Identifying Hypocalcemia in Dairy Cattle by Combining 3D Printing and Paper Diagnostics

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This paper describes the design, fabrication, and validation of a paper-based diagnostic device for the rapid diagnosis of hypocalcemia in dairy cattle at the point-of-care (POC). The device incorporates a 3D printed calcium ion-selective membrane (ISM) as the sensing element for free—unbound—calcium in real bovine whole blood samples. With a linear response range of 100 nM to 97.7 μM, the sensor covers the clinically relevant concentrations of Ca2+ associated with both healthy cattle as well as those suffering from hypocalcemia. The components of the Ca2+ ion-selective electrodes were successfully translated to a paper-based device to provide a sensing platform that is simple to use, disposable, and low-cost, and is therefore well-suited for applications at the POC. The paper-based calcium sensor showed a Nernstian response between 10 mM and 100 μM and required only 12 μL of sample to perform a measurement, which can be accomplished in less than two minutes without the need for time-consuming separation steps. The performance of the paper-based Ca2+ sensor was validated using the commercially available epoc Blood Analysis System, which provided results within 5% of the data obtained with 3D printed Ca2+-ISM integrated paper-based device.

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Hypocalcemia,1 commonly referred to as Milk Fever, is a metabolic disorder in dairy cattle defined as a blood ionized calcium (Ca2+) concentration below 4.0 mg dL−1 (1.0 mM)2 or a blood total calcium concentration below 8.0 mg dL−1 (2.0 mM).2 Clinical signs of hypocalcemia in dairy cattle range from reduced voluntary dry matter intake to paresis3 and can even lead to death.3 Hypocalcemia in dairy cows can lead to decreased milk production, reduced reproductive performance, and early herd removal.1–3 The economic burden for a 2,000-cow dairy farm with an incidence rate of 30% of subclinical hypocalcemia has been estimated at $50,000 annually.6 Moreover, its effect on a cow’s quality of life makes hypocalcemia one of the main welfare concerns among dairy farmers and veterinarians. Currently, diagnosing hypocalcemia requires blood samples to be sent off-site for lab testing, which is both expensive and time-consuming, delaying treatment for affected dairy cows.3 The USDA-National Animal Health Monitoring System reported that 77.2% of all dairy operations in the United States were affected by hypocalcemia.

Similarly, 47.6% of multiparous cows in German herds suffer from hypocalcemia within 48 h after parturition. The start of each new lactation significantly challenges a dairy cow’s ability to maintain normal blood calcium concentrations affecting up to 30% of the second or greater lactation of cows in a herd.4 Clinical hypocalcemia may be a life-threatening condition and requires timely intervention. Although treatment of hypocalcemia is expensive, it involves time-consuming intravenous injections of calcium gluconate (23%).8 Since treatment and diagnosis of hypocalcemia increase production cost9 on dairy operations, farmers need affordable solutions that are fast and reliable for diagnosis at the point-of-care (POC).9–11

This work developed a potentiometric sensor using a 3D printed ion-selective membrane (ISM) to determine Ca2+ in sodium (Na) heparinized bovine whole blood. Potentiometry12 is an ideal technique for the development of a POC diagnostic device13,14 because: (i) the ISM can be integrated into low-cost paper-based materials,15,16 (ii) ion-selective electrodes (ISEs) are highly selective for the target analyte through the incorporation of ionophores (molecular recognition elements),17–19 (iii) ISEs routinely have short response times (~1 s),20 and (iv) potentiometric sensors do not require intricate or expensive instrumentation for signal measurement and can easily be interfaced with both bench-top and portable potentiostats/potentiometer.31,32,36

In a recent review by Özbek et al., the authors highlight the rapid developments made in potentiometric analysis through paper-based devices.23 Based on the desire to develop low-cost sensors which are appropriate for routine utilization on a societal scale, paper, as a substrate, is ideal for fabricating analytical devices for various applications from environmental monitoring to disease diagnostics.24 The first entirely paper-based potentiometric sensor was developed by Novell et al.25,26 later, the Whitesides group proposed a paper-based sensor with microfluidic zones designed by patterning wax onto paper using wax-printing and directly pasting Ag/AgCl electrodes.27 Pairing potentiometric sensors with paper-based diagnostics simplifies disposal and minimizes the potential for contamination through incineration, making them more eco-friendly than other polymer-based sensing systems.27,28 paper-based devices are also highly portable, and easy to use in remote locations or on-site settings29 when paired with low-cost portable analytical instrumentation.29 In fact, various applications and advancements for paper-based potentiometric sensors have been reported, allowing for selective determination of many ionic species (e.g., blood electrolytes, pharmaceuticals, clinically relevant biomarkers, etc.)31–45 Recently, we reported on the fabrication of ISMs using a stereolithographic (SLA) 3D printing protocol and demonstrated its utility being incorporated in a paper-based device used to measure the tetrabutylammonium cation (TBA+).15

