J Fluoresc (2006) 16:809–816
DOI 10.1007/s10895-006-0124-6

ORIGINAL PAPER

Effect of Polystyrene Microsphere Surface to Fluorescence Lifetime Under Two-Photon Excitation

Marko E. Tirri · Rina Wahlroos · Niko J. Meltola · Juha Toivonen · Pekka E. Hänninen

Received: 22 June 2006 / Accepted: 9 August 2006 / Published online: 16 September 2006
© Springer Science+Business Media, Inc. 2006

Abstract Molecular assays such as immunoassays are often performed using solid carriers and fluorescent labels. In such an assay format a question can be raised on how much the fluorescence of the label is influenced by the bio-affinity binding events and the solid carrier surface. Since changes in fluorescence intensity as labels bind to surfaces are notoriously difficult to quantify other approaches are preferred. A good indicator, independent of the fluorescence intensity of the label, is the fluorescence lifetime of the marker fluorophore. Changes in fluorescence lifetime reliably indicate the presence of dynamic quenching, energy transfer or other de-excitation processes. A microsphere based assay system is studied under two-photon excitation. Changes in fluorescence lifetime are studied as labeled protein conjugates bind on microsphere surfaces – both direct on the surface and with a few nanometer distance from the surface. Fluorescence signal is measured from individual polystyrene microspheres and the fluorescence lifetime histogram is simultaneously recorded. The results indicate that self-quenching and quenching by the polystyrene surface are both present in such a system. However, the effect of the surface can be avoided by increasing the distance between the surface and the label. Typical distances achieved by a standard sandwich type of assay, are already sufficient to overcome the surface induced quenching in fluorescence detection.

Keywords Two-photon excited fluorescence · Fluorescence lifetime · Microparticle · Microsphere assay

M. E. Tirri (✉) · R. Wahlroos · N. J. Meltola · J. Toivonen · P. E. Hänninen
Laboratory of Biophysics, Institute of Biomedicine, University of Turku,
P.O. Box 123, Turku 20521, Finland
e-mail: marko.tirri@utu.fi

Introduction

Fluorescence lifetime measurement in nanosecond range is a standard method of characterizing fluorescent compounds in the field of spectroscopic research. It has been found to be an advantageous tool for monitoring the interactions of fluorescent molecules with surroundings as well as to monitor binding events to other molecules [1, 2]. Fluorescence lifetime is influenced by various parameters such as pH, solvent polarity and the presence of quencher molecules. Also, the de-excitation pathways, e.g. intersystem crossing, energy transfer and conformational changes can have an effect on the fluorescence lifetime [3].

Fluorescence lifetime measurements have many applications both in research and in routine bio-analytics. It has been found that fluorescence lifetime is dependent on the distance between fluorescent label and a metal surface or a dielectric interface [4, 5]. These phenomena have received growing interest over the past few years and has been utilized to control fluorescence lifetime and intensity with the presence of metallic surface or nanostructure on glass, silica or plastic [6, 7]. In bio-analytics, new fluorescence lifetime detection schemes and principles for lifetime-based fluorescent assays have been emerged [1, 8].

Fluorescence intensity and lifetime have been quantified in flow cytometry [9] using microspheres as a solid carrier surface [10–12]. In general, many fluorescence bio-affinity assays are based on the use of micro- or nanospheres [13–16]. Such an assay technique is also novel microvolume assay technique, TPX-technique [17, 18]. This technique is based on the measurement of single microspheres using two-photon excitation of fluorescence.

Two-photon excitation of fluorescence (TPE) is based on the simultaneous absorption of two photons by a fluorophore. Two-photon excitation is typically achieved by

focusing a long wavelength (near-infrared) laser light with a microscope objective. A 3-dimensional diffraction-limited excitation volume on the order of 1 μm^3 is created. As a consequence of the excitation scheme practically no background fluorescence signal is generated outside of this focal volume [19]. TPE is widely used in laser scanning microscopy and detection of fluorescent molecules within small volumes [20, 21]. TPE has been used to measure bioaffinity assays in microvolumes without separation steps [17] and it has been shown to perform excellently for example in fluorescence polarization measurements [22, 23].

In this paper, fluorescence lifetime of protein conjugates of dipyrrylmethene-BF_2 (BF-label) [24, 25] and R-Phycoerythrin (RPE) are measured to study the behaviour of these fluorescent conjugates on microsphere surfaces. Two approaches were used to compare the effect of polystyrene microsphere with fluorescence lifetime. One, where fluorescent conjugate was directly bound on the microsphere surface, and the other where a few nanometer distance between the surface and the conjugate was created by a standard sandwich assay (Fig. 1). Fluorescence lifetime changes were determined from individual microspheres using two-photon excitation. Single photon counting of fluorescence was measured in coincidence with laser back-scattering signal from the microspheres. Although our study aims to characterize the performance of two-photon excitation based microsphere assay system, we believe that the results are applicable to any particle or surface based assays.

