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Simple and inexpensive quantification of ammonia in whole blood

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ABSTRACT

Quantification of ammonia in whole blood has applications in the diagnosis and management of many hepatic diseases, including cirrhosis and rare urea cycle disorders, amounting to more than 5 million patients in the United States. Current techniques for ammonia measurement suffer from limited range, poor resolution, false positives or large, complex sensor set-ups. Here we demonstrate a technique utilizing inexpensive reagents and simple methods for quantifying ammonia in 100 μL of whole blood. The sensor comprises a modified form of the indophenol reaction, which resists sources of destructive interference in blood, in conjunction with a cation-exchange membrane. The presented sensing scheme is selective against other amine containing molecules such as amino acids and has a shelf life of at least 50 days. Additionally, the resulting system has high sensitivity and allows for the accurate reliable quantification of ammonia in whole human blood samples at a minimum range of 25 to 500 μM, which is clinically for rare hyperammonemic disorders and liver disease. Furthermore, concentrations of 50 and 100 μM ammonia could be reliably discerned with $p = 0.0001$.

1. Introduction

Hyperammonemia, a life-threatening condition, is characterized by elevated blood ammonia levels and causes severe neurodevelopmental and neurodegenerative complications. The condition originates from a variety of hepatic diseases. This includes metabolic disturbances in the urea cycle that are caused by several inborn errors of metabolism collectively referred to as urea cycle disorders, affecting approximately 1 in 35,000 births in the United States [1,2], as well as chronic hepatic diseases such as hepatic encephalopathy, carcinomas, cirrhosis and hepatitis. These diseases affect a large number of people, with 5 million in the U.S. alone having cirrhosis.

Current methods for blood ammonia detection lead to prolonged treatment of hyperammonemia due to the requirement of specific sample preparation and access to tandem mass spectroscopy in large central laboratories. Samples must be drawn, placed immediately on ice, separated into plasma, and frozen, as ammonia concentrations will increase in standing whole blood samples. Blood ammonia levels can also increase during the collection process if a tourniquet is used, making it difficult to accurately test for ammonia levels without previous training [3,4]. Point-of-care (PoC) detection and monitoring of blood ammonia rapidly and in under-equipped environments with limited sample preparation and training would improve the prognosis and disease management for these patients.

A majority of previously reported PoC techniques must first separate the ammonia from blood before analytically determining the concentration. A common approach is to take advantage of ammonia's volatility in alkaline conditions. In solutions with a pH higher than 10, ammonia primarily exists in its gaseous form NH_3 instead of NH_4^+. The alkalization of ammonia solutions gave way to distillation as a separation mechanism. These techniques have since been consolidated and miniaturized using microdiffusion [5,6]. An ammonia containing sample is taken up into a reservoir containing an alkaline tablet. The ammonia volatilizes from the solution and passes through a polymer membrane into another reservoir containing a second solution where analysis can be performed. These methods generally suffer from false positives caused by hydrolysis of proteins and amino acids such as glutamine in alkaline conditions which produces ammonia [3]. Once separated from blood by alkaline-based distillation, the ammonia must be measured using a quantitative, analytical technique. Titration, a non-specific approach, is frequently investigated for this purpose. The separated alkaline ammonia is added to an acidic solution and the resulting pH change is monitored by a colorimetric indicator such as bromocresol green or by the use of an electrode. The commercial product Blood Ammonia Checker II by Arkray utilizes this technology [7]. Gas sensing electrodes are also used post distillation, in which ammonia gas is quantified through impedance measurements. These methods are hindered by interference

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from changes in temperature or humidity [8]. Such drawbacks are compounded by the aforementioned issues associated with utilizing distillation as the separation mechanism. Other analytic techniques have focused on using the ammonia gas present in a patient's breath, measured by a polyaniline electrode, but these techniques have poor resolution and require fairly complex, large sensor setups [9,10].

Enzymatic reactions have also been investigated to measure ammonia, offering specificity as a means to avoid the challenges present in pH or distillation based approaches. Most enzymatic methods described in literature utilize glutamate dehydrogenase, which reductively aminates α-ketoglutarate in the presence of ammonia and the reduced form of nicotinamide adenine dinucleotide (NADH) [11]. The system can be probed either optically or electrochemically for the decreasing concentration of NADH. This enzymatic technique is very sensitive; however, measuring a loss in signal can be difficult, especially in consideration of the poor stability of NADH [12].

A variety of colorimetric reactions exist that respond to ammonia including ninhydrin, Nessler's reagent, and the indophenol reaction. Ninhydrin is a very sensitive chemical, which turns a brilliant purple color in the presence of primary and secondary amines. However, it will react with any and all primary and secondary amines, such as amino acids, making its use in a specific and selective ammonia sensor ineffective [13]. Nessler's reagent (potassium tetraiodomercurate (II)), is fairly selective and can be found in commercially available ammonia test kits for aquariums and waste water. This reaction generates different products depending on the concentration of ammonia present. The primary product is an opaque red–brown precipitate, which can pose processing issues [14].

