Potentiometric Analysis of Benzalkonium Chloride with 3D Printed Ion-Selective Membranes

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Benzalkonium (BA+) chloride is one of the most common preservatives used in prescription-based and over-the-counter eye drops. Knowing the concentration of BA+ in eye drops is important for both quality control (at the pharmaceutical preparation stage) and human health (ocular toxicity has been linked to BA+ use). This paper describes the design and fabrication of a benzalkonium-selective potentiometric sensor for the determination of BA+ in ophthalmic solutions. The sensor is composed of a 3D-printed ion-selective membrane (ISM) that selectively measures BA+ in the presence of potentially interfering ions routinely found in ophthalmic formulations (i.e., Mg2+, Ca2+, Na+ and K+). The 3D printed BA+-ion-selective electrodes (ISEs) produced a Nernstian response of 55 mV/Decade across a range of 1.0 mM to 31.0 μM BA+ along with an LOD of 8 μM, which covers the relevant concentration range found in ophthalmic solutions. The 3D printed BA+-ISEs proved to be highly stable with an average drift of 205 μV/hr. Successful measurement of BA+ in diluted ophthalmic solutions was completed from 100–500 μM. The mass production capability afforded by 3D-printing offers a unique and intriguing fabrication protocol for developing low-cost sensors which could be incorporated quickly and seamlessly by pharmaceutical companies or community-based pharmacies.

Ocular surface disease (OSD), a condition that affects between 25 and 50% of the population, can cause redness, tearing, burning, light sensitivity, intermittent blurred vision and even blindness. The prevalence of OSD in glaucoma patients can be significantly higher, where upwards of 75% report symptoms relating to OSD. The active ingredients in eye drops, which are used to lower intraocular pressure, are the primary cause of OSD, however, inactive ingredients such as preservatives also contribute to OSD, leading to destabilization of tear film, death of corneal and conjunctival epithelial cells, and other unwanted side effects. Development of these side effects is both time and dose dependent and many studies have found that long-term use of topical drugs may induce major and frequent ocular surface damage. The preservatives used in ophthalmic solutions come from a variety of chemical families, however, quaternary ammoniums have become one of the most common preservatives. Of the quaternary ammoniums used as an antimicrobial preservative in topical drugs, benzalkonium (BA+) chloride, a nitrogenous cationic surfactant, is the most common. Although BA+ is an excellent antibacterial which can dissolve bacterial cell membranes, eye drops that contain BA+ have been implicated as a major cause of ocular adverse effects. Many studies have also indicated a direct relationship between the presence of BA+ and the symptoms experienced during the anti-glaucoma therapy such as allergic or inflammatory reactions.

Besides its use in ophthalmic formulations, BA+ is also applied in disinfectants and sanitizers in sanitary products, textile-softener formulations, and hair conditioners. Proven toxicity to fish, invertebrates, and bacteria, coupled with the fact that significant amounts of BA+ (from wastewater) is discharged into rivers, highlights the need for the development of easily fabricated, reliable and low-cost analytical methods for the quantification of BA+ in diverse and often complex matrices.

The quantification of BA+ has been achieved using a variety of methods such as high-performance liquid chromatography (HPLC), gas chromatography (GC), ion-chromatography, mass spectroscopy (MS) and capillary electrophoresis (CE). Although these techniques proved to be sufficiently sensitive and accurate for the determination of BA+, expensive and bulky equipment, lengthy analysis times, and sample pretreatment prohibit rapid BA+ detection. A less common approach for BA+ detection is through electrochemistry, which is an ideal technique to obtain rapid and reliable on-site measurements. Voltammetric sensors for BA+ analysis based on carbon paste, glassy carbon, gold, and boron-doped diamond electrodes have been reported. Interestingly, potentiometric sensors for the determination of BA+ are much less common. In a comparative study, Gaber et al. fabricated solid contact (PVC-coated silver wire) and liquid contact ion-selective electrodes (ISEs) and used them determine BA+ levels in ophthalmic solutions. Although suitable detection limits and selectivity were reported, the sensing membranes (i.e., ion-selective membranes (ISMs)) did not contain ionophores, and as such the selectivity of the ISEs was determined by lipophilicity.

The use of potentiometry for the detection of ionic species has seen a drastic increase over the past two decades owing to its highly sensitive and selective measurements. As such, potentiometric sensors have seen utilization in a variety of applications ranging from biomedical analysis of important biomarkers, environmental monitoring of toxic ions and pharmaceuticals, Potentiometry relies on the selective partitioning of a charged species based on ion-exchanging salts and molecular recognition elements (i.e., ionophores), traditionally contained in plasticized (polyvinyl chloride (PVC) based ISMs. Recently, our group has reported on a new fabrication protocol for ISMs relying on stereolithographic (SLA) additive manufacturing (i.e., 3D printing).

