Biocatalytically initiated precipitation ATRP as a quantitative method for hemoglobin detection in biological fluids

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ABSTRACT: The hemoglobin content of blood is an important health indicator, and the presence of microscopic amounts of hemoglobin in places where it normally does not occur, e.g. in blood plasma or in urine, is a sign of diseases such as hemolytic anemia or urinary tract infections. Thus, methods to detect and quantify hemoglobin are important for clinical laboratories, blood banks, and for point-of-care diagnostics. The precipitation polymerization of N-isopropylacrylamide by hemoglobin-catalyzed atom transfer radical polymerization (ATRP) is used as an assay for hemoglobin quantification relying on the formation of turbidity as a simple optical read-out. Dose-response curves for pure hemoglobin, for hemoglobin in blood plasma, in urine, in erythrocytes, and in full blood are obtained. Turbidity formation increases with the concentration of hemoglobin. Concentrations of hemoglobin as low as low as $6.45 \cdot 10^{-3}$ mg mL^{-1} in solution, $4.88 \cdot 10^{-1}$ mg mL^{-1} in plasma, and $1.65 \cdot 10^{-1}$ mg mL^{-1} in urine could be detected, which is below the clinically relevant concentrations in the respective body fluids. Total hemoglobin in full blood is also accurately determined. The reaction can be regarded as a polymerization-based signal amplification for the sensing of hemoglobin, as the analyte catalyzes the formation of radicals which add many monomer units into detectable polymer chains. While most established hemoglobin tests involve the use of highly toxic reagents such as potassium cyanide, the polymerization-based test uses simple and stable organic reagents. Thus, it is an environmentally friendlier alternative to established chemical assays for hemoglobin.

KEYWORDS

Hemoglobin, ATRP, biocatalytic ATRP, polymerization-based amplification, anemia diagnostic, biosensing, precipitation polymerization.

INTRODUCTION

Methods to detect and quantify hemoglobin are essential in clinical diagnostics and for point-of-care testing. Total hemoglobin concentration in whole blood indicates the oxygen carrying capacity of the red blood cells (RBCs), and a decreased hemoglobin measurement is diagnostic of anemia, which can be caused by blood loss, nutritional deficiencies (iron, folate, vitamin B6, or vitamin B12), parasitic or viral infections, inflammatory and autoimmune diseases, genetic disorders, exposures to certain medications and toxic substances, and cancers. The World Health Organization (WHO) estimated 800 million women and children were affected by anemia in 2011 in both low and high income countries.[1] In severe anemia, an insufficient amount of oxygen is transported throughout the body by RBCs, which can lead to organ damage and even death. Oxygen is only transported by hemoglobin contained in RBCs, so the presence of free hemoglobin indicates a decrease in oxygen carrying capacity. Elevated free hemoglobin in blood plasma can indicate autoimmune hemolytic anemia and malaria, as well as other rare, potentially life-threatening dieseases.[2-3] In urine, blood (hematuria) or hemoglobin (hemoglobinuria) can be a sign for urinary tract or kidney infections,[4] glomerulonephritis and acute kidney injury,[5] and cancer of the kidney,[6] bladder,[7] or prostate.[8] Hemoglobin in stool may indicate invasive bacterial infections, inflammatory bowel disease, polyps, or colon cancer.[9] The quantification of hemoglobin is also important as a quality control for blood bank samples, especially if RBCs lyse and release their content into blood plasma during sample taking or sample storage.[10] Transfusion of lysed RBCs fails to improve oxygen transport, and can cause blood vessel injury, kidney injury, and blood clots.[10]

The hematocrit level measures the ratio of the volume of RBCs against the total volume of the blood after a centrifugation. Though this is a simple and fast method for anemia determination, it is highly dependent on the stability of the RBCs of a given sample,[11] and is not suitable to measure free hemoglobin. Quantification of total hemoglobin is a clinically useful measurement to diagnose anemia. Drabkin´s cyanomethemoglobin colorimetric assay is the most widely used and accurate method for hemoglobin quantification.[11] It is based on the oxidation and complexation of all major hemoglobin species with cyanides to the stable cyanmethemoglobin (HiCN).[12-13] The absorbance at 540 nm is used to determine the concentration of HiCN. The assay can be carried out manually, but is also the basis for automated hematology analysers.[14] However, this method employs highly toxic reagents, especially potassium cyanide,[15-16] and generates highly hazardous wastes.[11, 17-19] Accurate spectroscopic quantification of oxyhemoglobin has also been achieved,[20-22] though the Drabkin colorimetric assay still remains the international gold standard for hemoglobin quantification.[14] A variety of alternative methods are available for the quantification of hemoglobin that are based on the comparison of color of treated or untreated dry blood against standard color strips or glass slides.[11] Though these methods are well suited for use in low-resource settings, their main disadvantage is that color matching is subjective and can be dependent on the sample preparation. Moreover, these tests have difficulties to determine low concentrations of hemoglobin[14, 17] which may be useful for the detection of trace amounts in urine or mild hemolytic anemia. More sensitive methods include fluorimetry,[17, 23-28] electrochemistry,[29-33] colorimetry,[34-35] or chemiluminscence.[36] The main drawback of these assays is that they involve complex instrumentation and expensive chemicals such as DNA aptamers or fluorescent dyes.

