DIRECT HEMOGLOBIN MEASUREMENT BY MONOLITHICALLY INTEGRATED OPTICAL BEAM GUIDANCE

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ABSTRACT

We present a novel concept for optical beam guidance by total internal reflection (TIR) at V-grooves as retro reflectors which are monolithically integrated on a microfluidic "lab-on-a-disk". This way, the optical path length through a measurement chamber and thus the sensitivity of colorimetric assays is massively enhanced compared to direct (perpendicular) beam incidence. With this rugged optical concept, we determine the concentration of hemoglobin (*Hb*) in human whole blood. Outstanding features are a high degree of linearity ($R^2 = 0.993$) between the optical signal and the *Hb* together with a reproducibility of $CV = 2.9$ %, and a time-to-result of 100 seconds, only.

Keywords: hemoglobin, colorimetric assay, centrifugal microfluidics

INTRODUCTION

The transfer of clinical diagnostics to point-of-care applications is subject of various efforts concerning scientific aspects [1]–[5] as well as commercialization [6][7]. To meet the requirements of clinical diagnostics, so-called "lab-on-a-chip" or "micro total analysis" systems (µTAS) which feature a full process integration, reduced consumption of sample and reagents as well as short time-to-result and ease of handling are the most prominent candidates.

Among these "lab-on-a-chip" systems, we here consider centrifugal "lab-on-a-disk" technologies which exploit centrifugal and capillary forces for sample preparation, flow control in order to run fully process integrated assays [7]–[9]. Numerous lab-on-a-disk products have already been launched [10]–[12].

More precisely, we focus on the implementation of a disk-based, colorimetric hemoglobin assay. The *Hb* is defined as the amount of the protein hemoglobin in the erythrocytes with respect to the total blood volume. It is one of the most relevant markers in clinical diagnostics and routine blood screening, indicating, for instance, certain diseases (polycythemia vera, chronic hypoxia), genetic defects (thalessemia), and severe physiological conditions (blood loss, dehydration).

COLORIMETRIC ASSAY ON-DISK

Colorimetric assays are based on the measurement of the intensity I of a probe beam of an incident intensity I_0 after passing the measurement chamber containing the analyte solution. According to the law of Beer-Lambert, the optical density (or absorbance)

$$OD = -\ln\left(\frac{I}{I_0}\right) = \varepsilon \cdot Hb \cdot l_{\text{abs}} \qquad (1)$$

linearly depends on the molar extinction coefficient ε of the products, the initial *Hb*, and the optical path length l_{abs} through the measurement chamber.

In our novel approach, the optical beam of a standard laser diode (λ_{peak}= 532 nm) [13] impinges perpendicular to the flat upper side of the disk. Via total internal reflection (TIR) at the side facet of the triangular V-groove which is embedded into the reverse side of the chip (Fig. 1), the beam is then deflected by 90° into the plane of the flat measurement chamber. To ensure TIR, the angle of incidence α has to exceed the critical angle

$$\alpha_c = \sin^{-1}\left(\frac{n_{\text{air}}}{n_{\text{COC}}}\right) \qquad (2)$$

which is governed by the refractive indices of the polymer substrate $n_{\text{COC}} \approx 1.5$ and the surrounding air n_{air}. After passing the measurement chamber, the attenuated beam is reflected at another V-groove towards a spectrophotometer [14].

With this setup, the optical path length is appreciably extended to $l_{\text{abs}} = 5$ mm compared to the maximum path

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length of about 1 mm which can be realized for direct perpendicular incidence on typically flat microfluidic chips.

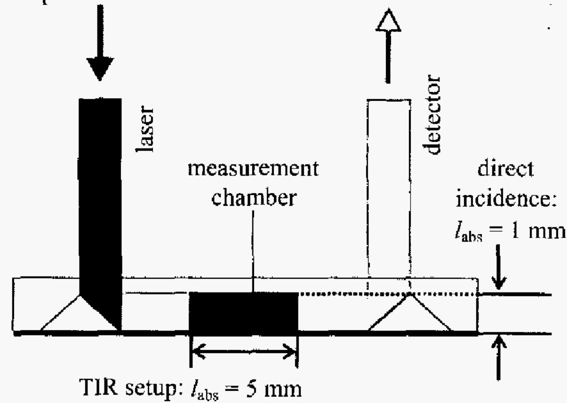
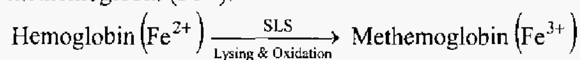


Fig. 1 Concept for an integrated optical beam guidance by total internal reflection (TIR). Next to the measurement chamber, integrated V-grooves act as retro reflectors to deflect the beam by 90° into the disk-plane. After passing the measurement chamber, the attenuated probe beam is reflected and then measured by a detector positioned above the disk. To ensure TIR, the incident angle α must be larger than the critical angle $\alpha_c \approx 41°$ governed by the refractive indices of the polymer $n_{COC} \approx 1.5$ and air n_{air} (equation 2).

