

Calmodulin Functionalized Porous Silicon Based Electrical Calcium Detector and its Comparison with Optical Detector

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Research Article

Keywords: electrical detector, porous silicon, calmodulin functionalized, high selectivity, comparative study

Posted Date: May 23rd, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1635553/v1>

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Abstract

In this work a bi-parametric, highly selective, electrical calcium detecting platform is fabricated. The fabricated detector consists of porous silicon substrate which is surface functionalized by calmodulin. The use of porous silicon provides many fold increment in surface area, facilitating effective absorption, where as calmodulin functionalization upon the porous surface provides excellent calcium attachment sites. The electrical parameters of this detecting platform are then tested with calcium and several other ionic solutions, at concentration relevant physiologically. The fabricated detector showed tremendous selectivity towards calcium ion. Even in mixture ionic solution the detector exhibited its high discrimination towards calcium ion. Thus the fabricated detecting platform can work as an effective calcium detector. Its bi-parametric approach provides enhanced calcium detection efficiency. Hence the detector imparts a highly selective, robust, label free and cost-effective alternative for calcium detectors, effective even in biologically relevant concentrations. Later a comparative study is performed between the optical and electrical response of the detector platform to provide a better understanding of the consistency of the detector developed that in future will facilitate multi-parametric approach to enhance the response, selectivity and thus the efficiency of the detector platform developed in this work.

1. Introduction

Calcium in ionic form is a crucial element of the human body. Besides being the most abundant cation in the human body, it plays a vital role in various biological activities [1-4]. Calcium plays an important role in cardiac and skeletal muscle excitation, neurotransmission, blood clotting, it is an important component of the skeletal minerals [5-6]. Moreover its predominance in cellular signaling pathways makes it one of the most essential cation in the human body [2-4]. Unusual alteration of calcium concentration in the human serum is a clear indication of numerous malfunctioning in the human body. Drastic increment in calcium concentration, namely hyper-calcemia is a clear indication for hyperparathyroidism, hyperthyroidism and malignancies [7]. In fact hyper-calcemia is a common anomaly associated with malignancies, being the almost inevitable in case of lung cancer [7-11]. Thus serum calcium detection becomes crucial in early detection and diagnosis of several malignant diseases but most importantly in case of lung cancer. Techniques developed so far for calcium detection are Spectroscopic technique [12-14], Potentiometric techniques [15-17], Chromatographic techniques [18] and electrochemical techniques [19-20]. Most of the techniques besides being costly requires huge system size, are complex instrumentally and pose limitations in selectivity and sensitivity.

In this work an electrical ion detector is designed using calmodulin functionalized porous silicon (PSi) substrate, capable of precise detection of calcium ion even in concentration relevant biologically. PSi is an emerging new material, having huge microelectronic application [22]. Unlike silicon, PSi shows a wide and direct band-gap due to quantum –confinement effect, for which it finds wide range of sensor applications [22]. Moreover PSi provides huge surface area to volume ratio making the material fit for bimolecular interaction and absorption [23-25]. Its cheap fabrication process and easily modulable porosity and pore diameter makes PSi a great choice for bio-molecule sensor [24, 26-30]. In this work PSi

is fabricated by electro-chemical etching process [24, 26-27], which is further functionalized using calmodulin. Calmodulin (CaM) is a common calcium binding protein common to all eukaryotic cells [31-34]. Besides taking part in important cellular functioning, CaM is effective in calcium binding [31-33]. Its great affinity towards calcium makes it's a perfect choice for a calcium binder [31-34]. The detector thus fabricated provides huge surface area for absorption due to the presence of P-Si, while CaM surface functionalization helps in efficient calcium attachment. The detector thus fabricated has been previously put to use in optical calcium detection [35], where parameters like reflectance, scattering and absorption losses were used as parameters for the detection of calcium ion. The optical detector exhibited appreciable response towards calcium ion detection. In this work the electrical parameter of the fabricated detector is studied. The detector showed dramatic change in electrical parameters when tested for calcium ion. Moreover the fabricated detector showed high selectivity towards calcium ion in comparison to other mono-valent and di-valent cations. The fabricated electrical calcium detector was tested successfully for calcium detection in composite salt aqueous solution. The detector was found to sensitive towards calcium ion in biologically relevant concentration. Later, a comparative study is conducted to compare the response as well as the consistency of the electrical detector fabricated in this work with the optical detector [35]. Appreciable level of consistency is observed for the electrical calcium detector developed in this work with the optical calcium detector developed previously [35]. Later a comparative study of the responses of both these detectors is conducted, that may facilitate multi-parametric approach, provide enhanced sensitivity for any future work in this field.

