

INTERFEROMETRIC PDMS-COATED OPTICAL FIBER BIOSENSORS

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Declaration

I hereby declare that this manuscript, entitled “Interferometric PDMS-coated optical fiber biosensors” is the result of my work except for quotations and citations which have been duly acknowledged.

I also declare that, to the best of my knowledge and belief, it has not been previously or concurrently submitted, in whole or in part, for any other degree or diploma at Nazarbayev University or any other national or international institution.

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List of Abbreviations

ALDH	Aldehyde dehydrogenase
APTMS	(3-Aminopryl)trimethoxysilane
Ab	Antibody
CSC	Cancer stem cells
CD_	Cluster of differentiation _
DMEM	Dulbecco's modified Eagle medium
EBF	Enhanced backscattering fiber
FBG	Fiber Bragg Grating
FBS	Fetal bovine serum
FISH	Fluorescence <i>in situ</i> hybridization
GEP	Gene expression profiling
GA	Glutaraldehyde
HER2	Human Epidermal Growth Factor Receptor 2
IHC	Immunohistochemistry
LoD	Limit of detection
MFI	Mean fluorescence intensity
m-PEG	Methoxypolyethylene glycol
PBS	Phosphate-buffered saline
PDF	Probability density function
PDMS	Polydimethylsiloxane
RI	Refractive index
RIU	Refractive index units
RPMI	Roswell Park Memorial Institute
SDI	Semi-distributed interferometer
SMF	Standard single-mode fibers
TNBC	Triple negative breast cancer

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Abstract

Cancer leads to millions of deaths worldwide, and the numbers are expected to rise in the future. Timely diagnosis of cancer improves the treatment effectiveness and increases survival rates. However, not many diagnostic tools are capable of accurately sensing the small amounts of present cancer biomarkers in clinical samples. Biosensor is an example of a promising tool that has proved to be efficient in early cancer diagnosis. Several types of biosensors exist, and their sensing capabilities differ based on design, working principle and purpose. The main advantage though, is that they have been proposed as portable, quick-responding, real-time tools that do not require the presence of trained personnel. This thesis work is based upon a fiber-optic semi-distributed interferometer (SDI), covered with PDMS, and is tested for detection of 3D cancer spheroids.

As was previously stated, the design affects the sensing capabilities of the biosensor. As SDI biosensors were proven to have good sensing properties of free biomarkers, when sensing cells their functionality is not the best. Thus, PDMS coating was proposed to increase the sensing surface area, additionally improving the stability of the biosensor. The formed spherical PDMS surface gets further functionalized with antibodies that bind with biomarkers present on cancer cell spheroids. Then the detection occurs using multi-channel Fiber-Bragg grating (FBG) analyzer, when biosensors first measure the cells in phosphate buffered saline (PBS), and later in serum-containing media.

The proposed biosensor exhibited better sensitivity and sensed CD44 proteins on the cancer cell spheroids, while remaining minimally reactive to negative control spheroids. The current PDMS-coated biosensor demonstrated a limit of detection (LoD) of 27.42 spheroids/mL in serum.

Keywords: Breast cancer, 3D cell culture, spheroid, biomarker detection, PDMS, SDI, biosensor, surface functionalization

Chapter 1 – (Introduction)

1.1 Human breast cancer: a review of characteristics and diagnosis

1.1.1 Breast cancer: Global Trends and Challenges

Cancer is characterized by uncontrolled cell proliferation and abnormal growth. Breast cancer is the most common type of cancer among women; it originates in the epithelial tissue [1]. Development of cancer is a complex process influenced by factors such as age, gender, lifestyle, and genetics [2]. Cancer is more prevalent for women with early menarche, later menopause, later age at first birth, less childbirths, less breastfeeding, excess body weight and unhealthy habits [3]. In the study of Mohammed, cancer was diagnosed at the mean age of 48.79 years. Globally, breast cancer was considered the second most occurring cancer overall and fourth leading cause of cancer-related deaths in 2022 [1]. It is the most occurring cancer type in women with the significant mortality burden [3]. According to Kim *et al.* [4] predictions, more than 3.2 million new cases of breast cancer are expected worldwide by 2050, which is a 38% increase compared to current years [4]. Statistically, the United States had the highest 5-year relative survival rate of 90.2%, while India had the lowest at 66.1% for the period of 2010-2014 [1]. The overall trend is that the survival gap is wide between developed and developing countries, but the survival rates are steadily improving [1].

Despite an increase in the breast cancer incidences, implementation of screening programs and advancements in cancer-related therapy lead to a drop in mortality cases. Current efforts of the Global Cancer Initiative is to lower cancer mortality by 2.5 % per annum, by focusing on three key aspects: early identification, timely diagnosis, and comprehensive care [3].

Early detection of cancer is critical, as it increases the survival chances and treatment effectiveness [4]. 5-year survival rates may exceed 90%, when diagnosed in the early stages [1]. Comparably, the net survival estimate dropped with later stage diagnoses: 74% at stage III and 23% at stage IV [6]. Thus, the stage at diagnosis is the important predictor of prognosis and demonstrates the importance of early stage detection of cancer.

1.1.2 Types of breast cancer

Variation in the transcriptional programmes lead to diverse human breast cancers. The World Health Organization (WHO) classifies breast carcinomas into no special type and special types. 75% of breast cancers belong to no special type group, while the rest are assigned to special subtypes [7]. Within special types, four main molecular subtypes exist: the

luminal A and B, the HER2-OE overexpressing and basal-like triple negative breast cancer (TNBC) [8]. The subtypes are distinguished by the presence or lack of expression of oestrogen, progesterone and HER2 neu receptors on the breast cancer tissue.

Luminal A cancer subtype is defined by the presence of estrogen and progesterone receptor expression, while in the luminal B subtype, besides the expression of these hormonal receptors, HER2 protein is overexpressed. Recently, the Ki-67 proliferative index was introduced to distinguish between these subtypes. If Ki-67 is less than 14%, then it is luminal type A. In the study of Mohammed [9], Luminal B type was diagnosed most frequently (43.73%) among the patients. The most occurring types of breast cancer, invasive ductal carcinoma and invasive lobular carcinoma, actually belong to the luminal A and B subtypes. In invasive ductal carcinoma, invasive cancer cells initiate in the breast ducts and then extend into nearby breast tissue, and may eventually spread to distant organs.. Mohammed reported that 89.1% of their patients were diagnosed with invasive ductal carcinoma of no special type. Regarding invasive lobular carcinoma, cancerous cells occur in the lobules and then metastasize to the surrounding breast tissue or any other place of the body [10].

In contrast, HER2-overexpressed cancer lacks the expression of the hormonal receptors, limited by HER2 protein overexpression alone. Paget's disease, which occurs rarely, is diagnosed in patients with HER2 enriched type.

In the basal-like TNBC, tumours are triple negative for the previously mentioned oestrogen, progesterone and HER2, but show distinct basal marker expression, including proteins such as cytokeratin (CK) 5/6 and epidermal growth factor receptor (EGFR). Compared to other subtypes, the mortality for basal cancer was the highest (17.4%) [9].

1.1.3 Biomarkers of breast cancer

Therapy depends on the cancer molecular subtype. The therapy prioritizes targeting cancer stem cells (CSC), which have stem-like characteristics, which account for cells' self-renewal and differentiation, granting tumours therapy resistance, invasiveness and malignancy. According to the CSC model, CSC within tumour tissues are the only proliferating cells and account for tumor growth, progression and metastasis. The main established CSCs markers are transmembrane glycoprotein clusters of differentiation (CD) - CD44 and CD24, and intracellular aldehyde dehydrogenase (ALDH), identified using immunohistochemistry or fluorescence *in situ* hybridisation (FISH) [11]. CD44 is a hyaluronic acid receptor [12] and it functions in normal cells during physiological processes, but also plays a crucial role in tumour initiation and development. CD44-mediated processes are

related with stemness, allowing for cell motility, proliferation and angiogenesis. CD44 is present in a range of cell types, including embryonic stem cells, differentiated cells and cancer cells [13], where it provides cell adhesion via binding to hyaluronic acid. CD24 expression serves for differentiated epithelial features [8]. While CD44 expression was found to be correlated with the molecular subtype of breast cancer, the correlation for CD24 was not significant [8]. In primary breast carcinoma, CD44 was expressed in 51.2% of the patients with breast cancer, whereas the expression for the other biomarkers was lower in the study of Ricardo *et al.* [8]. The profile for basal-like breast cancer is mostly the positive expression of CD44 (at least 10% of positive tumour cells) and negative or low expression of CD24 (0% or up to 25% of positive tumour cells) [14]. In luminal breast cancer, the expression profile is the opposite for CD44 and CD24. Additional CSC marker is ALDH1, which makes CSC highly tumorigenic. It was found that ALDH1 positive (>1% of cytoplasmic positivity [8]) CSCs can initiate tumour formation from as few as 20 cells, while ALDH1-negative CSCs are less invasive [8]. According to Ricardo *et al.* [8], 39.4% of their patients with basal-like carcinoma had positive cytoplasmic expression of ALDH1. However, about 1% of primary tumours are $\text{ALDH1}^+ \text{CD44}^+ \text{CD24}^{-/\text{low}}$. Thus, it is imperative to develop tools that are able to detect the small concentrations of biomarkers. Even though univariate analysis of CD44/CD24/ALDH1 expressions alone or in combination fail to predict overall survival, presence of more than 10% of $\text{CD44}^+ \text{CD24}^{-/\text{low}}$ cells in basal like carcinoma tumours showed less overall survival trend [8].

BRCA1 and BRCA2 genes are also considered as biomarkers. BRCA genes act as tumor suppressor genes, the proteins they synthesize play a pivotal role in repairing complex DNA lesions via homologous recombination [15]. When this DNA repair function is impaired by mutations, the risk for breast cancer development increases. They are inherited and increase breast cancer susceptibility risks. Individuals with mutations in BRCA1 typically develop basal-like carcinoma, while mutations in BRCA2 lead to luminal subtype progression [15]. BRCA genes are biomarkers for poly(ADP-ribose) polymerase (PARP) inhibitors, which inhibit the function of DNA repairing enzymes. Resulting unrepaired DNA double-strand breaks accumulate and lead to tumour cell death.