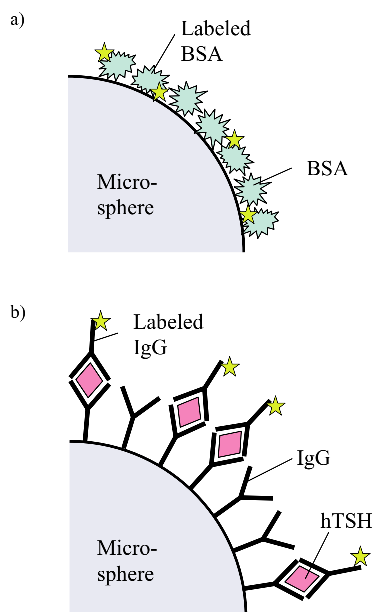

Fig. 1 Schematic representation of a) BF-BSA microspheres and b) hTSH sandwich assay microspheres. Fluorescent labels are bound on the polystyrene microsphere surface with two different distances: a) closely-spaced by BSA-conjugate or b) with few nanometer distance by hTSH sandwich assay and IgG-conjugate

Materials and methods

Reagents

Reagents were purchased either from Sigma or Fluka and used without further purification. N,N-dimethylformamide (DMF) was p.a. grade from Lab-Scan (Dublin, Ireland) and was dried over molecular sieves (4 Å). Water was Millipore™ Rios3 grade. Monoclonal mouse IgGs against human thyroid stimulating hormone (hTSH, clones 5404 and 5405) were purchased from Medix Biochemica (Kauniainen, Finland) and the hTSH standard from Scripps Laboratories (San Diego, CA, USA). Polystyrene microspheres (3.2 μm in diameter, carboxyl modified microspheres, PC05N, COOH-group/0.852 nm^2) were purchased from Bangs laboratories (Fishers, IN, USA).

The R-phycoerythrin-SPDP conjugate was purchased from Molecular Probes and bifunctional linker GMBS was from Pierce (Rockford, IL, USA). Dipyrrylmethene-BF_2 labeling reagents were synthesised according to previously published methods [24]. NAP-5 and Superdex-200 gel filtration columns were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Micro titration plates (384-well plate, TC-grade with black walls and clear bottom) were obtained from Greiner (Frickenhausen, Germany).

Labeling of IgG and BSA

Labeling of BSA and IgG (anti hTSH MAb clone 5405) with BF-labels was performed according to previously published method [25]. Briefly, the labeling reagent (BF530 or BF560 NHS esters) in anhydrous dimethylformamide was added to the IgG or BSA solution (1 mg/ml, 200 mM $NaHCO_3$, pH 8.3). The molar excesses of the labeling reagents in respect to the protein were 10 fold for BF530-BSA and 6 fold for BSA-BF560, IgG-BF530 and IgG-BF560. The reaction mixtures were incubated at room temperature for 3 h. The products were purified with NAP-5 gel filtration column using phosphate buffered saline (PBS; 50 mM phosphate, 150 mM NaCl, 10 mM NaN_3, pH 7.4) as eluent. The labeling degrees (dye/protein ratio) of the protein conjugates were determined spectrophotometrically using the following absorption coefficients (ε): BF530 (PBS) $\varepsilon_{533\ nm} = 48\ 000$ $cm^{-1}M^{-1}$; BF560 (PBS) $\varepsilon_{565\ nm} = 68\ 000\ cm^{-1}M^{-1}$; IgG (PBS) $\varepsilon_{280\ nm} = 210\ 000\ cm^{-1}M^{-1}$; BSA (PBS) $\varepsilon_{280\ nm} = 44\ 000\ cm^{-1}M^{-1}$ [25]. Following labeling degrees were obtained: 4.8 for BSA-BF530, 2.7 for BSA-BF560, 2.1 for IgG-BF530 and 3.6 for IgG-BF560.

RPE-IgG conjugate was prepared as described earlier [26] using SPDP and GMBS as cross-linking reagents. The RPE-IgG conjugate (labeling degree 1) was purified with Superdex-200 gel filtration column using the same phosphate buffered saline as for the BF-conjugates.

BF-BSA coated microspheres

The coupling of BSA on the microsphere surface was performed by using a standard EDC coupling method [18]. Microspheres with variable amount of labeled BSA (BF530-BSA or BF560-BSA) were prepared by using a mixture of labeled and unlabeled BSA for coating. The total concentration of BSA in the coating was kept constant. The BSA-mixture for coating contained 0, 5, 20, 50, 80 and 100% of labeled BSA.

