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Detecting free hemoglobin in blood plasma and serum with luminescent terbium complexes†

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Hemolysis, the rupturing of red blood cells, can result from numerous medical conditions (*in vivo*) or occur after collecting blood specimen or extracting plasma and serum out of whole blood (*in vitro*). In clinical laboratory practice, hemolysis can be a serious problem due to its potential to bias detection of various analytes or biomarkers. Here we present the first "mix-and-measure" method to assess the degree of hemolysis in biosamples using luminescence spectroscopy. Luminescent terbium complexes (LTC) were studied in the presence of free hemoglobin (Hb) as indicators for hemolysis in TRIS-buffer, and in fresh human plasma with absorption, excitation and emission measurements. Our findings indicate dynamic as well as resonance energy transfer (FRET) between the LTC and the porphyrin ligand of hemoglobin. This transfer leads to a decrease in luminescence intensity and decay time even at nanomolar hemoglobin concentrations either in buffer or plasma. Luminescent terbium complexes are very sensitive to free hemoglobin in buffer and blood plasma. Due to the instant change in luminescence properties of the LTC in presence of Hb it is possible to access the concentration of hemoglobin *via* spectroscopic methods without incubation time or further treatment of the sample thus enabling a rapid and sensitive detection of hemolysis in clinical diagnostics.

Introduction

The collapse of red blood cells (erythrocytes), followed by a release of their contents (mainly hemoglobin) into the surrounding environment, is called hemolysis. For several reasons this process can occur either *in vivo* or *in vitro*. Some diseases as well as genetic disorders or parasites are known to increase hemolysis. Detecting hemolysis is thus considered to be a diagnostics method.

Additionally, hemolysis is also a common problem in laboratory testing because it can potentially bias detection of various analytes and biomarkers.[1,2] Hemolysis is still one of the biggest challenges to face, because it appears incidentally, at different levels and it may impair assays performance. This could lead to serious problems when wrong biomarker determination followed by improper therapy risks a patient's life. Therefore sensitive and reliable pre-testing of samples is required to avoid the errors due to hemolysis. For a long period of time, the degree of hemolysis in biosamples has been assessed visually (by bare eye). This mostly unreliable method often led to underestimation of assay interferences, and therefore, has almost completely been replaced by automated processing. Automated testing is considered to be the most promising solution with regard to error rate, reproducibility and standardization.[3] Nevertheless, in regard to estimating the degree of hemolysis it has one main drawback: the sample must be prepared chemically in order to get reliable results.[4–7] That means both the sample is inevitably modified, being unusable for other measurements, and the approach is both time and material consuming.

In order to overcome these limitations, a reliable and hemolysis-specific sensor is needed, whose analytical signal is dependent on the level of hemolysis. At the same time, the sensor should be precise and easy to use.

In this study, we demonstrate that luminescent terbium complexes (LTC) can act as such sensors. For that purpose we evaluated the influence of free hemoglobin on the photophysical properties and sensing performance of several different LTC in buffer and blood plasma. Furthermore the interaction pathway between LTC and sample free hemoglobin was revealed, potentially leading to further improvement of the sensitivity of hemoglobin detection with adapted LTC.

Results and discussion

In order to obtain insight into the interaction between LTC and hemoglobin, we first performed steady-state (Fig. 1) and

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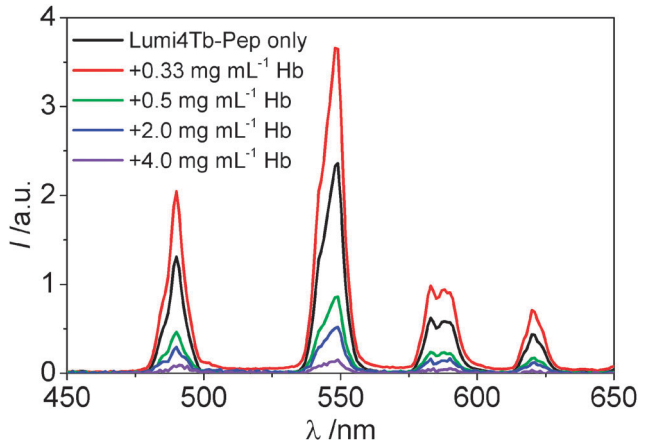

Fig. 1 Steady-state emission spectra of Lumi4Tb-Pep (c = 4 nM) in TRIS-buffer as a function of hemoglobin concentration (λ_{ex} = 340 nm).