1.1.4 Analytes quantification

Gene expression profiling (GEP) tests help in assessing the likelihood of disease recurrence and guiding individual treatment strategies [16]. However, GEP is costly, technically complex and impractical for routine clinical applications [9]. Also, there is an

increasing demand for trained personnel, who are able to conduct genetic counselling before and after testing [15]. A more available alternative is the immunohistochemical panel, using 6 IHC markers, which is able to track biomarkers expression. While, HER2-overexpressed and basal-like TNBC are discovered via IHC/FISH or GEP, luminal breast cancers can be also predicted with molecular assays such as 21-Gene Recurrence Score, Prosigna Gene Signature, MammaPrint, EndoPredict [7].

The Oncotype DX 21-gene Recurrence Score is a reverse transcription-polymerase chain reaction assay based on 16 genes that tests whether $ER^{+}HER2^{-}$ patient with ≤ 3 lymph node metastases faces post-endocrine therapy recurrence and evaluates the effect from adjuvant chemotherapy.

Prosigna Gene Signature analyses a microarray of 50 genes and is dedicated for ER^{+} patients with ≤ 3 lymph nodes to assess the risk of postoperative hormonal therapy for the next 5 years.

MammaPrint analyses a microarray of 70 genes in patients with stage I/II, $ER^{+/-}$ and $HER2^{-}$ with a tumor size ≤ 5 cm and up to 3 lymph nodes to estimate the tumor recurrence within the next 5-10 years.

EndoPredict assesses the activity of 12 genes for ER^{+} and $HER2^{-}$ patients with ≤ 3 lymph nodes to estimate the risk of distant metastasis within the next 10 years.

All the mentioned molecular assays have been approved by the US Food and Drug Administration (FDA). The outcomes help to manage the personalized treatment and predict postoperative patient prognosis.

1.1.5 Methods of cancer diagnosis

Clinical breast exam and breast-self exams. The most affordable and quick method for early cancer diagnosis, however the least reliable. A clinical breast exam is conducted by a healthcare provider, whereas a breast self-exam should be carried out by the patient. According to Huang *et al.* [17], there is no significant difference in detecting breast cancer when it is examined by a specialist or by the patient, but self examinations lead to more frequent discoveries of cancer. However, The American Cancer Society discourages using this method as there is not enough evidence, suggesting clear benefits for women with average risk of breast cancer at any age [18].

Mammography. Deriving an x-ray image of the breast by applying low radiation doses. Mammography is recommended for women older than 30 years old [18], and supports early

diagnosis of breast cancer prior to the onset of physical symptoms. Not entirely reliable, might miss some breast cancers. Sensitivity and specificity are estimated at 54.5% and 85.5%, respectively [19]. There are 2D and 3D mammography options available. 2D mammograms are the conventional type, while 3D mammography, or digital breast tomosynthesis produces the pseudo three-dimensional mammogram, it reduces the tissue overlap and significantly removes the masking effect to discriminate the lesions from tissue, their size and morphology [11]. There is evidence that 3D mammography is able to reveal more breast cancers in denser breasts and reduces the chance for a follow-up testing [18]. Nevertheless, 3D mammography utilizes slightly higher doses of radiation compared to traditional 2D mammograms, though the doses applied are lower than the limits specified in the European Guidelines for Screening Mammography [11]. Compared to magnetic resonance imaging (MRI), 2D and 3D mammograms are more affordable and are applicable when MRI has restrictions [11].

Ultrasound (US). Creates an image from the reflected acoustic pressure, created by piezoelectric elements (transducer) from the tissue interface [20]. Helps to differentiate between a solid tumour and a cyst with high frequency sound waves. Has the sensitivity of 67.2% and specificity of 76.8% for the identification of the disease [19]. Used for tumor size evaluation. Currently, handheld ultrasound (HHUS) and automated breast ultrasound (ABUS) are available, which operate with a computer, but do not provide quantitative data on tumors [20]. Other limitations are that the US have low contrast for soft tissues and cannot assess the peripherally located lumps [11], so additional screening tools are advised. Besides regular ultrasounding device, more advanced diagnostic tools such as photoacoustic (PA) tomography (PAT), Transmission Ultrasound Tomography Systems (TUST), and their combination - ultrasound/photoacoustic tomography (USPAT) system promise to overcome the aforementioned drawback and become the future of breast cancer imaging [20].

Magnetic resonance imaging (MRI). Provides a 3D image of the breast area without utilizing radiation energy. Integrating 3D mammography improves the inter and post-treatment response observations. MRI has a sensitivity of 87% and specificity of 60% [11]. The gold technique for determining disease extent, but not highly accurate in estimating the tumor size. While offering highest sensitivity compared to other traditional modalities, MRI is less utilized on a regular basis because it is more costly and takes more time to image.

Biopsy. The most accurate method and a gold standard in identifying breast cancer. Cells or tissues from the patient get examined for cancer cells with the help of a microscope. The lump, from where the tissue gets collected might be palpable or non-palpable, so

mammography or ultrasound imaging can help with correct location of the collection site. Several techniques for tissue biopsy exist: fine needle aspiration, vacuum-assisted core biopsy, a lymph node biopsy. The less invasive procedure is fine needle aspiration biopsy, where a healthcare professional inserts a fine needle with a syringe into the breast lump. The syringe then helps to suction samples, containing cells that later gets observed under a microscope. In vacuum-assisted core biopsy, a hollow needle gets inserted inside the breast region, then with the help of vacuum, tissue gets suctioned inside the needle. In lymph node biopsy, lymph nodes, containing breast cancer cells entered through a lymphatic system, get surgically extracted for further examination [21].

1.2 Positive and negative controls

1.2.1 HCC1806 and HEK293

HCC1806 is a TNBC epithelial cell line, has a higher expression of CD44 and E-cadherin compared to HEK293 cell line which was derived from an embryonic kidney. HCC1806 first-generation mammospheres of Laranjo *et al.* [22], had a phenotype of $CD44^{high}CD24^{low}$, while 2D cultures main populations is characterized by $CD44^{+}CD24^{-/low}$. With the formed mammospheres, the CD44 expressions decreased to 4045 (mean fluorescence intensity) MFI values, and CD24 expressions increased 3-fold compared to 2D parental cell lines. HEK293 cells have low expressions of CD44, but moderate expression levels of E-cadherin. All cell culture lines may differ in E-cadherin expression, which is an adhesion molecule, playing a role in spheroid formation.

1.2.2 2D and 3D cell culture models

Standard 2D cell cultures form homogenous monolayers. Also, 2D cell monolayers fail to provide insufficient CSC enrichment [23]. 3D cancer spheroid is a tumor-mimicking *in vitro* model, dedicated for solid tumor studies. Cell-cell interactions within cancer spheroids imitate the *in situ* organization of nonvascularized primary tumors [24] (Figure 1). Several 3D culturing methods exist, and they are either scaffold-based or not. For scaffold-based methods, the examples are matrix, spinner flasks or micro-patterned plates required methods. In contrast, the examples of scaffold-free methods are utilizing ultra-low attachment plates, hanging drop models, magnetic levitation and bioprinting (Figure 2). Among them, the most established and studied method is the hanging drop technique. In this technique, cells are deposited as droplets in their respective media, then those droplets are inverted for the cells to aggregate at the bottom. Hanging drop technique is simple and offers many other advantages such as no need of

specialized equipment, reduced reagent consumption, possibility of density and quantity regulation. On the other hand, this technique is laborious for mass production and is time-consuming. The 3D models mimic the tumor microenvironment, the cell niche, the complex molecular interactions, acting as a transitional model between traditional monolayer cultures and the tumor microenvironment [25]. Within the forming multicellular spheroids, gradients of nutrients, gases, and biochemical molecules develop. In result, spheroids develop necrotic areas in the center due to limited oxygen access, proliferative cells on the outer surface and have a quiescent cell layer in the middle. Fong *et al.* [26] and Chen *et al.* [27] reported that their cancer spheroids grown on scaffolds demonstrated a difference in expression of respective biomarkers. Spheroids may have diameters in the range of 100-1000 microns, depending on the initially introduced cellular density. Badea *et al.*'s 3D spheroids from 2500 cells per drop formed spheroids of $250.54 \pm 17.39 \mu\text{m}$ in diameter with circularity values of 0.85 ± 0.05 , almost reaching a perfect circularity of 1. In comparison, a single cell of standard cancer cell lines is $10\text{-}20 \mu\text{m}$. This morphology offers a unique platform for biosensor detection for irregular shaped targets. In spheroid form, cells are in stem cell form, compared to 2D cultures.

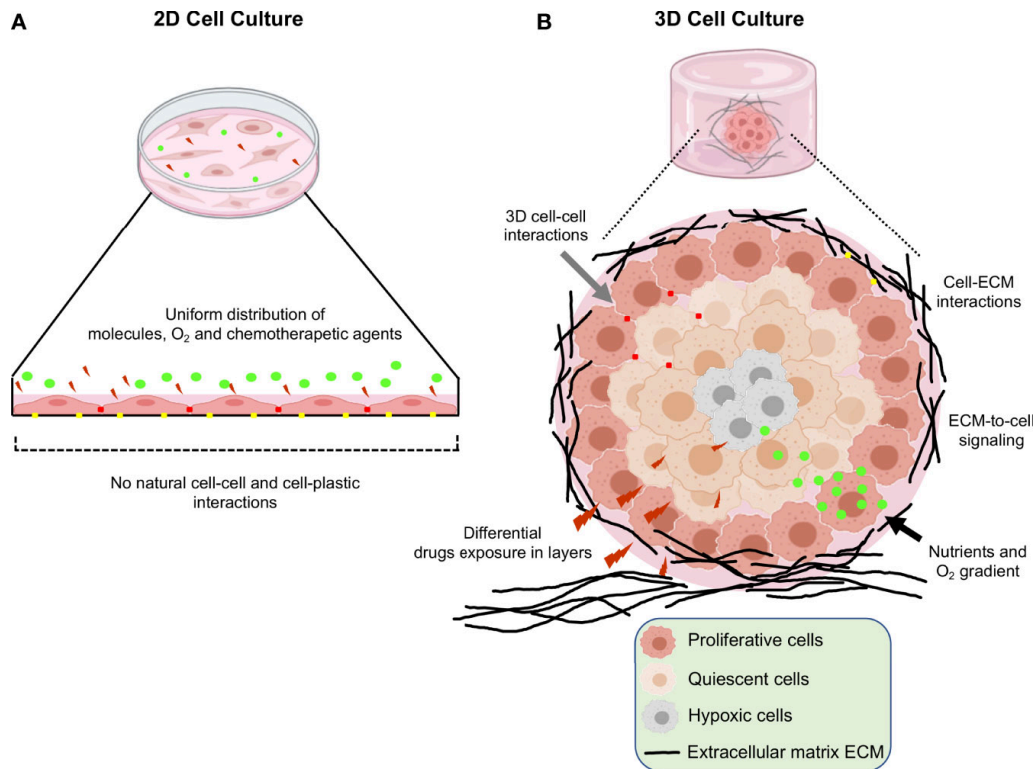


Figure 1. Structural and biological comparison between traditional 2D and 3D Cell Cultures.