Immunoassay

Mouse monoclonal IgG anti-hTSH (clone 5404) was covalently coupled to microspheres by using a standard EDC coupling method [18]. Concentration of stock suspension of coated microspheres was determined with Multisizer™ 3 Coulter Counter (Beckman Coulter Inc.), followed by dilution with assay buffer (TRIS-HCl 50 mM, NaCl 150 mM, 10 mM NaN_3, 0.5% bovine serum albumin, 0.01% Tween 20, pH 8.0) to concentration of 1×10^7 pcs/ml. Labeled anti-hTSH IgGs were diluted with assay buffer to concentration of 8 nM. hTSH analyte standard was diluted with assay buffer to concentrations of 100, 300 and 1000 mIU/l. Volumes of 5 μl of microsphere suspension and 5 μl of labeled IgG were dispensed into a well of micro titration plate followed by addition of hTSH standards (10 μl). The reaction mixtures were incubated for 3.5 hr at 20°C under continuous shaking (Variomag Monoshake, H + P Labortechnik AG, Germany) before measurements.

Measurement set-up

Figure 2 shows the fluorescence lifetime measurement set-up that is based on an optical module of ArcDia TPX-microfluorometer [27] (ArcDia Ltd., Turku, Finland). A mode-locked femtosecond diode pumped Nd:glass laser (Time-Bandwidth Products GLX-200, Zürich, Switzerland) was used as a light source to create two-photon excitation of fluorescence. The wavelength of the laser was 1057 nm with nominal pulse width of 140 fs ($sech^2$), repetition rate of 107 MHz and an average power of 150 mW. The laser beam at 1057 nm was reflected by a dichroic mirror through a beam scanner unit and focused with a microscope objective lens (Leica C-Plan 40×0.65, Leica Microsystems, Bensheim, Germany) through the cuvette bottom to the sample. Scattered laser light from objects entering the focal volume was directed by a beam splitter to a confocally arranged pinhole placed in the front of a photodiode detector. The fluorescence light from the sample was collected with the same microscope objective lens and directed within a range from 530 nm to 700 nm through the dichroic mirror and optical fibre to a monochromator (Oriel 77250, Grating 77911,

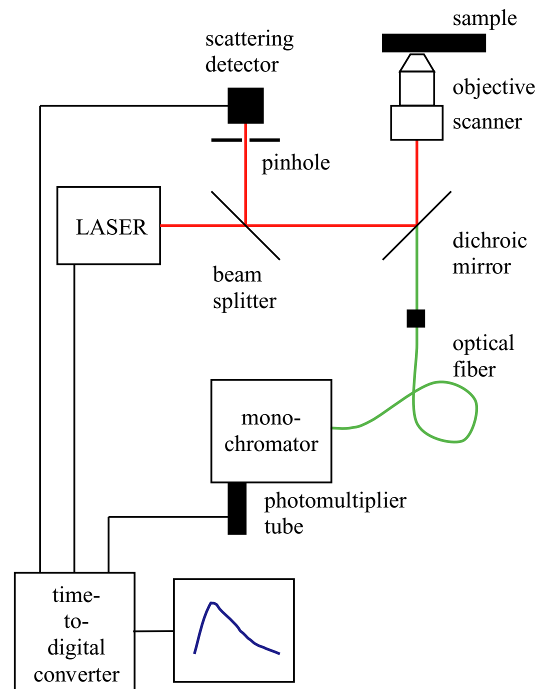

Fig. 2 Time-resolved fluorescence measurement set-up using two-photon excitation of fluorescence. Laser induced fluorescence from sample is detected with a photomultiplier tube and recorded with time-to-digital converter with excitation laser pulse detection and coincident with scattering signal. The measured data is plotted as fluorescence lifetime histogram

Stratford USA). A photomultiplier tube (R5600, Hamamatsu Photonics K.K., Sunayama-cho, Japan) was used as a detector in photon counting mode. Time-resolved fluorescence signal was recorded with time-to-digital converter (PAM and TimeHarp 200, PicoQuant Gmbh, Berlin, Germany).

Measurements

Fluorescence signal is collected coincident with scattering signal when a microsphere is in focal volume. Here, the standard TPX technique [17, 18] is expanded by fluorescence lifetime detection from single microspheres. The lifetime measurement was performed using the scattering detector signal from the spheres as an enable signal for time-to-digital converter (Fig. 2). The measurement of the fluorescence lifetime is based on the time-correlated single photon counting (TCSPC) method [28]. In this method time between the detected single photon of fluorescence (start signal) and the excitation laser pulse (stop signal) is measured. The measured data is plotted as fluorescence lifetime histogram. By measuring fluorescence in presence of microspheres, background signals, e.g. from assay buffer and dark counts from photomultiplier tube, are efficiently suppressed. As references, free BF-BSA and labeled IgG conjugates were measured by continuous fluorescent lifetime detection from a

homogenous liquid sample consequently without coincident scattering signal from microspheres. The fluorescence lifetimes were recorded at fluorescence emission maxima, 560 nm for BF530 and 580 nm for BF560 and RPE labeled samples.