Hemoglobin is not only an oxygen binding and transport protein, but has also a variety of catalytic activities. It can catalyze oxidations that are fueled by hydrogen peroxide,[37] initiate free radical polymerizations and RAFT polymerizations,[38] or catalyze atom transfer radical polymerizations (ATRP).[39-40] Radical polymerizations have proven to be powerful amplification reactions for biosensing.[41-43] Upon creation of a radical, a chain reaction links hundreds of monomers into a polymeric chain or network of high molecular weight, thereby concentrating the monomer at the location of the chain growth. These polymers can be detected by the formation of turbid spots on a sensor,[44-46] by turbidity in solution,[47] or by the formation of color or fluorescence and various other means.[48-56] In order to achieve polymerization-amplified biosensing and diagnostics, initiators can be linked to recognition motives such as antibodies or DNA fragments.[44, 48, 50-51] Alternatively, the catalytic activity of certain biomarkers can be exploited to initiate radical polymerizations, leading to a dual mode of amplification, the catalytic creation of radicals, followed by chain growth polymerization. Stevens and coworkers have developed a polymerization-amplified assay for peroxidases.[49] We have recently shown that hemozoin, the heme biocrystal that is produced by *Plasmodium sp.* parasites upon digestion of hemoglobin, can catalyze precipitation polymerization of the temperature-responsive polymer poly(N-isopropyl acrylamide) (PNIPAAm), resulting in an assay for malaria diagnosis.[47] Stable and cheap reagents for ATRP were used, and the polymerization was monitored by the formation of turbidity, which is a simple optical readout. Thus, the assay is suitable for malaria diagnostics in low-resource settings.

Here, we present that radical precipitation polymerization of NIPAAm can be used to detect and quantify hemoglobin in biological physiological fluids such as blood plasma and urine. Moreover, these polymerizations can also serve to determine the total hemoglobin content in whole human blood. The polymerizations were conducted as ATRP reactions at a temperature of 37 °C, i.e. above the lower critical solution temperature (LCST) of PNIPAAm. The rate of turbidity formation scaled with the concentration of hemoglobin. The assay has been thought as a low-cost alternative to other hemoglobin assays, with the main advantages of providing non-toxic wastes and a sensitivity that is able to quantify trace amounts of hemoglobin.

EXPERIMENTAL SECTION

Materials. Human blood samples (from healthy blood donors) were obtained on a weekly basis from Interregionale Blutspende SRK AG (Bern, Switzerland), stabilized with heparin. In accordance with the bio-safety level 2 certification, all individuals working with human blood were immunized against Hepatitis B and conducted their research in coherence with the specific standards of use and disposal necessary for this bio-safety level. Urine was collected from healthy volunteers on the day of the experiment. All chemicals were purchased from Sigma-Aldrich and were used as received except for NIPAAm which was recrystallized from hexane. The Drabkin´s reagent solution was prepared by dissolving 0.61 mM $K_3Fe(CN)_6$, 0.77 mM KCN, and 1.03 mM KH_2PO_4 in ultrapure water. Buffers were prepared as follows: ACK lysis buffer: 155 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA in MilliQ water; PBS buffer: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 in 800 mL MilliQ water. The pH was adjusted to 7.4 with 1 M HCl and volume was adjusted to 1 L; Polymerization buffer (0.1 M sodium phosphate buffer pH 6 with saline salts): 1 M Na_2HPO_4 and 1 M NaH_2PO_4 solutions in MilliQ water were made. 120 mL of the Na_2HPO_4 were measured and then 880 mL of the NaH_2PO_4 were added. Solutes were added to a total concentration of 137 mM NaCl and 2.7 mM KCl.

Isolation of RBCs. RBCs were isolated using an established protocol.[57] After full blood centrifugation (at 4 °C, 800 x g for 5 min), the RBC fraction was isolated and then aliquoted (5 mL) in 50 mL Falcon tubes. PBS was added to a final volume of 50 mL and the re-suspended RBCs were centrifuged for 2 min at 500 x g at 25 °C. The supernatant was discarded and the process was repeated three more times. The resulting pellets were then re-suspended in PBS to a final volume of 45 mL and stored at -20 °C, inducing partial haemolysis.

Isolation of blood plasma. Blood plasma was isolated from full human blood. To this end, the blood was centrifuged at 1000 x g for 20 min at 25 °C. The supernatant was passed into new Falcon tubes and centrifuged for 8 min at 500 x g at 25 °C to remove any possible cells. The supernatant was separated from the pellets and frozen in liquid nitrogen. The samples were further stored at –20 °C to be used in further experiments.

Isolation of human hemoglobin. Samples of 45 mL isolated RBCs in PBS buffer were incubated for 30 min with 50 mL ACK lysis buffer on ice. They were then centrifuged at 4696 x g for 10 min at 1 °C to obtain a pellet of RBC membranes and cell debris. The supernatant was concentrated to a volume of 5 mL by spin diafiltration using a Macrosep Advance Centrifugal Device (Pall Life Sciences) with a molecular weight cut-off of 10 kDa. Then, PBS buffer was added to the complete volume

of the tube, and the sample was concentrated again by centrifugation. The process was repeated twice to wash away the ACK lysis buffer. Finally, the sample was concentrated to a volume of 4 mL. The fresh solution was used for further experiments within 24 h.

Determination of human hemoglobin concentration by the Drabkin assay. The hemoglobin concentration was determined using the Drabkin colorimetric assay.1 1µl, 2 µl, 5 µl, 10 µl and 20 µl of the human hemoglobin were added to a Drabkin´s reagent solution to reach a total volume of 1 mL in semi-micro poly(methyl methacrylate) (PMMA) UV-Vis cuvettes. The solutions were vortexed and incubated for 20 min at room temperature. UV-vis measurements were then performed at 540 nm. Taking into account the dilutions and the molar absorptivity of HiCN (11000 L mol^{-1} cm^{-1} referring to one heme subunit),[12,57] the mean of the measurements was obtained, giving the final Hb concentration.