Adapted from [28].

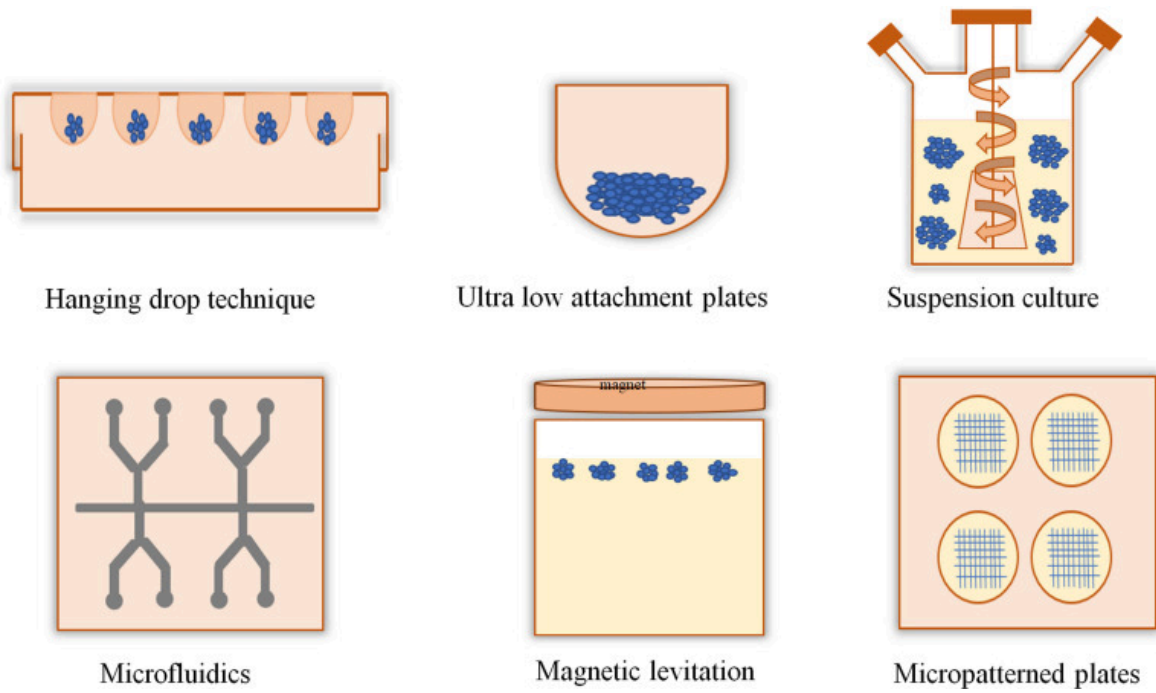


Figure 2. Common laboratory techniques for spheroid generation. Adapted from [29].

1.3 The future of the optical fiber biosensors

1.3.1 SDI Interferometer

Traditionally, the status of cancer has been monitored with imaging modalities such as mammography; MRI; computed tomography (CT scan); ultrasound, and molecular diagnostic techniques [30]. Despite their effectiveness, all the mentioned diagnostic tools require trained healthcare professionals, are time-consuming, and sometimes costly to obtain the results; this can be a limiting factor for some vulnerable populations from low-resource healthcare settings [30]. Immense efforts are being put into advancing current technologies to accommodate patients with a rapid and precise diagnosis of cancer. One of the prominent developments is a biosensor that is supposed to identify cancer-related biomolecules in various samples. The focus of this work will be on optical fiber biosensors.

Many fiber-optic biosensors are moving towards clinical adoption due to their unique advantages. Optical biosensors are often preferred over the traditional electronic sensors because they are immune to electromagnetic interference, chemically inert [31], non-toxic, and cost-effective. One of the most sensitive optical sensing techniques is the interferometry method.

Interferometry is a powerful technique in biosensing because it offers high sensitivity to changes in the surrounding medium. This sensitivity to refractive index (RI) variations

comes from the interference of light waves, which travels through the branches of the interferometer. The interference occurs due to the superposition of at least two waves traveling through the arms, each with its own phase and path length. When these waves combine, the resulting spectrum exhibits either constructive or destructive interference [32].

Traditional Fabry-Perot interferometers are being continuously improved over many years. With current research, interferometric fiber optic biosensors are achieving even higher sensitivities, especially with surface modifications [33]. Polydimethylsiloxane (PDMS) is an example of a biocompatible, chemically inert [34], and low-cost material that has proven to improve the sensor performance, as an example.

1.3.2 PDMS introduction

PDMS provides an encapsulation of the interferometer's measuring and reference arms, serving as an adhesive packaging and a protective layer that holds together all the internal compartments [35]. Additionally, PDMS has a high Poisson's ratio and a low elastic modulus [35], contributing to its flexibility and durability. All these characteristics make PDMS particularly attractive for biosensor optimization applications. Depending on the PDMS application method, the final dimensions and shapes of the coating may vary, affecting the final design and performance of the biosensor. However, Duo *et al.* [35] stated that the dimensional variations of the PDMS coating had a negligible effect on the sensor's functionality.

While extensive research has been conducted on PDMS-coated multimode-core fibers, few-mode fibers, photonic crystal fibers [36], taper fibers, exposed-core fibers, etc., many micro-structured fiber structures are costly and complex to fabricate [37]. Thus, PDMS coating will be tested on a semi-distributed interferometer (SDI), a recent and simplified version of the Fabry-Perot configuration. The SDI sensor, presented by Kazhiyev *et al.* [38], demonstrates an excellent sensitivity of 787 dB/RIU, but with the assembly of PDMS, the sensitivity is expected to give even better results. On average, the RI sensitivity in the RI interval of 1.39–1.41 is 5400 nm/RIU [39].

$$R(RI) = \frac{\Delta n \times \Delta \lambda_{min}}{\Delta \lambda_{peak}} [40]$$

1.4 Framework of the study

In this work, we propose PDMS-coated SDI biosensors for 3D cancer spheroid detection. PDMS-coating promises to provide better stability to the bare SDI, thus increasing sensitivity. The sensitivity parameter is especially important in detecting the early stages of cancer, where only small concentrations of biomarkers are available. Besides that, 3D spheroids offer a unique platform for studying biosensor performance, as the irregular shape and relatively large size play a significant role in signal propagation. This is the first proof-of-concept work, aiming to evaluate the PDMS biosensor's performance in detecting 3D cancer spheroids in a Phosphate-Buffered Saline (PBS) and human serum environment. Experiments using PBS exclude the potential interfering variables, setting a base for controlled measurements. Measurements in human serum further tests biosensor selectivity for targets. PDMS coating is expected to enhance the SDI performance for CD44 detection on spheroids by improving antibody immobilization efficiency, increasing effective sensor–spheroid contact area, and reducing nonspecific adsorption in sensing media.

Aims and objectives are:

- To fabricate and functionalize the PDMS-coated SDI biosensor;
- To test the biosensor performance for cancer detection in PBS and human serum;
- To evaluate the sensitivity and specificity of the proposed biosensor;
- To evaluate the specificity of the biosensor in detecting CD44 of 3D cancer and normal cultures.

Chapter 2 – (Materials and Methods)

2.1 Materials

Cell culture work: Dulbecco's Phosphate-buffered saline (DPBS) (Capricorn Scientific, #PBS-1A), fetal bovine serum (FBS) (Sigma Aldrich, #F4135), RPMI 1640 (Capricorn Scientific, #RPMI-HA), DMEM (Capricorn Scientific, #DMEM-HPA), Trypsin-EDTA (Sigma Aldrich, #T4049), Trypan blue stain (Sigma Aldrich, #T8154)

For fabrication and functionalization: sulfuric acid (Sigma Aldrich, #258105), hydrogen peroxide (Sigma Aldrich, #18304), SYLGARD 184 Silicone Elastomer Kit (Dow), methanol (Sigma Aldrich, #179337), (3- Aminopropyl)trimethoxysilane (APTMS) (Sigma Aldrich, #281778), glutaraldehyde (GA) (Thermo Scientific, #A10500), methoxypolyethylene glycol amine (m-PEG) (Meryer, #M85744-25G), Anti-CD44 monoclonal antibodies (Sigma-Aldrich, #SAB4700179-100UG).

2.2 Semi-distributed interferometers (SDI) fabrication

The fabrication methodology for the SDI was adapted from Kazhiyev *et al.* The so-called “splice-and-cleave” methodology was applied to a single-mode fiber (SMF-28) with an enhanced backscattering fiber (EBF). Initially, SMF's protective outer jacket gets manually removed for the cladding exposition. Then SMF gets cleaved with a single fiber cleaver to obtain a straight cut at the fiber end. Next, EBF, which has a MgO nanoparticles doped core, gets prepared. The protective layer of EBF also gets removed, and the tip gets cleaved to obtain a straight end. Prepared SMF and EBF get spliced with standard telecom splicers (Fujikura 12-S) as shown in Figure 3.A. Finally, the EBF side gets cleaved for it to remain 1 mm long. This 1 mm part of the SDI becomes the interferometric cavity, providing scattering elements that enhance the light backscattering.