2. Experimental

2.1 Materials:-

Hydrofluoric acid (HF, 48%), Ethanol (C₂H₅OH, 99.9%), N, N-Dimethylformamide (DMF) (HCON(CH₃)₂, 99.8%), Silicon wafer (P-Type, <100>, resistivity 10–20Ω), Sulphuric acid (H₂SO₄, 98%), 2-Amino-2 (Hydroxymethyl)-1,3-Propanediol (Tris buffered saline (TBS)), Calmodulin (CaM) Bovine (Lyophilized powder, ≥ 98%), Calcium Chloride (CaCl₂, 99.9%), Magnesium Chloride (MgCl₂, 99.9%), Manganese Chloride (MnCl₂, 99.9%), Sodium Chloride (NaCl, 99.5%), Potassium Chloride (KCl, 99%), Ultrapure water (deionized (DI) water ~ 18MΩ resistivity) (Milipore Co.). The chemicals were obtained from Sigma-Aldrich.

Fabrication of sample:-

P-type < 100 > Silicon wafer of resistivity 10–20Ωcm and dimension 1.5×1.5 cm is acid polished and cleaned thoroughly. At the lower surface of the wafer, metal is deposited by vapour deposition method to establish ohmic contact. The wafer is further cleansed multiple times with > 99% ethanol for electrochemical etching process. P-Si is fabricated upon the silicon wafer by electrochemical etching. HF resistant electrochemical bath of any acid resistant material like Teflon is used for the process. Electrolyte solution of HF 48% and > 99.8% DMF is prepared in the ratio 1:3 and electrochemical etching is initiated within this electrolyte with silicon wafer acting as anode and graphite rod as cathode. Current density of

22mA/cm² is maintained and the etching is continued for 30min. hereafter the prepared PSi is washed multiple times with >99% ethanol and then with deionized water. The prepared PSi is then left to be dried at room temperature. For ohmic contact on the detector surface, Silver-nitrite paste is applied on the detector surface, dried for 12 hrs at room temperature and then heat treated at 450°C for 45–50 seconds for stabilization of the PSi structure as well as for the stabilization of the ohmic contact established. Electrical continuity is checked multiple times over the ohmic contacts to ensure error free connection.

CaM solution is prepared by diluting CaM in trizma base saline solution (TBS) in 1:1 ratio and stirring it in magnetic stirrer at 1900rpm for 10 minutes. The pH of the CaM solution is modulated to 7.3. The sensor surface is prepared by drop casting the CaM solution of pH 7.3 upon the prepared PSi surface. The sensor is then incubated for 16–18 hours at 4⁰C which ensures complete CaM absorption within the pores of the PSi substrate. CaM is thus physisorbed upon the PSi surface and is hence immobilized. The incubated sensor surface is then cleansed multiple times with de-ionized water to remove excess CaM. The sensor is dried at room temperature for 3hours before it is made ready for further testing. The overall schematic process of the fabrication of the detecting platform is shown in the Fig. (1).

Ion solutions namely Ca²⁺, Mg²⁺, Mn²⁺, Na⁺, K⁺ and the salt mixture solution containing all the above salts were prepared by dissolving 0.01mM of each salt in 0.5M TBS and magnetic stirring the same at 1900rpm for 10 minutes. The pH of each salt solution is maintained at 7.3.

The prepared detector surface is used for data acquisition, without any ion incubation and the reading obtained is treated in the experiment as the reference level reading. For the ionic solution incubation of the detector surface, ionic solution of 40µl was drop casted on the surface. The ionic solution is left on the detector surface for complete physisorption for 30 minutes. The detector surface is then thoroughly washed multiple times with de-ionized water to remove excess solution. It is then dried completely at room temperature before it is used for data acquisition. The entire process is carried out under ambient pressure and temperature conditions. After the data acquisition for each ionic solution, the detector sample is dipped in calcium chelating solution to remove the calcium (if any) attached to the surface. After which the detector is washed multiple times with de-ionized water, dried completely at room temperature. Readings are obtained again with this washed detector and tallied with the reference level reading to check for any buffer effect present. On the detection of any buffer effect, the washing routine is repeated from the step of dipping in calcium chelating agent. The detector is now ready for next the ionic solution data acquisition.