Determination of hemoglobin concentration by precipitation polymerizations.

(1) Polymerizations with purified human hemoglobin: In a typical experiment, NIPAAm (1.429 g, 12.63 mmol) and (+)-sodium L-ascorbate (NaAsc) (250 mg, 1.26 mmol) were weighted in a glass vial and 10 mL of polymerization buffer were added to form a stock solution. 0.7 mL of this solution were aliquoted into semi-micro PMMA cuvettes. These solutions were then spiked with different volumes of the human hemoglobin solution (0 to 0.25 mL) to obtain different final hemoglobin concentrations and then filled to a final volume of 0.95 mL with polymerization buffer. The solutions were gently vortexed avoiding spilling of the cuvette and were sealed with 0.5 mL mineral oil. The cuvettes were immediately placed in a thermostatted six-cell holder at 37 °C of an UV-Vis spectrophotometer. After 3 min of thermal equilibration, 50 µl of a 375 mM 2-hydroxyethyl 2-bromoisobutyrate (HEBIB) solution in dimethylformamide (DMF) was added to the aqueous phase. Spectral scans were recorded every 30 s between 300 and 800 nm at a speed of 20 nm s^{-1}. Time scans were recorded at 600 nm every 30 s (integration time 1 s, slow time scan) during 2 h. The final concentration in each cuvette were as follow: NIPAAm (767 mM), NaAsc (76.7 mM), and HEBIB (18.7 mM). Extinction versus time curves obtained by UV-Vis measurements were processed via a script using Wolfram Mathematica software to calculate the rate of turbidity for each polymerization. The script drew a line between two points (at 30 and 40 % of the maximum amplitude of the extinction) of the curves and gave the slope of the line. This value $\Delta E/\Delta t$ was then used as the assay read-out, e.g. to obtain dose response curves. Each polymerization catalyzed by a given concentration of Hb was performed in triplicate and the mean value as well as the standard deviation are reported.

(2) Polymerizations with human blood plasma that contained hemoglobin: The same stock solution of monomer with NaAsc as in 1 was employed. 0.7 mL of this stock solution was aliquoted into PMMA cuvettes, followed by the addition of 0.1 mL of isolated human blood plasma. Then, the samples were spiked with a solution of hemoglobin in the polymerization buffer. Polymerization buffer was added to a final volume of 0.95 mL. Finally, the assay was performed as in described in point 1.

(3) Polymerizations with human urine that contained hemoglobin: The same procedure was applied as in 4, substituting the addition of human blood plasma by human urine.

(4) Polymerizations with isolated RBCs: The same procedure was applied as in 1, substituting the addition of purified human hemoglobin by the addition of a dispersion of isolated RBCs in PBS buffer.

(5) Polymerizations with full blood: The same procedure was applied as in 1, substituting the addition of purified human hemoglobin by the addition of full human blood. As the hemoglobin concentration in full blood is much higher than the working range of the assay, full blood was diluted with polymerization buffer (0.1 M sodium phosphate buffer pH 6 with saline salts) to a hemoglobin concentration between $5 \cdot 10^{-3}$ mg mL^{-1} to 0.50 mg mL^{-1} to yield a reliable read-out.

The concentration of hemoglobin in the provided blood bank samples was usually around 340 mg mL^{-1} by Drabkin colorimetric determination. Therefore, in all tests the samples needed to be diluted to quantifiable hemoglobin concentrations by our assay to a concentration within the range of 10^{-3} mg mL^{-1} to 0.50 mg mL^{-1}.

Precipitation polymerization assay in anaerobic conditions. A typical polymerization mixture as described for the determination of hemoglobin, containing 0.1 mg mL^{-1} of hemoglobin was poured into a semi-micro 1 mL PMMA cuvette. The solution was sealed with 0.5 mL of mineral oil and subjected to bubbling under an argon flow for a period of 30 min. In parallel, a glass vial containing a 1 mL solution of HEBIB (375 mM) in DMF was also subjected to bubbling under an argon flow. Then, the glass vial was transferred to a UV-Vis spectrophotometer thermostatted at 37 °C and the reaction was initiated by the addition of 50 µL of the initiator solution.

Microscopy imaging of the polymerization assay in the presence of RBCs. RBCs were isolated from a human donor and added in a proportion of 1 % v/v to solutions as described in the supporting information. For these experiments, the buffer consisted of 0.1 M phosphate buffer (pH 6.5, 150 mM NaCl). 5 µL of the suspensions were added to a glass slide and covered by a cover slip thermostatted in an incubator at 37 °C. Images were recorded every minute for a period of up to 1 hour.

RESULTS AND DISCUSSION

Determination of hemoglobin concentration by precipitation polymerizations. The assay for hemoglobin is based on the hemoglobin-catalyzed precipitation polymerization of NIPAAm at 37 °C, using HEBIB as ATRP initiator and NaAsc as reducing agent. Even though oxygen inhibits radical polymerizations, neither the reagent solution nor the reaction mixtures were deoxygenated in order to allow for a simple handling of the assay. ATRP was chosen as the polymerization of choice for this assay instead of hydrogen peroxide-initiated free radical polymerizations, because the dehalogenation of the initiator is a specific reaction to metalloproteins, thereby decreasing the chance of unwanted initiation by other biomolecules. Moreover, the ATRP initiator is much more stable than hydrogen peroxide. The latter tends to decompose upon storage in the presence of light and at elevated temperatures. Finally, we found in initial experiments that hydrogen peroxide caused a noticeable blank reaction in the assay mix.