2.3 SDI calibration

A calibration step is required to evaluate the sensor's sensitivity. Calibration procedure is based on testing the sensor's response to the change of the surrounding media. Optically, this can be done by changing the refractive index (RI) in the sensing media. In the calibration of SDI, six concentrations of sucrose get prepared: 1.3476 RIU, 1.3497 RIU, 1.3522 RIU, 1.3546 RIU, 1.3570 RIU and 1.3585 RIU. The initial measurement is performed in 10% sucrose solution, which accounts for 1.3476 RIU. The subsequent concentrations are

obtained by adding 400 μl of 40% sucrose solutions. Optical sensing interrogator (Micron Optics si255, VA, US) records the return loss in dBm for peaks and dips across wavelengths ranging from 1460 to 1620 nm. This interrogator device is connected to a fiber bragg grating (FBG), which provides a reference signal. Then the recorded data gets analyzed to obtain the sensitivity value.

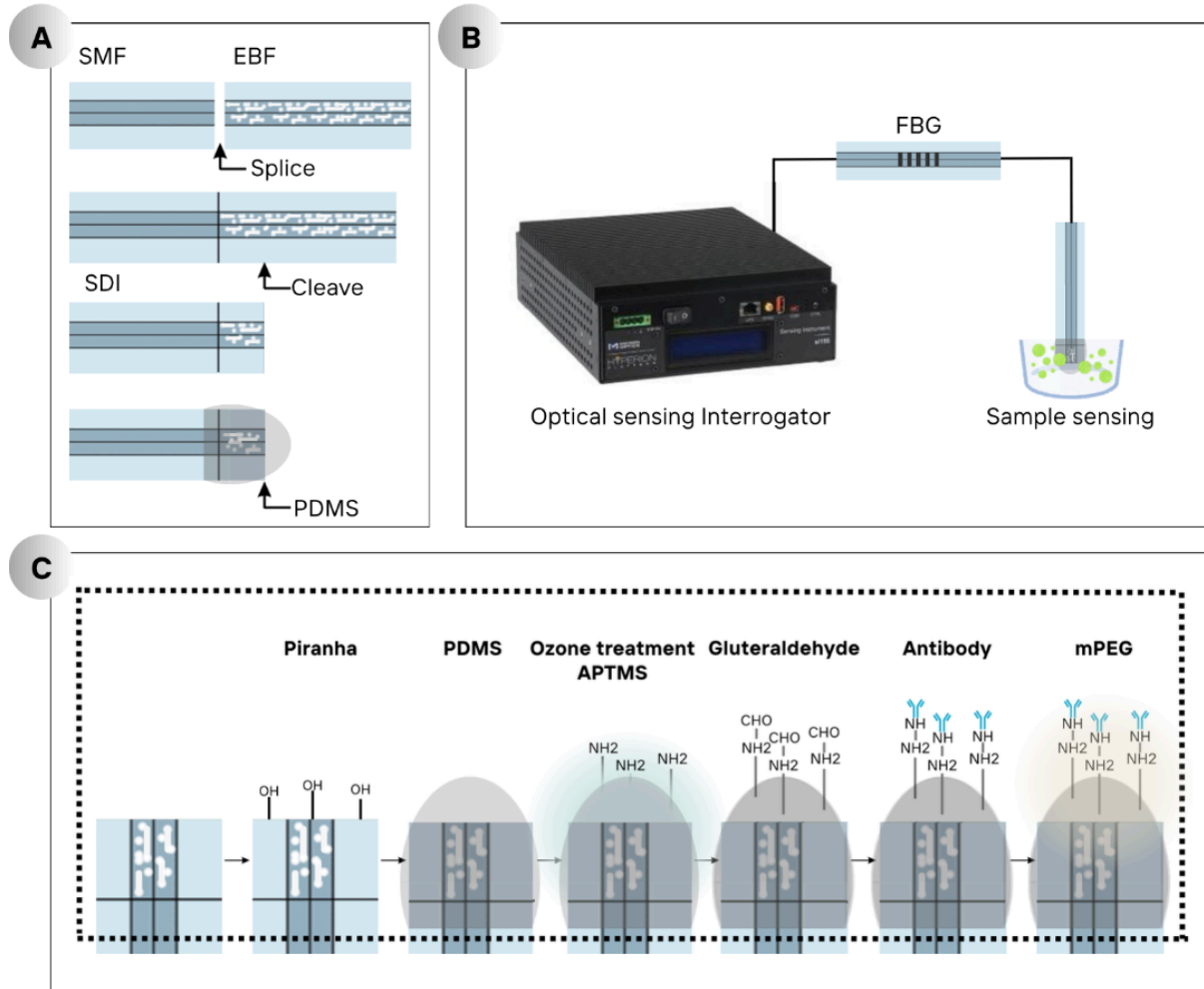


Figure 3. Biosensor fabrication and functionalization. (A) The SDI sensor fabrication and PDMS coating. (B) Biosensing experimental setup. (C) PDMS application and biofunctionalization procedure.

2.4 PDMS coating and further functionalization

The initial step is a 15 minute treatment of the SDI tip with a Piranha solution (a mix of sulfuric acid (H_2SO_4) and hydrogen peroxide (H_2O_2) at a 4:1 proportion) to clean the surface from organic residues and incorporate hydroxyl groups ($-\text{OH}$) [41]. The tip is then rinsed in DI water and dried under a stream of nitrogen gas (N_2). PDMS is prepared by mixing the liquid PDMS with curing glue at 10:1 ratio, followed by stirring for 5 minutes. It is also advisable to

place the PDMS mix into a vacuum chamber to avoid air bubbles formation [42]. Prepared PDMS then is applied onto the SDI, using a loop method to form a semi-spherical cap at the tip. Then the PDMS cap is cured in an oven at 110°C for an hour. PDMS-coated SDI is then visually inspected for defects and calibrated once again to see the sensitivity change.

The next step is the biofunctionalization of the PDMS surface. First, the hydrophobic PDMS surface is irradiated with ultraviolet-ozone (UVO) for 20 minutes, to induce oxidized molecules - specifically hydroxyl and carbonyl species [43] - that later aid with APTMS attachment [44]. After, the surface can be silanized in 1% (3-aminopropyl)trimethoxysilane (APTMS) solution in methanol for 20 minutes. When completed, methanol is used to wash off remaining extra silane. Then the sensor is placed inside of an oven at 110°C for 1 hour. Next, the sensor is placed into a 50% glutaraldehyde (GA) solution in PBS for 1 hour, to acquire an aldehyde group for antibody (Ab) binding. The sensor is then placed in an anti-CD44 antibody solution (4 µg/mL in PBS) for 1 hour. Finally, the sensor gets incubated in a 10% m-PEGPBS-based solution for 30 minutes to saturate non-specific binding sites. Prepared biosensors are then kept in PBS at 4°C for further usage.

2.5 3D cell spheroid preparation

3D cell spheroids are prepared for two cell lines: epithelial breast cancer (HCC1806) and embryonic kidney (HEK293T) cell lines via hanging drop method. HCC1806 cell line is grown inside a 75 cm² flask with RPMI 1640, containing 10% FBS, while HEK293 cell line grows in DMEM 10% FBS. The flasks with cells are incubated at 37°C in a 5% CO₂, until 90% confluence is reached. Cell detachment is achieved after treatment with 0.05% trypsin-EDTA for 5-10 minutes. Then respective complete media get added to the flasks to stop trypsinization. Cells in suspension get collected inside 15 mL conical tubes and get centrifuged for 5-10 minutes at 130 XG. Supernatant gets removed, and the pellet gets resuspended in the respective complete culture media. Then the cell concentration is calculated with a hemacytometer to obtain 125×10^3 cells/mL. Next, PBS gets added into a 150 mm sterile Petri dish, and its lid gets used for droplet deposition. Each droplet is 20 µl and contains 2500 cells. After the lid gets populated with droplets, it gets carefully inverted onto the PBS filled Petri dish. Hanging drops get maintained inside an incubator under standard physiological conditions (37°C and 5% CO₂) for five to seven days until spheroids form. Once spheroids form, they get collected with either PBS or human serum inside a conical tube for the detection experiments.

2.6 Detection of 3D spheroids in media

The experimental setup is illustrated in Figure 3.B. The biosensor tip gets immersed inside a vial with samples, and the other end gets connected to the interrogator, which automatically transmits the data to the computer. Later, all the recorded data was analyzed in MATLAB for biosensor performance. The detection interval per specific concentration of a sample is 20 minutes and data is saved each minute. The various concentrations of the sample get prepared beforehand. In this work, the biosensor tests for 0 spheroids (sph)/mL, 10 sph/mL, 20 sph/mL, 40 sph/mL and 80 sph/mL for both HCC1806 and HEK293 cell lines. Each spheroid is formed from 2500 cells.

Table 1. Spheroid concentrations

Time intervals	Spheroid concentrations	Approximate cell count
0 - 20 min.	0 sph/mL (PBS/serum)	0 cells
20 - 40 min.	10 sph/mL	25'000 cells
40 - 60 min.	20 sph/mL	50'000 cells
60 - 80 min.	40 sph/mL	100'000 cells
80 - 100 min.	80 sph/mL	200'000 cells

2.7 Data analysis

- Limit of detection (LoD) was obtained with:

$$y_{LoD} = (y_{blank} + 3\sigma_{max}) \quad (1)$$

where,

y_{LoD} - LoD concentration; y_{blank} - average blank of signal; 3 - the statistical factor (99.7% confidence); σ_{max} - maximum value of the standard deviation.

Since the maximal concentration of spheroids did not exceed 100 spheroid/mL, a linear model was applied for detection in PBS and for detection in serum.

- Repeatability was assessed using multiple biosensors fabricated under identical conditions. Each biosensor performed the same set of measurements, and their responses were compared to evaluate consistency across device

Chapter 3 – (Results and Discussion)

3.1 Characterization of PDMS-coated SDI biosensor

Several biosensors were visually characterized through a Scanning Electron Microscopy (SEM). The visualization confirmed the formation of a uniform PDMS spherical cap at the tip (Figure 4), which was functionalized for biosensing. The average diameter for the widest part of the PDMS biosensor for 9 independent sensors was measured to be $354 \pm 80 \mu\text{m}$. In comparison, SDI's diameter is around $120 \mu\text{m}$. This geometric scale is intentionally designed to ensure that the sensor's active area is sufficiently large to capture the refractive index changes from 3D spheroids.

