

680 **Table 3.** Nanotechnology-based methods used for the diagnosis of oral carcinoma

Technique	Nano biosensor component	Sample	Biomarker	Range of detection	Limit of Detection/ Sensitivity	Response Time (in min.)	Ref.
SERS	Ag-TiO <sub>2</sub> SERS substrate	OSCC tissue	-	100 $\mu$ M to 1 nM	1 nM	15–20	(Girish et al., 2014)
	silver-coated silicon nanopillar substrates (SERStrates™)	Saliva	-	-	89%	-	(Connolly et al., 2016)
	Nt.BstNBI/AgNCs /HAuE	Saliva	-	10 fM - 1 nM	3.1 fM	60	(Liu et al., 2021)
	AuNP/DNA probe/MGITC	Saliva	S100P mRNA	Free solution- 1.2–200 nM; Vertical flow chip-, 10–100 mM	Free solution - 1.1 nM ; Vertical flow chip - 10 nM	53	(Han et al., 2019)
Differential pulse voltammetry	anti-CYFRA-21–1/ APTES/ ZrO <sub>2</sub> -rGO/ITO	Saliva	Cyfra-21-1	2–22 ng mL <sup>-1</sup>	0.122 ng mL <sup>-1</sup>	16	(Kumar et al., 2016b)

	BSA/anti-CYFRA- 21-1/Ser/ nZrO <sub>2</sub> /ITO	Saliva	Cyfra-21-1	0.01–29 ng mL <sup>-1</sup>	0.01 ng mL <sup>-1</sup>	6	(Kumar et al., 2016a)
	BSA/anti-Cyfra-21-1/Cys-La(OH) <sub>3</sub> /ITO	Artificial Saliva	Cyfra-21-1	0.001–10.2 ng mL <sup>-1</sup>	0.001 ng mL <sup>-1</sup>	5	(Tiwari et al., 2017)
	BSA/anti-CYFRA- 21-1/APTES/nHfO <sub>2</sub> @RGO/ITO	Saliva	Cyfra-21-1	0–30 ng mL <sup>-1</sup>	0.16 ng mL <sup>-1</sup>	15	(Kumar et al., 2018a)
	Anti-IL-8/AuNPsrGO/ITO	Saliva	IL-8	500 fg mL <sup>-1</sup> – 4 ng mL <sup>-1</sup>	72.73 pg mL <sup>-1</sup>	9	(Verma et al., 2017)
	BSA/Anti-IL-8/β-Ag <sub>2</sub> MoO <sub>4</sub> /ITO	Saliva	IL-8	1 fg mL <sup>-1</sup> – 40 ng mL <sup>-1</sup>	90 pg mL <sup>-1</sup>	10	(Pachauri et al., 2020)
	Anti-IL8/ZnO–rGO/ITO	Saliva	IL-8	100 fg mL <sup>-1</sup> – 5 ng mL <sup>-1</sup>	51.53 pg mL <sup>-1</sup>	10	(Verma and Singh, 2019)
Square wave voltammetry	Anti-IL6/GO/GCE	Serum	IL-6	0.002–20 ng mL <sup>-1</sup>	1 pg mL <sup>-1</sup>	-	(Li and Yang, 2011)



Amperometry	Anti-IL8/GSH-AuNP/pyrolytic graphite	Serum	IL8	1–500 fg mL <sup>-1</sup>	1 fg mL <sup>-1</sup>	-	(Munge et al., 2011)
	8 AuNP array	Serum	IL-6, IL-8, VEGF, and VEGF-C	0-1000 fg mL <sup>-1</sup>	IL-6, IL-8, -10 and 15 fg mL <sup>-1</sup> VEGF, and VEGF-C -8 and -60 fg mL <sup>-1</sup> respectively	50	(Malhotra et al., 2012)
	anti-IL6/SWNTs/graphite	HNSCC cell lines/ Serum	IL-6	0.5–30 pg mL <sup>-1</sup>	calf serum-0.5 pg mL <sup>-1</sup>	60	(Malhotra et al., 2010)
Cyclic voltammetry	anti-CYFRA-21-1/APTES/ZrO <sub>2</sub> /ITO	Saliva	Cyfra-21-1	2–16 ng mL <sup>-1</sup>	0.08 ng mL <sup>-1</sup>	20	(Kumar et al., 2015)
	BSA/anti-CYFRA-21-1/APTES/nHfO <sub>2</sub> /ITO	Saliva	Cyfra-21-1	2–18 ng mL <sup>-1</sup>	0.21 ng mL <sup>-1</sup>	15	(Kumar et al., 2016a)
Electrochemical impedance spectroscopy	BSA/anti-CYFRA-21 –	Saliva	Cyfra-21-1	0–12 ng mL <sup>-1</sup>	0.24 ng mL <sup>-1</sup>	35	(Kumar et al., 2018b)