Advancements in 3D printing are rapidly altering the landscape of many scientific disciplines, including analytical sensing. For example, microfluidic devices have been fabricated with unique and novel designs of channels and device structures. Electrodes and sensors have also been fabricated with 3D printing, relying heavily on the fused-deposition modelling (FDM) approach, with the ability to print metallic and carbon-based electrodes for electroanalysis. 3D printing is a robust technology that has the following benefits, i) it is a user-friendly technique, ii) it allows for rapid fabrication times, iii) it allows for user-defined control over size and shape of printed material and iv) 3D printers are relatively low-cost. Taking advantage of the capabilities of 3D printing, this paper describes the development of a 3D-printed ISM for the rapid determination of BA+ in ophthalmic solutions.

Experimental

Materials and equipment—2-nitrophenyl octyl ether (NPOE, Selectophore, C12H24NO3S >99.0%), potassium tetraakis-(4-chlorophenyl) borate (KTCPB, Selectophore, C20H10BCl4K >98.0%), potassium chloride (KCl), calcium chloride (anhydrous powder, 99.0%), potassium tetrakis(4-chloro-phenyl) borate (KTCPB, Selectophore, C20H10BCl4K >98.0%), potassium chloride (KCl), calcium chloride (anhydrous powder, 99.0%), magnesium chloride hexahydrate (MgCl2•6H2O >99.0%), sodium chloride (NaCl, >99.0%), calcium chloride (CaCl2, 99.0%), and disodium hydrogen phosphate (Na2HPO4, 99.0%).

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Preparation of 3D printable ISM cocktail.—Commercial flexible UV resin (80 A, Formlabs) was used in the replacement of PVC ISM. NPOE was the primary plasticizer, meanwhile KTCPB was the ion exchanging salt along with the ionophore C6X. The BA-ISE cocktail was composed of 96.2% wt of 80 A 3D resin, 2.0% wt NPOE, 0.5% wt of KTCPB. The ionophore, C6X, was included based on 1:2 mol ratio (1.3% wt) of 80 A 3D resin, 2.0% wt NPOE, 0.5% wt of KTCPB. The ionophore, C6X, was included based on 1:2 mol ratio (1.3% wt) with the ion salt exchanger, KTCPB.

CAD and 3D printing.—A computer aided design (CAD) was generated using Fusion 360. The CAD design was a 10 mm diameter circle with a thickness of 400 μm. The design was then transferred to an open-source slicing software compatible with the 3D printer and finally transferred to the 3D printer. Once the CAD design is uploaded into the 3D printer it is then printed using the ISM cocktail previously described. Afterwards the design is removed from the build plate and washed in an isopropyl alcohol (IPA) to remove excess resin. At this point the design is ready to be affixed to PVC tubing and prepared as an ISE.

Preparation of 3D LC-ISE.—Liquid-contact (LC)-ISEs are constructed using a small amount of excess resin on the membrane to bind it to PVC tubing where it is post cured using the UV lamp for 15 min. Any excess resin remaining after the post cure is removed in an IPA bath for 30 seconds and then rinsed with DI water. Next, inner filling solutions (0.5 mM BA+ and 0.5 mM KCl at pH 8) were added with an Ag/AgCl wire and then conditioned in 1 mM BA+ solution overnight (~12 h).

Calibration of 3D-ISE.—The calibration of LC-ISEs was carried out by performing a series of 1:1 dilutions beginning with 1 mM BA+ solution at pH 8 and diluted with a phosphate buffer (pH 8). All measurements were performed with 3 BA+ ISEs.

Determination of selectivity coefficient.—LC-ISEs were conditioned in 100 μM KCl at pH 8 overnight for unbiased selectivity coefficient analysis. After conditioning, the emf value was recorded for 100 μM solutions of KCl, NaCl, CaCl2, MgCl2 and BaCl2 at pH 8. By using the selectivity equation with the obtained emf values, the log of selectivity coefficients was calculated.

Stability analysis.—LC-ISEs were conditioned in 1 mM Ba2+ solution at pH 8 for 3 h. The stability test was then carried out over 14 h.

Shelf life.—LC-ISEs were stored in 1 mM Ba2+ solution in the dark at room temperature. Every 2–4 days, an emf measurement was recorded in a 62.5 μM Ba2+ solution to observe any deviation from previous emf readings. The measurements were carried out for 15 days before the emf values drastically changed.