2.2 :- Characterization

The prepared detector surface is structurally investigated by performing X-ray diffraction spectroscopy and FESEM. The electrical properties, namely conductive and capacitive property of the sensor are performed using an electrical parameter analyzer (kethiley) and a capacitive parameter analyzer under air- ambient condition. Two ohmic contacts were established upon the detector surface and the electrical parameter data were collected using a data acquisition system. The sensitivity of the detector is tested by

monitoring the change in current (I) of the sensor surface against increasing voltage (V) applied across the detector surface. The voltage was applied across the established ohmic contacts while the detector surface is incubated with respective cationic solution. The frequency (f) versus capacitance (C) data of the detector is monitored by applying voltage of varying frequency across the detector surface and respective capacitive readings were monitored, while the detector surface is incubated with respective cationic solutions. Both the electrical parameters were measured using ionic solutions of concentrations that are biologically relevant. The detector surface is also tested with the mixture ionic solution and the results were tallied accordingly.

3. Results And Discussion

The structural investigation of the sample prepared is performed first by X-ray Diffraction spectroscopy (XRD) (Bruker D8 Advance Diffractometer). The 2θ value for the sample is varied from 10° to 85° , at a scan speed of 0.1s and step size of 0.02° . A source of CuK α ($\lambda=1.5418\text{\AA}$) is used for the purpose. Generator parameters were fixed at 40KV and 40mA. Fig(2) shows a sharp peak at $\sim 69.9^\circ$ which is characteristic for silicon based sample, PSi sample in this case. The morphological investigation of the sample was performed by FESEM (FEI INSPECT F50). Fig 3(a) confirms the development of PSi sample with uniformly distributed pores, of diameter ranging from 780 to 1073nm visible on the silicon surface. Fig 3(b) shows the detector surface after surface-functionalization. CaM can be seen clearly attached to the PSi surface.

The schematic diagram of the final detecting platform structure is shown in the fig (4). The electrical response of the detector is tested with and without Ca $^{2+}$ ion incubation of the detecting platform. The current (I) response of the detecting platform is calibrated by simultaneous variation of the applied voltage (V) across it, within a fixed voltage range. As shown in fig 5(a), the detector's current response is seen to be almost exponential, when the platform is incubated with Ca $^{2+}$ ion, in the voltage range of 2.5-5V, clearly visible by the red line in the graph, in comparison to the response for the detector without ion incubation, depicted by the black line in the same graph. The substantial increment in the current is attributed to the binding of the Ca $^{2+}$ ion to CaM functionalized PSi detecting platform. Due to the binding affinity of CaM towards Ca $^{2+}$ ion, Ca $^{2+}$ ion gets attached to the detecting platform, which creates conduction pathways in the detector platform, causing resistance to decrease drastically resulting in the exponential rise in the current value. The detector's selectivity towards Ca $^{2+}$ ion is further tested by the calibration of the current versus voltage characteristics of the detector for other bi-valent and mono-valent cations such as Mg $^{2+}$, Mn $^{2+}$, Na $^+$ and K $^+$ ions (those which are commonly present in human body). Fig 5(a) shows no drastic current change of the detector for other ions. For the mixture ion solution, the detector's response closely mimics its response in the case of Ca $^{2+}$ ion, as shown by the turquoise line in fig 5(a). The detector thus prepared shows high discrimination towards Ca $^{2+}$ ion within all the other ions, which is evident from the detector's response for the mixture ion solution. Fig 5(a) shows the exponential increment of the current response in the case for detector incubated with Ca $^{2+}$ ion, in comparison to the

other ions. The detector's highly discriminative response for Ca^{2+} ion is also demonstrated from the closely mimicking graphs of current response for Ca^{2+} ion and mixture ion incubation.

The change in capacitance value for changing input voltage frequency is calibrated for the detector. As shown in fig 5(b), the detector shows high capacitive response for Ca^{2+} ion incubation at low input voltage frequency, in comparisons to other ions. The Capacitance value however diminishes with increase in voltage frequency. For the mixture salt incubation the capacitance response of the detector closely mimics the response for Ca^{2+} ion, once again demonstrating the high selectivity of the detector towards Ca^{2+} ion among all the other ions under consideration.

