DEVELOPMENT OF A BIOSENSOR FOR THE EARLY DETECTION OF CERVICAL CANCER

Project Reference No.: *45S_BE_2168*

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Keywords:
Cervical Cancer, Pap Smear, Human Papilloma Virus (HPV), Colposcopy, Cervicovaginal Fluid, Biosensor, Chemiresistive, Polymer, Crosslinker, Antibody, ELISA, CIN, Bradford's test, Resistance, SEM

Introduction:
Cervical cancer is one of the common causes of death among women in developing countries. According to the 2020 cancer statistics of WHO, in India, cervical cancer is the 3rd most cancer with respect to new cases and ranks 2nd with respect to death. Despite being one of the treatable cancers, the mortality rate is high because of its diagnosis. The Pap smear test which is a routine test to detect cervical cancer is based on the detection of morphological changes in cervical mucosal cells. Unfortunately, the low sensitivity of this test can result in cervical cancer being diagnosed at a late stage.

As a result, despite having great advances in surgical and adjuvant therapy, the overall survival of cervical cancer patients, especially that of advanced patients, is still very poor. Apart from pap smear tests, Human papillomavirus (HPV) testing is also done as HPV is one of the major causes of cervical cancer. However, the HPV test has low specificity and a high false-positive rate for cervical cancer, mainly because the virus may or may not lead to cancer as 80% of the HPV-positive patients spontaneously clear the virus. Hence treatment based on the HPV test may lead to the overtreatment of patients leading to further complications. Additionally, colposcopy examination is labour-intensive, difficult to automate and vulnerable to inter-and intra-observer variation.

The reason why the development of a diagnostic biosensor is based on cervicovaginal fluid (CVF) is, CVF is a potential source of biomarkers for diseases of the lower female reproductive tract and the fluid can easily be collected by the individual which is a fast and simple sample process. It is estimated that only 5% of women in low-resource countries are screened appropriately for cervical cancer. Lack of healthcare infrastructure and financial cost are the main reasons why cytology-based programs are not implemented. Since alternative methods are currently investigated in these regions, use of a CVF-based diagnosis test could be considered.

Hence the project aims to develop a biosensor which has the potential to detect cervical cancer at its precancerous stage helping with the early diagnosis of the disease using

cervicovaginal fluid as the sample. Chemiresistive biosensors are being chosen as the biosensor type for this project. These biosensors rely on a direct chemical interaction between the sensing material and the analyte.

The first step in the fabrication of this biosensor is the selection of the candidate biomarker. This candidate biomarker is carefully chosen and an Elisa test is performed to validate the biomarker's quantity in the normal and cancerous cervicovaginal fluid samples. A printed circuit board (PCB) is employed as the substrate and a conducting polymer is coated on top of this substrate.

In the fabrication process, polymer nanomaterial is prepared and mixed with binder and binder solvent which results in a paste consistency which is then coated on PCB substrate. Crosslinker of a specific concentration is coated on top of the sensing material. Further antibody which is the biological recognition element is immobilized on the biosensor for the detection of the biomarker. In the fabrication of the biosensor, the initial experimentation and optimization are carried out using human IgG antibodies and IgG from Blood serum. At a later stage, these will be replaced by the candidate biomarker specific antibody for the detection of candidate biomarker in the CVF sample.

Overall, the designed biosensor is expected to possess high specificity towards the detection of cervical cancer at its early stage with enhanced sensitivity and robustness and thereby strengthening the health care system.