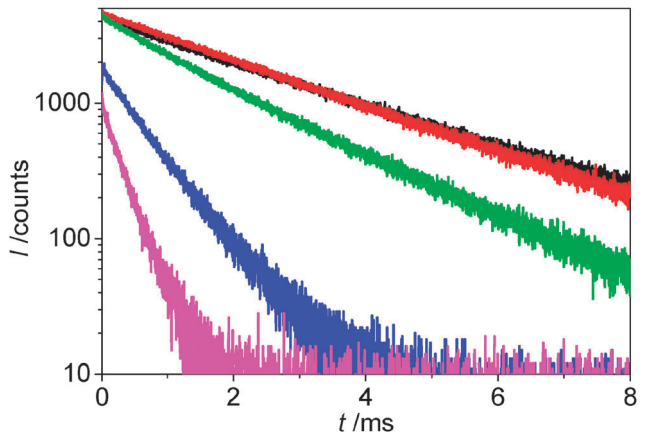

Fig. 2 Luminescence decay curves of Lumi4Tb-Pep in the presence of hemoglobin in buffer, LTC concentration c = 0.2 nM, increasing hemoglobin concentrations (black – 0 mg mL^{-1}, red – 0.03 mg mL^{-1}, green – 0.5 mg mL^{-1}, blue – 2.0 mg mL^{-1}, magenta – 4.0 mg mL^{-1}), λ_{ex} = 337 nm, λ_{em} = 495 nm.

time-resolved (Fig. 2) luminescence measurements with two LTC (Lumi4Tb® and Tb-Al086 [data not shown]) at various hemoglobin concentrations in TRIS-buffer (pH 7.4).

The emission spectra reveal an inconsistent picture of hemoglobin influence on luminescence intensity of LTC. At lower hemoglobin concentrations an increase in intensity can be observed, whereas at higher concentrations hemoglobin is a strong quencher of LTC luminescence (Fig. 1). Due to a spectral overlap between LTC emission and hemoglobin absorption (see ESI,† Fig. S1) this interference could be considered as reabsorption. Apparently, that is not the case because the shape of the emission spectrum remains unchanged, while emission intensity is decreasing (see ESI,† Fig. S2). More importantly, reabsorption would also not change the LTC luminescence lifetime.[8] Noticeably, the lifetime changes when hemoglobin interferes with LTC. With increasing hemoglobin concentration LTC luminescence lifetime decreases significantly. For the LTC Lumi4Tb-Pep there is decrease of luminescence lifetime from τ_0 (luminescence lifetime in the absence of hemoglobin) = 2.6 ms to τ (luminescence lifetime in the presence of 4 mg mL^{-1} hemoglobin) = 0.3 ms observable (Fig. 2, other Lanthanide complexes see ESI,† Fig. S3).

For hemoglobin dependent measurements of LTC luminescence lifetimes intensity averaged lifetime τ_D was used. A decrease in luminescence intensity, together with a change in luminescence lifetimes, could be induced by an energy transfer *via* donor–acceptor-pairing as well as by collisional (dynamic) quenching.[8,9] In order to distinguish between an energy transfer mechanism and dynamic quenching, we compared the decrease in luminescence intensity to the decrease in luminescence lifetime.