The change in capacitance value in lower frequency range ($10\text{-}10^5$ Hz) can be attributed to differential polarization effect [36-37]. In lower frequency range of the input voltage the space-charge polarization and orientational polarization results in the change of dielectric constant, characteristics for nano-structured materials that leads to the change in the capacitance value [37, 38]. A nano-structured material like PSi possesses a large number of interfaces and numerous defects within these interfaces. On application of external electric field, the change in the positive and negative space charge region that occurs through these defects produces dipole moment. The developed dipole moment is termed as space-charge polarization. The rotation of these dipole on application of external electric field results in orientational polarization, effecting the dielectric constant of the material, hence the capacitance value gets effected [36, 37, 39]. In frequencies greater than 10^5 , the dipoles are unable to orient themselves as rapidly as the externally applied electric field changes, resulting in the nullification of the orientational polarization effect. At higher frequency only space charge polarization effect is present, which too saturates at even higher frequencies that explains the behavior of the frequency versus capacitance graph in higher input voltage frequency range.

Comparative Study:-

The electrical response of the detector developed in this work is that of the I-V response and the C-F response of the detector for different cations. Mathematically the I-V response of the detector is obtained by using the following formula:

$$R_i = (I_i - I_{wi}) / (I_{wi}).$$

For the C-F response of the detector:

$$R_c = (C_i - C_{wi}) / (C_{wi}).$$

Where, I_i = Current value at 5V with ion incubation

I_{wi} = Current value at 5V without ion incubation

C_i = Capacitance value at min frequency with ion incubation

C_{wi} = Capacitance value at min frequency without ion incubation

In the Optical calcium detector [35], developed previously the fall in reflection peak intensity, absorption loss and the scattering loss of the detector platform were under taken as parameters to study the response of the detector platform [35]. The detector showed appreciable response and selectivity towards calcium ion. In the mentioned optical detector the optical response, namely reflection peak intensity fall, scattering loss and absorption loss of the detecting platform was found to be 8.49%, 13.09% and 2.16% for calcium ion, 8.65%, 17.88% and 3.60% for mixture ion solution, 1.25%, 2.49% and 0.10% for Magnesium, 0%, 0.80% and 0.26% for Manganese, 1.40%, 2.25% and 0.13% for Potassium and 0.76%, 0.32% and 0.07% for sodium [35]. In this work, the electrical response of the detector is obtained by calibrating the change in the I Vs V response of the detector as well as the C Vs F change of the detecting platform. The I-V response of the detector is found to be 303.84% for calcium ion, 253.84% for mixture ion solution, 7.69% for magnesium, 100% for manganese, 69.3% for potassium and 42.3% for sodium. The C Vs F response of the detector shows a response of 178% for calcium ion, 175% for mixture ion solution, 37.5% for magnesium, 115% for manganese, 92.55% for potassium and 95% for sodium. Thus both the electrical response is observed to be highest for calcium ion and mixture ion solution in comparison to all the ions. It is clearly visible from the response obtained from the electrical parameters that the detector developed in this work shows appreciable discrimination towards calcium ion, that is more evidently established when the electrical calcium detecting platform is tested with mixture ion solution and the response being consistent with the response of the electrical detector for calcium ion only, as depicted in fig 6. Both the electrical parameter response of the detector shows highest response for calcium ion and for mixture ion solution, showing evidence of the selectivity of the detector towards calcium ion.

Comparing the optical response [35] with the electrical response of the detector surface developed in this work, it is found that the response of the electrical detector is more profound and appreciably consistent with the response of the optical detector, as clearly visible in fig 6. The electrical detector developed shows considerably selective towards calcium ion as in the case of the previously developed optical detector. This may find use in the designing of multi-parametric calcium sensor with better selectivity and sensitivity.

4. Conclusion

Calmodulin surface-functionalized PSi based electrical detecting platform provides an effective alternative for calcium detection. Besides being highly selective towards calcium ion, the detecting platform exhibits enhanced sensitivity due to its bi-parametric approach in calcium detection. The comparative study establishes that the electrical detector developed here exhibits high level of consistency with the existing optical calcium detector [35] developed previously. Due to the detector's effectiveness in calcium detection even in biologically relevant concentration, it may find immense use in calcium detection in numerous fields, especially for calcium detection in clinical diagnostics, drinking water and food reagents. Moreover, the detecting platform being cost effective and its robust structure

renders it as an effective alternative for calcium detection. The electrical detector developed in this work and the optical detector developed previously may provide a highly sensitive, precise, robust, cost-effective and multi-parametric approach towards calcium sensing.