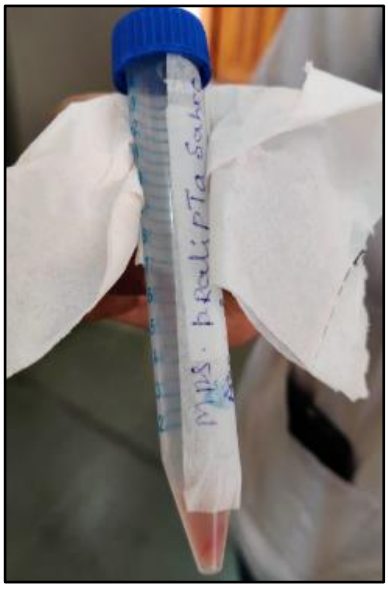
Objectives:

- To identify and validate the potential cellular based biomarker for the early detection of cervical cancer.
- To fabricate a biosensor with high specificity towards cervical cancer diagnosis at its early/precancerous stage based on the candidate biomarker concentration in the cervicovaginal fluid sample.
- To test and optimize the sensitivity of the biosensor in real time by using cervicovaginal fluid samples.
- To publish the outcomes of the study in reputed journals.

Methodology:

Cervicovaginal fluid sample collection methodology:

- The required CVF samples were collected from Bhagawan Mahaveer Jain Hospital, Bangalore.
- The pap smear test sample procedure was employed to collect the sample using cervical broom and the collected CVF was suspended in 1ml of PBS solution.

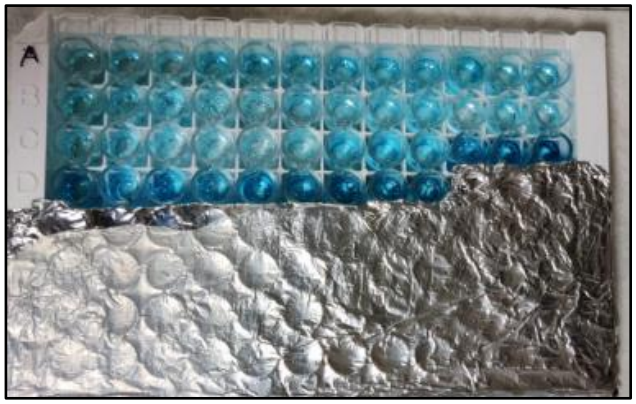
CVF samples collected from Hospital

Transportation and storage methodology:

- Once the sample is collected, it was transported to the college laboratory using ice box.
- Further centrifugation was carried at 3000 rpm for 20 -25 mins.
- Aliquoted and stored at -70 degree Celsius.

ELISA: Elisa test was performed using the Candidate biomarker specific Elisa kit from Krishgen Biosystems to quantify the Candidate biomarker in the cervicovaginal fluid sample procured from the hospital. This test will validate the candidate biomarker concentration in the obtained samples.

Candidate biomarker specific Elisa Kit from Krishgen Biosystems



Positive wells turned blue after adding TMB substrate

Blue to yellow color after adding stop solution

Bradford's test (total protein concentration): Bradford's test was performed to know the total protein concentration in CVF samples.

Bradford's Test

Biosensor Fabrication: In the fabrication of the biosensor, the initial experimentation and optimization is carried out using human IgG antibodies and IgG from Blood serum. At a later stage, these will be replaced by candidate biomarker specific antibody for the detection of candidate biomarker in the CVF sample.

- **Polymer nanoparticle preparation:**
  The polymer nanoparticle base for the biosensor was prepared by an aqueous polymerization pathway methology.
- **Coating of nanoparticles onto the substrate:**
  Polymer nanomaterial is prepared and mixed with binder and binder solvent which results in a paste consistency which is then coated on PCB substrate by drop casting method.
- **Coating of crosslinker:**
  Crosslinker is coated on the polymer nanoparticle layer by the drop-casting method as crosslinker between polymer nanoparticles and antibody. So 3 different concentrations (2%, 2.5% and 3%) and volumes of crosslinker are being experimented with for the optimization.
- **Serum complement inactivation:**
  Since the initial experimentation and optimization are carried out using human IgG antibodies and IgG from Blood serum, Blood was collected from hospital and centrifuged. Then the serum is complement inactivated.
- **Coating of antibody:**
  Once the crosslinker is coated, the IgG antibody is coated on the crosslinker coated layer and incubated. Further, it is blocked using 1% BSA and and later washed with PBS to remove the unbound antibodies.
- **COATING AND INCUBATION OF SERUM igg FROM SERUM SAMPLE:**
  Once the antibodies are coated, blocked and washed, the assay is carried out by adding serum to the sensor and was incubated. The change in the resistance will be measured. In the fabrication of a biosensor, after each step, the change in the resistance will be noted by giving constant current input and a change in the voltage is obtained as output from which the resistance will be calculated.