The Stern–Volmer analysis can be applied to the systems studied because energy transfer in samples with dilute donor and acceptor concentrations, as in the experiments here, reveals linear Stern–Volmer behaviour.[10] If the ratio of intensities F_0/F (intensity in absence to intensity in presence of Hb) equals the ratio of lifetimes τ_0/τ (decay time in absence to decay time in presence of Hb), the quenching is purely dynamic. If $F_0/F > 1$ and $\tau_0/\tau = 1$, the quenching is purely caused by energy transfer. If $F_0/F > \tau_0/\tau > 1$ the quenching is a combination of energy transfer and collisional deexcitation.[9] Fig. 3 shows typical Stern–Volmer plots for luminescence quenching of both lifetime and intensity data of the LTC used in our experiments. Whereas the lifetime ratio τ_0/τ rises linearly with increasing hemoglobin concentration, the ratio of intensities F_0/F indicates an approximately quadratic increase with concentration. Due to $F_0/F > \tau_0/\tau > 1$ quenching character of this interaction is a combination of energy transfer from LTC bound to hemoglobin and collisional quenching. In contrast to these findings and as mentioned above (see Fig. 1) for lower concentrations of hemoglobin interacting with LTC there is no intensity quenching of LTC emission but an increase. This increase in intensity influences Stern–Volmer analysis too, leading to intensities ratios $F_0/F < 1$ for lower hemoglobin concentrations (Fig. 3). It follows that there has to be a mechanism which

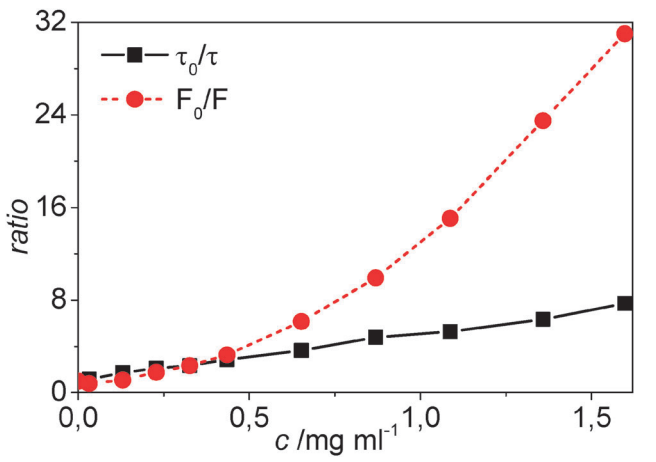

Fig. 3 Stern–Volmer plots for changes of LTC emission (Lumi4Tb-Pep shown) in presence of hemoglobin in TRIS-buffer. Lumi4Tb-Pep concentration c = 2 nM, increasing hemoglobin concentrations (0–1.6 mg mL^{-1}) for luminescence lifetime as well as for luminescence intensity.

superimposes intensity quenching of LTC emission especially for lower hemoglobin concentrations. Metal–ion centered porphyrins, such as hemoglobin or chlorophyll, are known to act as antennas in energy transfer.[11,12] Thus, binding of hemoglobin to terbium complexes could add another antenna unit to the latter, leading to an increase in absorbance of the LTC and therefore to enhanced luminescence emission.

Linearization of the Stern–Volmer relationship for combined quenching as given in eqn (1)

$$\frac{F_0}{F} = \frac{\tau_0}{\tau}(1 + K_S[Q]) = (1 + K_D[Q])(1 + K_S[Q]) \qquad (1)$$

with K_D the Stern–Volmer constant for dynamic quenching, K_S the Stern–Volmer constant for static quenching and $[Q]$ the concentration of the quencher, leads to eqn (2)

$$\left(\frac{F_0}{F} - 1\right)\frac{1}{[Q]} = (K_D + K_S) + K_D K_S[Q] \qquad (2)$$

and allows for determination of the quenching constants. The Stern–Volmer constants K_D for dynamic and K_S for static quenching were determined to be 4.1 ± 0.1 mL mg^{-1} (or 2.7 × 10^5 ± 3.3 × 10^3 L mol^{-1}) and 2.8 mL mg^{-1} (or 1.8 × 10^5 L mol^{-1}), respectively. The bimolecular quenching constant k_q for dynamic quenching is given by eqn (3)

$$k_q = \frac{K_D}{\tau_0} \qquad (3)$$

with K_D the Stern–Volmer constant for dynamic quenching and τ_0 the luminescence lifetime of the unquenched donor. k_q reflects the efficiency of quenching and/or the accessibility of fluorophores to the quencher. For quenching of LTC luminescence by hemoglobin k_q is ~10^8 M^{-1} s^{-1}. Diffusion-controlled quenching typically results in values of k_q is ~10^{10} M^{-1} s^{-1}, meaning that efficiency of quenching of LTC luminescence by hemoglobin is low.