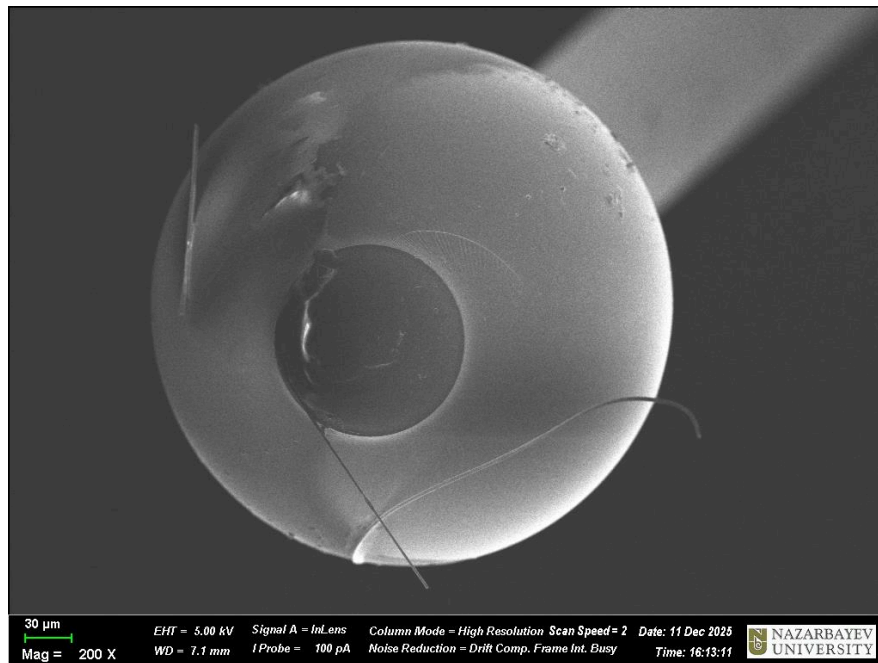


Figure 4. SEM image of the PDMS-coated SDI biosensor.

3.2 Preparation of 3D spheroids using the hanging drop technique

The morphology of the spheroid aggregates was characterized after 6 days of culture using the hanging drop technique. While starting with an identical initial seeding density of 2,500 cells per drop, the two cell lines exhibited different growth profiles. The target HCC1806 spheroids reached a compact diameter of $245 \mu\text{m}$, highly correlating with dimensions obtained in [45]. In contrast, the HEK293T aggregates formed significantly larger structures with a mean diameter of $580 \mu\text{m}$ (Figure 5), resulting from different levels of N,E-cadherin expression. While these models represent a stabilized "bulk" tumor environment and the current study successfully

demonstrates the detection of multicellular spheroids, it is important to note that the diameters of the spheroids used (245–580 μm) are larger than what is realistically encountered in early-stage cancer. In future works, these spheroids could be intentionally dissociated to better study the Epithelial-Mesenchymal Transition (EMT). During EMT, cells transition into a mobile, mesenchymal state. Sensing these mesenchymal state cells would provide more clinically relevant data. Furthermore, moving from static measurements to a dynamic sensing system would allow for real-time monitoring, closer approximating the physiological conditions.

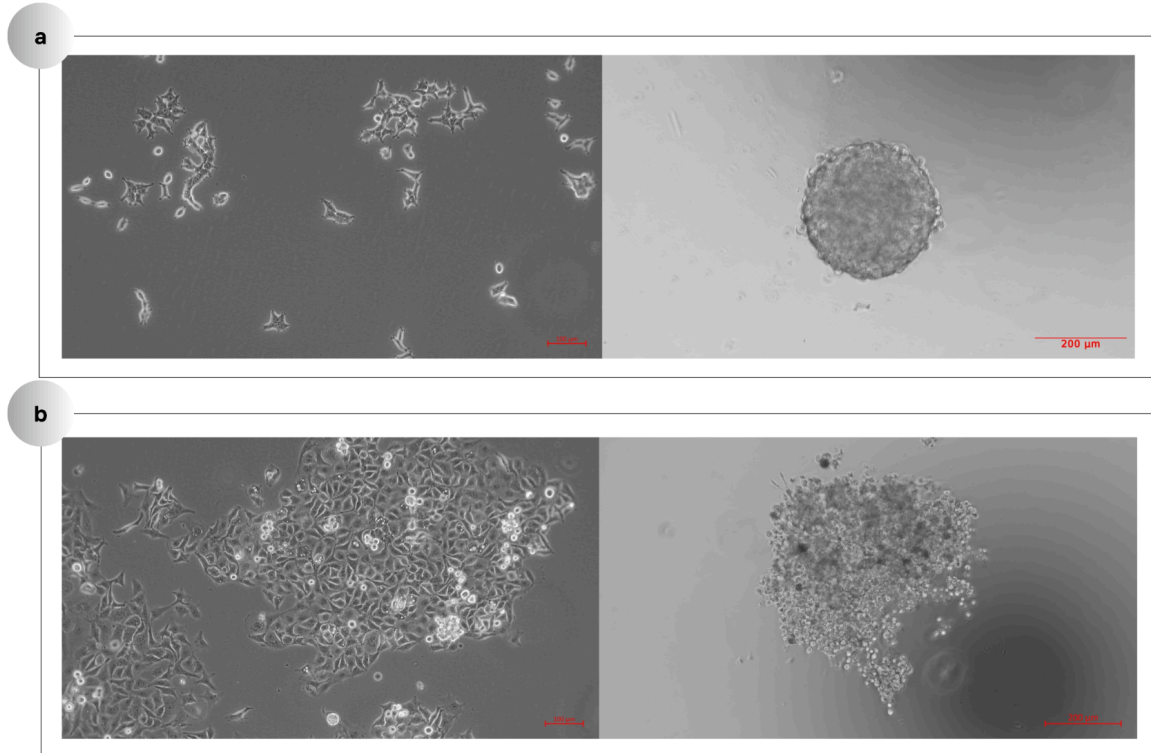


Figure 5. Comparison of morphologies of 2D monolayer and 3D spheroids, produced by hanging drop technique for (a) HCC1806 and (b) HEK293 cell lines from 2500 cells after 6 days of culture.

A multi-Resonance analysis was implemented to process the interference signals generated by the 3D spheroids. Figure 6(a) demonstrates the spectral envelope for the raw transmission spectrum, which identifies individual resonance peaks (red dots) and valleys (green dots) across a 150 nm bandwidth. Fig. 6(b) illustrates the intensity shift of all identified peaks (blue) and valleys (green), plotted as a function of HCC1806 spheroid concentration (0 to 80 sph/mL). The downward slopes across the majority of features indicates a consistent increment in the RI due to the introduction of biological targets. The resulting Probability Density Function (PDF) and its Gaussian estimate in Fig. 6(c) shows a comparison between the baseline (blue) and high concentration (green). The distinct leftward shift and broadening of the distribution provide

statistical evidence of the sensor's response. Sensitivity Distribution (Boxplots) will reveal a statistical summary of the sensitivity (slope) for the most significant features to check for consistency between peaks and valleys, and demonstrate the robustness of the multi-resonance method.

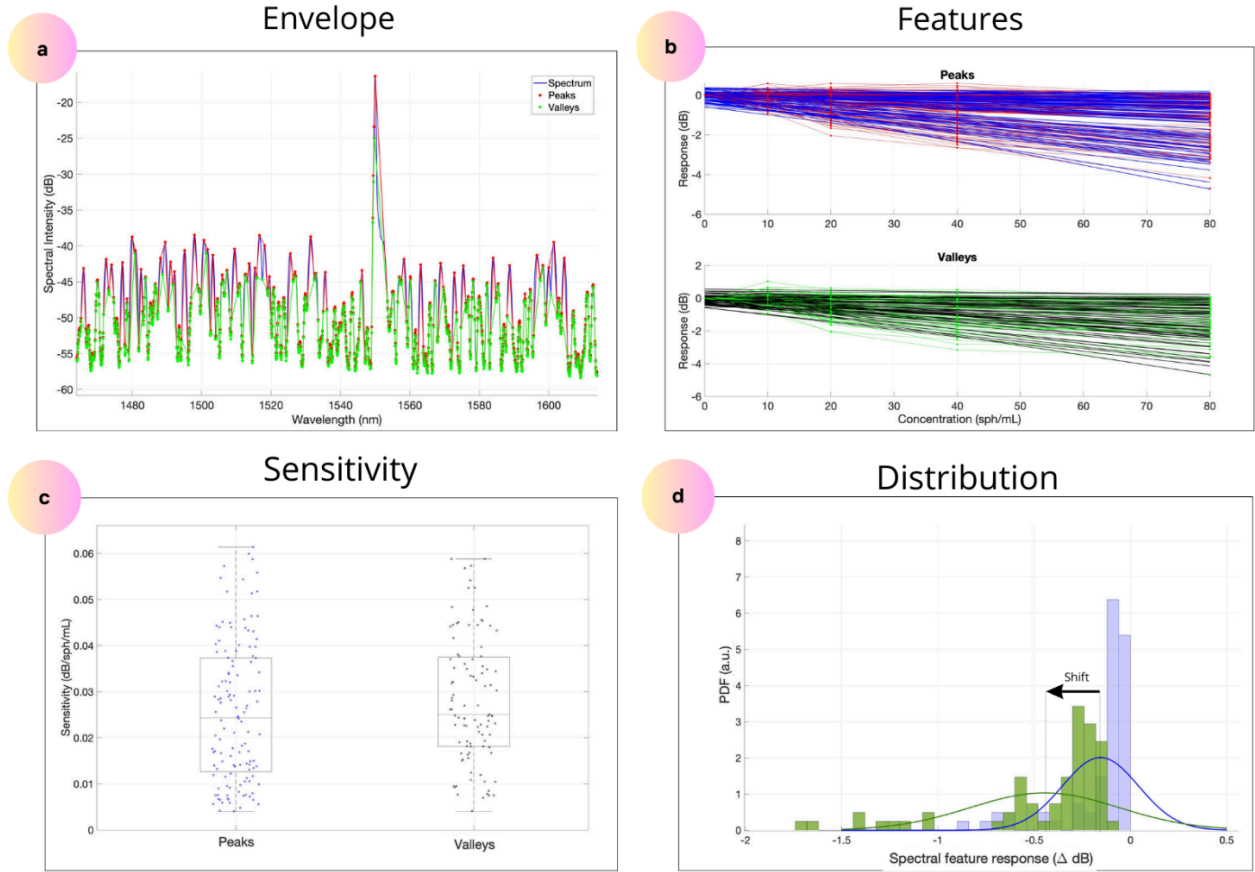


Figure 6. Multi-resonance spectral feature analysis for cancer spheroid detection. (a) Spectral envelope tracking resonance peaks (red dots) and valleys (green dots). (b) Feature response trends plotted as a function of cancer spheroid concentration (0 to 80 sph/mL). (c) Sensitivity (slopes) distribution boxplot for all tracked features. (d) A Probability Density Function (PDF) comparison between the baseline (blue) and high concentration (green).