The indophenol reaction consists of ammonia, hypochlorite and phenol, which produce a deep blue, water-soluble compound [15]. The largest challenge with this reaction is negative interference from proteins and reducing agents found in whole blood. There have been previous efforts to overcome this challenge and utilize the indophenol reaction to measure blood ammonia. Some approaches utilized cation exchange resins to extract the ammonia from blood, which was then quantified using the indophenol reaction. The reported process was very complex and required multiple washing and extractions steps [16–18]. Another interesting approach used a variation of the indophenol reaction in a photonic crystal hydrogel matrix [19]. However, it has a long response time and was not demonstrated with human whole blood samples. Due to its inherent sensitivity and selectivity, the indophenol reaction was investigated as a potential means for sensitive blood ammonia detection.

In the following work the indophenol reaction was modified to contain unconventionally large concentrations of hypochlorite to greatly diminish negative interference from small blood-borne reducing agents. The modified reaction was then utilized in tandem with a polyelectrolyte cation-exchange membrane, Nafion. The long-range, negatively charged pores provide a means to separate ammonia directly from whole blood without any sample preparation [20]. As seen in Scheme 1A, placing the blood in a Nafion bisected well prompts an ion-exchange between ammonia in the blood and sodium ions in the opposing side of the well. To produce Nafion bisected wells, 3D printed, modular, well-halves were fabricated, which allowed for the quick assembly of multiple wells containing different blood samples, bisected with a cation exchange membrane (Scheme 1B). After the ion-exchange has occurred, the extracted ammonia solution can be mixed with the indophenol reagents and the resulting color measured using a plate reader (Scheme 1C). This detection scheme can rapidly quantify ammonia in multiple whole blood samples, allowing for faster diagnosis and management of hyperammonemia.

2. Experimental section

2.1. Stability studies

Aqueous solutions of 0.25% hypochlorite, 500 mM sodium hydroxide and a solution of 59 mM 2-phenylphenol in ethanol were stored with limited exposure to light. At intervals of 3, 5, 7, 15, 21, 28, 35, 50, 75 and 100 days the reagents were utilized to develop a standard curve using ammonium chloride concentrations ranging from 0 to 500 μM. To a 384 well-plate, 10 μL of 2-phenylphenol, 10 μL of NaOH, 5 μL of hypochlorite and 35 μL of ammonium chloride were mixed. Concentrations ranging from 7 μM to 3 mM of sodium nitroprusside can be used as the coupling agent to initiate the blue color generation. The resulting color was measured using a SpectraMax M5 plate reader at a wavelength of 635 nm after 10 min. Significant deviations from the original standard curve indicated the degradation of the stored reagents.

2.2. Response to amino acids

The selectivity of the indophenol reaction was determined towards ammonia against other primary amines such as amino acids. 1 mM solutions of each of the 21 proteinogenic amino acids were prepared in 1× PBS and tested using the previously described indophenol reaction. 10 min after the indophenol reagents and amino acid solution was mixed, absorbance at 635 nm was measured using a plate reader. The response was directly compared to the response measured from a 1 mM solution of ammonium chloride and expressed as a percentage of the ammonium response.

2.3. Sensor design and construction

A bisected well containing whole human blood in one section and a solution of sodium acetate in the other provide a means for cation-exchange of the whole blood to occur, yielding a high recovery of the ammonium. Modular well-halves were 3D printed from acrylonitrile–butadiene–styrene thermoplastic. The pieces snap together with the 1 cm [2] Nafion 111 membrane in the middle, forming a Nafion bisected well. This design was chosen to provide a uniform platform for all future experiments involving this sensing method. A silicone gasket, at a 1/64 in. thickness, was glued to the inner face of each well-half to ensure a water tight seal.

2.4. Extracting ammonia

Sodium acetate was utilized to extract ammonia through ion-exchange. Concentrations of 0.1, 0.5 and 1 M sodium acetate were prepared using fresh Milli-Q water (18.5 MΩ), to ensure no ammonia contaminants were present. Bisected wells were prepared, with 100 μL of 500 μM of ammonium chloride in one section and 45 μL of the sodium acetate solution in the other. Ion-exchange took place for 20 min before 35 μL of the now ammonia-enriched sodium acetate was tested.

2.5. Reducing interference from blood-borne small molecules

Reducing agents in blood such as uric acid can negatively interfere with the indophenol reaction. Increasing concentrations of the hypochlorite were utilized in a modified version of the indophenol reaction to eliminate this negative interference. 500 μM solutions of ammonium chloride were prepared in PBS and in whole human blood. The ammonia was extracted from these samples using previously described ion-exchange protocol. The concentration of hypochlorite was varied between 0.25 and 2.5% to examine its effectiveness in reducing interference.

2.6. Sensor response to ammonia in whole blood

The 3D printed wells were constructed with 1 cm [2] pieces of Nafion membrane. Whole human blood was spiked using ammonia chloride to generate concentrations of ammonia of 25, 50, 75, 100, 150, 200, 250, 300, 400 and 500 μM. In one section of the well, 100 μL of the ammonia containing blood sample was added. In the opposing Section 1 M sodium acetate was added. Ion-exchange of ammonia was