The reaction conditions are similar to our previously reported assay for hemozoin,[47] with the main differences that the analytical samples were prepared differently and that a different buffer was used in the polymerizations. The polymerization buffer used in the hemoglobin assay resembled PBS buffer but had a pH of 6.0. It contained sodium chloride and potassium

chloride at physiological concentrations to account for the fact that also the analytical samples (e.g. blood plasma and blood) contain these salts. The presence of chloride ions influences ATRP kinetics and the stability of the deactivation species of ATRP catalysts.[58] It is therefore important to work under constant salt concentrations within one set of assays. Buffered solutions of a hemoglobin-containing sample, NIPAAm, sodium ascorbate and hemoglobin were sealed with a layer of mineral oil and were thermostatted in cuvettes for spectroscopy at 37 °C. The subsequent addition of HEBIB in DMF started the polymerization (Figure 1a). The reactions were carried out above the LCST of PNIPAAm. As the polymer chains precipitated, they scattered light. Thus, the polymerization could be followed qualitatively by naked eye and quantitatively with an UV-Vis spectrophotometer. Spectral scans from 300 to 800 nm were recorded every 30 s for a reaction mixture that contained 0.1 mg mL^{-1} purified human hemoglobin (Figure 1b). The first spectral scan consisted of the Soret band at 409 nm and two visible bands at 539 and 574 nm which indicate that most of the hemoglobin had oxygen bonded to its prosthetic groups and was in the oxyhemoglobin form.[59] An additional band at 630 nm could be observed indicating that inactive methemoglobin was present. Two successive phenomena could then be distinguished. First, upon addition of the initiator, the Soret band decreased and shifted to 416 nm. In the meantime, the visible bands became less pronounced and a new band at 673 nm appeared. Probably, upon addition of HEBIB, oxyhemoglobin released its oxygen while abstracting a bromine, thus creating a radical, generating an oxidized Fe(III)-bromine species. Both sodium ascorbate and the growing polymer chain end could reduce this Fe(III) species to an activating Fe(II) state. However, as the spectra represent a superposition of the individual species, it is difficult to assign the absorption bands unambiguously. In a second phase, once this equilibrium of hemoglobin species was reached, the extinction increased over the whole range of wavelengths with time meaning that the precipitation of the formed polymer chains scatter light. To minimize the influence of the hemoglobin absorbance and to increase throughput, further polymerizations were monitored by a time scan at a single wavelength, i.e. at 600 nm.

Control experiments showed that all ATRP reagents were needed to obtain a significantly fast formation of turbidity (Figure S1). The reactions also show that blank reactions happen, albeit at much lower rates than the hemoglobin assay. Importantly, the blank reaction without catalyst only sets in after 3000 s, when the hemoglobin assay is already over. The oil layer on top of the reaction mixtures is important to avoid diffusion of additional oxygen from the headspace into the reaction mixtures, which quenches the reaction.

Once the reaction conditions had been established, the correlation between turbidity formation and hemoglobin concentration was investigated. Polymerizations were run with known amounts of purified human hemoglobin from blood bank donors.