The total measurement times for BF-BSA spheres was 180 s and for hTSH assay spheres 300 s per sample. In average, one microsphere per second is detected. Thus an average of 200 and 300 microspheres were measured. The measurement time for a single particle is about 50 ms on average [27] and the effective fluorescence signal integration time was approximated to be about 10 s and 15 s for BF-BSA microspheres and hTSH assay, respectively. The measurement time for free labeled conjugates was 60 s.

The fluorescence lifetime data was analyzed using MicroGraph Origin™ software. Single exponential fitting was used to determine average lifetimes from the fluorescence lifetime histogram and the fitting was applied to the data between 1 ns and 6 ns after the excitation pulse. The BF-BSA microspheres and hTSH assay samples were measured with ArcDia™ TPX Platereader to evaluate the validity of the samples (data not shown).

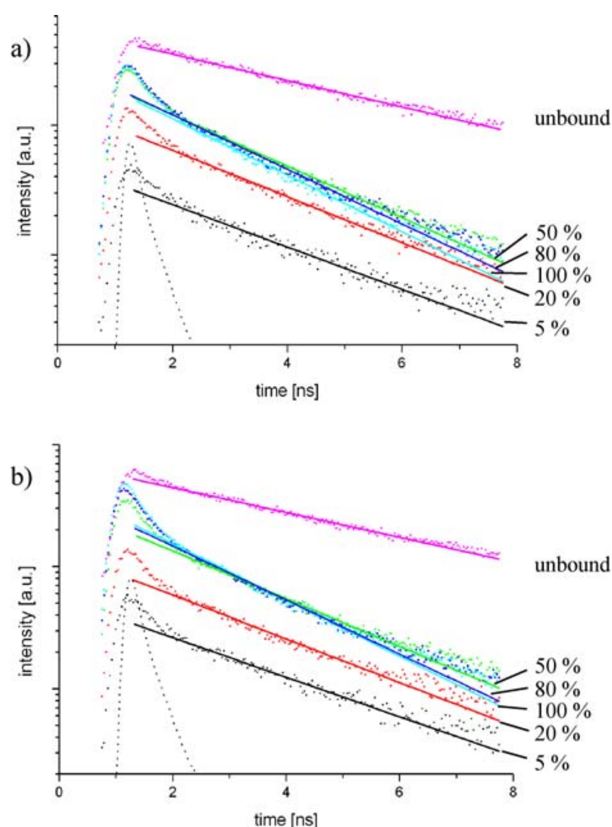

Fig. 3 Fluorescence lifetime histograms of BF-BSA microspheres and unbound conjugates with a) BF530 and b) BF560 labels. Percentage values represent the proportion of labeled BSA of all BSA in the microsphere coating procedure. Solid lines show single-exponential fitting to time-window of 1–6 ns after laser pulse. The instrument response curve is shown with dotted line

Results and discussion

Choice of data analysis

The fluorescence lifetime τ is a characteristic constant in time-resolved fluorescence intensity function: $I = I_0 e^{-t/\tau}$, where I denotes fluorescence intensity, I_0 initial value of intensity, t time and e the natural base. When only single exponential decay is present, solving of the lifetime τ is quite straightforward. However, in most cases samples exhibit multiple lifetimes with the presence of different populations of fluorescent labels due to existent de-excitation pathways e.g. self-quenching caused by a high antibody labeling degree [29]. Various types of fitting algorithm have been developed and compared to solve single- [30] and multi-exponential decays [31]. However, multi-exponential curve fitting has its limitations since the higher requirements on the quality of measured data than with single exponential fitting. Multi-exponential fitting requires significantly higher total number of photons than in the single exponential case to obtain reliable results with respect to the lifetimes and relative intensities of each lifetime component [32]. Additionally, the acquisition time-window should be longer in case of multi-exponential fitting to ensure the separation between longer lifetime sections and background level. Therefore, single exponential curve fitting is suitable for the acquired TCSPC data in this study.

With chosen single exponential curve fitting our excitation pulse repetition rate of 107 MHz is in applicable range [33]. Single exponential decay analysis with least-squares fitting method yields to a low systematic deviation of lifetimes without a requirement of high statistical precision for the recorded data [31]. Also, the question of the total effect of the solid phase surfaces to the fluorescent properties of labels can be answered by the observation of the average lifetime rather than analyzing multiple lifetimes.

The instrument response was measured by exposing the photomultiplier tube directly to an attenuated laser beam. The measured instrument response curve is shown for reference with dotted line in Figs. 3 and 4. The downward slope yields decay of 0.26 ns for the instrumental response, thus the fitting is possible directly without de-convolution the instrumental response when the fitting time-window of 1 ns to 6 ns is used. The background was determined to be extremely low and therefore no background subtraction was necessary.