3D printing is an extremely useful fabrication methodology which is seeing an exponential rise in many scientific disciplines, including analytical chemistry.46–50 Interestingly, over the past five years, there has been an increased number of researchers utilizing 3D printing for the fabrication of electrochemical sensors.51 This increase in 3D printed sensors stems from the inherent benefits of 3D printing, namely: (i) the ability to rapidly print, prototype and iterate device designs, (ii) mass production capabilities, and (iii) precise control over size and shape of printed material.52,53 Using

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photocurable resins as the structural support for the membranes functional components (i.e., ion-exchanging salt, plasticizer, and ionophore), as opposed to polyvinyl chloride (PVC) used in conventional ISMs, ISMs can be rapidly fabricated, tested, iterated and optimized. This work develops a potentiometric sensor composed of a 3D printed Ca2+ ISM. Importantly, this potentiometric sensor is translated to a paper-based device used to measure Ca2+ in dairy cattle blood, offering a low-cost potentiometric device for the reliable measurement of Ca2+ at the point-of-care.

**Experimental**

**Chemicals and reagents.**—Biis-2-ethylhexyl sebacate (DOS, Selectrophore, C20H32O4 >97.0%), potassium tetrakis(4-chlorophenyl) borate (KTCIPB, Selectrophore, C22H16Cl4K, >98%), hydrochloric acid (HCl, 6.0 M), potassium chloride (KCl), isopropyl alcohol (IPA, C3H8O), sodium sulfoacetate (Na2SO3·H2O, >98%), sodium bicarbonate (ACS reagent, CHNaO3·H2O, >99.7%), magnesium sulfate (MgSO4, 99.0%), calcium ionophore IB (C52H100N2O3, Selectrophore grade), creatinine hydrochloride (C4H7N3O3·HCl, ≥97%), urethane dimethacrylate oligomer, 3%–6%; photoinitiator-undisclosed, <1.5% was purchased from FormLabs. 1/2 in. PVC tubing was purchased from Tygon S3.081 mm dia. Whatman filter paper was purchased from GE Healthcare Life Sciences. Ag wires were purchased from Integrity Beads. All solutions were prepared with deionized (DI) water.

**Measurements and equipment.**—An Elegoo Mars 2 stereolithography printer was purchased from Elegoo and was used for the fabrication of ion-selective membranes (ISMs). A 365 nm UV oven was purchased from Melody Susie and was used for the post processing procedure for the 3D printed ISMs. A wax printer (Xenox 8560) was used in the fabrication process of the paper devices. A 16-channel potentiometer was purchased from Lawson Labs and was used for the collection of emf vs time data for each ISE.

**Fabrication of the Ca2+ ISE.**—A commercial UV resin (Flexible 80 A, FormLabs) was used as the support material. Plasticizers used was DOS. Ion exchanging salt was KTCIPB as well as an ionophore Calcium IV. The ISM composition consists of 78.25% wt. UV resin, 19.25% wt. DOS, 0.5% wt. KTCIPB, and 2% wt. Calcium IV ionophore. These components were combined and made homogeneous through mixing/stirring. Once homogeneously mixed the ISM composition was then added to the 3D printer (Elegoo Mars 2). A computer aided design (CAD) was uploaded to the 3D printer and the ISM was then printed with a diameter of 10 mm and a thickness of 200 μm. Ca2+-ISEs were constructed using a small amount of excess resin on the ISM to bind it to the PVC tubing where it is post cured using a UV lamp (365 nm) for 5 min. Any excess resin is removed in a 100% isopropyl alcohol (IPA) bath for 30 s and then rinsed with DI water. Next, inner filling solutions (1 mM CaCl2, pH 7.4) were added with an Ag/AgCl wire (prepared through chronopotentiometric deposition) and then conditioned in 100 μM CaCl2, pH 7.4 overnight (~12 h).

**Fabrication of the paper device.**—A paper device design (5.0 cm × 1.5 cm) was created using Microsoft word and printed using a wax printer (Xenox). After the devices were printed on filter paper (Whatman 1), they were placed in an oven at 60 °C for 15 min to allow the wax to wick through the filter paper and define microfluidic channels. Ag/AgCl paste was applied to the reference well and IFS well and left to dry before using. 3D printed ISMs were fabricated and conditioned using the same parameters as the Ca2+-ISE. Theses membranes were conditioned separately from the paper device prior to experiment.