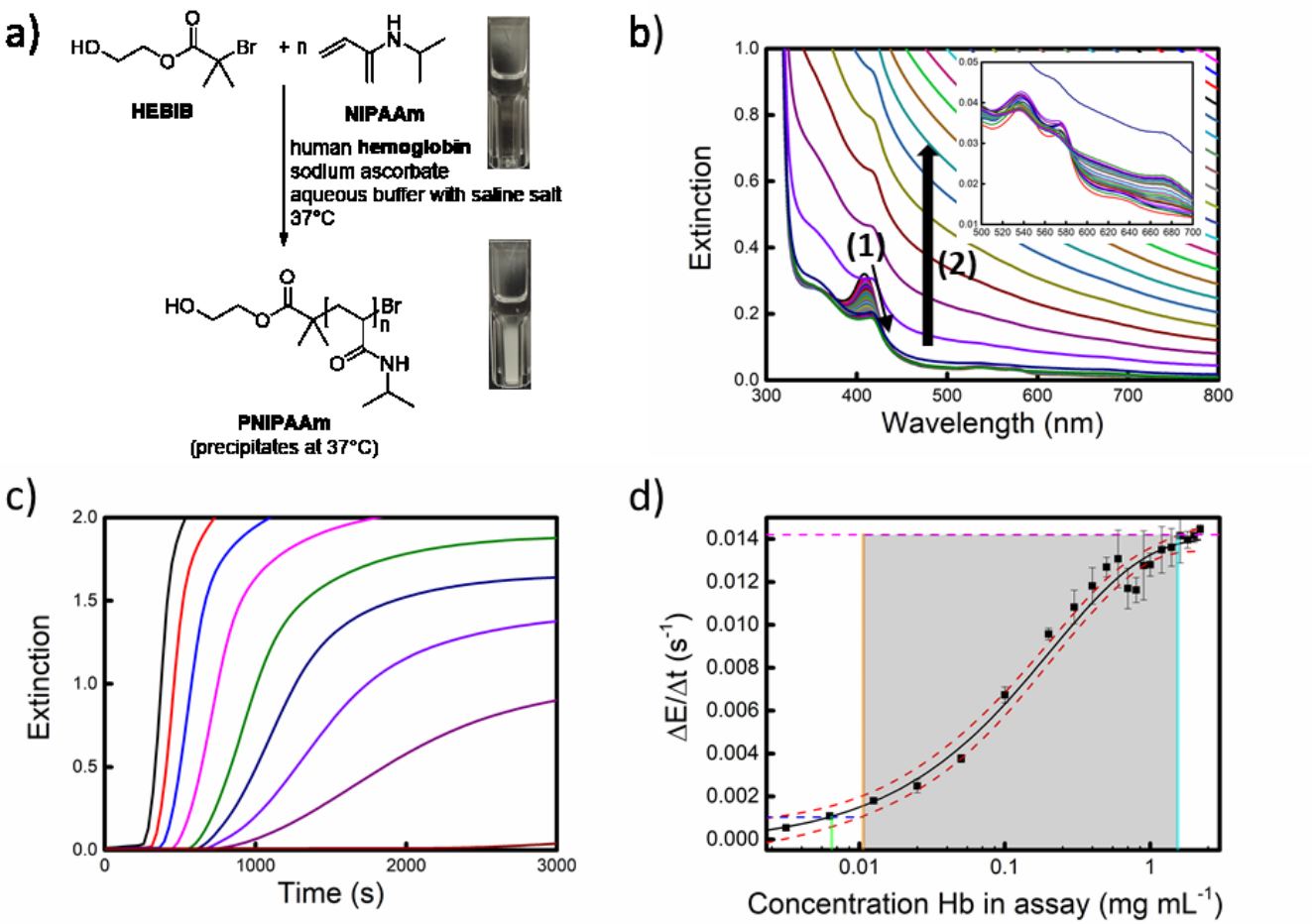

Figure 1. Hemoglobin-quantification assay based on precipitation polymerization of NIPAAm at 37 °C. a) Reaction scheme of the polymerization and photos before and after NIPAAm polymerization catalyzed by 0.1 mg mL^{-1} Hb. b) Time-dependent UV-vis spectral scans of NIPAAm polymerization catalyzed by 0.1 mg mL^{-1} Hb, spectra were taken every 30 s. Arrow 1 indicates the direction of the shift of the

soret band of hemoglobin and arrow 2 indicates the increase in extinction throughout the polymerization reaction. c) Turbidity formation (measured as extinction at 600 nm) during NIPAAm polymerization with different concentration of purified human hemoglobin: 0.4 mg mL^{-1} (black), 0.2 mg mL^{-1} (red), 0.1 mg mL^{-1} (blue), 5·10 2 mg mL^{-1} (pink), 2.5·10 2 mg mL^{-1} (green), 1.2·10 2 mg mL^{-1} (dark blue), 6.2·10 3 mg mL^{-1} (violet), 3.1·10 3 mg mL^{-1} (purple) and no catalyst (brown). A linear fit in the near-to-linear region of the curves was employed to correlate the rate of turbidity formation with the concentration of catalyst. d) Dose-response curve between hemoglobin concentration with a logarithmic scale and the rate of turbidity formation (average of n = 3 and SD). The black line represents a fit of the data following equation (1), the red dotted lines indicate the 95% confidence interval, the green vertical line indicates the detection limit, the grey area corresponds to the quantifiable range delimited by an orange vertical line and a cyan vertical line which indicate the lower and upper limit of quantification, respectively. The magenta dotted vertical line corresponds to the saturation.

The resulting curves of extinction at 600 nm versus time exhibited a lag phase, which was followed by an almost linear increase in extinction. Finally, a plateau was reached (Figure 1c). The slope of the linear region was taken as the read-out of the assay. It increases with the concentration of hemoglobin. A dose-response curve was obtained by plotting the increase in extinction over time versus the concentration of full hemoglobin protein in the assay volume (Figure 1d and Figure S2).

The formation of turbidity did not scale linearly with the concentration of hemoglobin but can be quantified with a 95% confidence level in the range between $1.04 \cdot 10^{-2}$ mg mL^{-1} and 1.55 mg mL^{-1} using the following equation (parameters can be found in SI):

$$f(x) = a + b\,(1 - \exp[-c\,x^{d}]) \quad (1)$$

At low Hb concentrations, a limit of detection of $6.45 \cdot 10^{-3}$ mg mL^{-1} (100.0 nM) was obtained. At high Hb concentrations the turbidity formation reached a plateau. One possibility to explain this trend is that once that the polymerization reaction is initiated, the formation of hydrophobic PNIPAAm above LCST induces the precipitation of hemoglobin. This effect is enhanced at increasing concentrations of hemoglobin where the initiation and propagation rate of the polymerization is higher.

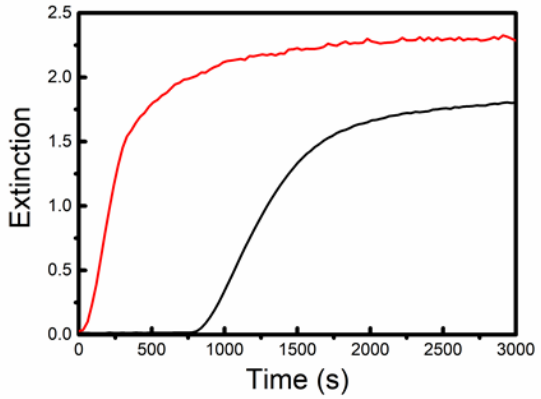

Figure 2. Effect of oxygen on the hemoglobin-catalyzed precipitation polymerization of NIPAAm. Reaction catalyzed by 0.1 mg mL^{-1} Hb in anaerobic conditions (red) and in the standard test conditions, i.e. without deoxygenation (black).