The detecting platform can find great use in calcium detection in human serum samples, for clinical diagnosis. Since magnesium ion is the second most abundant cation after calcium, magnesium provides the highest amount of interference for calcium detection in human serum. However for the detecting platform developed in this work, no such interference is observed in the results obtained. The results obtained clearly demonstrates the detector's discrimination towards calcium ion to be sufficiently high, hence it provides label free, highly sensitive alternative for calcium detection in numerous fields and most importantly in human serum. Since serum calcium level is an eminent marker for a lot of health conditions such as lung cancer, the detector may provide early detection and better prognosis for numerous health conditions and play a key role in early treatment of such diseases. As the comparative study of the two calcium detectors response is found to be consistent, it can provide a new approach towards multi-parametric detection of calcium and provide better efficiency and sensitivity towards calcium ion detection in various fields.

Declarations

Acknowledgment

This work was supported by the RUSA program, Govt. of India. The authors wish to acknowledge Department of Physics, IEST Shibpur, for providing support as and when required.

Conflict of Interest

The authors declare that there are no known conflicts of interest associated with the work presented here and there has been no significant financial support for this work that could have influenced its outcome.

Author Contribution

Kaustav Sen and Tanusree Sarkar performed the experiments. Deeparati Basu helped in data analysis and prepared the figures. Kaustav wrote the main manuscript text. Syed Minhaz Hossain and Jayoti Das reviewed the manuscript thoroughly.