**Potentiometric measurements.**—**Measurement with LC-ISEs.**—Each LC-ISE was calibrated using successive dilutions (1:1) starting at 100 mM Ca2+ and adjusted to a pH of 7.4 with phosphate buffered solution. A phosphate buffered solution (pH 7.4) was used for dilutions.

**Determining selectivity.**—The selectivity of Ca2+-ISEs were obtained by conditioning a 3D printed Ca2+-ISM with the same composition and IFS as previously stated in 1 mM KCl at pH 7.4 overnight. After conditioning, emf values were recorded for magnesium, sodium, potassium, ammonium, dopamine, creatinine, and calcium all with concentrations of 1 mM at pH 7.4. The emf values were entered into the selectivity equation below and the log of selectivity coefficient was calculated and reported.

$$\log K_{ist}^\text{pot} = \frac{z_a (E_x - E_a)}{59.2} + \log \left( \frac{[C_{x}]}{[C_{a}]} \right)$$

Where $K_{ist}^\text{pot}$ is the selectivity coefficient, $z_a$ is the charge of the analyte, $z_i$ is the charge of the interfering ion, $[C_x]$ is the concentration of the analyte, $[C_a]$ is the concentration of the interfering ion, $E_x$ is the emf of the interfering ion, and $E_a$ is the emf of the analyte.

**Stability analysis.**—The stability of the 3D printed Ca2+-ISE was recorded by fabricating a Ca2+-ISE with the same composition and IFS as above and conditioned overnight. The conditioned Ca2+-ISE is placed in 10 mM Ca2+ at pH 7.4 and the emf was continuously monitored (1 data point every 6 s).

**Determination of shelf-life.**—The shelf-life of the 3D printed Ca2+-ISM was determined by fabricating a Ca2+-ISE with the same composition and IFS as above. The ISE is stored in 1 mM Ca2+ at pH 7.4 overnight. The Ca2+-ISE is then monitored daily over the span of 22 d. The Ca2+-ISE is tested with a 10 mM Ca2+ concentration daily, after testing the Ca2+-ISE is placed back in the conditioning solution and stored at room temperature (20 ± 2 °C) in the absence of light.

**Measurement with paper devices.**—To perform a measurement, 12 μl of reference solution (1 M KCl, pH 7.4) was applied to the reference well. 12 μl of the analyte solution was applied to the sample well and 12 μl of the inner filling solution (IFS) (1 mM CaCl2, pH 7.4) was added to the IFS well. A 10 mm diameter disk 3D-Ca2+-ISM was placed on top of the sample well and folded with the IFS well over top and then clamped together to allow for liquid contact from the membrane to the ion-electron transducer (Ag/AgCl paste). A salt bridge occurs between the reference well and the analyte well to allow a potentiometric measurement to be acquired. A schematic of the paper-based device is shown in Fig. 1B.

**Blood collection.**—All procedures involving live dairy cows were approved by the Washington State University Institutional Animal Care and Use Committee (protocol # 6855). Blood was sampled from the coccygeal vessels using 20 gauge × 2.54 cm needles and blood collection tubes (Becton, Dickinson and Company Becton Drive Franklin Lakes, New Jersey) containing 158 USP of Na heparin anticoagulant for plasma separation. Immediately after collection, blood samples were inverted five times to ensure a homogeneous mixture between anticoagulant and blood sample. Then, all blood samples were placed on ice until analysis within 1 h of collection using the epoc® Blood Analysis System (Siemens Healthcare GmbH, Erlangen, Germany). All epoc Test Cards used in this study were kept at room temperature following manufacturer instructions and were not expired. An epoc Test Card was removed from the Card pouch and inserted immediately into the reader. Then the Test Card and reader start a 165 s calibration step. After the calibration step is complete, the reader indicates that the Test Card is ready.
ready. Then, approximately 0.3 ml of Na heparinized whole blood sample was introduced into the Test Card using a 1 ml needleless luer slip syringe following manufacturer instructions. Around 40 s after the sample was introduced, the display showed the Ca$^{2+}$ blood concentration.