- **Dot ELISA**
  Dot ELISA is done with human IgG antibodies and IgG from Blood serum on nitrocellulose membrane and exact steps were followed same as in the case of biosensor where the first the step is antibody coating, with a hypothesis that if the colour change is obtained in the dot ELISA then the biosensor will also produce the same result. This was done to replicate the working of a biosensor.

Future steps:

Optimization of antibody and antigen: Further, on the optimized Crosslinker concentration, different concentrations and volumes of antibody and sample (antigen/biomarker) will be experimented to standardize the protocol.

Replacement of IgG antibody and IgG from serum by candidate biomarker specific antibody and candidate biomarker from CVF sample: Once the biosensor fabrication protocol is optimized/standardized, IgG antibodies and IgG from serum will be replaced by candidate biomarker specific antibody and candidate biomarker from the CVF sample for which the biosensor is developed.

Further, the developed biosensor will be subjected for **testing with large sample size** and possibly **miniaturisation and optimization** will be done to make the biosensor portable.

Results and Conclusions:

ELISA RESULTS:

Patient	**Age**	**Condition and History of Treatment**	**Conc of the biomarker in CVF (pg/ml)**
P-1	58	**Ovarian Cancer - Stage -3 (Advanced)** Pain-abd, CT scan shows ovarian malignancy, uterus is normal	90
P-2	35	**Endometriosis** uterus and cervix is normal	184
P-3	67	A case of **Cancer of Endometrium** but has undergone Radical Hysterectomy in Dec-2021. There is no evidence of disease now. Now under follow up.	150
P-4	48	**CIN 1 (precancerous cervical cancer) - test sample** Has undergone hysterectomy for CIN in 2017. Now under follow up.	142
P-5	-	**Endocervical and endometrial growth** vaginally discharge and bleeding	130

Candidate Biomarker concentration obtained from ELISA results

From the Elisa results towards quantification and validation of biomarker, P-4 with CIN (precancerous cervical cancer) shows high concentration of candidate biomarker which proves that the biomarker is overexpressing at the precancerous stage of cervical cancer. P-1 with stage-3 of ovarian cancer shows the presence of candidate biomarker in CVF sample. However, the candidate biomarker is not overexpressed in CVF with respect to ovarian cancer patients. P-5 with endocervical and endometrial has good candidate biomarker concentration but less than P-4 (CIN). Since candidate biomarker is one of the protein which involves in cytoskeletal mechanisms to maintain the integrity of the cell, so when the cells divide in these types of growth, the concentration of candidate biomarker also increases which explains the increase in case of P-5. P-2 (endometriosis) and P-3 (endometrium cancer) has high candidate biomarker concentration than P-4 which might question the specificity of the biomarker. In endometriosis, the cells in the endometrial layer of the uterus grows outside the uterus and might shed few cells into the CVF which might be the reason for the candidate biomarker increase. However, the P-4 has undergone hysterectomy as the treatment of cervical cancer which might involve the removal of certain part of cervix or entire cervix. So based on the results obtained, the complete validation of the candidate biomarker cannot be done as proper untreated cervical cancer of different stages have been studied yet. So **more of cervical cancer samples of different stages is needed** to know the concentration range of the candidate biomarker in the CVF samples.