	1/APTES/TiO <sub>2</sub> /ITO						
	Anti CIP2A/VANTAGIDE	Saliva	CIP2A	1–100 pg mL <sup>-1</sup>	0.24 pg mL <sup>-1</sup>	35	(Ding et al., 2018)
Field effect transistor	Array of SiNW sensor	Artificial saliva	IL-8 and TNF-α	1 ng mL <sup>-1</sup> – 1 fg mL <sup>-1</sup>	Buffer, saliva -10 and 100 fg mL <sup>-1</sup> respectively	120	(Zhang et al., 2015)
Fluorescence	Anti-Cyfra-21-1/Al <sub>2</sub> O <sub>3</sub> -coated 3DN-CNTs	Saliva	Cyfra-21-1	1–1000 ng mL <sup>-1</sup>	0.5 ng mL <sup>-1</sup>	30	(Song et al., 2018)
	MFBP coated with QDs	Saliva	CD63	500 to 10 <sup>5</sup> particles/μL	500 particles/μL	30	(Wu et al., 2021)
	NGO-BBN-AF750	HSC-3 cell line	GRPR	-	-	40	(Li et al., 2020)
Colorimetric	RB-GNR	OSCC cell line CAL27 and Tca8113	-	2.2 X 10 <sup>3</sup> to 30.3 X 10 <sup>3</sup> cells/mL	2000 cells/mL	-	(Wang et al., 2013)

	Au NP	-	PKC- $\alpha$	0-0.2 $\mu\text{g mL}^{-1}$	-	-	(Kang et al., 2010)
	Au NP	HOC 313 clone 8 and HSC 3 cell lines	-	-	-	-	(El-Sayed et al., 2005)
Modified Immunoassay	Au nanorods; Au nanosphere	Saliva	osteopontin	0.31-20 $\text{ng mL}^{-1}$	AuNRs-0.02 $\text{ng mL}^{-1}$ ; AuNPs-0.03 $\text{ng mL}^{-1}$	-	(Chakraborty et al., 2018)
Optical coherence tomography (OCT)	AuNP/EGFR	cheek pouch tissues	-	-	-	-	(Kim et al., 2009)
MRI	fol-cht coated magnetic PLGA (fcMagP)	KB oral cancer cells	-	-	-	-	(Shanavas et al., 2017)
Photoacoustic imaging	molecularly activated plasmonic nanosensors (MAPS)	Lymph node tissue	EGFR	-	-	30	(Luke et al., 2014)

Diffusion AuNRs OSCC EGFR - - 15 (Ankri et  
reflection tissue al., 2016)  
imaging

681

## 682 **5. Bench-to-bedside transition of nanosensing platforms: challenges and** 683 **limitations**

684 Nanotechnology has tremendous promise in the field of oral cancer diagnostics, but so far only a few  
685 studies have been translated into commercial prototypes. The biosensing technologies require further  
686 fine-tuning to support clinical applications (Singhal et al., 2021). The specificity and sensitivity of a  
687 biosensing technique must be evaluated in the form of a receiver operating characteristic (ROC) curve  
688 before it can be applied to clinical practices. The efficacy can be represented as an area under the  
689 curve (AUC) and a minimum value of 0.8 is essential for successful adoption (Brocklehurst and Speight,  
690 2018). Several bottlenecks such as ethical aspects, low sample requirements, portability, sensitivity,  
691 and automation require immediate redressal. This section discusses the challenges involved with each  
692 of the nano biosensing methods (Noah and Ndangili, 2019).