**Results and Discussion**

Figure 1A shows the general fabrication scheme to rapidly prototype 3D printed Ca$^{2+}$-ISMs and then translate them to LC-ISEs for testing and optimization. The 3D printable Ca$^{2+}$-ISM cocktail contains i) a support material (photocurable resin (FormLabs) composed of an acrylate monomer, urethane dimethacrylate and a photoinitiator), ii) a plasticizer (DOS), iii) a cation exchanging salt (potassium tetrakis(4-chlorophenyl) borate, KTCIPB), and iv) an ionophore (Calcium Ionophore IV). Once the ISM cocktail is made homogenous, it is introduced to the 3D printer. Using computer-aided design (CAD) software, we designed disk shape membranes with a thickness of 200 $\mu$m for the ISM. Although the resolution of the 3D printer allows for thinner membranes to be printed, membranes with 200 $\mu$m thickness have sufficient structural integrity that they can be easily tested in both inner-filling based liquid contact ISEs and paper-based devices. Once printed, the Ca$^{2+}$-ISMs are removed from the build plate and rinsed with IPA to remove excess resin and are then ready for ISE fabrication. Through an iterative process the composition of the ISM cocktail can be adjusted and optimized rapidly using this method. It should be stated that we previously demonstrated the capability of 3D printing to produce ISMs for incorporation into both solid-contact and LC-ISE configurations, although in the current report, we chose to focus on LC-ISEs owing to their convenient translation into paper-based devices, as shown in Fig. 1B.14

$$E = E^\circ + \frac{2.3RT}{zF} \log (a) = E^\circ + \frac{59.2 mV}{z} \log (a) \quad [2]$$

The schematic representation of the complete potentiometric setup to test the performance of the 3D printed ISMs is shown in figure 2.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** (A) A schematic representation of the 3D printing process for fabricating Ca$^{2+}$-ISMs. An iterative process can be used to rapidly optimize the ISM composition for the fabrication of LC-ISE. (B) A schematic illustration of a Ca$^{2+}$ paper-based device. (i) a descriptive schematic of a wax printed paper device. (ii) Translation of the optimized Ca$^{2+}$-ISM onto a paper device. (iii) A schematic of the experimental setup for Ca$^{2+}$ detection using a paper-based device.

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Schematic representation of the Ion-Selective Membrane for a 3D printed LC-Ca$^{2+}$-ISE.
Fig. 1A, where the potentiometer measures the electrical potential difference (i.e., \( \text{emf} \), electromotive force) between the reference electrode (sample independent potential) and the ISE. The sensing mechanism relies on the creation of a separation of charges (Ca\(^{2+}\) and Cl\(^{-}\)) at the interface of the ISM and sample shown in Fig. 2. The magnitude of the \( \text{emf} \) (which is the sum of all interfacial potentials) is dependent on the activity of Ca\(^{2+}\) in the sample and is given by the Nernst equation (Eq. 2), where \( E^0 \) is the standard potential, \( T \) is the temperature, \( R \) is the universal gas constant, \( F \) is Faraday’s constant, \( z \) is the charge of the analyte of interest (here 2\(^+\) for calcium ions), and \( a \) is the activity of Ca\(^{2+}\) in the sample. From the Nernst equation, an order-of-magnitude decrease in Ca\(^{2+}\) activity would decrease the \( \text{emf} \) by 29.6 mV. Importantly, all interfacial potentials are assumed to be constant, except for the interface between the ISM and the sample. Using the LC configuration shown in Fig. 1A, Fig. 3 presents the calibration curve obtained for varying concentrations of Ca\(^{2+}\) in a phosphate buffer of pH 7.4. We report \( \text{emf} \) vs concentration (as opposed to \( \text{emf} \) vs activity) since the phosphate buffer ensures a constant ionic strength, allowing us to assume a constant activity coefficient for Ca\(^{2+}\). Here we see a linear relationship between measured \( \text{emf} \) and the logarithm of Ca\(^{2+}\) concentration over 3 orders of magnitude, from 100 mM to 97.7 \( \mu \)M. We chose the pH of 7.4 for our phosphate buffer to closely mimic the pH of the biological fluid of dairy cattle. The slope of calibration curve was found to be 28 ± 2 mV decade\(^{-1}\), which aligns well with the theoretical value predicted by the Nernst equation.

Using the optimized 3D printed ISM, the selectivity of the Ca\(^{2+}\)-ISEs were investigated against common cationic species routinely found in the blood of dairy cattle. Figure 4 shows the experimentally observed \( \text{emf} \) responses of an unbiased Ca\(^{2+}\)-ISE to various potential interferants. Using Eq. 2, selectivity coefficients were determined and are included in Table I. As shown in Table I, the selectivity of the Ca\(^{2+}\)-ISE towards calcium ions is sufficiently high to ensure that calcium ions are preferentially sensed over other positively charged species in solution, even though they may be present at much higher concentrations than calcium (e.g., Na\(^+\) and Mg\(^{2+}\)). Compared to previously reported Ca\(^{2+}\)-ISEs which incorporate photocurable polymers, the 3D printed Ca\(^{2+}\)-ISEs resulted in an

![Figure 3. Linear calibration of Ca\(^{2+}\)-ISEs with a Nernstian response of 28 ± 2 mV Decade\(^{-1}\) across a range of 100 mM to 97.7 \( \mu \)M Ca\(^{2+}\) at pH 7.4.](image)