BF-BSA microspheres

BSA was labeled and coupled on microsphere surfaces as shown in Fig. 1a. The BF-BSA conjugate bound to

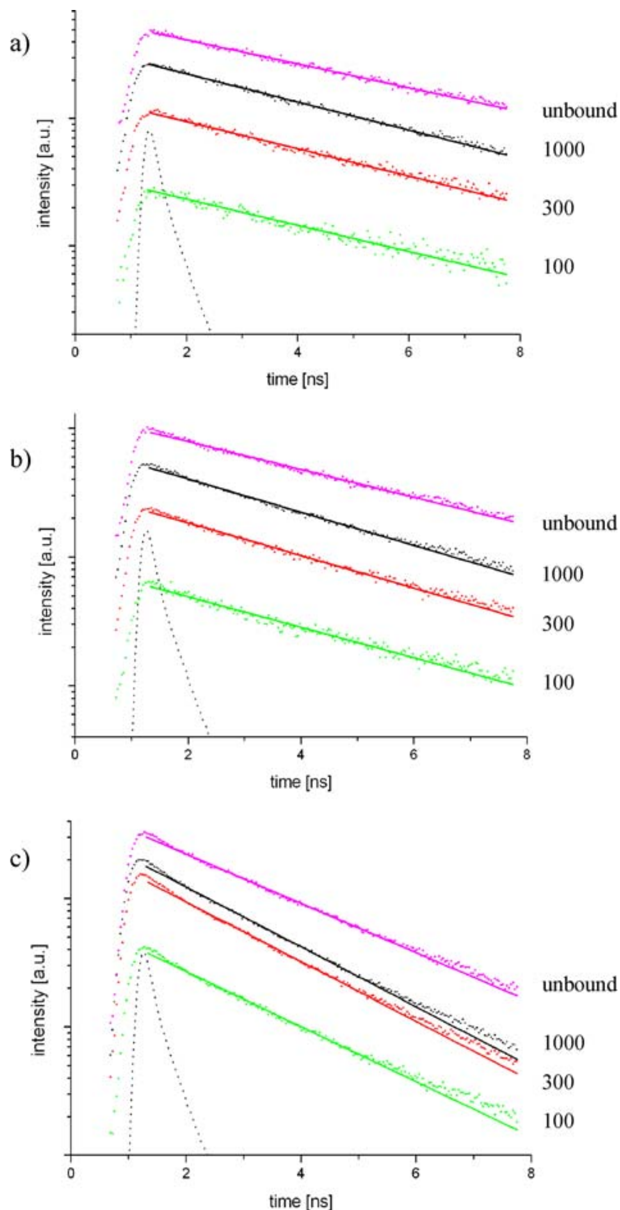

Fig. 4 Fluorescence lifetime histograms of hTSH assay microspheres and unbound conjugates with a) BF530, b) BF560 and c) RPE labels. In the hTSH assay the analyte concentrations were 100, 300 and 1000 mIU/l. Solid lines show single-exponential fitting to time-window of 1–6 ns after laser pulse. The instrument response curve is shown with dotted line

microsphere were measured in 5 different concentrations denoted as 5, 20, 50, 80 and 100 % representing the proportion of labeled BSA of all BSA in the coating procedure. Figure 3 presents the raw fluorescence intensity decay as histogram. Table 1 shows the determined average lifetimes and changes in lifetimes in respect of the unbound BF-BSA.

BF530 and BF560 labeled unbound BSA molecules were measured both to have fluorescence lifetime of 4.26 ns. Already the smallest relative amount of labeled BSA (5%) on microsphere surface leads to remarkable changes in fluorescence lifetimes: from 4.26 ns down to 2.65 and 2.68 ns for BF530 and BF560, respectively. When increasing the relative amount of labeled BSA the lifetimes are shortened. With maximal relative BF-BSA amount (100%) lifetimes are 1.99 and 1.89 ns for BF530 and BF560 labels, respectively. Fluorescence lifetimes from these BF-BSA microspheres were measured with different central wavelengths of 540, 560, 580 and 600 nm, but no significant changes in lifetimes with respect to emission maxima were observed indicating that the observed quenching processes are not wavelength dependent.

Two separate fluorescence lifetimes can be seen in Fig. 3. The ratio of the fast decay is increasing with relative amount of BF-BSA and being somewhat higher with BF560 label – this fast decay suggests a very fast quenching channel among some population of conjugates. The labels that lay within very short distances from the microsphere surface are assumed to be quenched heavily. Similarly, a small fragment of the fast decay can be seen in unbound conjugation samples suggesting a population of the labels having self-quenching within BSA molecules [34].