Several experiments were conducted to gain a fundamental understanding of the hemoglobin-catalyzed precipitation polymerization. The lag phase was probed by carrying out the polymerization in oxygen-free conditions (Figure 2). Hemoglobin was deoxygenated prior to this experiment. The polymerization started upon the addition of the initiator and the rate of turbidity formation was higher than under aerobic conditions. This indicates that the lag phase was due to the presence of oxygen in the reaction mixture, which first had to be consumed at the beginning of the reaction by radicals that formed in the initiation step. This also consumed some of the initiator. As a result, the subsequent formation of turbidity was lower because fewer polymer chains form. Similar observations were made in our previous work on hemozoin-catalyzed polymerizations,[47] but the rate of turbidity formation increased less between deoxygenated reaction and normal assay conditions. The difference between hemozoin and hemoglobin is that the latter carried oxygen when introduced into the reaction mixture. Thus, additional oxygen was introduced into the reaction and the inhibition effect was increased.

Once the polymerization starts, PNIPAAm precipitates and forms nanoparticles. Dynamic light scattering allowed to study the evolution of these nanoparticles (Figure 3). During the polymerization, they grew in size. The more hemoglobin present, the faster the particles grew. Moreover, particle size scaled with turbidity. If left to react for an hour, which is longer than the usual assay time, the polymer partially sedimented (Figure S3). The precipitate did not did not dissolve when the reaction mixture was cooled to room temperature, which would be the expected behavior of pure PNIPAAm. Thus, the hemoglobin-catalyzed precipitation polymerization resulted in cross-linked PNIPAAm. This is not detrimental to the assay, as sedimentation only occurs at reaction times that are longer than the usual assay time. However, it is an indication that larger crosslinked aggregates formed. The presence of catalyst resulted in the formation of insoluble hydrogels throughout the polymerizations. Probably, the formation of a hydrophobic environment by PNIPAAm induces denaturation of hemoglobin, thereby producing a gel with the polymer chains. In addition, hemoglobin may act as a crosslinker for aggregating polymer chains. The observations described above are different from the previously reported hemozoin-catalyzed polymerizations, where the particle size grew at a similar rate independently of the concentration of the catalyst, and the particles remained suspended in solution even at long reaction times.[47] Most likely, this is because hemozoin does not undergo a transition toward an insoluble species.

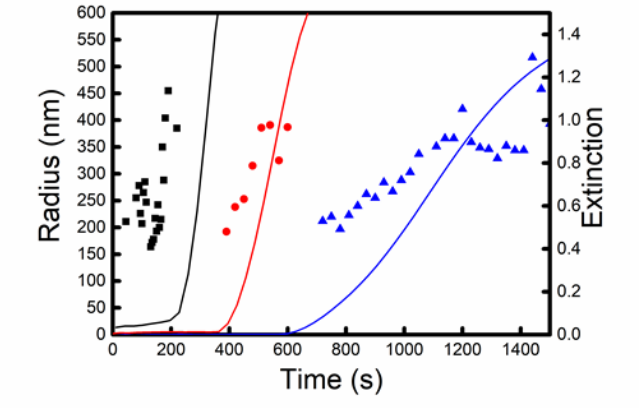

Figure 3. Evolution of particle size during the hemoglobin-catalyzed precipitation polymerization of NIPAAm. Dynamic light scattering measurements of NIPAAm polymerizations catalyzed by 1 mg mL^{-1} Hb (black squares), 0.1 mg mL^{-1} Hb (red circles), and 0.0125 mg mL^{-1} Hb (blue triangles), correlated to extinction measurements of polymerizations with equal Hb concentrations (respective to their color).

The characterization of the resulting polymer from hemoglobin-catalyzed precipitation polymerizations by NMR spectroscopy and gel permeation chromatography was difficult as not enough material was created with low concentration of catalyst while higher concentrations of catalyst yielded insoluble polymer.

Influence of biological fluids on hemoglobin-catalyzed precipitation polymerizations. In most applications, hemoglobin needs to be detected and quantified in a physiological fluid such as blood plasma, urine, or whole blood. For example, free hemoglobin is scarce in plasma. Its concentration is around 0.05 mg mL^{-1}.[60] If the hemoglobin concentration is found to be higher, this indicates hemolysis. However, these plasma and other biological fluids contain proteins and biomolecules that might influence the precipitation polymerization, e.g. by acting as chain transfer agents or as colloidal stabilizers. Therefore, dose-response curves for hemoglobin from blood bank samples spiked in various physiological fluids were recorded (Figure 4 and Figure S4a).