![Figure 4. Experimental \( \text{emf} \) values for biologically relevant cations found in dairy cattle blood for selectivity analysis using a Ca\(^{2+}\)-ISE.](image)

![Figure 5. (A). An \( \text{emf} \) trace illustrating the stability of a Ca\(^{2+}\)-ISE over a 15 h timeframe with an average drift of 166 \( \mu \)V h\(^{-1}\) and a total drift of 2.493 mV. (B). Experimental \( \text{emf} \) values of a Ca\(^{2+}\)-ISE for determination of the shelf-life calcium doped LC-ISE over 24 d.](image)

### Table I. Selectivity coefficients for biologically relevant cations in cow blood vs calcium and the physiological ranges.

| Interfering Ion | Concentration | Selectivity Coefficient (logK) | Normal physiological range (mM) |
|----------------|---------------|-------------------------------|---------------------------------|
| Mg\(^{2+}\)    | 1 mM          | -5.66                         | 0.8 – 1.2                       |
| Na\(^+\)       | 1 mM          | -5.82                         | 139 – 147                       |
| K\(^+\)        | 1 mM          | -2.96                         | 3.6 – 5.8                       |
| NH\(_4\)\(^+\) | 1 mM          | -2.13                         | <0.294                         |
| Dopamine\(^+\) | 1 mM          | -2.54                         | 5.9 \times 10\(^{-7}\)         |
| Creatinine\(^+\) | 1 mM         | -2.51                         | 0.085-0.0919                  |
| Ca\(^{2+}\)    | 1 mM          | 0                             | 2.2–3.0                        |

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and a similar selectivity over K

CaCl₂ solution. Here we see a total drift over the course of the obtained over 15 h of a fully conditioned Ca₂⁺/ISEs have shown an increased selectivity over Na

term stability, and shelf-life. Figure 5A shows the explored two important characteristics of analytical devices, long-

Before translating the Ca²⁺-ISM to a paper-based device, we explored two important characteristics of analytical devices, long-term stability, and shelf-life. Figure 5A shows the emf time-series obtained over 15 h of a fully conditioned Ca²⁺-ISE in a 10 mM CaCl₂ solution. Here we see a total drift over the course of the experiment of under 2.5 mV, which corresponds to an average drift of 166 μV h⁻¹. To evaluate the shelf-life of the calcium sensor, the Ca²⁺-ISE was conditioned overnight and then used to measure the emf of a 10 mM CaCl₂ solution. Measurements were repeated daily until the sensor began to fail (i.e., large deviations in observed emf) and the results are presented in Fig. 5B. The sensors maintain their performance over a period of 22 d before the observed emf begins to significantly decrease.

To demonstrate the point-of-care potentiometric sensing of Ca²⁺, we combined the 3D printed Ca²⁺-ISM into a paper-based device inspired by previous designs. Microfluidic paper analytical devices (μPADs) have revolutionized low-cost diagnostics owing to their affordability, ease of modification and established mass-production infrastructure. However, paper devices routinely experience less than desirable reproducibility, highly drifting signals, and decreased detection limits. The schematic representation of our paper-device is shown in Fig. 1B. Briefly, to perform a measurement using this device 12 μl of reference solution (1 M KCl, pH 7.4) and 12 μl of inner-filling solution (1 mM CaCl₂) are pipetted on the Reference Zone and Inner-Filling Solution Zone, respectively. 12 μl of sample is then pipetted onto the sample zone where the sample solution and reference solutions are transported to the Salt Bridge zone where the make ionic contact. The Ca²⁺/ISM is then placed onto the sample zone, followed by a folding of the inner-filling solution zone, effectively sandwiching the Ca²⁺-ISM between the sample and inner-filling solutions zones. Connection to the potentiometer via painted Ag/AgCl electrodes, completes the circuit and yields an emf response based on concentration. The time to complete the entire procedure is under 2 min. Figure 6A shows the emf drift observed with the Ca²⁺ paper-based ISE over 4 min. Here, a gradual increase in emf is observed, corresponding to an average drift of ~370 μV min⁻¹ with the total drift of 1.66 mV. A photograph of an actual paper-based device is shown in the inset of Fig. 6A. Figure 6B shows the linear relationship observed with the paper-based device, which exhibits a Nernstian response of 29 ± 1 mV Decade⁻¹ between 10 mM and 100 µM, covering the required range for normal physiological and hypocalcemic levels of Ca²⁺ in dairy cattle. Table II highlights some of the previously reported analytical characteristics for various sensing approaches for Ca²⁺ detection. To test the ability of our device to give comparable results with the current state-of-the-art POC analyzer epoc® available to veterinarians, we obtained 3 whole blood samples from local dairy cattle. These samples were tested for Ca²⁺ using the epoc® Blood Analysis System. Using the 3D printed Ca²⁺-ISM integrated paper-based device, we analyzed the samples within one hour of the blood being drawn. The obtained data is presented in Table II. Here we see that our device gives similar results to the handheld analyzer, with all 3 measurements being within 5%, with an average % difference between the 3 samples of 2.18%. These results suggest that the proposed 3D printed Ca²⁺-ISM integrated paper-based device is a