Ophthalmic solution analysis.—The Equate™ lubricant eye drops was diluted with phosphate buffer (pH 8) using a 1:10 ratio. Then the diluted eye drop solution was used to make standard solutions of 0.1, 0.3, and 0.5 mM for Ba2+. An “unknown” Ba2+ concentration was analyzed and compared against the previously determined calibration curve. Results were obtained using 3 different electrodes.

Results and Discussion

Figure 1A shows a schematic representation of the protocol used to fabricate BA+-ISEs using stereolithographic (SLA) 3D printing. With this approach, the component of the BA+ISE which is 3D printed is ISM, which is also the element that performs the sensing. The BA+-ISM cocktail consists of four components, i) 2-nitrophenyl octyl ether (NPOE), a plasticizer used to improve solubility of membrane components, ii) potassium tetrakis(4-chlorophenyl) borate, an ion-exchanging salt, iii) Calix[6]arene, an ionophore to provide selectivity, and iv) a photocurable resin consisting of an acrylate monomer, diurethane methacrylate, and a photo-initiator. Once the BA+-ISM cocktail is introduced to the 3D printer, a computer-aided design (CAD) file, to specify the physical dimensions of the BA+-ISM, is then uploaded. We chose to print planar disk-shaped membranes with a thickness of 400 μm and a diameter of 10 mm. Once printed, the ISMs are rinsed with IPA to remove any uncured resin and then affixed to a PVC tube followed by a final 5-minute curing in the UV oven. Lastly, a Ag/AgCl wire is placed inside the PVC tubing, which contains an inner-filling solution. The complete potentiometric setup used to perform measurements pairs the 3D printed BA+-ISE with a reference electrode. Figure 1B shows a schematic representation of the experimental setup used for the measurement of BA+ using 3D printed LC-ISEs. A simplified graphic of the 3D printed BA+-ISM is also included in Fig. 1B.

Potentiometric measurements rely on the generation of a charge separation at the membrane/solution interface, which results in a potential difference (electromotive force, emf) between the reference electrode and the ISE.29–31,42 Importantly, this emf is logarithmically related to analyte activity as described by the Nernst equation (Eq. 1), where \( E^0 \) is the standard potential, \( T \) is the temperature, \( F \) is Faraday’s constant, \( z \) is the charge of the ion (Ba^+) has a +1 charge) and \( a \) is the activity of Ba^+.

\[
emf = E^0 + \frac{RT}{zF} \ln a = E^0 + \frac{59.2 \text{ mV}}{z} \log a
\]  

According to the Nernst equation, an order-of-magnitude change in Ba^+ concentration should result in a 59.2 mV change in the observed emf. Figure 2A shows the emf response of the BA^+-ISE to decreasing concentrations of Ba^+ in solution, where stable signals are obtained within 6 seconds. Figure 2B shows the linear response of the 3D printed Ba^+-ISE to changing concentrations of Ba^+ in a phosphate buffer of pH 8.0. Since the phosphate buffer maintains a constant ionic strength, we assume a constant activity coefficient, and thus report emf vs concentration, as opposed to activity. The slope of the emf vs logarithm of Ba^+ concentration was 55 mV/decade, which is close to the theoretically expected slope predicted by the Nernst equation for a monovalent cationic species.43 The linear response range of the 3D printed Ba^+-ISE was determined to be from 1 mM to 31 μM with a limit of detection (LOD) of 8 ± 3 μM, which more than covers the normal therapeutic concentration of Ba^+ in ophthalmic solutions (∼100–600 μM).

In order to be an effective sensor, especially when the target analyte is routinely found in complex aqueous environments such as ophthalmic solutions which contain various active and inactive ingredients, the ISE must exhibit sufficient selectivity for the analyte of interest. As mentioned above, to ensure our ISE responds selectively to Ba^+, the 3D printable ISM cocktail was doped with the ionophore calix[6]arene. In previous work, calixarenes have been shown capable of providing remarkable complexity with...
choline-type molecules (i.e., quaternary ammonium containing molecules), which are structurally similar to the BA$^{+}$. To investigate the selectivity of the 3D printed BA$^{+}$-ISE we measured the emf response of an unbiased sensor towards the cationic species present in ophthalmic formulations which include benzalkonium chloride as the preservative: Na$^{+}$, K$^{+}$, Mg$^{2+}$, and Ca$^{2+}$. Figure 3 shows the observed emf results for these cations and BA$^{+}$ (anions were excluded as this sensor only responds to cationic species). To quantify the selectivity, selectivity coefficients were calculated using Eq. 2, where $z_{BA}$ and $z_x$ are the charge of BA$^{+}$ and interferant, respectively, $K$ is the selectivity coefficient, $C_{BA}$ and $C_x$ are the concentrations of BA$^{+}$ and interferant, respectively, $m$ is the slope of the BA$^{+}$-ISE and the term $E_{BA} - E_x$ is the measured emf difference between the interferant and BA$^{+}$, respectively. The results are presented in Table I where we see that the 3D printed BA$^{+}$-ISE responds very selectively to BA$^{+}$. By incorporating calix[6]arene as an ionophore, the sensor displays higher selectivity for BA$^{+}$ compared to results presented in a previous work using ISEs that did not contain an ionophore.