693 The enhancement factors for SERS substrates are not reproducible. Numerous studies have indicated  
694 inconsistent results where the same substrate was fabricated and used in multiple laboratories.  
695 Furthermore, non-specific binding and interfering species can obscure the SERS signal from the target  
696 analyte (Bantz et al., 2011).

697 In fluorescent nano biosensors, it can be challenging to achieve a lower limit of detection due to the  
698 restricted extinction coefficients or quantum yields of organic dyes and a lower ratio of dye-to-reporter  
699 molecule labeling (Zhong, 2009).

700 Colorimetric assays suffer from limited accuracy due to the hindrance caused by the matrix effect of the  
701 sample, which makes it difficult to convert minute signals into a colour readout (Yu et al., 2021).  
702 Additionally, stability issues due to in high ionic strength of the experimental samples result in non-  
703 specific nanoparticle aggregation, triggering associated colour change (Aldewachi et al., 2018).

704 The primary issue of electrochemical nano biosensors is the miniaturization of the device into a portable  
705 system for POC diagnosis (Mani et al., 2021). Other constraints include the complex fabrication

706 techniques, lack of mechanistic transparency, and stability issues while using single or reusable  
707 electrodes (Shandilya et al., 2019).

708 Several *in vivo* nano-contrast agents are commonly used for sentinel lymph node biopsy, tumor contour  
709 imaging, and, enhanced visualization of lesions (Kircher et al., 2003; Kobayashi et al., 2004). It has  
710 been reported previously that the contrasting efficiency of MRI nanoprobe such as that of  
711 superparamagnetic iron oxide NPs (SPIONs) is affected by the biofouling of the NPs surface due to  
712 protein corona formation (Amiri et al., 2013, Gao et al, 2016). Anti-biofouling coatings such as  
713 polyethylene glycol (PEG), zwitterions, and fluorinated coatings can suppress the unwanted protein  
714 corona formation and enhance the targeting ability of the nanoprobe (Sanchez-Cano et al., 2020).  
715 Additionally, arrays of nano-cantilevers, tubes, and wires are emerging as promising tools for the  
716 detection of multiple serum markers. A Low-signal-to-noise ratio due to interference from serum matrix  
717 limits the applicability of such nano sensing platforms (Ferrari, 2005).

718 Therefore, a prospective nano biosensor for early diagnosis and monitoring of OC is still in its infancy.  
719 Significant research is required to improve them and expand their application from the bench- to-  
720 bedside.

## 721 **6. Conclusion and future work**

722 Diagnostics, along with vaccines and therapeutics, form the essential and often overlooked "third  
723 imperative" of reducing any disease burden. Early diagnosis regarding oral carcinoma, can be a real  
724 game-changer as it critically determines a patient's quality of life. In this article, we have attempted to  
725 cover different diagnostic innovations and methods that are aimed at screening oral carcinoma.  
726 Achieving better and more feasible nanosensors that are portable, reusable, and most importantly non-  
727 invasive can be highly beneficial for OC detection. Technical modifications of the current nanosensing  
728 methods and other future research advances that can change the current oral cancer diagnosis  
729 scenario are discussed below (Fig. 14).

- 730 • The development of new techniques such as bioplasmonic paper using advanced biomimetic  
731 nanoparticles can be easily deployed in resource-limited settings. Gold nanorattles with higher  
732 refractive index sensitivity as compared to other anisotropic gold nanostructures can form  
733 efficient biodiagnostic platforms (Hu et al., 2019). However mechanical instability of paper,

734 unidirectional flow of the analyte, and water adsorbing property hinder the transitioning of such  
735 systems into efficient lateral flow assays and microfluidic paper-based analytical devices  
736 ( $\mu$ PADs). The use of synthetic polymers, such as polymer polydimethylsiloxane (PDMS),  
737 conducting polymers (CPs), and molecularly imprinted polymers (MIPs) can solve such issues  
738 and can be beneficial in the development of highly sensitive systems for OC diagnosis.