After the fast decay ($t < 2$ ns) a remarkable lifetime shortening compared with the free reference sample can also be seen already with 5% spheres. This change compared with unbound labeled BSA cannot originate from direct self-quenching – the distance between adjacent labeled BSA molecules on the microsphere surface is too large (in the order of tens of nanometers). More probable factors are the interactions of the conjugate with the microsphere. Quenching may occur, as the binding of the conjugate is in direct contact with polymer surface. Also, the presence of microsphere as an optical resonator creates various quenching channels [35]. These phenomena are strengthened with higher relative amount of labeled BSA as the distances between labeled protein conjugates are approaching scale (<10 nm) that enables Förster type of energy transfer.

BSA molecule has a size of approximately 5 nm yielding a label distance from 0 nm to maximally of 5 nm from the particle surface depending on the position of the label within the BSA molecule. This distance can be considered as a ‘near-field’ thus the optical resonances or quenching by polymer material are clearly possible as well as the self-quenching of the conjugates having a labeling degree greater than 1 [29, 34].

hTSH assay spheres

Above-mentioned BF-BSA microspheres demonstrates the situation where fluorescent conjugate was directly bound on the microsphere surface. Human thyroid stimulating (hTSH) assay [36] was selected to study the effect of surface on the fluorescence lifetime when the distance between the labels

Table 1 Results of lifetime measurements

	BF530		BF560		RPE	
	t (ns)	*dt* (%)	*t* (ns)	*dt* (%)	*t* (ns)	*dt* (%)
% of labeled BSA[a]						
unbound BSA	4.26 ± 0.05		4.26 ± 0.05			
5	2.65 ± 0.11	38%	2.68 ± 0.13	37%		
20	2.44 ± 0.04	43%	2.43 ± 0.05	43%		
50	2.19 ± 0.02	49%	2.23 ± 0.02	48%		
80	2.06 ± 0.02	52%	1.97 ± 0.02	54%		
100	1.99 ± 0.02	53%	1.89 ± 0.02	56%		
hTSH [mIU/l][b]						
unbound IgG	4.61 ± 0.04		4.02 ± 0.04		2.26 ± 0.02	
100	4.21 ± 0.19	9%	3.66 ± 0.14	9%	2.03 ± 0.03	10%
300	4.07 ± 0.05	12%	3.45 ± 0.04	14%	1.87 ± 0.01	17%
1000	3.92 ± 0.02	15%	3.37 ± 0.02	16%	1.86 ± 0.01	18%

[a]BF-BSA microspheres are labeled with fluorescent labels BF530 or BF560. The average fluorescence lifetimes and standard errors (*t*) are presented with changing relative amount of labeled BSA.

[b]hTSH assay microspheres were measured with fluorescent IgG conjugates containing BF530, BF560 or RPE. The average fluorescence lifetimes and standard errors (*t*) were determined with different hTSH analyte concentration. Lifetime shortenings (dt) are calculated in relation to unbound conjugate.

and the surface is larger than 10 nm. hTSH is a standard sandwich type of assay (Fig. 1b) where the separation from the surface was created by primary binding antibody. The lifetimes of IgG coupled BF530, BF560 and RPE labeled unbound IgG molecules are shown in Table 1 and Fig. 4. The unbound conjugates were determined to have lifetimes of 4.61, 4.02 and 2.26 ns, respectively. With the BF530 label, the assay lifetimes were from 4.21 to 3.92 ns with increasing analyte concentration from 100 to 1000 mIU/l, the highest value approaching the capacity of the assaying reagents. The observed lifetime decreases of BF560 and RPE labels were within the used assay range from 3.66 to 3.37 ns and from 2.35 to 1.86 ns, respectively. The BF530 labeled hTSH fluorescence lifetime histogram in Fig. 4a appears to follow single exponential decay much closer than the results of the BF-BSA measurements. Despite the fluorescent lifetime histogram of BF560 conjugate in Fig. 4b is single exponential, a very small fraction of fast decay is found in the first part of the lifetime histogram. In the RPE-hTSH histogram RPE seems to have several lifetime components in Fig. 4c: a clearly faster (in the range of 1 ns) and slower (5 ns) components compared with the determined average lifetimes.