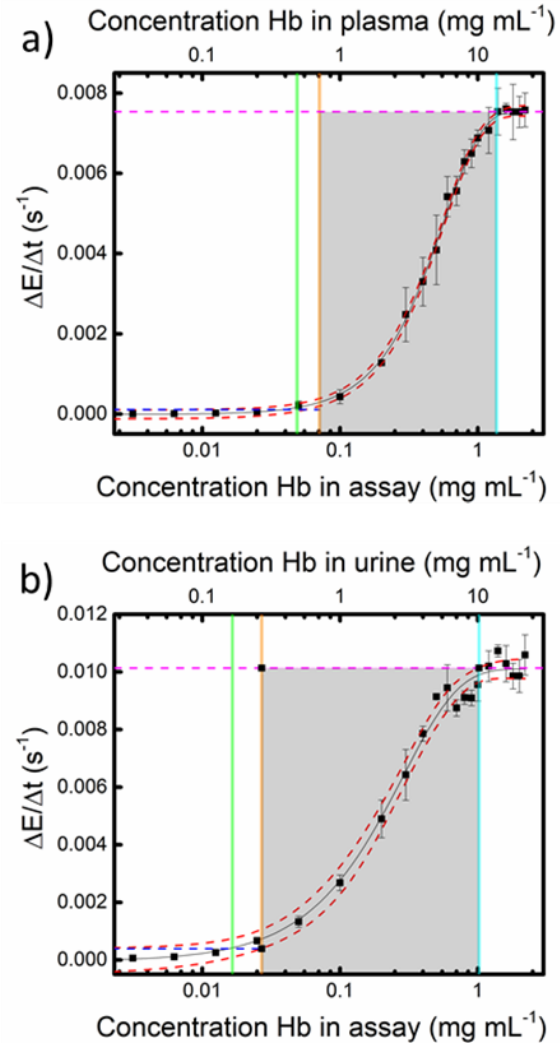

Figure 4. Dose-response curve of NIPAAm polymerization catalyzed by hemoglobin for different biological samples. a) With 10% volume plasma. b) With 10% volume urine. (average of n = 3 and SD) The black line represents a fit of the data following equation (1), the red dotted lines indicate the 95% confidence interval, the green vertical line indicates the detection limit, the grey area corresponds to the quantifiable range delimited by an orange vertical line and a cyan vertical line which indicate the lower and upper limit of quantification, respectively. The magenta dotted vertical line corresponds to the saturation.

The rate of turbidity formation of hemoglobin-catalyzed polymerizations in the presence of plasma from three donors can be quantified from $7.09 \cdot 10^{-2}$ to 1.37 mg Hb mL^{-1} in the assay using equation (1) (Figure 4a and Figure S4a). The turbidity formation was slower than in buffer and the limit of detection went up to $4.88 \cdot 10^{-2}$ mg mL^{-1} in the assay. The sample was diluted 10-fold when introduced into the assay volume. Thus, the limit of detection is $4.88 \cdot 10^{-1}$ mg mL^{-1} in the plasma sample. This is not problematic as the analytically relevant concentration of free hemoglobin in plasma is above this value (vide supra). The difference to the polymerizations with purified hemoglobin could be explained by the presence of antioxidants in blood plasma. Serum albumin, glutathione and cysteines are effective radical scavengers.[61] Therefore, these molecules consumed radicals before polymerization reactions could take

place. Moreover, albumins are known to be effective transporters of hydrophobic compounds, i.e. they act as surfactants.[62] Therefore, albumin might solubilize hydrophobic PNIPAAm polymers and thus decrease the rate of turbidity formation.

Microscopic amounts of hemoglobin or blood in urine can be benign but also a sign of lethal disease such as bladder[63] or kidney cancer.[64] Thus, we investigated the hemoglobin assay in the presence of urine from a human donor (Figure 4b and S4b). The precipitation polymerization detected hemoglobin as low as $1.65 \cdot 10^{-2}$ mg mL^{-1} in the assay. The sample was diluted 10-fold when added to the assay volume, resulting in limit of detection of $1.65 \cdot 10^{-1}$ mg mL^{-1} in the urine sample. The dose response curve enables quantification of Hb in the assay from $2.70 \cdot 10^{-2}$ mg mL^{-1} to 1.02 mg mL^{-1}, corresponding to $2.70 \cdot 10^{-1}$ mg mL^{-1} to 10.2 mg mL^{-1} in urine samples. The rate of turbidity formation is higher than in plasma, but lower than with purified hemoglobin, allowing to conclude that urine does contain fewer interfering biomolecules than plasma. The assay can detect trace amounts of hemoglobin in urine in a range which is relevant for clinical diagnosis.[65]

Quantification of total hemoglobin in full blood. The total amount of hemoglobin in blood is an important health indicator. A hemoglobin concentration below the normal range is diagnostic of anemia. Hemoglobin levels that are higher than normal concentrations (polycythemia) can be caused by blood disorders (primary polycythemia), by hypoxia-induced erythropoiesis, or by dehydration (relative polycythemia). Usual hemoglobin levels vary from 130 to 180 mg mL^{-1} for an adult man and are slightly lower for women and children.[11] Mild anemia can be detected when the hemoglobin concentration is below 110 mg mL^{-1} and becomes very severe when this concentration goes below 70 mg mL^{-1}. We explored if the precipitation polymerization assay required the addition of lysis agents. To this end, we observed RBCs in the presence of the polymerization reagents in a light microscope. While the RBCs were stable in the presence of the polymerization buffer and DMF (Video S1), the presence of the polymerization reagents degraded the cell membranes and erythrocytes became unstable, observing a decreased number within 2 min due to precipitation from the focal plane (Video S2). In the course of the polymerization, microscopic polymer particles formed (Video S2). This observation was confirmed by microscopy images before and after the polymerizations (Figure S6). Intact RBCs were initially visualized, whereas degraded RBCs in the presence of polymer particles were generated after 10 min. The degradation of RBCs could be either because of the lysing activity of the reagents, or because the formation of polymers inside the RBCs induced lysis. Thus, RBCs were exposed to the test conditions in the absence of sodium ascorbate, i.e. in conditions where the polymerizations do not occur. Video S3 shows collapsed RBCs in focus. Therefore, we conclude that the polymerization reagents act as lysing agents. In a further step, to develop the precipitation polymerization assay toward the quantification of hemoglobin in full blood, we explored isolated RBCs from blood bank donors as catalysts in the polymerizations to decipher the effect of the cell components on the polymerization (Figure 5a). The dose response curve follows the same trend as for purified hemoglobin, but with lower rates of turbidity formation, possibly because intraerythrocytic glutathione (1.4 mM ± 0.5 mM)[66] quenches some of the radicals and the lipids from the membrane could act as surfactants. However, given the low volume of blood which was fed into the assay, the total concentration of glutathione is negligible in comparison of the number of radicals generated by the polymerization reagents, minimizing the effect of glutathione variability between patients.