3.3 HCC1806 and HEK293 spheroid detection in PBS

Figure 7 summarizes the sensor performance for cancer spheroid detection using multi-resonance valley tracking in a PBS environment. Fig.7 (a) represents the raw transmission spectrum of the fiber sensor, and includes the inset of a magnified view of the resonance valley at 1564.6 nm, where intensity decreases as cancer spheroid concentration increases. Panel (b) showcases the time-resolved spectral heatmap of a valley at 1601.2 ± 0.1 nm, which captures the

transition from the initial baseline (0 sph/mL) to a high-concentration state (80 sph/mL) over the course of 100 minutes. The consistent horizontal bands indicate a highly synchronized intensity shift of more than 1dB. PDF plot in (c) shows the negative shift along the response axis as concentration increases from 0 to 80 sph/mL. This visualization clearly demonstrates the increasing separation of the signal from the baseline. Fig. 7(d) displays a step-response for HCC1806 spheroid detection versus time: The real-time intensity change (Δ dB) as concentrations are increased in stages every 20 minutes. The red "Time-Average" line highlights the distinct averages for each concentration step, and reaches 3 dB, which is an absolute change in the response value. A linear calibration curve for this stepwise progression is the mean intensity shift vs. concentration graph. The sensor exhibits high linearity ($R^2=0.963$) and a calculated Limit of Detection (LOD) of 10.60 sph/mL in PBS for a most significant valley. Sensitivity distribution or the slopes for all tracked valleys are shown in Fig.7(f), and a median sensitivity is approximately 0.025 dB/sph/mL.

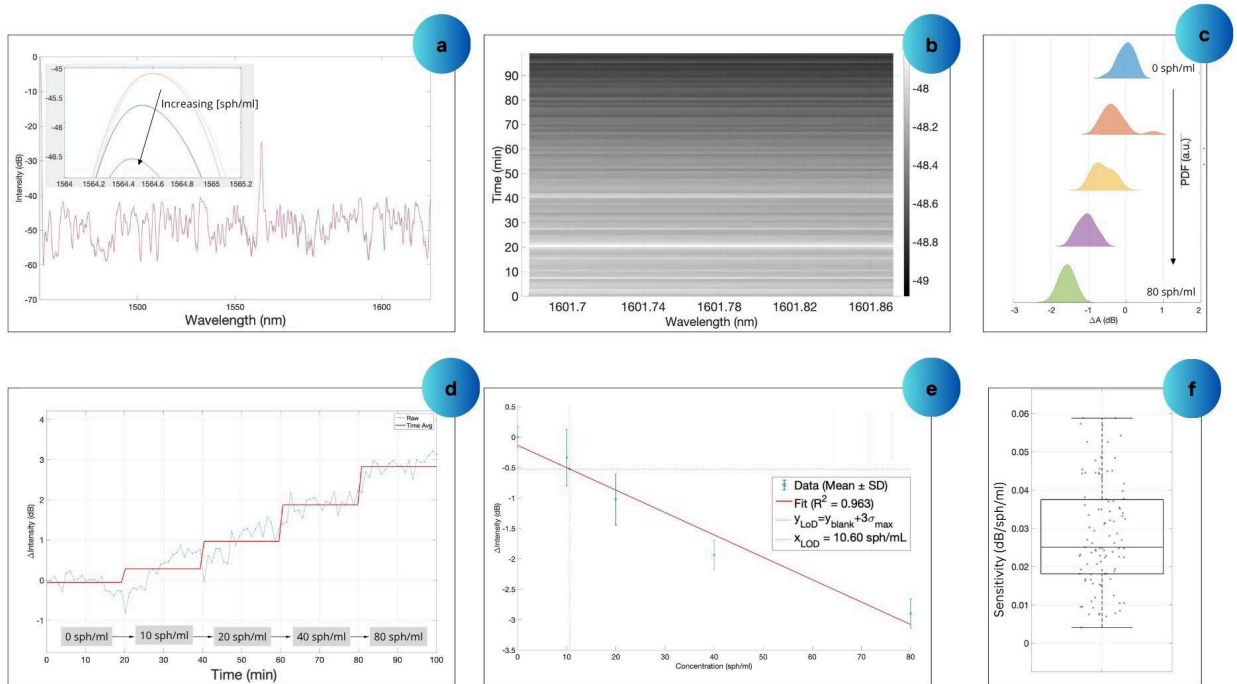


Figure 7. Multi-resonance valley tracking in PBS. (a) Representative spectrum and a magnified resonance detail at 1564.656 nm. (b) Time-resolved spectral heatmap over a 100-minute duration. (c) PDF comparison showing the progressive distribution shift from 0 to 80 sph/mL. (d) Real-time intensity variation(Δ dB) and time-averaged line for each concentration step. (e) Linear regression analysis of the mean intensity shift and standard deviation vs. cancer spheroid concentration with LoD estimation. (f) Box-and-whisker plot summarizing the sensitivity (dB/sph/mL) for all tracked valley features.

Figure 8 is the representation of application of the same analysis, but to the most significant resonant peaks. Panel (a) contains Gaussian function based PDF, where resonance peaks PDFs demonstrate a consistent distribution shift toward negative ΔA values (around -1.5 dB) as the cancer spheroid concentration increases from 0 to 80 sph/mL. Fig. 8(b) illustrates the absolute real-time peak intensity variation (Δ dB) over a 100-minute duration. The gray "Block Avg" line tracks the sensor's average transition between concentration states, highlighting the responsiveness to the increasing concentrations of the target. The calibration curve of the mean peak intensity shift in Fig. 8(c) has worse linear relationship ($R^2=0.831$) than for valleys, but the LoD of 13.87 sph/mL is near to the valley LoD value. Box-and-whisker plot for the tracked peak population in Figure 8(d) shows similar median sensitivity as for the valley, only varying in the spread of the data points.

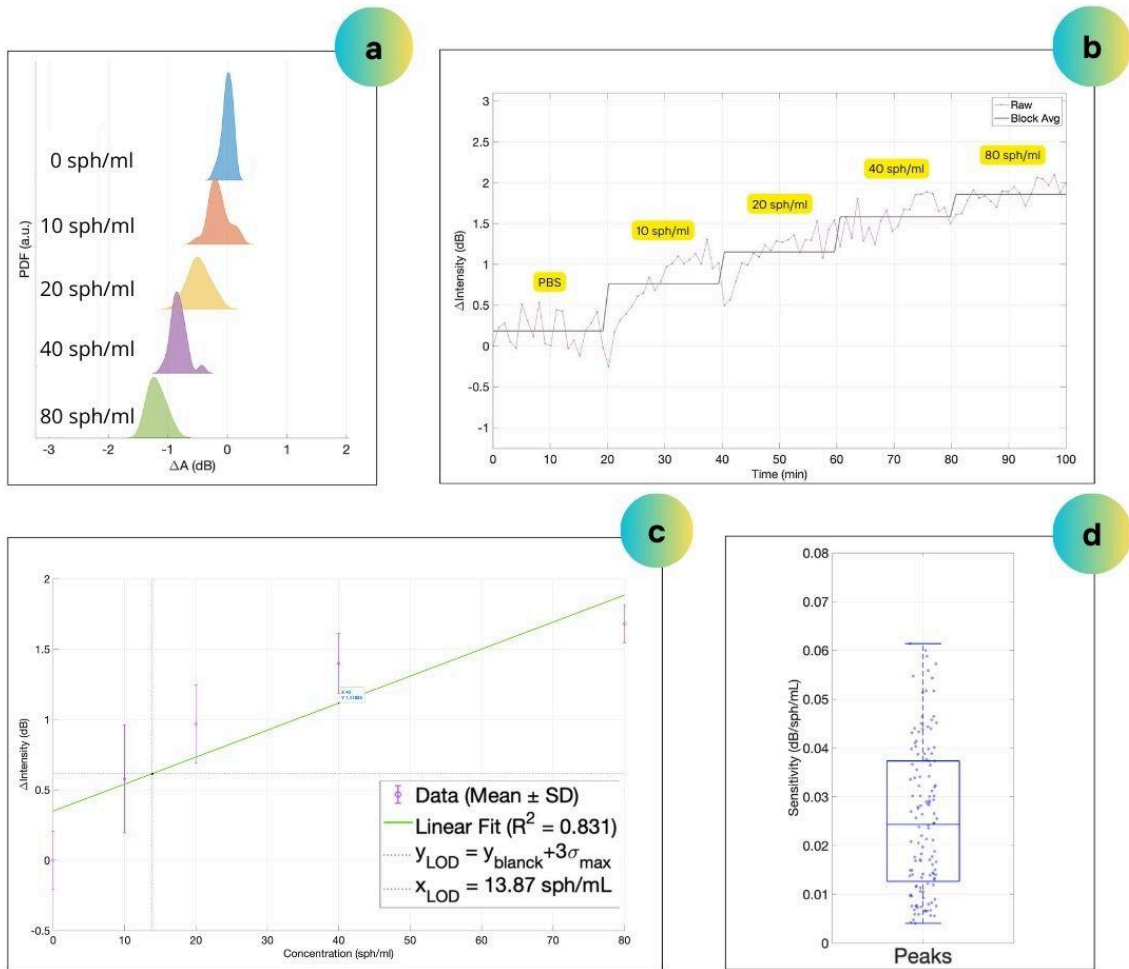


Figure 8. Multi-resonance peak tracking in PBS. (a) PDF comparison for resonance peaks for each cancer spheroid concentration. (b) Raw peak intensity variation (Δ dB) over a 100-minute duration and a gray "Block Average" line for each cancer spheroid concentration block (20 min.). (c) Calibration Curve: Linear regression analysis of the mean peak intensity shift. (d) Sensitivity Distribution for the tracked peak population.