Fluorescence lifetime decay near surface

There are a few possibilities that can explain observed hTSH assay decay behaviour and further confirm our earlier conclusions about the BF-BSA conjugates. Firstly, the distance between label molecule and the microsphere in the case of hTSH assay is on average considerably larger than BF-BSA conjugates on microspheres. The sandwich type of assay limits the quenching, which is caused by direct contact with the surface, assuming that the sandwich structure is formed perpendicular to the microsphere surface. hTSH analyte is small in size about 1 nm, but the used IgGs have a size of approximately 12 nm leading in sandwich assay to a distance even up to 25 nm between microsphere and label. In the case of labeled BSA the distance was evaluated to vary between 0 and 5 nm. The long distance resulting from the sandwich assay rules out also other near-field related phenomena yielding again single exponential decay, distances over 10 nm are relatively far to allow any near-field related phenomena to take place. However, the decrease in fluorescence lifetime even with the concentration of 100 mIU/l indicates changes in the environment of individual labels in any antibody to antigen binding reaction. The shortening of the lifetime with increasing analyte concentration is the most likely due to the self-quenching as the labeled IgG is concentrated on the microsphere surface and therefore the distance between individual label molecules is decreased. Although RPE label differs largely from BF labels by its size and quantum efficiency, RPE conjugate exhibits similar kind of behaviour as the BF conjugates. Since multi-exponential fluorescence decay can be observed from the measured fluorescence lifetime histogram of RPE conjugate, more complex phenomena, which are related to the fluorescent protein itself can be accounted for.

Relative lifetime changes of the different labels are about the same in the BF-BSA microspheres and in the hTSH assay microspheres as percentual figures indicate in Table 1. The quenching of the labeled BSA shows that the polystyrene surface effects strongly to the labels in close vicinity. The polystyrene surface induced quenching is clearly minimized in the hTSH assay as the label distance from the surface

is extended. Although our specific excitation approach, we believe that the results can be used as a guideline in other assays based on different excitation schemes since emission by definition is independent of the excitation scheme [19, 37].

Conclusions

We chose to study the temporal behaviour of fluorophores in a two-photon excitation based assay system. The results of this study indicate that fluorescence lifetimes of labels near solid surfaces are influenced by complex phenomena such as direct interaction with the surface and self-quenching. The magnitude of these phenomena, however are heavily dependent on the distances between the label and the surface as well as the distance between adjacent labels. With a separation layer between the label and the surface as in sandwich type of assay, these surface induced effects, like quenching, can be avoided whereas proper measurement design or calibration techniques need to be used to compensate the self-quenching induced by near-by labels.

Acknowledgement This work was supported by Finnish Graduate School of Chemical Sensors and Micro Analytical Systems, and Turku University Foundation (M.T.), Academy of Finland (R.W. and P.H.), Arctic Diagnostics Inc. and European Commission (N.M. and J.T.).

