Preconcentration of charged molecules on paper pads using greenly synthesized smart nano-composite membranes

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Abstract
Low concentration of biomarkers (proteins, nucleic acids, metabolites) is a major concern in early disease diagnosis due to the very low concentration of these biomarkers at the early stage of any disease. This challenge can be addressed by preconcentrating the biomarkers to improve the sensitivity of the diagnostic device. This paper describes a novel method to preconcentrate the charged molecules on paper using greenly synthesized iron nanoparticles. The iron nanoparticles were synthesized by utilizing plant extract as stabilizing agent, which both minimizes the cost and protects the environment. Three different samples of the nanoparticles were prepared by changing the charge or attaching a functional group (amine) to the surface. The iron nanoparticles act as a charged nanoporous membrane when deposited on the paper pads inducing electrostatic forces around the membrane leading to the concentration of the charged analytes near the boundary of the membrane. Crystal violet was used as a surrogate for the charged biomolecule. We report ∼24-fold increase in the concentration of crystal violet dye within 120 seconds using the positively charged membrane and the 0.2 mM dye solution. The developed experimental set-up eliminates the need for external pumping device and complex fabrication processes making the proposed method cost-effective, environment-friendly, and simple to use.

Introduction
The COVID 19 pandemic has once again highlighted the need for rapid, easy-to-use, sensitive, and cost-effective point-of-care (POC) diagnostic devices, especially in resource-poor countries.[1–4] One of the key challenges of POC diagnostics is the low concentration of specific biomarkers in the test samples. [5, 6] Charged biomolecules like metabolites, proteins and nucleic acids are regularly used as biomarkers to monitor the physical wellbeing of the patients. Biomarker concentrations fluctuate over the course of the disease are often found in low abundance at the early stages of the disease or an exposure event.[7] This challenge can be addressed by preconcentrating the biomolecules in a specific region for easy and rapid visual detection.

Lab-on-a-chip devices (traditional microfluidic devices) are sensitive and versatile platforms that can be used for POC testing and help overcome this obstacle. POC testing not only ensures safe, personalised, and effective care of patients but carrying out these diagnostic (analytical) processes at the microscale also allows for use of lower reagent and sample volumes, rapid detection time, and cost-effectiveness.[8–12] To further improve sustainability and cost-effectiveness, paper-based microfluidic devices have gained popularity in recent years. Paper-based diagnostics devices are cheap, portable, easy to use and dispose of (environment friendly), and more importantly, eliminate the need for an external pumping system as the capillarity drives the fluid flow and complex microfabrication procedures [13–16].

Recently isotachophoresis (ITP) [17–21] and electrokinetics [22–30] have emerged as techniques of choice for preconcentration at nano and microscale. ITP exploits the differences in the electrophoretic mobility of the
analytes in an electric field. ITP uses two buffers to achieve simultaneous separation and concentration of charged molecules in a fluidic system. The leading electrolyte (LE) has higher electrophoretic mobility compared to the trailing electrolyte (TE) and the focusing of analytes happens at the interface of the LE and the TE [31].

The electrokinetic preconcentration, on the other hand, is achieved by applying an electric field across a nanoporous ion-selective membrane in a microchannel. The membrane allows the ions of the same charge to concentrate when its dimension becomes comparable to that of the electrical double layer (EDL) [32, 33]. The ion concentration polarization (ICP) occurs at the membrane/electrolyte interface leading to the development of an ion depletion zone and the concentration occurs at the boundary of the ion depletion zone regardless of the size of the charged molecules [30, 32].

ICP is characteristic of cation-selective membranes with poly(3,4-ethylenedioxythiophene)-poly(styrenesulfonate) (PEDOT:PSS) [34, 35], Nafion® [22, 25, 28], silica beads [33, 36, 37], N-[3-(Trimethoxysilyl)propyl]-N-(4-vinylbenz)ethylenediamine hydrochloride (TMSVE) and polyE-323 coated nanochannels [38] being the most commonly used membranes. Most of these membranes are commercially available but expensive. Moreover, PEDOT:PSS and TMSVE are hazardous [39, 40].