BRADFORD'S TEST RESULTS:

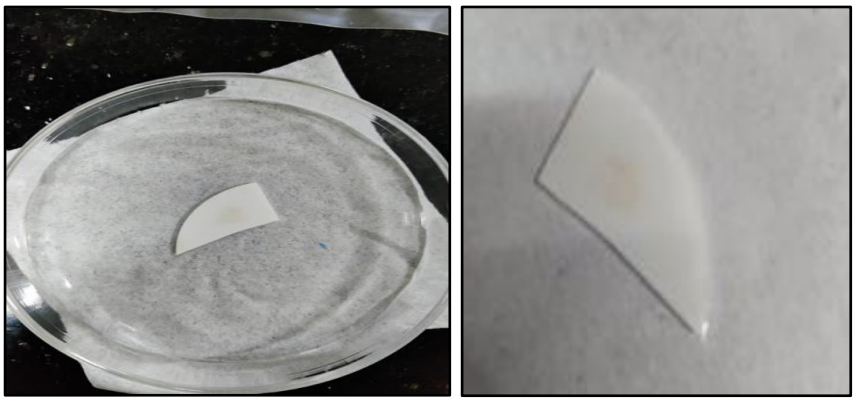
Patient Sample	**Total protein of the sample (µg/ml)**	**Conc of Candidate biomarker in total protein of the sample (pg/ µg)**
P-1	72	1.25
P-2	96	1.916
P-3	65	2.307
P-4	**40**	**3.55**
P-5	62	2.129

Bradford's test Result

Among 5 samples, **P-4 has the highest concentration of Candidate Biomarker** per microgram of the total proteins in the sample which paves the way for further investigation.

Dot ELISA

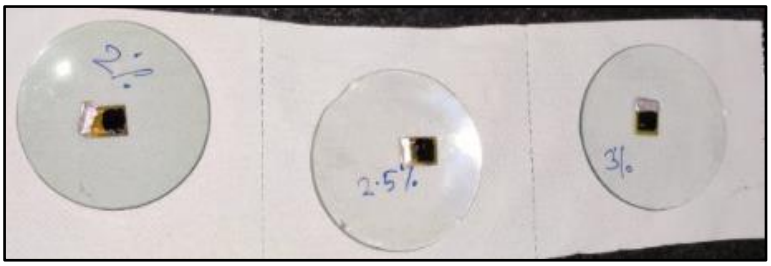
Colour change was observed on nitrocellulose paper



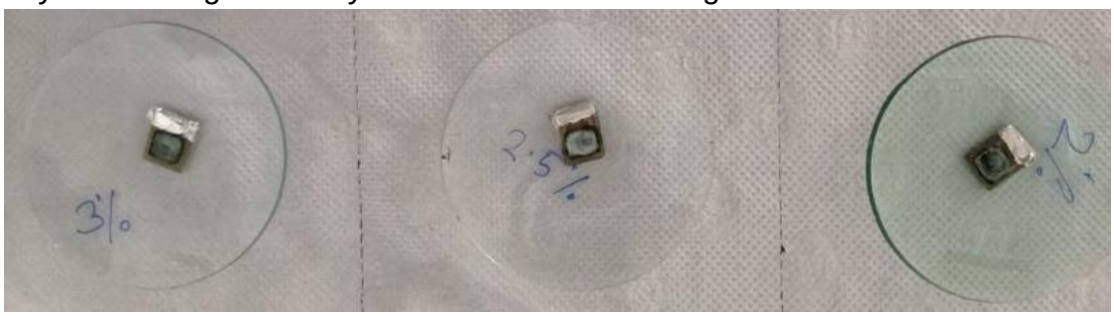
Light Brown dot was observed on nitrocellulose paper**.**

Drop casted sensors:

Polymer coating



Polymer + crosslinker coating at 3 different concentrations



Polymer + crosslinker at 3 different concentrations + Antibody coating (blocked with 1% BSA and washed)

Base resistance analysis: Biosensor being measured using the nano voltmeter and a current source, the resistance was measured, the current was set to 1 milliampere. The two terminals of the sensor were connected to different polarities of the probe and the resistance was measured.