$$\log K_{BA,x}^{\text{pot}} = \frac{z_{BA} (E_{BA} - E_x) - E_A}{m} + \log \left( \frac{C_{BA}}{C_x} \right)$$  \[2\]

An important parameter of any sensor is its stability. Previous work by our group reported on the very high stability (a drift of $\sim 17 \, \mu \text{V/hr}$ over 15 hr) of ISEs composed of 3D printed ISMs (in a solution of tetrabutylammonium, TBA$^+$). Figure 4 shows the measured emf in a solution of 1 mM BA$^{+}$ taken over 14 h. Here we see that the emf remains fairly constant throughout the measurement with a total drift of 2.87 mV over the course of the experiment, corresponding to a drift of just 205 $\mu \text{V/hr}$. Such stability would...
allow the 3D printed BA+ -ISE to provide accurate and reliable results over long time periods if incorporated into flow-cells or to give continuous monitoring capabilities to pharmaceutical companies for quality control applications. Table II compares previously reported analytical approaches for BA+ detection to our potentiometric sensor. To test the usable lifetime of the 3D printed BA+ -ISE, we performed a series of measurements of a solution containing 62.5 μM BA+ over a period of ∼3 weeks. Between measurements the sensors were stored in the absence of light at room temperature. Interestingly, the sensors retained their performance for 15 days before the measured emf response became unreliable.

To test the ability of the 3D printed BA+ -ISE to measure BA+ levels in a real ophthalmic solution, we tested the Dry Eye Relief Eye Drops by Equate™. Figure 5 shows a calibration performed in a diluted ophthalmic solution containing differing concentrations of BA+ and phosphate buffer (pH 8.0). As can be seen, a linear relationship between emf and logarithm of the BA+ concentration remains, with an observed Nernstian slope of 57.8 mV/decade. Then, an unknown solution containing 235 μM BA+ was tested with three different 3D printed BA+ -ISEs. The measured emfs correspond to a BA+ concentration of 230 ± 3 μM, which resulted in a percent recovery of 98 ± 6%. These results suggest that the 3D printed BA+ -ISEs can provide rapid and accurate measurements of BA+ in real ophthalmic formulations.

### Conclusions

This work describes the design and fabrication of a potentiometric sensor for the determination of benzalkonium chloride in ophthalmic formulations. 3D printing was used to fabricate the sensing element (i.e., ISM) of the BA+ -ISE. By incorporating the ionophore calix[6]arene, the 3D printed BA+ -ISE showed excellent selectivity over common cationic ingredients of ophthalmic solutions and showed a linear Nernstian response, with a slope of 55 mV/decade between 1 mM and 31 μM, which covers the concentration range of BA+ in eye drops. Furthermore, the sensor proved itself to be stable, having an average drift of ∼205 μV/hr over a 14 h timespan. This stability and selectivity suggest that the 3D printed BA+ -ISE would be capable performing continuous monitoring of BA+ in complex pharmaceutical formulations. Future work will look to expand on the ability of 3D printing potentiometric sensors for measuring the levels of prescription pharmaceuticals and their metabolites in both dosage form and in biological fluids.

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**Table II. Comparison of analytical techniques for the detection of BA+.**

| Detection method                  | Linear range (μM) | LOD (μM) | References |
|-----------------------------------|-------------------|----------|------------|
| CE-ESI/MS                        | —                 | 2        | 45         |
| CE-ESI/MS/MS                     |                   | 4        |            |
| BDDB electrode                   | 215–11            | 1        | 26         |
| Glassy Carbon                    |                   | 2        |            |
| Kinetic enzymatic method         | 500000–7          | 1.9      | 21         |
| HPLC                             | 700–270           | —        | 46         |
| LC/MS/MS with QueChERS           | 0.5–0.03          | 0.03     | 47         |
| Potentiometry                    | 1000–63           | —        | 30         |
| Potentiometry                    | 10000–0.02        | 0.02     | 48         |
| Potentiometry                    | 1000–31           | 8        | Current Work |

a)—Capillary electrophoresis-electrospray ionization. b)—Boron-doped diamond.