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Figures

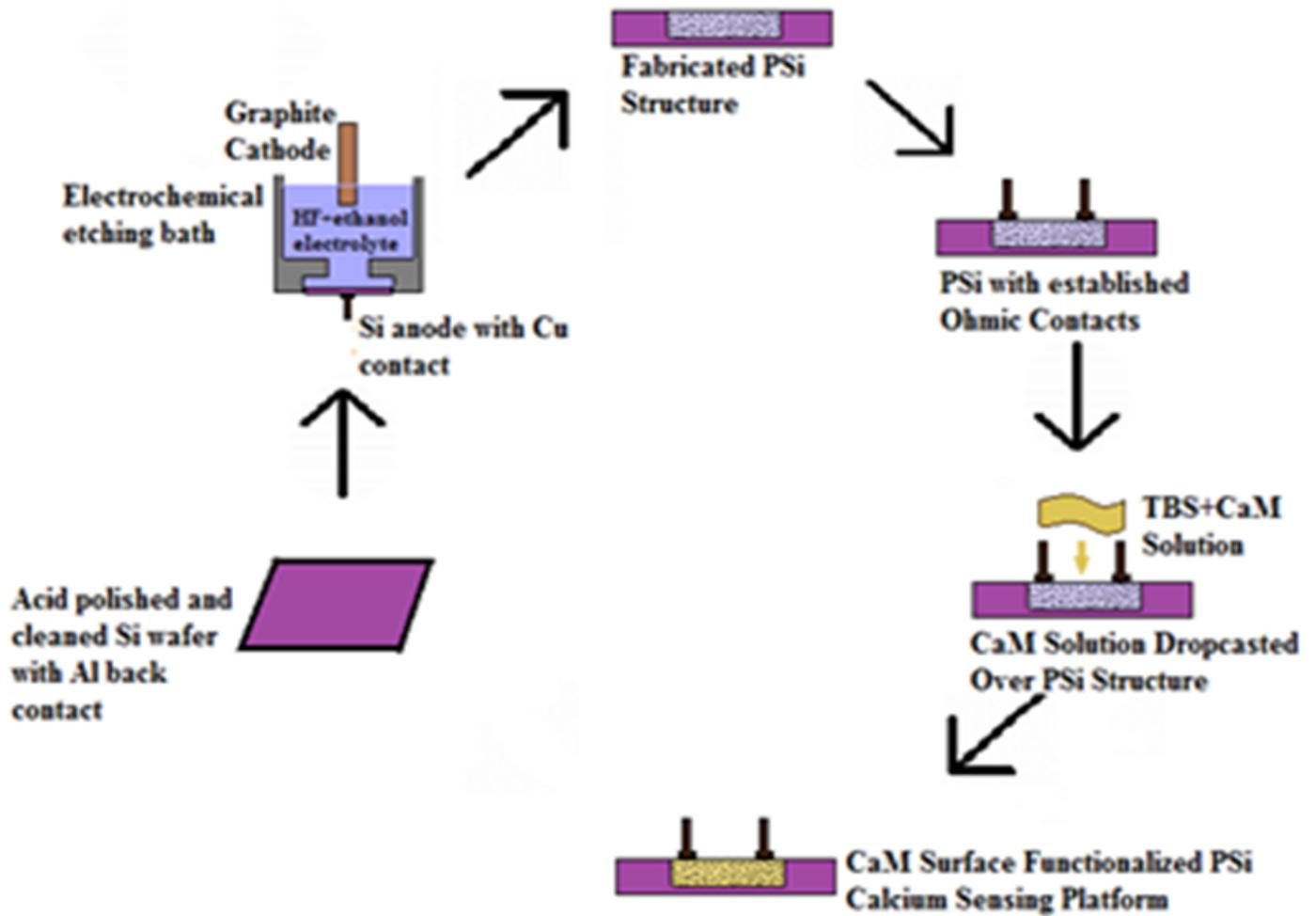


Figure 1

Schematic of the fabrication of CaM-Functionalized P_{Si} calcium detecting platform.

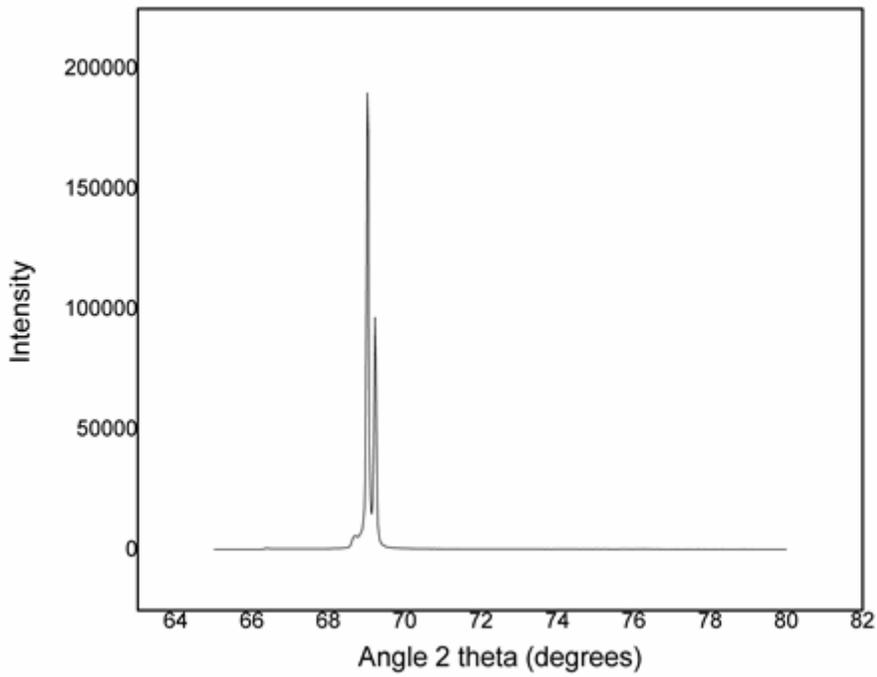


Figure 2

X-Ray Diffraction pattern for porous silicon.