- 739 • Nanoparticle-based bio barcode assay wherein magnetic beads and AuNPs are used for  
740 ultrasensitive detection of target analyte using minimal sample volume, is a classic example of  
741 nanogold mediated diagnostic technology. Magnetic bead ensures analyte separation and  
742 barcode DNA conjugated AuNPs serve the purpose of signal amplification in such sensing  
743 systems. The use of bimetallic nanomaterials that possess unique peroxidase-like activity that  
744 can catalyze sensitive “turn on” fluorescence signals in presence of the target molecule can  
745 simplify the overall detection strategy. (Lin et al., 2017)
- 746 • Another major technical obstacle is the lower abundance of critical biomarkers during the early  
747 stages of OSCC. Lower availability of ctDNA, miRNA, ctRNA, long non-coding RNA (lncRNA),  
748 and exosomes in saliva samples have been reported previously (Soda et al., 2019). High-  
749 quality pre-isolation and amplification techniques are mandatory prerequisites (Lousada-  
750 Fernandez et al., 2018). In this regard, integrated systems that perform sample enrichment and  
751 sensing can be highly beneficial in terms of saving time, cost and improving the analytical  
752 performance of the nanosensors (Gorgannezhad et al., 2018). Multiplexed systems such as  
753 PCR/SERS assays wherein, magnetic beads mediated enrichment of the analyte is performed  
754 followed by imaging via SERS nanotag can effectively increase the sensitivity of the detection  
755 by 10 folds as compared to commercially available PCR (Wee et al., 2016). Another SERS-  
756 based recent advancement that promotes high-throughput analysis is the development of  
757 SERS-fluorescence joint spectral encoded (SFJSE) methods. This technique improves the  
758 sample encoding capacity by employing a multi-layered nanoprobe containing a magnetic core  
759 (for analyte enrichment), metallic nanoparticle (SERS generator), and quantum dots  
760 (fluorescence signal)(Wang et al., 2014). Such modified systems can be well adapted for  
761 sensitive detection of several diagnostic, prognostic and intra/post-operative biomarkers related  
762 to OC.

- 763 • Wearable sensors have revolutionized the concept of daily health monitoring and play a crucial  
764 role in disease prediction and treatment. Wireless Graphene/silk nanobiosensors have  
765 previously shown promising results for remote monitoring of pathogens in saliva(Mannoor et  
766 al., 2012). Typical architectural modifications by using similar components can be applied for  
767 the fabrication of a mouth-guard type sensing device for recording different types of salivary  
768 OC markers.
- 769 • Amongst the recent emerging trends is the introduction of AI-based specific behavioral changes  
770 for monitoring the health status of an individual. Such detection mechanism works on the core  
771 principle of machine learning with advanced AI, nudge theory for behavioral alterations, and  
772 social impact bonds (SIB)(Misawa et al., 2020). The advanced method can be utilized for the  
773 early prediction of OC in large heterogeneous populations.

774 Further, saliva-based POC adjuncts are gaining tremendous momentum in recent years. The  
775 development of a proper biomarker panel exclusively for oral malignancy can promote the fabrication  
776 of several multiplexed bioassay systems. It can be suggested that with a better understanding of the  
777 concepts of machine learning and artificial intelligence, as well as their applications in data analysis,  
778 precise and accurate determination of oral malignancy at early stages will be possible in near future.

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## 782 **Author Contributions**

783 **Debolina Chakraborty and Debayan Ghosh:** Conceptualization; writing-original draft; writing-review  
784 and editing. **Sanjit Kumar:** Writing-review and editing. **David Jenkins:** Writing-review and editing.  
785 **Natarajan Chandrasekaran:** Editing, supervision. **Amitava Mukherjee:** Conceptualization, writing-  
786 review, editing, and supervision.

## 787 **Conflicts of interest**

788 There are no conflicts of interest to declare

789

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## 1195 **Figure legends**

1196 **Fig. 1.** Schematic representation of the causative factors, stages of disease progression, and  
1197 government program design for oral malignancy