Due to a significant spectral overlap between the LTC emission and the absorption of the porphyrin ligands of the hemoglobin units (see ESI,† Fig. S1) energy transfer can take place from terbium complexes to the porphyrin molecules.[13] Experimental data provides evidence for such energy transfer (see ESI,† Fig. S4).

In contrast to theoretical expectations, energy transfer sensitized emission of hemoglobin could not be observed (data not shown). A possible reason may be the quenching effect of the central iron(III) ion, which is known to quench very efficiently luminescence of metal chelating molecules such as the porphyrin ligands of hemoglobin.[14,15] Therefore in the experiments performed, hemoglobin acts as a dark quencher for LTC luminescence and the quantification of the degree of hemolysis is limited to LTC luminescence quenching.

Noticeably, the interaction between terbium complexes and hemoglobin induces a change in terbium luminescence lifetimes even at very small amounts of hemoglobin (<0.05 mg mL^{-1}) (Fig. 4). It can be distinguished between normal and elevated Hb concentrations by measuring luminescence decay times of LTC. That implies a sensitivity of the method presented here close to that of established methods to estimate free

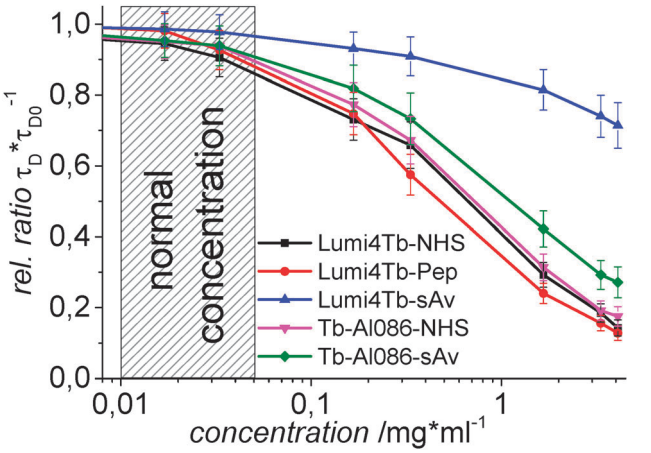

Fig. 4 Change in luminescence lifetimes τ of LTC dependent on hemoglobin concentration in TRIS-buffer in relation to luminescence lifetime τ_D in the absence of hemoglobin.

hemoglobin concentration in plasma.[16–18] Moreover, LTC luminescence is sensitive for Hb over a wide range of concentrations from 0.05 mg mL^{-1} to at least 4 mg mL^{-1} following that hemoglobin induced change of LTC luminescence should be able to cover the whole range of abnormal increased hemoglobin concentrations in plasma.[16,19] In Fig. 4 it is clearly visible that the decrease in LTC luminescence lifetimes depends not only on hemoglobin concentration, but on the size of the LTC-labeled biomolecule, too. LTC incorporated in streptavidin (overall diameter of approximately 5 nm) exhibit significantly longer luminescence lifetimes in the presence of hemoglobin than unlabeled LTC or LTC labeled to peptide, each with an overall molecule diameter of around 1–2 nm.

This observation stays in agreement with the quenching mechanisms we proposed. Collisional quenching as well as quenching *via* energy transfer is distance dependent.[8,9] The bigger the molecules engaged the lower is the diffusion coefficient, and the lower is the energy transfer efficiency due to an increased average distance between donor and acceptor as well. It follows that smaller LTC-labeled biomolecules should be preferably used when detecting hemolysis. The difference in the degree of luminescence lifetime quenching between both LTC incorporated into streptavidin (sAv) can be a result of different labeling procedures. Lumi4Tb-sAv was acquired from Lumiphore with a labeling ratio of around 4.4 Lumi4Tb per streptavidin, whereas Tb-Al086-sAv was labeled on site with a labeling ratio of around 2.6 Tb-Al086 per streptavidin. Hence there are differences in the allocation of LTC inside streptavidin resulting in differences in distance between LTC and hemoglobin.