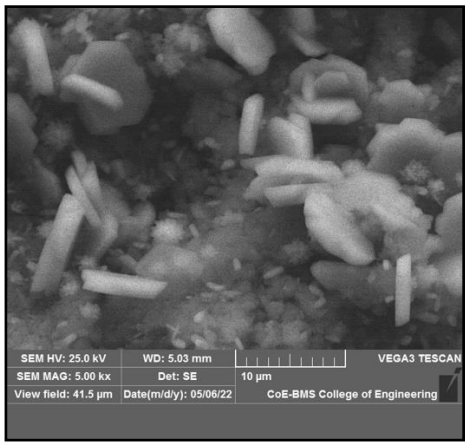
Resistance measurement

Sl.no	Sample	Base Resistance measured	After blood Serum Sample
1	Polymer+crosslinker-2%	75 k **Ω**	-
2	Polymer+crosslinker -2.5%	73 k **Ω**	-
3	Polymer+crosslinker -3%	77 k **Ω**	-
4	Polymer+crosslinker-2% + Antibody	85 k **Ω**	45k **Ω**
5	Polymer+crosslinker-2.5% + Antibody	84k **Ω**	2.5k **Ω**
6	Polymer+crosslinker 3% + Antibody	90.8 k **Ω**	2.2k **Ω**

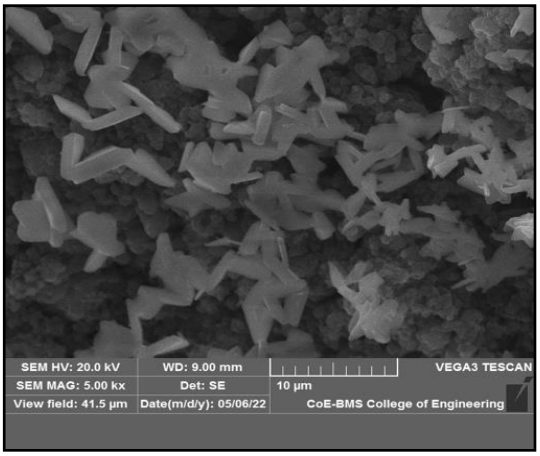
Base resistances after crosslinker and antibody coating and resistance reading after adding serum sample.

The resistance of the crosslinker coating is around 75 kiloohms and it increases in the presence of antibodies to approximately 85 kiloohms. Once protein from blood serum binds to the antibody, the resistance decreases drastically which indicates the binding reaction.

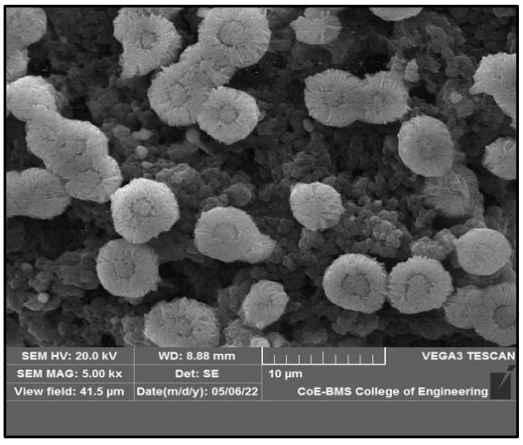
SEM analysis: 3 biosensors were coated with different concentrations (2%,2.5% and 3%)