**Table II. Comparative table of analytical techniques and paper-based devices used to detect Ca²⁺.**

| Technique | Concentration Range (μM) | LOD (μM) | References |
|-----------|--------------------------|----------|------------|
| FES       | 15 – 300                 | 0.387    | 56         |
| PL        | 0.001 – 0.01             | 0.000077 | 57         |
| Fluorescence | 0.25 – 50              | —        | 58         |
| Colorimetry | 1000 – 4000             | —        | 59         |
| AAS       | —                        | 249      | 60         |
| Potentiometry | —                    | 0.01     | 61         |
| Potentiometry | 1 – 100               | 0.12     | 62         |
| Potentiometry | 97.7 – 100000          | —        | Current Work |

**Calcium paper Device**

| Technique | Concentration Range (μM) | LOD (μM) | References |
|-----------|--------------------------|----------|------------|
| Coulometry | 50 – 50000              | 50       | 63         |
| Potentiometry | 12 – 130000           | —        | 45         |
| Potentiometry | 1000 – 100000         | —        | 14         |
| Potentiometry | 100 – 100000          | —        | Current Work |
potential low-cost alternative for rapid diagnosis of hypocalcemia at the point-of-care.

Conclusions

A rapid and reliable method to diagnose hypocalcemia in dairy cattle at the POC is needed. This research developed the first 3D printed Ca2+ ion-selective membrane for the detection of Ca2+ in biological samples. The potentiometric Ca2+ sensor had a linear response range between 100 mM and 97.7 μM at pH 7.4, which covers the concentration ranges found in both healthy and hypocalcemic dairy cattle. We showed that the sensor selectively responds to Ca2+ in the presence of potentially interfering cations commonly found in biological fluids (e.g., K+, Na+, Mg2+, etc.), without the need to perform time-consuming separation steps. Furthermore, the sensor displayed sufficient stability, reproducibility, and longevity, with a shelf-life of approximately 3 weeks when stored in a solution of CaCl2.

Integrating the 3D printed Ca2+ ion-selective membrane with paper-based microfluidics resulted in a low-cost and disposable sensor which could be used at the POC (i.e., local farms, veterinary laboratories, etc.). Importantly, analysis performed with the 3D printed Ca2+-ISM integrated paper-based device covered the diagnostically relevant concentrations of Ca2+ in dairy cattle, requiring only 12 μl of sample to perform the measurement, and can be completed in under two minutes. Validation of our sensor was achieved against a commercially available handheld analyzer epoc, which provided results within 5%. The 3D printed Ca2+-ISM integrated paper-based device offers a low-cost and reliable alternative to diagnosing hypocalcemia in point-of-care settings. Cost analysis of the devices described in this paper results in a cost per device of approximately $6.60 USD, where 95% of that cost is due to the costly ionophore. Although the Ca2+-ISMs are reusable, disposal of entire devices is recommended when using biological samples to minimize the potential for contamination. Simple miniaturization of the Ca2+-ISMs within the resolution capabilities of commercially available 3D printers, such as the Elegoo Mars 2 used here, to 5 mm diameter and 100 μm thickness decreases the cost of individual devices to approximately $0.94 USD. In comparison, individual tests using the epoc analyzer cost upwards of $25 USD, although the analyzer provides data on additional blood components such as Mg2+, K+, and Na+. Our future work will focus on developing multiplexed devices by incorporating 3D printed ISMs for various electrolytes and biomarkers of dairy cattle health, building towards full panel diagnostic assays. We believe that with the affordability and mass production capabilities of all sensor components, (approximately 20 paper-devices with the dimensions of 30 mm × 10 mm can be printed on a single circular Whatman filter paper in less than 1 min, and the 3D printer can fabricate 50 membranes in ~20 min) will allow for greater data collection capabilities to be placed in the hands of the end-users (i.e., dairy farmers).