Human blood is a complex cocktail containing many different substances, which can be classified mainly into two components: cells (40–45% of volume) and plasma (55–60% of volume).[20] As disease-specific biomarkers are found in plasma, this part of blood is most interesting for clinical diagnostics using point-of-care to high-throughput-screening. Hemolysis is one of the effects that can bias those measurements and therefore must

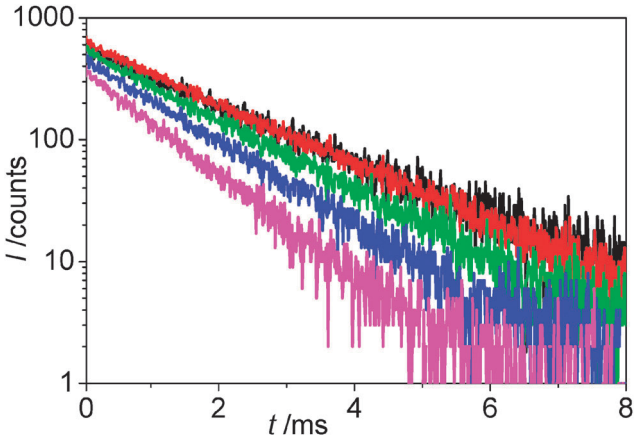

Fig. 5 Representative luminescence decay curves of LTC (Lumi4Tb-NHS shown) in the presence of hemoglobin in blood plasma, LTC concentration c = 2.5 nM, hemoglobin concentrations (black – 0 mg mL^{-1}, red – 0.2 mg mL^{-1}, green – 0.4 mg mL^{-1}, blue – 1.0 mg mL^{-1}, magenta – 1.5 mg mL^{-1}).

be monitored carefully. LTC luminescence is sensitive to hemoglobin concentration in buffer and for this reason might be a sensor to detect hemolysis in plasma, too.

Blood plasma is by far the most complex media in immunoanalysis to deal with as it is a mixture of different cells, proteins, peptides, ions and other molecules in water.[21] That is why innumerable interactions between plasma components and sensor molecules can take place. In particular, non-specific and specific binding of plasma components to sensor molecules can alter the characteristics of the sensors intended to use.[22–24] Fig. 5 shows the LTC luminescence changes with an increase in hemoglobin concentration in blood plasma. One can observe that in plasma hemoglobin has similar influence on LTC luminescence as it could be observed in buffer. With increasing hemoglobin concentrations a decrease both in LTC luminescence intensity and lifetime can be observed, even though the effect is not as pronounced as in buffer (Fig. 5 *vs.* Fig. 2).

This reduction in sensitivity most likely is a consequence of the plasma viscosity, which is higher when compared to buffer. Higher viscosity results in lower diffusion of the molecules and therefore reduces influence from collisional quenching. As a result luminescence decay times of LTC in the presence of Hb become longer with increasing viscosity (see ESI,† Fig. S5). Furthermore, blood plasma always contains hemoglobin binding proteins such as haptoglobin, which decrease concentration of free hemoglobin to detect *via* binding.[25] These characteristics of blood plasma reduce the sensitivity of LTC as sensors to detect hemolysis. Nevertheless, the LTC sensitivity in plasma is more than sufficient especially when taking into account that the plasma we used already contained hemoglobin due to marginal hemolysis as a result of the extraction process.

Assuming that the decrease in LTC luminescence lifetime in plasma in the absence of hemoglobin (τ_D = 1.9 ms) compared to those in buffer in the absence of hemoglobin (τ_D = 2.76 ms) is caused by an initial concentration of hemoglobin in the plasma sample only, we can estimate this initial concentration to correct our experimental results (see ESI,† Fig. S6). In our experiments the initial hemoglobin concentration in our plasma samples was determined to be 0.22 mg mL^{-1} (see ESI,† Fig. S6), which is in excellent agreement with the concentration of 0.24 mg mL^{-1} estimated by TMB, a method widely used for hemoglobin analysis.[7] The concentration estimation was done based on the luminescence decay of LTC in buffer. The additional interactions between Hb and blood plasma, as mentioned above, are not taken into account here. Nevertheless these interactions exist as the change in luminescence decay times dependent on the Hb concentration is less pronounced in plasma compared to those in buffer as mentioned above. Therefore in a next step the estimation of a calibration function for the measurements in blood plasma is needed.

