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Measuring calcium content of human milk on a microfluidic chip

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Abstract

Current standard human milk analyzers based on near-infrared analysis cannot detect calcium concentration, important for bone growth of low birth weight infants (LBWI). The presented microfluidic chip provides a simple colorimetric determination of calcium concentration within minutes, requiring a sample volume of only 5 µL. This fast and simple device is the basis for further clinical investigations on the influence of calcium content in human milk, leading to an increased knowledge and optimization of LBWI nutrition.

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Keywords: human milk; calcium; microfluidic; phaseguide; absorbance; colorimetric assay

1. Introduction

While human milk is the ideal feeding for all infants, it does not meet the special nutritional needs of low birth weight infants (LBWI). Nevertheless, it is beneficial because human milk provides unique bioactive factors, e.g. hormones, enzymes, antibodies and immunoglobulins. Feeding human milk instead of formula to neonates significantly decreases the risk of necrotizing enterocolitis [1], a disease that is associated with an in-hospital mortality of 16 % to 42 %, depending on birth-weight [2]. Schanler et al. report on improved general health of

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premature infants, i.e. less sepsis and less necrotizing enterocolitis, if fed with human milk instead of preterm formula [3]. Therefore, human milk is unequivocally preferable to formula. Nevertheless, it is recommended to be fortified with proteins and minerals in order to meet the nutritional needs of LBWI and to achieve best possible clinical outcomes in terms of survival, neurological development, growth and long-term health.

Supplying calcium is of high importance for the infant's bone mineralization. Commercially available human milk analyzers based on near infrared analysis, e.g. MIRIS Human Milk Analyzer (MIRIS AB, Uppsala, Sweden), cannot detect calcium. For this reason, human milk is often fortified in a standardized way, not knowing either the initial or the resulting fortified calcium concentration.

The proposed microfluidic chip is able to measure calcium concentration, enabling an individualized fortification of calcium in human milk to further optimize the nutrition parameters and therefore, the growth of LBWI.

2. Materials and methods

In order to measure multiple milk samples simultaneously, before and after fortification, a chip contains 16 individual analysis chambers, each measuring approximately 3.5 x 4 mm. Microfluidic channels are structured using a dry film resist (Ordyl SY300, Elga Europe, Milan, Italy), sandwiched between two standard microscope slides (Fig. 1a).

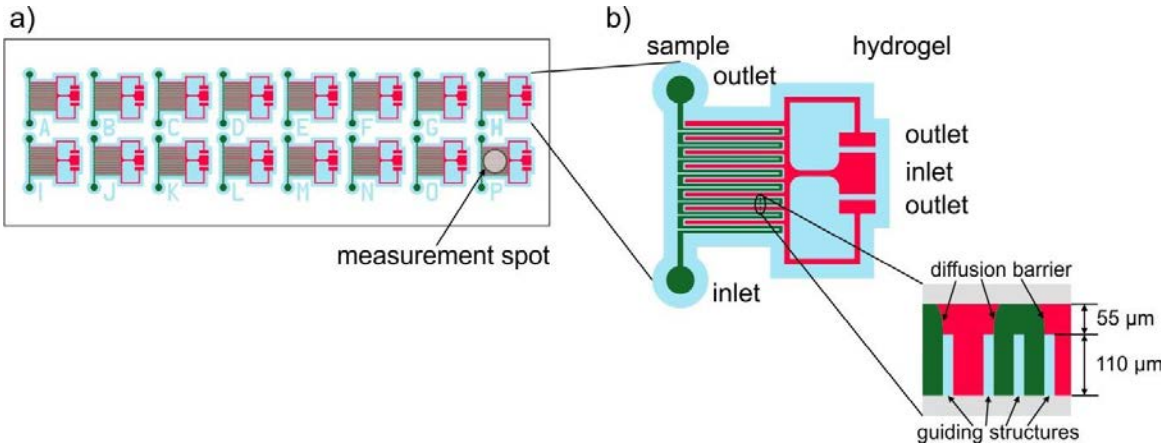

Fig. 1. (a) Schematic of the microfluidic chip with visualization of a measurement spot; (b) individual analysis chamber with cross sectional view, showing sample (green), guiding structures (blue) and hydrogel diffusion barrier (red).

Before measurement, each chamber is prefilled using a micropipette with a hydrogel (Agarose Low Melt, Carl Roth, Karlsruhe, Germany) that incorporates the calcium-sensitive color reagent Arsenazo III (Sigma Aldrich, St. Louis, USA) [4]. Utilizing the concept of microfluidic phaseguides [5, 6], this reagent is introduced into a predefined finger structure within the analysis chamber. The gel is formed by cooling the microfluidic chip down to 4 °C. The guiding structures cover two thirds of the channel height, enabling a diffusion barrier between sample and color reagent, as shown in the cross sectional view in Fig. 1b.

Human milk samples are collected from mothers of preterm babies. After expressing the milk using an electric milk pump the sample is refrigerated. Within 24 hours the human milk sample is homogenized, aliquoted into 1 mL volumes and frozen. Prior to analysis, the sample is thawed at room temperature and homogenized. Homogenization of human milk samples is accomplished by an ultrasonic bath heated to 38 °C and gentle vortexing.

After sample introduction the diffusive mixing into the hydrogel starts. Small diffusion lengths provide mixing in a reasonable short time, typically 30 s to 3 min. The optical absorbance measurement is performed with a miniaturized spectrometer (Torus, Ocean Optics, Dunedin, USA) at $\lambda = 655$ nm as the spectrum exhibits a maximum absorbance at this wavelength. For sample illumination a halogen light source (HL-2000-HP-FHSA, Ocean Optics) is utilized. The measurement spot size is adjusted to match the area of an analysis chamber, see Fig. 1a.