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References

1. J. P. Goff, Vet J., 176, 50 (2008).
2. A. L. Schafer and D. M. Shoback, Endotext (2016).
3. M. Santell, Proceedings of the Four-State Dairy Nutrition and Management Conference 80 (2012).
4. R. L. Horst, J. P. Goff, T. A. Reinhardt, and D. R. Buxton, J. Dairy Sci., 80, 2012.
5. M. R. Wilkens, C. D. Nelson, L. L. Hernandez, and J. A. McArt, J. Dairy Sci., 103, 2909 (2020).
6. A. R. Domino, H. C. Korzec, and J. A. McArt, J. Dairy Sci., 100, 9681 (2017).
7. R. C. Neves, B. M. Leno, K. D. Bach, and J. A. McArt, J. Dairy Sci., 101, 9321 (2018).
8. M. S. Cooper and N. J. Gittos, Brit. Med. J., 336, 1298 (2008).
9. F. B. Malcata, P. T. Pepler, E. L. O’reilly, N. Brady, P. D. Eckersall, R. N. Zadoks, and L. Viora, J. Dairy Res., 87, 60 (2020).
10. P. F. Turner, ECS Sensors Plus, 1, 011601 (2022).
11. S. K. Das, K. K. Noyak, P. R. Krishnaswamy, V. Kumar, and N. Bhat, ECS Sens. Plus., 1, 031601 (2022).
12. E. Zdrazilek and E. Bakker, Anal. Chem., 93, 72 (2021).
13. M. Santell, M. P. S. Mousavi, and M. K. A. El-Rahman, J. Electrochem. Soc., 165, B835 (2018).
14. W. J. Lan, X. U. Zou, M. M. Hamedi, J. Hu, C. Parolo, E. J. Maxwell, P. Buhlmann, and G. M. Whitesides, Anal. Chem., 86, 9548 (2014).
15. R. E. Glassco, N. H. B. Ho, A. M. Mamari, and J. J. Bell, Anal. Chem., 48, 15826 (2021).
16. D. L. Glassco, A. M. Mamari, N. H. B. Ho, A. Sheelam, and J. G. Bell, J. Electrochem. Soc., 169, 073513 (2022).
17. L. Deng, J. Zhai, X. Xu, and X. Xie, ACS Sens., 6, 1279 (2021).
18. R. Canovas, S. P. Sanchez, M. Parrilla, M. Cuartero, and G. Crespo, ACS Sens., 4, 2529 (2019).
19. N. K. Joon, J. E. Barnsley, R. Ding, S. Lee, R. Latonjen, J. Bobacka, C. Gordon, T. Ogawa, and G. Lisak, Sens. Actuators, B, 305, 127311 (2020).
20. C. Maccà, Anal. Chim. Acta, 512, 183 (2004).
21. A. Ainla, M. P. S. Mousavi, M. N. Tsagloglou, J. Redston, J. G. Bell, M. T. Fernandez-Abedul, and G. M. Whitesides, Anal. Chem., 90, 6240 (2018).
22. A. Scott, R. Pandeley, S. Saxena, E. Osman, Y. Li, and L. Soleymani, ECS Sens. Plus., I, 014601 (2022).
23. O. Ozbek and C. Berkel, SI, 3, 100189 (2022).
24. M. Sher, R. Zhuang, U. Demirci, and W. Asghar, Expert Rev. Mol. Diagn., 17, 351 (2017).
25. M. Novell, M. Parrilla, G. A. Crespo, F. X. Rius, and F. J. Andrade, Anal. Chem., 84, 4965 (2012).
26. M. Novell, T. Guinovart, P. Blondeau, F. X. Rius, and F. J. Andrade, Lab Chip, 14, 1308 (2014).
27. M. Santell, E. W. Nery, G. P. Santos, and L. T. Kubota, Biosens. Bioelectron., 6, 89 (2014).
28. G. Bhattacharya, S. J. Fishlock, S. Hussain, S. Choudhury, A. Xiang, B. Kandola, A. Pritam, N. Soin, S. S. Roy, and J. A. McLaughlin, ACS Appl. Mater. Interfaces, 14, 27 (2022).
29. E. W. Nery and L. T. Kubota, Anal. Bioanal. Chem., 405, 7573 (2013).
30. M. Dryden and A. R. Wheeler, PLoS One, 10, e0140349 (2015).
31. A. H. Kamel, A. E. G. Emir, A. A. Almehizia, and A. H. Kamel, J. Dairy Sci., 110, 12227 (2017).
32. R. Ding, M. Fiedoruk-Pogrebniak, M. Pokrzywnicka, R. Koncki, J. Bobacka, and G. Lisak, Sens. Actuators, B, 323, 1269 (2022).