The effect of bilirubin on the luminescence of LTC was not within the scope of this study, too. Bilirubin as a degradation product of hemoglobin is present in blood plasma in its free as well as conjugated form. It is known to influence hemoglobin measurements.[16] The investigations of the cross sensitivity of the luminescence LTC on bilirubin is therefore important. A corresponding study is currently in progress.

Experimental section

The LTC "Lumi4Tb" labeled to streptavidin (1.24 mg mL^{-1} [21 μM]), *ca.* 4.4 Lumi4Tb/sAv) was provided by Lumiphore Inc. (Richmond CA, USA).[26] For peptide labeling, *N*-hydroxysuccinimide-activated Lumi4Tb (NHS-Lumi4Tb) was provided by Lumiphore. The peptide utilized here consisted of the sequence H2N-G·SGAAAGLS·(His)6-amide. The N-terminal amine extending from the G residue provides a unique site-specific handle for labeling with the NHS-Lumi4Tb. The peptide was labeled using the manufacturer's suggested protocol and then purified, desalted, lyophilized and stored at −20 °C in a desiccator until used as previously described in detail.[27] Labeling ratio was 1 Lumi4Tb/peptide. A concentration of 0.31 mg mL^{-1} (100 μM) was determined by absorption spectroscopy.

The Terbium complex Tb-Al086 (see ESI,† Fig. S7 for structure) was obtained by modifications of the procedure describe in ref. 28. The concentration of the complex was estimated with absorbance spectroscopy to be 0.5 mg mL^{-1}.

Hemoglobin from bovine blood (lyophilized powder) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and 5,10,15,20-tetrakis-(4-carboxy-phenyl)-21,23-porphyrin was purchased from TriPorTech GmbH (Lübeck, Germany).

The solvents used were 10 mM TRIS-buffer (pH 7.4) without any further additives and human plasma collected from fresh blood (pH ~ 7.4). The latter were collected in accordance with all institutional regulations. The plasma was extracted from fresh blood before each measurement. For this purpose the blood was centrifuged for 30 minutes at 2500*g* directly after extraction and then the supernatant was used for measurements. Because of the strong plasma absorption (especially in the UV), it was diluted with water four times for absorption measurements. For all other measurements undiluted plasma was used. All experiments were performed at room temperature.

Luminescence spectra and temperature-dependent luminescence decays were recorded in 3 mm quartz cells with a fluorescence lifetime spectrometer (FLS920, Edinburgh Instruments, UK). For the lifetime measurements the samples were excited with a Xe flashlamp (50 Hz repetition rate at 340 nm). All other luminescence lifetime measurements were performed on an immunoanalysis platereader (IOM Nanoscan LF500, Berthold Detection Systems, Germany) and an immunoanalysis platereader (KRYPTOR, Cézanne/ThermoFisher Scientific, France), both with two photomultiplier tube (PMT) detection channels using changeable bandpass filters (Semrock, USA). All samples were excited with a pulsed 337.1 nm nitrogen laser system (SpectraPhysics, USA) with 128 shots at 30 Hz repetition rate (laser pulse duration *ca.* 7 ns) and a pulse energy of *ca.* 30 μJ (at the sample). For all samples the LTC and the hemoglobin or porphyrin emission were recorded simultaneously in the two PMT channels (LTC channel and hemoglobin/porphyrin channel). Decay time curves were recorded from 0 to 10 ms after the excitation pulse in 1000 bins of 10 μs (IOM Nanoscan LF500) and from 0 to 8 ms after excitation pulse in 4000 bins of 2 μs (KRYPTOR), respectively.