3. Results and discussion

Fig. 2a shows two analysis chambers after gelation, ready for sample introduction. Samples are injected with a micropipette. After incubation time of 2 min the color has changed depending on the calcium concentration of the sample. Fig. 2b compares a blank sample and a sample with a calcium concentration of 5 mmol/L, which reflects the measurement range of the microchip.

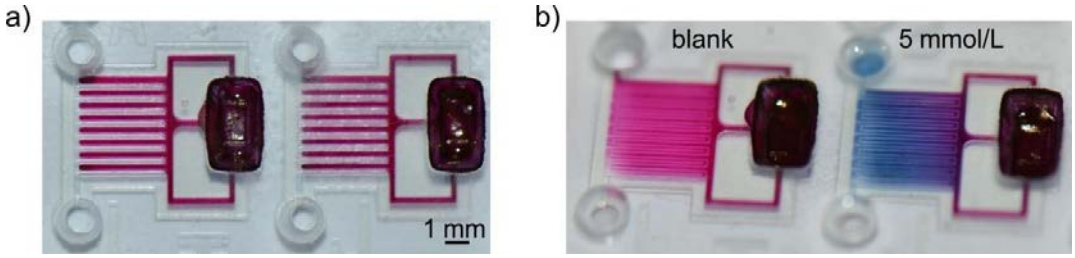

Fig. 2. (a) Microfluidic chip after filling and gelation of hydrogel, ready for sample injection; (b) analysis area after incubation time (2 min) of a blank sample and a sample with 5 mmol/L (right) calcium concentration.

Calibration, depicted in Fig. 3, is performed by diluting a standard human milk fortifier (Aptamil FMS, Milupa, Bad Homburg, Germany), also used as a nutritional supplement in prenatal care. Measurement points ranging up to 3 mmol/L were captured. In this range a linear fit of $R^2 = 0.998$ is reached. Above a concentration of 3 mmol/L saturation begins, resulting in a non-linear relation between absorbance and calcium concentration.

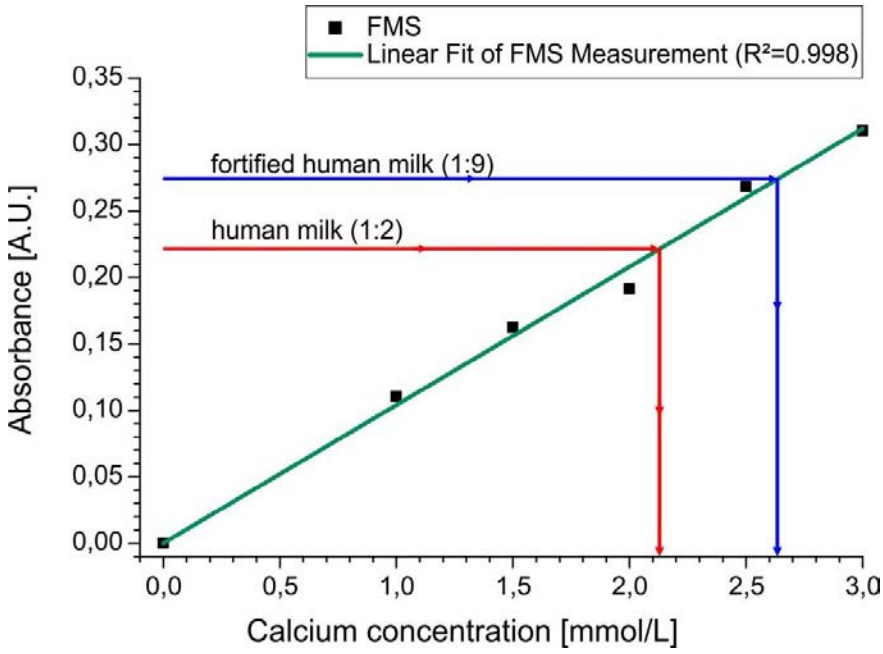

Fig. 3. Calibration curve based on dilution rates of human milk fortifier (FMS) (black dots) including linear fit (green, $R^2 = 0.998$) and absorbance measurement of human milk (red, volume dilution ratio 1:2) and fortified human milk (blue, volume dilution ratio 1:9). Absorbance is measured at $\lambda = 655$ nm.

Exemplarily, the calcium concentration of a human milk sample (volume dilution ratio 1:2 with deionized water) is determined (red line) to be 6.4 mmol/L. This is within the expected measurement range of non-fortified human milk (5.5 - 7.5 mmol/L [7, 8]). After fortification, which is performed according to standard instructions including a mixing and homogenization step, the fortified milk was diluted (volume dilution ratio 1:9 with deionized water) to shift the calcium concentration within the linear range of the assay. The enriched sample shows an increased calcium concentration of 26.5 mmol/L (green line), corresponding to typical calcium concentrations of fortified milk samples fed to neonates in intensive care units.

4. Conclusion and outlook

A simple colorimetric assay to determine calcium concentration of human milk in a microfluidic device was fabricated and demonstrated. The utilization of microfluidic phaseguides in combination with diffusion based mixing of a hydrogel with the sample is proven.

The device shows a linear behavior in the range up to 3 mmol/L calcium concentrations. Higher concentrations can be measured after dilution of the sample. Concentrations of human milk and fortified human milk samples were successfully determined with the device. This approach offers the potential to measure additional milk constituents, such as protein [9], lactose [10] and phosphate [11].

Accurate determination of calcium concentration in human milk samples is the basis for further clinical studies to monitor the correlation to lactation period and the influence on LBWI growth.

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