References

1. Pfeifer L, Stein K, Fink U, Welker A, Wetzl B, Bastian P, Wolfbeis OS (2005) Improved routine bio-medical and bio-analytical online fluorescence measurements using fluorescence lifetime resolution. J Fluoresc 15(3):423–432
2. Turconi S, Bingham RP, Haupts U, Pope AJ (2001) Developments in fluorescence lifetime-based analysis for ultra-HTS. Drug Discov Today 6(12):S27–S39
3. Valeur B (2002) Molecular Fluorescence, Principles and Applications, Wiley-VCH, Weinheim, Germany
4. Lukosz W, Kunz RE (1977) Fluorescence lifetime of magnetic and electric dipoles near a dielectric interface. Opt Commun 20(2):195–199
5. Lukosz W, Kunz RE (1979) Changes in fluorescence lifetimes induced by variation of the radiating molecules optical environment. Opt Commun 31(1):42–46
6. Lakowicz JR (2001) Radiative decay engineering: Biophysical and biomedical applications. Analyt Biochem 298(1):1–24
7. Aslan K, Badugu R, Lakowicz JR, Geddes CD (2005) Metal-enhanced fluorescence from plastic substrates. J Fluoresc 15(2):99–104
8. Hoefelschweiger BK, Pfeifer L, Wolfbeis OS (2005) Screening scheme based on measurement of fluorescence lifetime in the nanosecond domain. J Biomol Screen 10(7):687–694
9. Horan PK, Wheeless LL (1977) Quantitative Single Cell Analysis and Sorting. Science 198(4313):149–157
10. Steinkamp JA, Keij JF (1999) Fluorescence intensity and lifetime measurement of free and particle-bound fluorophore in a sample stream by phase-sensitive flow cytometry. Rev Sci Instrum 70(12):4682–4688
11. McHugh TM, Stites DP, Casavant CH, Fulwyler MJ (1986) Flow cytometric detection and quantitation of immune-complexes using human C1q-Coated Microspheres. J immunol Methods 95(1):57–61
12. Fulton RJ, McDade RL, Smith PL, Kienker LJ, Kettman JR (1997) Advanced multiplexed analysis with the FlowMetrix^{TM} system. Clin Chem 43(9):1749–1756
13. Jolley ME, Wang CHJ, Ekenberg SJ, Zuelke MS, Kelso DM (1984) Particle Concentration Fluorescence Immunoassay (PCFIA) - a new, rapid immunoassay technique with high-sensitivity. J Immunol Methods 67(1):21–35
14. Lovgren T, Heinonen P, Lehtinen P, Hakala H, Heinola J, Harju R, Takalo H, Mukkala VM, Schmid R, Lonnberg H, Pettersson K, Iitia A (1997) Sensitive bioaffinity assays with individual microparticles and time-resolved fluorometry. Clin Chem 43(10):1937–1943
15. Hakala H, Virta P, Salo H, Lonnberg H (1998) Simultaneous detection of several oligonucleotides by time-resolved fluorometry: the use of a mixture of categorized microparticles in a sandwich type mixed-phase hybridization assay. Nucleic Acids Res 26(24):5581–5588
16. Kulmala S, Suomi J (2003) Current status of modern analytical luminescence methods. Anal Chimica Acta 500(1–2):21–69
17. Hanninen P, Soini A, Meltola N, Soini J, Soukka J, Soini E (2000) A new microvolume technique for bioaffinity assays using two-photon excitation. Nat Biotechnol 18(5):548–550
18. Waris ME, Meltola NJ, Soini JT, Soini E, Peltola OJ, Hanninen PE (2002) Two-photon excitation fluorometric measurement of homogeneous microparticle immunoassay for C-reactive protein. Anal Biochem 309(1):67–74
19. Lakowicz JR (1999) Principles of Fluorescence Spectroscopy, 2nd edn. Kluwer Academic/Plenum Publishers, New York
20. Denk W, Strickler JH, Webb WW (1990) Two-photon laser scanning fluorescence microscopy. Science 248(4951):73–76
21. Berland KM, So PT, Gratton E (1995) Two-photon fluorescence correlation spectroscopy: method and application to the intracellular environment. Biophys J 68(2):694–701
22. Lakowicz JR, Gryczynski I, Gryczynski Z (1999) High throughput screening with multiphoton excitation. J Biomol Screen 4(6):355–361
23. Tirri M, Huttunen R, Toivonen J, Harkonen P, Soini J, Hanninen P (2005) Two-photon excitation in fluorescence polarization receptor-ligand binding assay. J Biomol Screen 10(4):314–319
24. Meltola NJ, Wahlroos R, Soini AE (2004) Hydrophilic labeling reagents of dipyrrylmethene-BF2 dyes for two-photon excited fluorometry: syntheses and photophysical characterization. J Fluoresc 14(5):635–647
25. Meltola NJ, Kettunen MJ, Soini AE (2005) Dipyrrylmetheneboron difluorides as labels in two-photon excited fluorometry. Part I-Immunometric assays. J Fluoresc 15(3):221–232
26. Meltola NJ, Soini AE, Hanninen PE (2004), Syntheses of novel dipyrrylmethene-BF2 dyes and their performance as labels in two-photon excited fluoroimmunoassay. J Fluoresc 14(2):129–138
27. Soini JT, Soukka JM, Soini E, Hänninen PE (2002) Two-photon excitation microfluorometer for multiplexed single-step bioaffinity assays. Rev Sci Instrum 73(7):2680–2685
28. Becker W, Hickl H, Zander C, Drexhage KH, Sauer M, Siebert S, Wolfrum J (1999) Time-resolved detection and identification of single analyte molecules in microcapillaries by time-correlated single-photon counting (TCSPC). Rev Sci Instrum 70(3):1835–1841
29. Deka C, Lehnert BE, Lehnert NM, Jones GM, Sklar LA, Steinkamp JA (1996) Analysis of fluorescence lifetime and quenching of FITC-conjugated antibodies on cells by phase-sensitive flow cytometry. Cytometry 25(3):271–279

30. Good HP, Kallir AJ, Wild UP (1984) Comparison of fluorescence lifetime fitting techniques. J Phys Chem 88(22):5435–5441
31. Nishimura G, Tamura M (2005) Artefacts in the analysis of temporal response functions measured by photon counting. Phys Med Biol 50(6):1327–1342
32. Kollner M, Wolfrum J (1992) How many photons are necessary for fluorescence-lifetime measurements. Chem Phys Lett 200(1–2):199–204
33. Good HP, Kallir AJ, Wild UP (1984) Optimum pulse repetition rates for single Photon-counting experiments. J Luminescence 29(4):491–496
34. Hemmilä IA (1991) Applications of fluorescence in Immunoassays. John Wiley & Sons, Inc., New York, USA
35. Lakowicz JR (1991) Topics in Fluorescence Spectroscopy, vol 3, Biochemical Applications, Anonymous Plenum Publishers, New York
36. Hanninen P, Waris M, Kettunen M, Soini E (2003), Reaction kinetics of a two-photon excitation microparticle based immunoassay - from modelling to practice. Biophys Chem 105(1):23–28
37. Xu C, Webb WW (1996), Measurement of two-photon excitation cross sections of molecular fluorophores with data from 690 to 1050 nm. J Opt Soc Am B-Opt Phys 13(3):481–491 !