In this paper, we report using electrostatic forces to preconcentrate charged molecules on paper pads using greenly synthesized iron nanoparticles (FeNPs). Recently, iron nanoparticles have attracted much research attention in many areas such as the removal of organic and inorganic pollutants from water, catalysis, and antibacterial applications. This interest in FeNP stems from the simplicity of synthesis, low cost, and possibility of applying green chemistry to get an eco-friendly product. Furthermore, FeNP has many other attractive features such as the high surface area, ease of handling and separation, and the possibility of controlling the charge of the surface by simple acidic or basic treatment [42, 45].

The FeNPs used in this work were synthesized from Acacia nilotica seedless pods extract, the detailed synthesis of these NPs is published elsewhere [45]. We used FeNPs deposited on paper pads as a nano-composite membrane and crystal violet (a positively charged dye) as a surrogate for the charged molecules to demonstrate the proof of concept.

**Experimental section**

**Device fabrication and membrane deposition**

GE Whatman CHR 1 paper was used as the paper substrate. The paper pad has a test channel (3.5 × 0.5 cm) and two pads (0.5 × 0.5 cm) at each end to hold the pad in place during experimentation. To make the membrane, 1 μL of FeNPs was deposited thrice (the paper was allowed to dry in between each deposition) at the centre of the test channel on the top side and once on the bottom side. This was done to make sure that the membrane fills the entire cross-section of the test channel and to stop the diffusion of the charged molecule from the edges and the bottom layers of the device. The schematic of the experimental setup is shown in figure 1(A).

**Membrane characterization**

Three different FeNP suspensions were used to prepare membranes on paper, by changing the charge or attaching an amine group to the surface. (i) 1 mg FeNP in 5 ml deionized (DI) water and 1 drop of concentrated HNO₃ (1 M) for positively charged membrane, (ii) 1 mg FeNP in 5 ml DI water and 1 drop of concentrated NaOH (1 M) for negatively charged membrane, and (iii) 1 mg of Fe-DA (diamine) in 5 ml of DI water for a neutral membrane. The suspensions were sonicated at room temperature for 60 min prior to use. Scanning electron microscopy (SEM, figure 1(B)) and Transmission electron microscopy (TEM) were used to characterize the FeNPs (figures 1(C), (D)). The SEM image of FeNP (1B) shows that the size of nanoparticles is around 200 nm, and this is mainly because of the tendency of the nanoparticles to aggregate under the effect of the magnetic dipole–dipole interactions. The same observation about aggregation is clear in the TEM image (1C). However, figure 1(D) shows that the unaggregated particles have an average size of 40 nm.

**Sample preparation**

0.2, 0.4, 0.8, 1.6, and 3.2 mM solutions of crystal violet were prepared in DI water for the concentration experiments. Once the membrane was dry, 17 μl of the dye was added thrice on one side of the paper pad. The setup was left undisturbed for two minutes and then images were taken using a OnePlus6 mobile phone (20 MP rear camera) inside a box with flash on. Images were analysed with NIH ImageJ and the concentration of the dye was estimated as the mean intensity of the image.
Results and discussion

Calibration curve of crystal violet
The calibration curve was prepared by measuring the image mean intensity of crystal violet at 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, and 12.8 mM concentrations (figure 2). It is clear from the figure that the relation between the concentration and the colour intensity is described by an exponential relation with an $R^2$ of 0.99.

Device characterization
When treated with 1 M HNO$_3$ and 1 M NaOH, the FeNPs show positive and negative surface charges, respectively. The NPs treated with diamine are neutral. Figure 3 shows the results of the concentration experiments. For control experiments, 51 µl (17 µl × 3) of the 0.2 mM dye was added to the paper pads without the membrane. The dye covers the entire test channel within two minutes (figure 3(A)). Figure 3(B) shows the paper pads with the FeNPs deposited in the centre of the test channel before adding the dye. These NPs act as a nanoporous membrane resulting in the electrostatic repulsion or attraction between the membrane and the dye.
molecules. When the dye is added to the paper channel with positively charged FeNPs, the membrane repels the positively charged crystal violet molecules (solute) but lets the water (solvent) pass through leading to the concentration of the dye molecules near the boundary of the membrane (figure 3(C)). Figure 3(F) shows the slightly clear area near the boundary of the positively charged membrane confirming the repulsive forces.