Reproducibility and statistical repeatability estimations of sensor response in PBS are shown in Figure 9. Firstly, individual sensor calibration curves with intensity response (Δ dB) as a function of cancer spheroid concentration are given for seven independent sensor units (Sensor 1–7). Each unit demonstrates a consistent linear downward trend, confirming the reliability of the bio-functionalization process across multiple fiber probes. Sensor 2 has a much steeper slope (reaching -4 dB) compared to others. This behavior may result from a more dense antibody coating on that specific fiber, or the overall sensitivity.

The last panel (HCC Repeatability ($n=7$)) contains normalized aggregate response of all 7 tested sensors. The solid blue line represents the mean response, while the shaded area indicates the standard deviation. The shaded area is wider at low concentrations (10–20 sph/mL) but narrows at 80 sph/mL, suggesting that the sensor is most precise when it reaches its full binding capacity.

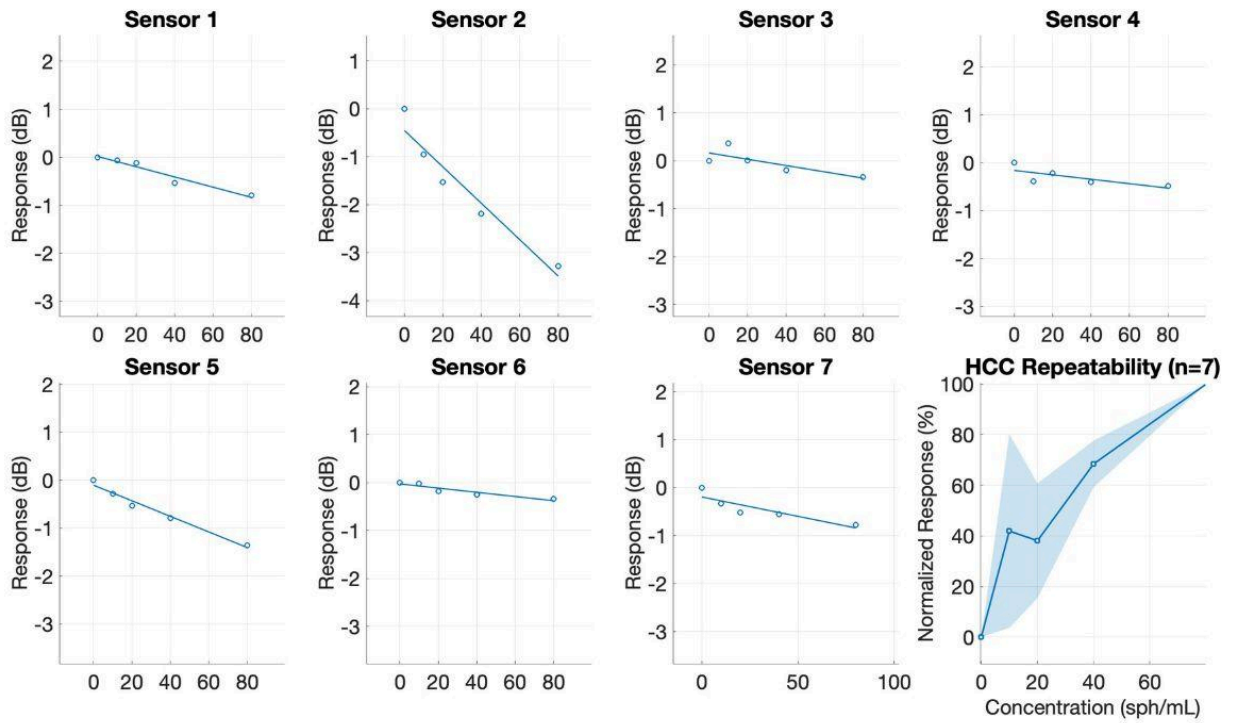


Figure 9. Reproducibility and statistical repeatability of sensor response in PBS. (a) Intensity response (Δ dB) as a function of cancer spheroid concentration for seven independent bio-functionalized sensors ($n=7$). HCC1806 Repeatability ($n=7$): Normalized aggregate response of all tested sensors with the solid blue line representing the mean response, and the shaded area indicating the standard deviation (error bound) (0% - lowest concentration; 100% = highest concentration).

Figure 10(a) is a representative spectrum of the biosensor testing for HEK297 spheroids in a PBS environment. Figure 10 (b) proves selectivity because the biosensor is specifically responding to the cancer spheroids (HCC1806) and not to control spheroids (HEK293) or the PBS buffer. Figure 10 (b) is the stacking of absolute intensity change vs increasing concentrations of HEK293 (purple) and HCC1806 spheroids (green). The target spheroids exhibit a clear, dose-dependent stair-step response, while the non-target spheroids remain within the noise floor of the baseline, demonstrating negligible non-specific binding and high sensor selectivity.

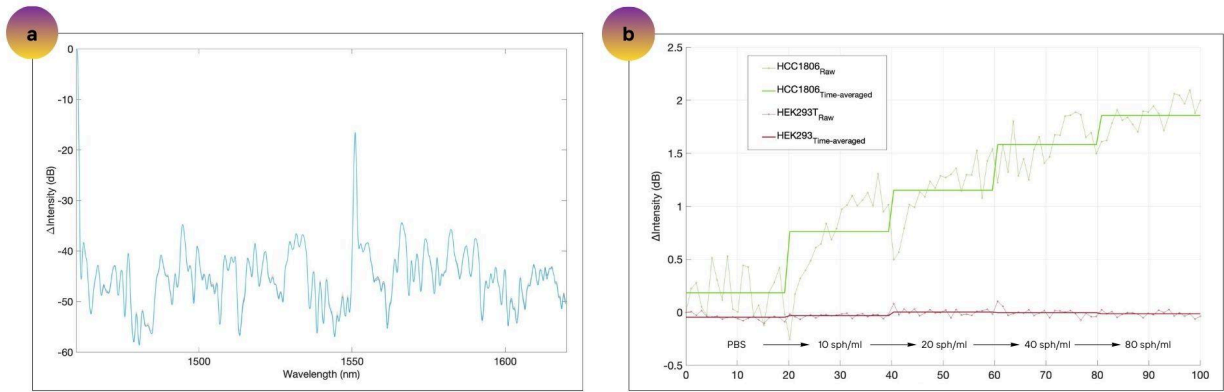


Figure 10. Selectivity analysis of bio-functionalized sensors against non-target (HEK293) spheroids in PBS. (a) Representative Spectrum of HEK293 spheroid detection. (b) Comparative real-time intensity variation (Δ dB) for target HCC1806 cancer spheroids (green) versus non-target HEK293T cells (purple).

3.4 HCC1806 and HEK293 spheroid detection in media with serum

Figure 11 provides a comprehensive analysis of sensor performance in a diluted 10% serum containing media. Panel (a) exhibits a comparative transmission spectra for HCC1806 (blue) and HEK293 (purple) spheroid detecting sensors in a serum environment. For HCC1806 spheroid detection, PDF shift graph (Figure 11(b)) compares the statistical distribution of the sensor response from baseline (serum) up to 80 sph/mL. A clear shift of about -1 dB is observed when concentration of introduced targets is increased. Similarly to PBS analysis, panel (c) provides a comparative selectivity timeline, showing the clear divergence between target HCC1806 (red) and non-target HEK293T (green) spheroid sensing in a serum-containing environment. While the non-target response remains near baseline, proving high selectivity even in complex media, the absolute response for positive control reaches almost 1 dB change. Figure 11(d) presents a horizontal sensitivity distribution boxplot for the tracked features, illustrating

the range of sensitivities, achieving a median slightly higher than 0.01 dB/sph/mL across the spectral comb. This sensitivity is lower than in PBS, which is expected due to the damping effect of the serum refractive index. Panel (e) shows a serum calibration curve with a positive slope and linearity of $R^2 = 0.898$. The calculated LoD in serum is 27.42 sph/mL and it is almost three-times higher, compared to the PBS LoD, which might be an input from non-specific protein adsorption, coming from serum. Panel (f) assesses the multi-sensor reproducibility ($n=5$) across several experimental batches. In general, individual calibration curves for five independent sensors and a normalized aggregate repeatability plot confirm the consistency of the sensor performance.