33. T. Sakata, M. Hagiyo, A. Saito, Y. Mori, M. Naka, and K. Nishi, Sci. Technol. Adv. Mater., 18, 1 (2017).
34. R. Ding, N. K. Joon, A. Ahamed, A. Shafaat, M. Wagner, T. Ruzgas, J. Bobacka, and G. Lisak, Sens. Actuators, B, 344, 130200 (2021).
35. H. S. Abd-Rabboh, A. E. G. Emir, A. A. Almehizia, and A. H. Kamel, ACS Omega, 6, 27755 (2021).
36. A. M. Yehia, M. A. Farag, and M. A. Tantawy, Anal. Chim. Acta, 1104, 95 (2020).
37. H. S. Abd-Rabboh, A. E. G. Emir, A. Y. Sayed, and A. H. Kamel, RSC Adv., 11, 12227 (2021).
38. R. Kawahara, P. Sahatya, S. Badulika, and S. Uno, JAAAF, 57(4S), 04FM08 (2018).
39. E. Chow, D. T. Liana, B. Raguse, and J. J. Gooding, Aust. J. Chem., 70, 979 (2017).
40. J. G. Bell, M. P. Mousavi, M. K. Abd El-Rahman, E. K. Tan, S. Homero-Vanniasinkam, and G. M. Whitesides, *Biosens. Bioelectron.*, **126**, 115 (2019).
41. S. Dolai and M. Tabib-Azar, *Med. Devices Sens.*, **3**, e10112 (2020).
42. M. Bouri, J. C. Zazahnur-Gordona, M. Novell, P. Blondeau, and F. J. Andreade, *Electroanalysis*, **33**, 81 (2021).
43. M. Borràs-Brull, P. Blondeau, and R. . J. Riu, *Electroanalysis*, **33**, 181 (2021).
44. K. Yamaoka, J. Eguchi, and S. Uno, *Telkomnika*, **15**, 836 (2017).
45. E. W. Nery and L. T. Kubota, *Anal. Chim. Acta*, **918**, 60 (2016).
46. H. Zhou, H. Yang, S. Yao, L. Jiang, N. Sun, and H. Pang, *Chinese Chem. Lett.*, (2021), In Press.
47. A. Yakob, S. Chaiyo, W. Siangproh, and O. Chailapakul, *ACS Sens.*, **4**, 1211 (2019).
48. B. Schmidt, D. King, and J. Kariuki, *J. Chem. Educ.*, **95**, 2076 (2018).
49. X. Liu, M. N. George, S. Park, A. L. Miller II, B. Gaihre, L. Li, B. E. Waletzki, A. Terzic, M. J. Yaszemski, and L. Lu, *Acta Biomater.*, **111**, 129 (2020).
50. M. Sharafeldin, K. Kadimisetty, K. S. Bhalerao, T. Chem, and J. F. Rusling, *Sensors*, **20**, 4514 (2020).
51. D. L. Glasco, A. Sheelam, N. H. B. Ho, A. M. Mamaril, M. King, and J. G. Bell, *ECS Sens. Plus*, **1**, 010602 (2022).
52. N. H. B. Ho, D. L. Glasco, and J. G. Bell, *ECS Sens. Plus*, **1**, 020601 (2022).
53. C. Ocanne, N. Abramova, A. Bratov, T. Lindfors, and J. Bobacka, *Talanta*, **186**, 279 (2018).
54. L. Y. Heng and E. A. H. Hall, *Anal. Chem.*, **72**, 42 (2000).
55. A. K. Singh and S. Mehtab, *Sens. Actuators B*, **123**, 429 (2007).
56. J. Yue, L. Li, L. Cao, M. Zan, D. Yang, Z. Wang, Z. Chang, Q. Mei, P. Miao, and W. F. Dong, *ACS Appl. Mater. Interfaces*, **11**, 44566 (2019).
57. S. R. Ankireddy and J. Kim, *Sens. Actuators B*, **255**, 3425 (2018).
58. X. Hu and Z. Zhang, *Microchim. Acta*, **159**, 255 (2007).
59. S. Kim, J. W. Park, D. Kim, D. Kim, I. H. Lee, and S. Jon, *Angew. Chem.*, **121**, 4202 (2009).
60. S. P. Raman et al., *J. Supercrit. Fluids*, **153**, 104545 (2019).
61. T. Sokalski, A. Ceresa, M. Fibbioli, T. Zwickl, E. Bakker, and E. Pretsch, *Anal. Chem.*, **71**, 1210 (1999).
62. J. Zhai, Y. Zhang, D. Zhao, L. Kou, and G. Zhao, *Anal. Chim. Acta*, **1191**, 339209 (2022).
63. H. Shibata, Y. Hiruta, and D. Citterio, *Analyst*, **144**, 1178 (2019).