On the other hand, the negatively charged membrane binds to the positively charged dye molecules stopping their flow whilst letting the water molecules flow through the membrane leading to the concentration of the dye molecules (figure 3(D)). There is no clear area near the boundary of the membrane as seen with the positively charged membranes. The neutral FeNPs fail to show any concentration as the dye molecules flow through the membrane (figure 3(E)) proving our hypothesis that the concentration is caused by the electrostatic forces.

**FeNPs as a nanoporous membrane**

Electric double layer (EDL) forms at the interface of two conducting phases, the charge of one phase being balanced by an opposite charge of same magnitude. The EDL is approximately equal to the Debye screening length ($\lambda_D$) [32] which is inversely proportional to the square root of the ionic strength and is given by

$$\lambda_D = \frac{\varepsilon k_B T}{q^2 c}$$

($\varepsilon$: dielectric permittivity of the media; $k_B$: Boltzmann’s constant; $T$: absolute temperature; $q$: electron charge; $c$: ionic strength of the electrolyte).

Typical $\lambda_D$ ranges from 1–10 nm for most physiological ionic concentrations. 1, 0.1, and 0.01 phosphate buffer saline (PBS) solutions have a Debye length of 0.7, 2.4, and 7.4 nm respectively [46]. High ionic strength results in smaller $\lambda_D$ due to charge screening leading to lower pre-concentration and vice-versa.[32]

To demonstrate that the FeNPs deposited on the paper pads act as charged nanoporous membrane, three solutions of 0.4 mM crystal violet were prepared in (i) deionized (DI) water, (ii) 0.125 M phosphate buffer (PB) and (iii) 0.25 M PB. When dye in DI water solution was added to the paper pads with positively charged membrane, the dye did not pass through the membrane (figure 4(A)) while as when dye in 0.125 (figure 4(B)) and 0.25 M solutions (figure 4(B)) were added, the dye molecules leaked through the membrane with the most leakage seen in the later solution.
Preconcentration of crystal violet on paper pads

Figure 5(A) shows the results of preconcentration results for FeNP in HNO\textsubscript{3} (positively charged membrane). 0.2 mM dye shows the highest, 24-fold increase in concentration which then decreases as the concentration of the analyte increases. This is because the paper reaches saturation easily with the higher initial concentrations of the dye leading to lower preconcentration. For FeNP in NaOH (negatively charged membrane), the highest preconcentration, 16-fold, was observed in 0.4 mM dye solution (figure 5(B)). As the initial concentration of the dye increased, the overall preconcentration decreased showing a similar pattern to the positively charged membrane. The lowest preconcentration was observed in 3.2 mM dye solution (3-fold) in both cases. It worth mentioning that results in figures 5(A) and (B) show how effective this technique is since it works well with both negatively and positively charged membrane which make it applicable for preconcentration of both cationic and anionic reagents.

Preconcentration of charged molecules on paper-based devices using ICP reported previously have shown a range from 10-fold — 1000-fold concentration \cite{22, 24-27, 29} but the setup requires an external voltage source to induce ICP. With our setup, we can achieve comparable results without a voltage source thus making it truly ‘equipment free’, a criterion used by the World Health Organisation (WHO) \cite{47} for benchmarking low-cost diagnostic devices for resource-limited environments.

Conclusion

In this work, we demonstrated a passive preconcentration method using greenly synthesized FeNPs on paper pads. The experimental setup is easy to use and fabricate and removes the need of an external pumping system and a voltage source. A 24-fold increase in analyte concentration was observed within 120 seconds. This device has the potential to be used as a universal platform for low-cost diagnostics in resource-limited settings.

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Data availability statement

All data that support the findings of this study are included within the article (and any supplementary files).

Competing interests

The authors declare no competing interests.

Author contributions

AS and ED conceived and designed the experiments; ED and AT synthesized the iron nanoparticles; AS and ED performed the experiments; AS analysed the data and wrote the paper; ED reviewed the paper.

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