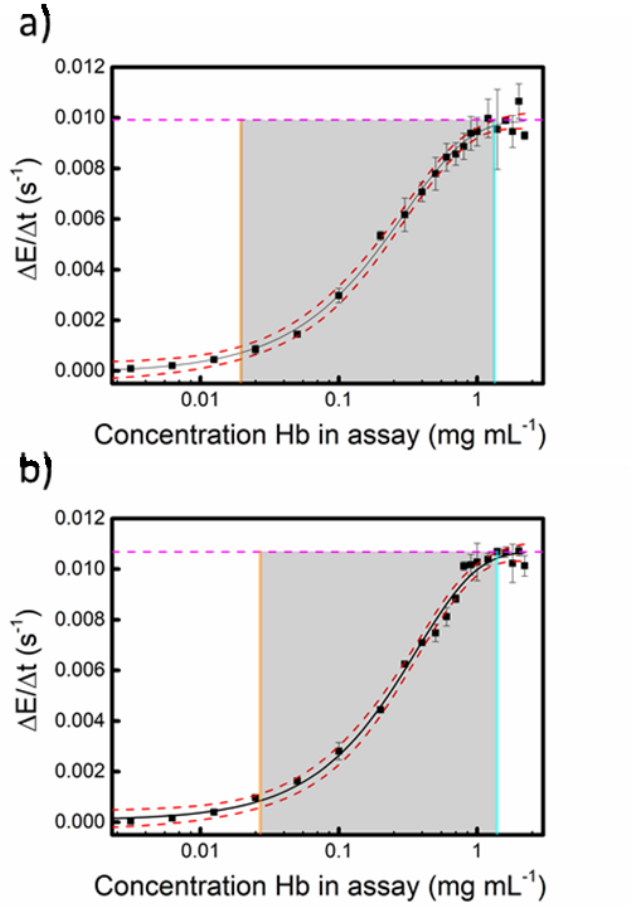

Figure 5. Dose-response curve of NIPAAm polymerization catalyzed by hemoglobin contained in red blood cells. a) With hemoglobin in isolated RBCs in buffer. b) With hemoglobin in RBCs contained in full blood. (average of n = 3 and SD) The black line represents a fit of the data following equation (1), the red dotted lines indicate the 95% confidence interval, the grey area corresponds to the quantifiable range delimited by an orange vertical line and a cyan vertical line which indicate the lower and upper limit of quantification, respectively. The magenta dotted vertical line corresponds to the saturation.

Next, whole blood from human donors was used as the catalyst for the precipitation polymerization, with the aim to use the assay to quantify the hemoglobin concentration in blood. To bring the hemoglobin concentration into the working range of the assay, three independent blood bank samples were subjected to three dilution series between 130000-fold and 184-fold. Figure 5b shows the dose-response curve for full blood. It is similar to the one of isolated RBCs in buffer solution, with slightly lower read-outs. The error bars are small, which indicates a low degree of variability between the samples stemming from different individuals. A possible reason for the lower read outs could be that plasma was introduced into the assay in addition to RBCs, which contributes to the quenching of the turbidity formation (vide supra). Due to the similarity of the results to the polymerizations with isolated RBCs we can conclude that the only relevant catalyst in full blood for the polymerizations reactions is hemoglobin. As hemoglobin contained in RBCs is in

huge excess in comparison to the possible concentration of hemoglobin in plasma, the precipitation polymerization assay could be an accurate method for the quantification of full blood. For this, the sample needs to be highly diluted. Thus, the whole clinical range of concentrations can be quantified using equation (1) and by diluting a sample to a Hb concentration between $2.71 \cdot 10^{-2}$ mg mL^{-1} to 1.39 mg mL^{-1}. In addition, we compared the accuracy of our precipitation polymerization test against the gold standard Drabkin colorimetric assay with a blood bank sample that was enriched in erythrocytes (Figure S7). The concentration of hemoglobin was determined to be 413 ± 31 mg ml^{-1} by the precipitation polymerization assay and 411 mg ml^{-1} by the Drabkin colorimetric assay, showing the accuracy of the polymerization test.

CONCLUSION

We have developed an assay to detect and quantify hemoglobin in solution and in physiological fluids that is based on the biocatalytic precipitation ATRP of NIPAAm. The assay can measure hemoglobin concentrations as low $6.45 \cdot 10^{-3}$ mg mL^{-1} in solution, $4.88 \cdot 10^{-1}$ mg mL^{-1} in plasma, and $1.65 \cdot 10^{-1}$ mg mL^{-1} in urine. This is lower than the detection limit needed for clinical testing of hemoglobin in these liquids, which makes the assay viable for potential diagnostic applications, for example to detect hemolytic anemia or microscopic hematuria, and to assess the quality of blood bank samples. Furthermore, the assay can also be used to quantify the total concentration of hemoglobin in blood and blood bank samples. Even though the experiments with full blood bank samples indicate that the assay is not substantially influenced by variations in sample composition from different individuals, the patient-to-patient variability has to be studied in greater detail in future studies with a larger number of individual samples and for all types of samples. Importantly, the assay does not require toxic cyanide reagents. Thus, the precipitation polymerization assay might become an environmentally friendly alternative to the commonly used Drabkin assay, especially if it is integrated into diagnostic devices for clinical and point-of-care hemoglobin testing.

ASSOCIATED CONTENT

Supporting Information. Experimental details and addition results (PDF, Videos). The Supporting Information is available free of charge on the ACS Publications website.

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Competing interests

The authors declare that they have submitted a patent application that covers the technology. N. B., O. R.-G., and J. P. are the inventors on this patent. Moreover, the authors are in the process of establishing a spin-out company with the aim to commercialize this and similar tests.

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