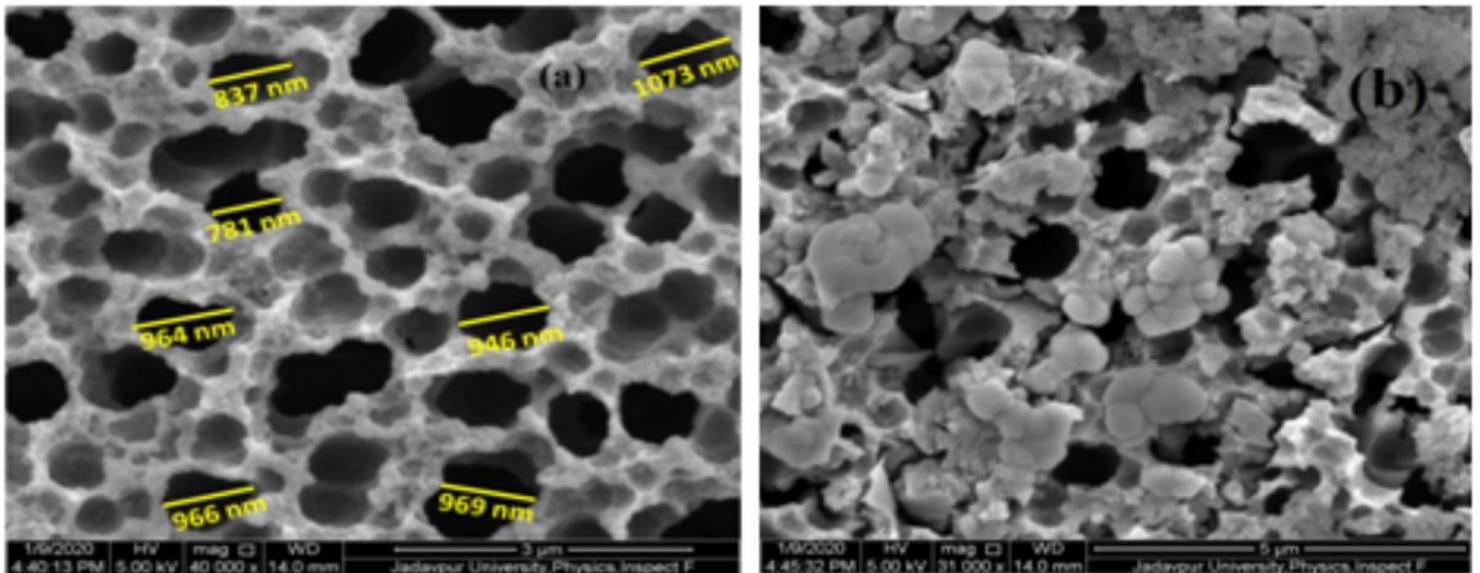


Figure 3

FESEM image of PSi surface with pore diameter (a) FESEM image of surface-functionalized PSi surface (b).

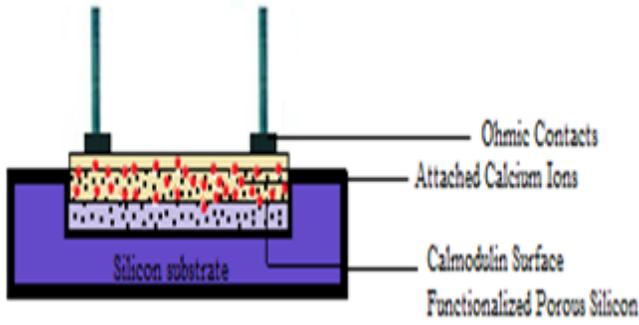


Figure 4

Schematic representation of the fabricated detecting platform.