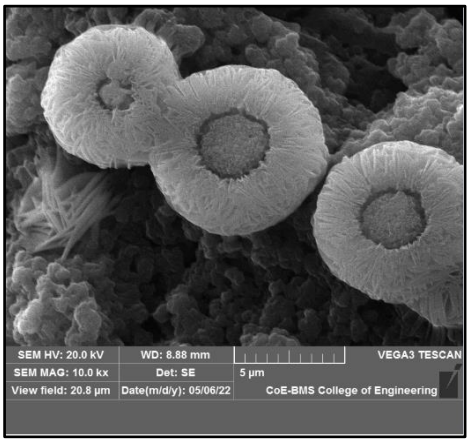
Polymer+crosslinker-2% at 10 µm resolution



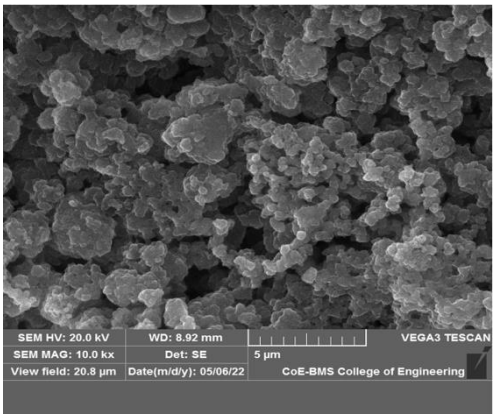
Polymer+crosslinker-2.5% at 10 µm resolution



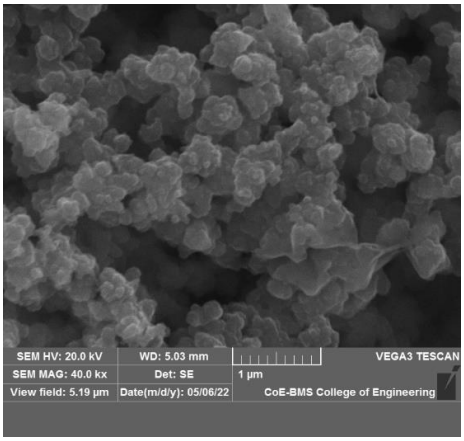
Polymer+crosslinker-3% at 10 and 5µm resolution



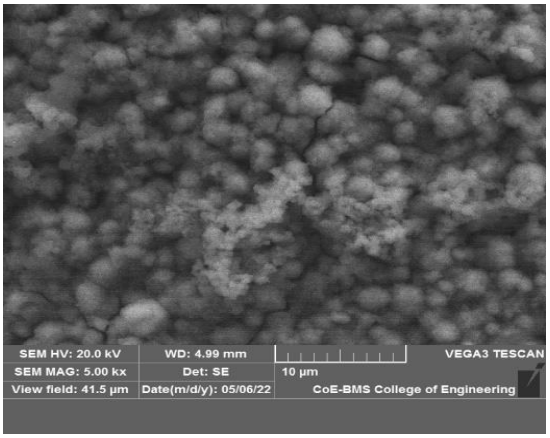
3 biosensors were coated with antibodies on 2%, 2.5% and 3% different concentrations of crosslinker



Polymer+crosslinker-2% + Antibody at 5µm resolution



Polymer+crosslinker-2.5% + Antibody at 1µm resolution



Polymer+crosslinker-3% + Antibody at 10 µm resolution

According to the base resistance analysis, 3% crosslinker coating has shown a high variation in resistance and can be used in sensors to clearly show the variation. The various crosslinker concentrations (2%, 2.5%, 3%) were compared using SEM analysis. The sensor with 3% crosslinker coating showed a flower-like structure and uniform coating. Hence we have optimised crosslinker concentration to 3%. Further we would be optimising the concentration of antibody and antigen in sample on 3% crosslinker coated sensors.

Scope for future work:

1. We have filed for the **provisional patent**.
2. We plan on doing characterization of selected crosslinker concentration with FTIR.
3. We have applied for ethical clearance in HCG for a larger sample size.
4. We plan on optimising the concentration of antibody on 3% crosslinker Testing with clinical samples to check and identify the LOD of the sensor.
5. Publishing paper in a reputed journal.