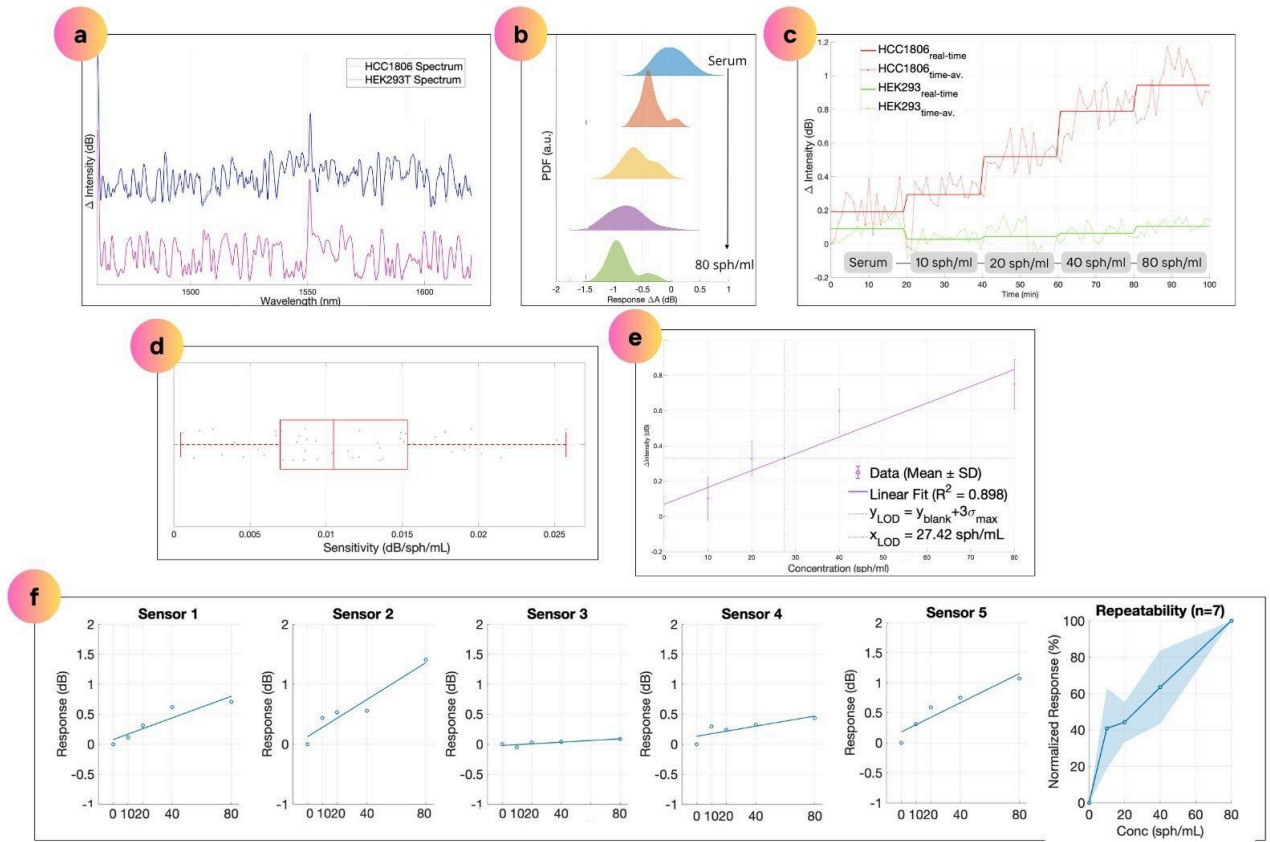


Figure 11. Analysis of biosensor performance for cancer spheroid detection in serum containing media. (a) Comparative transmission spectra for HCC1806 and HEK293T spheroid detection in a serum environment. (b) Sequential PDF comparison of the statistical distribution of the sensor response from baseline (serum) up to 80 sph/mL. (c) Comparative step-response timeline between target HCC1806 (red) and non-target HEK293T (green) in serum. (d) Horizontal sensitivity distribution for the tracked features. (e) Serum Calibration Curve: Linear regression analysis of the aggregate biosensor response. (f) Calibration curves of the most significant features of five independent sensors and a normalized multi-sensor ($n=5$) repeatability plot.

Figure 12 provides a repeatability and aggregate response analysis in serum for biosensors tested in the single experimental batch for HCC1806 spheroid detection. It highlights that while individual sensors show some non-linear fluctuations (Biosensors 1-3), the aggregate trend remains distinguishable. Figure 12(a) shows individual biosensor calibration curves for three representative biosensors (1–3), which include the intensity response to cancer spheroid concentrations in a diluted serum environment. The rightmost panel displays the normalized aggregate repeatability ($n=3$), with the shaded area representing the standard deviation across the sensors. Panel (b) includes a normalized response of biosensors in serum media with HEK293 spheroids, where HCC1806 response is set to 100%. The significantly higher variance, lack of a clear dose-dependent trend compared to panel (a), response mark being below the 40% confirm that the observed shifts in the functionalized biosensors are driven by specific target interactions rather than serum-induced drift.

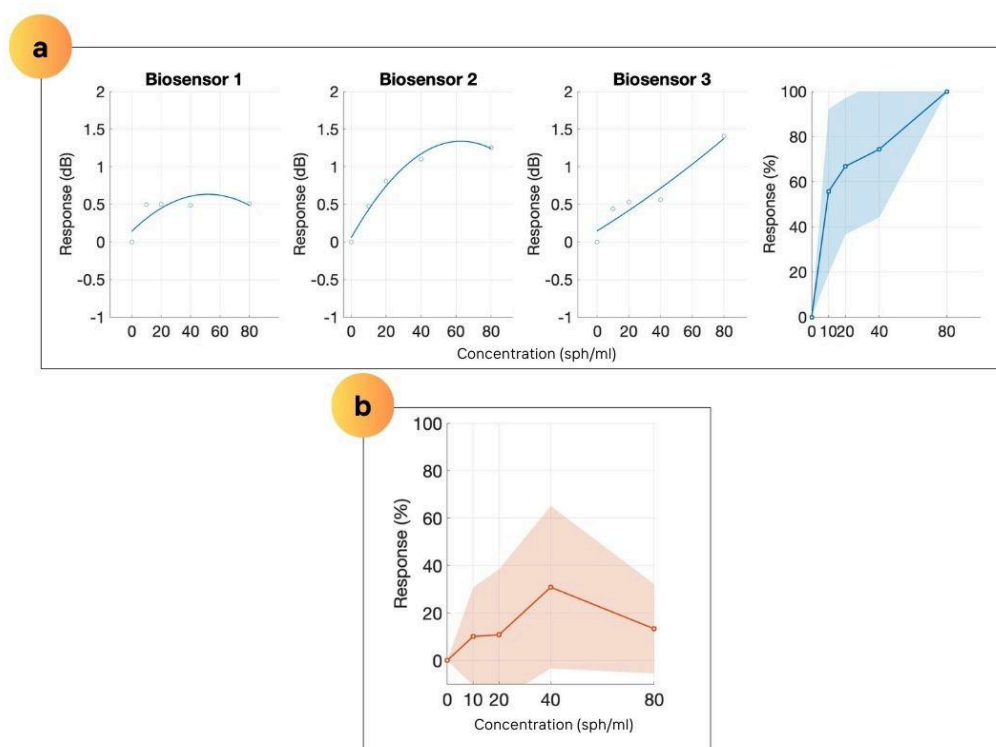


Figure 12. Batch reproducibility and selectivity analysis in serum.(a) **Target (HCC1806) detection:** The most significant spectral feature intensity response curves for three biosensors fabricated in a single experimental batch and a normalized aggregate response ($n=3$), where the blue shaded area represents the standard deviation and the solid line is the average. Data is normalized to the maximum HCC1806 response (100%) to establish a baseline for relative sensitivity. (b) **Relative Cross-Reactivity (HEK293T):** Aggregate response of sensors ($n=3$) exposed to non-target cells in serum, normalized against the target maximum. The orange shaded area represents the non-specific binding ratio.

3.5 Relative performance of the PDMS-coated SDI biosensor

Table 2 summarizes the sensitivity performance of several PDMS-coated optical fiber sensors. Considering the maximum achieved sensitivity for the proposed biosensor of 801 dB/RIU allows estimating the wavelength resolution. Assuming that the average amplitude resolution per an optical sensing interrogator (OSI) is 0.01 dB, the wavelength resolution becomes:

$$\Delta n = \frac{\Delta I}{S} = \frac{0.01 \text{ dB}}{801 \text{ dB/RIU}} = 1.248 \times 10^{-5} \text{ RIU}$$

Δn - change in the RI (RIU)

ΔI - amplitude resolution (dB)

S - Sensitivity (dB/RIU)

Table 2. Comparative performance analysis of different PDMS-coated optical fiber sensors

Sensor structure	PDMS layer	Sensitivity	Wavelength Resolution	RI change	Source
A two-parameter PCF-SPR Biosensor	Part	18,600 nm/RIU	5.38×10^{-6} RIU	1.33 - 1.41	[46] (2025)
A Single-Core Dual-Channel Sensor	Part	9600 nm/RIU	NA	1.33 ~ 1.41	[47] (2025)
SPR-based MOF sensor	Part	28225.7 nm/RIU	NA	1.35 - 1.42	[39] (2024)
PDMS-PCF biosensor	Entirety	13,000 nm/RIU	7.69×10^{-6} RIU	1.2 - 1.28	[48] (2024)
PDMS-filled Au/GST SPR HCF	PDMS core	10 758 nm/RIU	1.8591×10^{-6} RIU	1.333 - 1.373	[49] (2024)
Dual-channel SPR sensor	Entirety	2938 nm/RIU	NA	1.333 - 1.385	[50] (2024)

NCF SPR sensor	Part	5200 nm/RIU	2.87×10^{-5} RIU	1.34 - 1.39	[51] (2022)
Dual-core PCF SPR sensor	Part	20,000 nm/RIU	5×10^{-6} RIU	1.36 - 1.42	[40] (2022)
No core D-type fiber	Part	12530 nm/RIU	NA	1.33 - 1.44	[52] (2021)
PDMS-coated SDI	Entirety	801 dB/RIU	1.25×10^{-5} RIU	1.33 - 1.38	This work

NA - not applicable

Chapter 4 – (Conclusion)

In summary, the proposed PDMS-coated biosensor demonstrates a robust platform for the detection of the CD44 biomarkers present on 3D multicellular cancer spheroids. The fabrication of a $354 \pm 80 \mu\text{m}$ PDMS cap provided a necessary spatial match for the target HCC1806 spheroids ($245 \mu\text{m}$), and improved the overall stability for biosensing. This intentional scaling consequently allowed the sensor to capture bulk refractive index changes. The biosensor achieved a LoD of 10.60 sph/mL in PBS and 27.42 sph/mL in 10% serum. Furthermore, the biosensor maintained high target specificity, consistently distinguishing between target HCC1806 cells and significantly larger HEK293T aggregates ($580 \mu\text{m}$). The non-target response remained below 40% of the target maximum in complex serum media, thereby confirming that the signal is driven by CD44-antibody affinity rather than non-specific protein adsorption. Notably, the wavelength resolution was determined to be approximately 1.248×10^{-5} RIU, offering competitive results in biosensing. Despite these promising results, the current study utilizes 3D spheroids, representing a stabilized "bulk" tumor environment, as a proof-of-concept. Accordingly, a critical future direction involves adapting this platform for the Epithelial-Mesenchymal Transition (EMT). By intentionally dissociating these spheroids into smaller, mesenchymal-state clusters or single cells, the sensor can be validated against the highly mobile units responsible for early-stage metastasis. Moreover, transitioning from the current static incubation to a dynamic flow-based sensing system will allow for real-time monitoring under physiological conditions. Such an advancement would transform the PDMS-coated SDI biosensor into a practical tool, offering rapid, cost-effective, and label-free diagnostics suitable for both clinical laboratories and resource-limited settings.

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