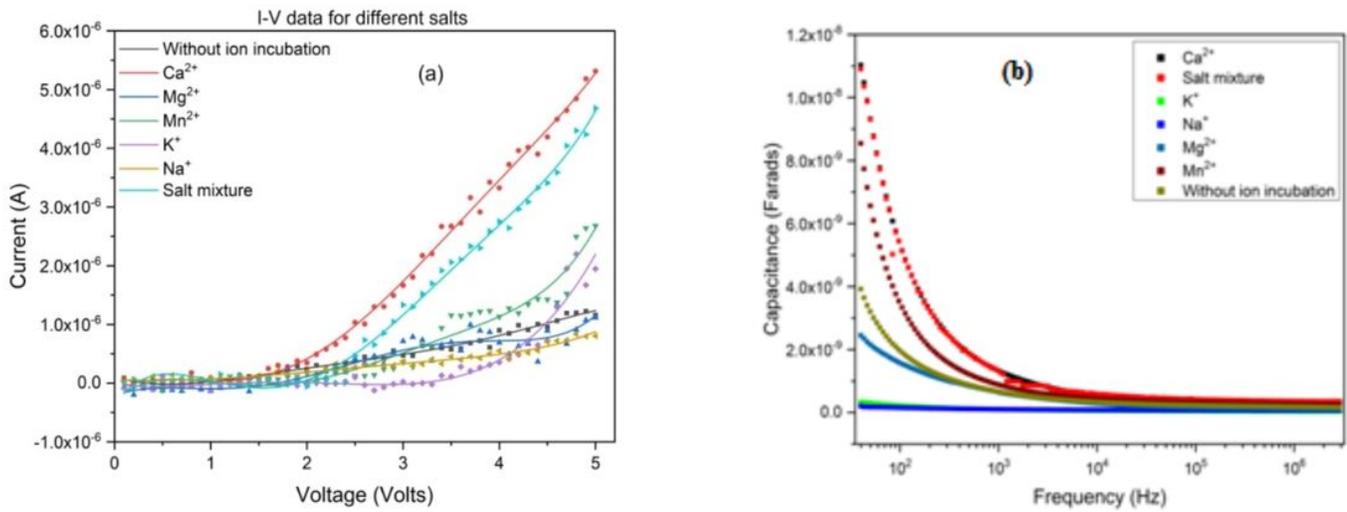


Figure 5

(a) Current (I) Vs Voltage (V) response of the sensing platform for different ion incubation and (b) Capacitance Vs Input voltage frequency response of the sensor for different ion incubation.

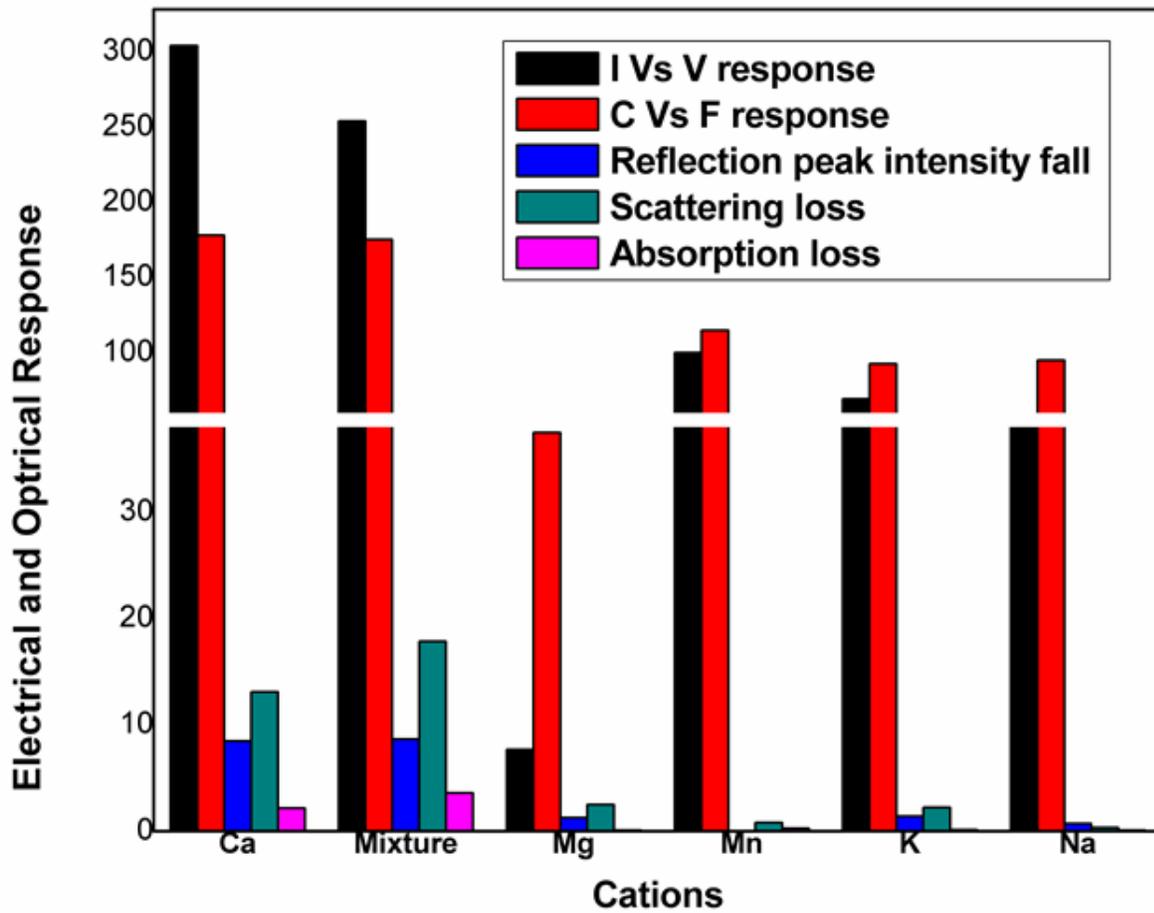


Figure 6

Individual Electrical response and Optical response of the CaM functionalized PSi Calcium detector.