BIOCHEMICAL CONCEPT

In our colorimetric reaction scheme, *Hb* is directly quantified in an untreated human whole blood sample where it reacts with sodium lauryl sulfate (SLS) dissolved in a detergent. Compared to the reference method for *Hb*-determination [18] using cyanide, the non-toxic SLS-method [19] is advantageous for point-of-care applications.

The reagents (V_r = 98 µL) first lyse the erythrocytes of the blood sample (V_{sample} = 2 µL) and then oxidize the heme-groups (Fe^{2+}) of the released hemoglobin to methemoglobin (Fe^{3+}):



In contrast to hemoglobin located in the erythrocytes, the released methemoglobin exhibits a distinct absorption band matching the emission of the laser diode (Fig. 2).

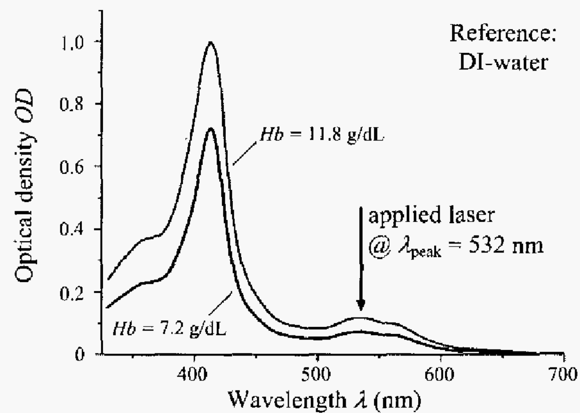


Fig. 2 Characteristic absorption spectra of processed blood with *Hb* at two different concentrations. A distinguishable side maximum is located near the wavelength of the probe laser (λ_{peak} = 532 nm).

ASSAY PROTOCOL

In our lab-on-disk concept, the hydrodynamic actuation is delivered by the reusable and robust macroscopic centrifuge drive. On the other hand, the microstructured disk which is made of Cyclic Olefin Copolymer (COC) in the format of a conventional data CD features passive microstructures, only. It can hence be fabricated in a very economic fashion. In our prototyping technology, microfluidic and optical elements are micromachined into the disk. Next, the surface is hydrophilized and coated by a dip-coating process with PetOx (poly(2-ethyl-2-oxazoline) dissolved in methanol to prevent from protein-adsorption.

The frequency protocol $\nu(t)$ (Fig. 3) which conducts the assay comprises an initial ramp-up to a maximum frequency ν_{max} = 20 Hz for transporting the sample and the reagent mixture into the measurement chamber. To enhance the diffusion-limited speed of the reaction, we then apply our previously developed "shake-mode", i.e. a frequent change of the sense of rotation between the maximum ν_{max} = 8 Hz at an acceleration ramp $d\nu/dt$ = 32 Hz / s [20][21]. Within one second, the disk goes through a complete clockwise *and* counter-clockwise revolution. After an 80-second period of such inertially induced mixing and reacting, the optical density *OD* is measured at constant spinning (ν_{det} = 8 Hz) to eventually quantify the *Hb* (equation 1).

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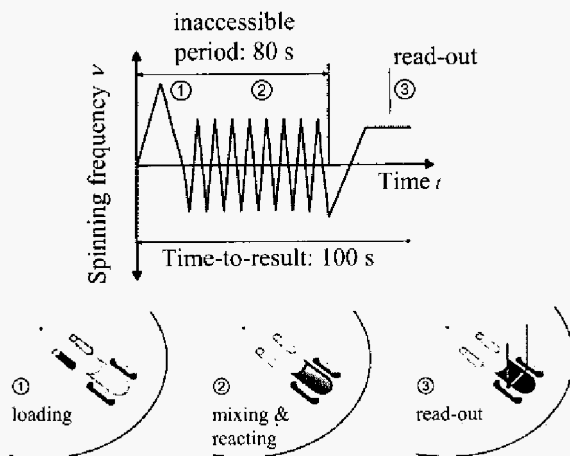

Fig. 3 Frequency protocol ν(t) to perform an on-disk hemoglobin-assay within a time-to-result of 100 s. (1) After the sample and the reagents are loaded into the inlet reservoirs, the disk is spun to transport the liquids into the measurement chamber. (2) While applying the "shake-mode", convective currents are induced to accelerate mixing, lysing of the erythrocytes, and the oxidation of the hemoglobin. (3) The assay result is quantified under constant spinning.