For spectroscopic measurements in buffer and in plasma the sample volumes were 150 μL and 20 μL, respectively. All samples were mixed within the used media. Concentrations for luminescence measurements were 4 nM for Lumi4Tb labeled peptide (Lumi4Tb-Pep) and 0, 0.0325, 0.25, 0.5, 1.0, 2.0, 3.0 and 4.0 mg mL^{-1} for bovine hemoglobin, respectively. Luminescence lifetime measurements in buffer in the presence of hemoglobin were performed with constant concentrations of 0.2 nM for Tb-Al086, Lumi4Tb-Pep, Lumi4Tb-sAv and Lumi4Tb-NHS, respectively. Luminescence lifetime measurements in plasma in the presence of hemoglobin were performed with concentrations of 2.5 nM for Lumi4Tb-Pep and Lumi4Tb-NHS. Hemoglobin concentrations for all measurements varied between 0 and 1.56 mg mL^{-1}.

Measurements of temperature-dependent luminescence lifetimes were carried out with a concentration of 2 nM Lumi4Tb-Pep and a hemoglobin concentration of 0.325 mg mL^{-1}.

Luminescence lifetime measurements in the presence of porphyrin were performed with LTC concentrations of 2 nM and porphyrin concentrations in the range of 0–19 μg mL^{-1}.

Luminescence decay times of all LTC were calculated from the luminescence decay curves by bi-exponential fitting procedures ($I = \sum(A_x \exp(-t/\tau_x)$ with I the measured luminescence intensity, A_x the intensity fraction and τ_x the luminescence decay time of the x-th lifetime component). Numerical fits were performed with FAST™ software (Edinburgh Instruments, UK). Stern–Volmer analysis was done by using the calculated intensities and luminescence decay lifetimes of the LTC.

Conclusions

In conclusion, we described the interaction of LTC with hemoglobin in buffer and blood plasma for the first time. Different LTC, unlabeled or labeled to biomolecules of different sizes, were used to quantify the hemoglobin influence on photophysical properties of the LTC. The impact of hemoglobin on LTC luminescence was characterized in TRIS-buffer (pH 7.4) using steady-state and time-resolved measurements. Experimental results show that LTC were influenced by both a dynamic quenching process due to interactions with the hemoglobin and static quenching *via* pairing of LTC with porphyrin ligands of the hem-units of hemoglobin, respectively. The interaction of LTC with hemoglobin in TRIS-buffer is very sensitive and allows for determination of hemoglobin concentrations from 0.01 mg mL^{-1} up to several mg mL^{-1}. Having in mind that hemoglobin concentration in blood plasma of healthy persons is around 0.01 mg mL^{-1}, the hemoglobin-dependent terbium luminescence is able to cover the whole range of abnormal hemoglobin concentrations possible in plasma samples. Moreover, it is an easy and very fast method. Unlabeled or small LTC molecules should be preferred as they are more sensitive to appearance of hemoglobin than LTC labeled onto biomolecules.

Hemoglobin dependent LTC luminescence in blood plasma compared to experimental results in buffer showed similar response although sensitivity was decreased due to lower interference between hemoglobin and terbium complexes. This reduced interference has its origin in higher viscosity of plasma compared to buffer as well as in less efficient binding between LTC-donors and porphyrin acceptors. The latter is most likely due to a non-specific binding between plasma components and LTC or hemoglobin. Those interactions can separate LTC from hemoglobin and thus inhibit binding. Nevertheless, within our experiments the decrease in luminescence lifetimes in the absence of hemoglobin in plasma compared to those in the absence of hemoglobin in buffer could be explained by an initial concentration of hemoglobin in blood plasma. Initial free hemoglobin concentrations estimated by TMB were in excellent agreement with initial free hemoglobin concentrations measured by LTC. Therefore it could be assumed that free hemoglobin is the main reason for luminescence lifetime decrease of LTC in plasma. For sure it is not the only one as plasma is a complex mixture of many molecules and ions. Some of those (*e.g.* serum albumines or glycoproteins) are known to interact with LTC.[22,23] Hence, further experiments to quantify interference of hemoglobin detection *via* LTC by plasma components are essential. Nonetheless our results clearly demonstrate that LTC could be suitable sensors to detect the degree of hemolysis in biosamples as they combine sensitivity with the possibilities to measure hemolysis rapidly, cost-effectively and user-friendly.

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