1198 **Fig. 2.** (i) Schematic representation of patient's workup for screening oral carcinogenesis. (ii) VELscope  
1199 VX model, Reproduced from Cicciù, M., Early diagnosis on oral and potentially oral malignant lesions:  
1200 a systematic review on the VELscope® fluorescence method. Dentistry journal 7, 93, 2019, MDPI (iii)  
1201 The use of Identafi optical system on a suspicious lesion showing loss of fluorescence and increase  
1202 vascularity. (A) Picture of a non-healing ulcer on the right lateral border of the tongue of a 62-year-old  
1203 male taken with the white reflectance (regular light) Identafi 3000 DentalEZ optical device. (B)  
1204 Application of the Identafi DentalEZ device with violet fluorescent light shows dark area (loss of auto-  
1205 fluorescence) in suspicious areas. (C) Application of the Identafi DentalEZ with the green amber  
1206 reflectance light show increase vascularity in the suspicious areas. A biopsy taken from this area  
1207 showed a moderately differentiated squamous cell carcinoma. Reproduced from D.V. Messadi,  
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1210 **Fig. 3.** The number of PubMed records for terms "nanoparticles for oral cancer detection" from 2007 to  
1211 2021

1212 **Fig. 4.** (i) Represents the schematic illustration of the A) Collective oscillation of the free electrons after  
1213 illuminating the nanoparticles with the monochromatic light with resonance wavelength, B) The role of  
1214 electromagnetic and chemical enhancement factor in SERS. Reprinted from (Panikar et al., 2021) with  
1215 permission from Elsevier (ii). SERS-based analysis using a label-free SERS approach (left) or SERS  
1216 tags (right). In label-free SERS, the spectroscopic signal results from analyte adsorption onto the SERS  
1217 substrate, whereas in SERS tags-based specific recognition assays, the spectroscopic signal results  
1218 from the reporter molecules on the SERS tags (Zhang et al., 2019).

1219 **Fig. 5.** Schematic diagram of the proposed SERS biosensor for DNA detection. Reprinted from (Liu et  
1220 al., 2021) with permission from Elsevier

1221 **Fig. 6.** (i) Physical and chemical properties of AuNPs and schematic illustration of AuNPs  
1222 (aggregation/dispersion) colorimetric based detection systems. The unique properties of AuNPs, such

1223 as their high absorption coefficient, scattering flux, luminescence and conductivity, as well as their ability  
1224 to enhance electromagnetic fields, quench (or enhance) fluorescence and catalyse reactions, provide  
1225 numerous possibilities to exploit these particles for sensing and quantification purposes. Reprinted with  
1226 permission from (Aldewachi et al., 2018). Royal Society of Chemistry. (ii) Schematic of the GNP-based  
1227 assay for cancer diagnosis. This system is based on the non-crosslinking aggregation mechanism and  
1228 a cationic PKC- $\alpha$ - specific substrate peptide is used as a coagulant of citrate-coated GNPs with anionic  
1229 surface charges. Reprinted from (Kang et al., 2010) with permission from Elsevier.

1230 **Fig. 7.** Jablonski diagram and a timescale of photophysical processes for organic molecules. (Berezin  
1231 and Achilefu, 2010)

1232 **Fig. 8.** Schematic illustration of the 3DN-CNTs sensor for Cyfra 21-1 detection. Reprinted from (Song  
1233 et al., 2018) with permission from Elsevier.

1234 **Fig. 9.** Schematic representation of nanoparticle-based electrochemical biosensor

1235 **Fig. 10.** Synthesis of  $\beta$ -Ag<sub>2</sub>MoO<sub>4</sub> NPs and immunoelectrode fabrication. Reprinted from (Pachauri et  
1236 al., 2020) with permission from Elsevier.

1237 **Fig. 11.** Tentative procedure for synthesis of Cys-La(OH)<sub>3</sub> NPs and fabrication of BSA/anti-Cyfra-21-  
1238 1/Cys- La(OH)<sub>3</sub>/ITO immunoelectrode. Reprinted from (Tiwari et al., 2017) with permission from  
1239 Elsevier.

1240 **Fig. 12.** (i) Schematic representation of PLGA nanoparticle contrast agents for advanced MRI detection  
1241 of oral cancer (ii) Schema representing the various components and working methodology of  
1242 nanomaterial based photoacoustic imaging of oral cancer tissues

1243 **Fig. 13.** (i) Amplified signal generation via nano-ELISA method for detection of oral cancer specific  
1244 biomarker (ii) Graphical representation of nanosensor-based microfluidic approach for sensitive  
1245 detection of oral cancer biomarker

1246 **Fig. 14.** Pictorial diagram suggesting the possible futuristic developments in the field of oral cancer  
1247 sensing.