RESULTS

According to the predictions of Poisson statistics, it is beneficial to keep the signal level I of a detector at the upper limit of its working range because the CV is proportional to $\left(\sqrt{I}\right)^{-1}$. This way, the appropriate adaptation of the integration time τ, which is proportional to I (Fig. 4), allows to keep I rather constant, even when the OD increases with higher Hb.

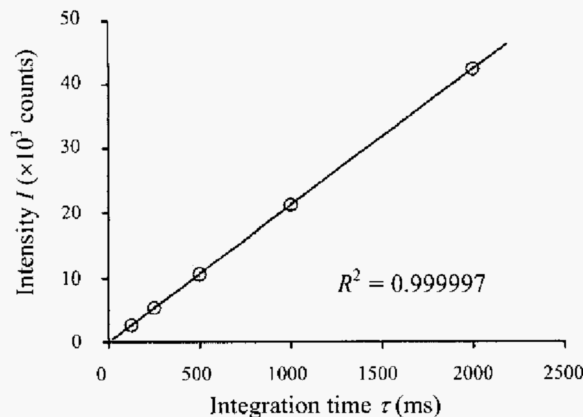

Fig. 4 Calibration curve of the spectrophotometer at $\lambda = 532$ nm. The recorded curve features an excellent linearity ($R^2 = 0.999997$).

The disk-based experiments are calibrated by a series of optical density measurements with a standard sample at known Hb [22]. With increasing Hb, the integration time τ is doubled twice to elevate the output signal (Fig. 5, A). The displayed absorption characteristics clearly comply with the law of Beer-Lambert (equation 1). We obtain a CV of 2.9 %, a lower limit of detection of $Hb_{\mathrm{min}} = 0.29$ g/dL together with a high resolution ($\Delta Hb = 0.38$ g/dL) and a good linearity between the hemoglobin concentration and the optical signal ($R^2 = 0.993$) (Fig. 5, B). Additionally, by adapting τ, the CV was improved from 5 % at constant τ to less than 3 %.

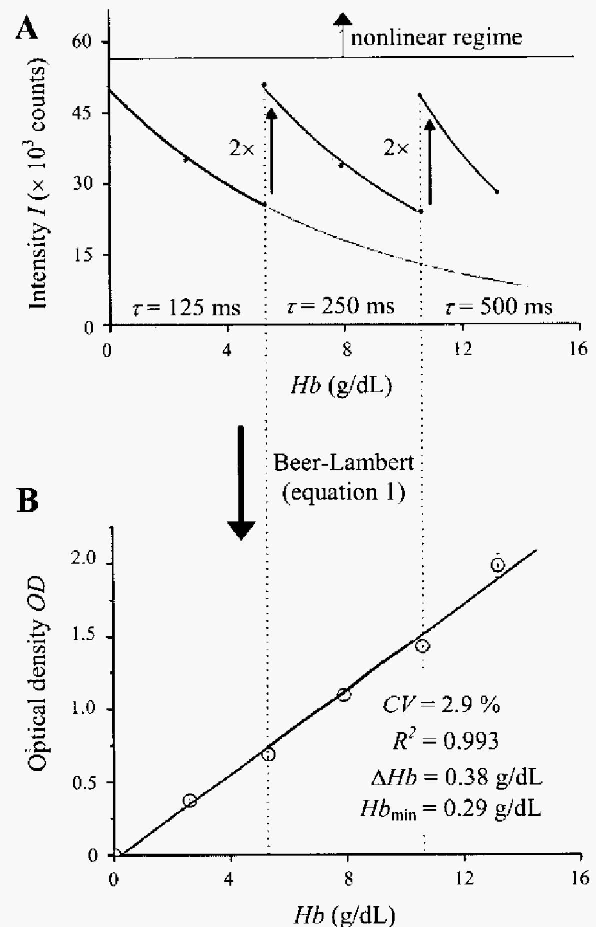

Fig. 5 Results of the Hb determination: (A) The integration time τ of the spectrophotometer is increased in discrete steps (τ, 2τ, 4τ) to keep the output signal I of the detector in the upper of the working range (i. e., lower than 55,000 counts). (B) The conglomerate calibration curve is obtained by successive dilution of a calibrated sample.

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CONCLUSION AND OUTLOOK

We introduced a chip-based, non-invasive read-out method for common colorimetric assays with a conventional laser diode and detector. The method is based on the total internal reflection (TIR) at monolithically integrated beam-guidance structured in a possibly disposable polymer substrate.

The principle is implemented by means of designated microstructures and spinning protocols on our centrifugal lab-on-a-disk platform. We successfully demonstrated the direct quantification of hemoglobin in whole blood.

In the future, the *Hb*-assay will be combined with other colorimetric assays at different probe wavelengths [15] or even fluorescence immunoassays (FIA) [16] in one read-out device for disk-based point-of-care applications [17].

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