Homogeneous Luminescence Decay Time-Based Assay Using Energy Transfer from Nanospheres

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Following a study on the feasibility of resonance energy transfer (RET) from carboxylated nanospheres with an incorporated phosphorescent donor to a cationic polyelectrolyte/acceptor aggregate on their surface, a novel scheme for homogeneous assays is presented that is based on RET from phosphorescent biotinylated nanospheres to fluorescently labeled streptavidin (SA). The phosphorescent nanospheres, with a diameter of well below 50 nm, are made from carboxylated polyacrylonitrile and dyed with ruthenium(II)–tris-4,7-diphenyl-1,10-phenanthroline dichloride (Ru(dpp)). Due to the small size of the nanospheres and the complete extraction of the ruthenium dye into the nanospheres during the precipitation process, RET occurs from Ru(dpp) to the label if labeled SA binds to the surface of the nanospheres. Luminescence quenching by oxygen or other species present in the sample can be neglected due to the shielding effect of the polymer matrix. Based on this finding, a competitive binding assay was established, where avidin and labeled SA compete for the biotin binding sites on the nanosphere. The process of binding to the surface can be detected by measurement of the luminescence intensity or the apparent decay time which is in the order of 2.5 – 4.5 μs.

Luminescence techniques are becoming more and more attractive for immunoassays.1–4 Among the existing concepts for immunoassays are (1) radioimmunoassays (RIAs), (2) enzyme-linked immunosorbent assays (ELISAs), (3) dissociation-enhanced lanthanide fluoroimmunoassays (DELFIs), (4) fluorescence polarization immunoassays (FPIAs), (5) time-resolved immunoassays, and (6) energy-transfer immunoassays.5 ELISAs and DELFIs are heterogeneous and require several washing steps, while bioassays based on energy transfer, measurement of polarization, or apparent decay time can be homogeneous, making them faster and less prone to sources of error caused by the number of working steps. To further improve the limit of detection (by eliminating interferences by shortwave fluorophores), we use long-wave-emitting dyes and combine this with measurements of apparent decay times in the microsecond regime, thus enabling time-resolved or gated measurements.

We recently presented a simple encapsulation technique to prepare highly phosphorescent nanospheres, which are viable luminescent markers.6 The beads precipitate in the form of very small aggregates of spherical shape. The most important characteristic of the nanospheres is their very low cross-sensitivity to oxygen due to the efficient shielding effect of the polyacrylonitrile (PAN) copolymers used.

Such nanospheres have now been used to establish a novel scheme for a homogeneous binding assay based on long-lived luminescence.7 Resonance energy transfer (RET) becomes possible from those nanospheres to an acceptor bound to the surface because of the small size of the nanospheres and their fractal structure, which warrants that the major fraction of the donor is accessible from those nanospheres to an acceptor bound to the surface.

**EXPERIMENTAL SECTION**

**Chemicals, Reagents, and Dyes.** All chemicals and solvents were of analytical grade and were used without further purification. Poly(acrylonitrile-co-acrylic acid-co-ethylene glycol) containing 5.0% (w/w) acrylic acid and 5.0%(w/w) ethylene glycol (PAN-COOH/OH) and poly(acrylonitrile-co-acrylic acid) containing 5.0%(w/w) acrylic acid (PAN-COOH) were obtained from Optosense GmbH (Würth, Germany). The SA/Alexa Fluor 633 (AF633) conjugate, avidin/egg white, and N-(2-aminoethyl)biotinamide hydrobromide (biotin ethylenediamine) were purchased from Molecular Probes (Eugene, OR). The cationic polyelectrolyte Superfloc C-587 (20% polymer concentration in water, MW 200 000) was from Cytec (Rotterdam, The Netherlands). The sodium salt of bromophenol blue (BPB) was purchased from Fluka (Buchs, Switzerland). The preparation of ruthenium(II)–tris-4,7-diphenyl-1,10-phenanthroline dichloride (Ru(dpp), MW 1169.20) is described elsewhere.8–10 Ru(dpp) is now available from Fluka.
Preparation of Phosphorescent Donor Nanospheres. The precipitation procedure to prepare the phosphorescent nanospheres was described in detail elsewhere. In essence, a solution of PAN derivatives and Ru(dpp) is coprecipitated from a solution in N,N-dimethylformamide (DMF). The preparation of the PAN-COOH/OH nanospheres (for the avidin assay) and the PAN-COOH nanospheres (for the polyelectrolyte binding study) is virtually identical.

While stirring vigorously, 250 mL of a 1 mM solution of sodium hydroxide was added dropwise to a solution of 250 mg of PAN-COOH/OH or PAN-COOH (equivalent to 0.5% (w/w) matrix/DMF) and 7.5 mg of Ru(dpp) (3.0% (w/w) dye/matrix) in 52.7 mL of DMF. Then, 1 M hydrochloric acid was added to the flask until a pH of 4.0 was reached. This caused the precipitation of finely dispersed nanospheres. The DMF/water mixture was centrifuged at 3000 rpm for 15 min and the colored precipitate thoroughly washed with water. All washing solutions remained colorless. The precipitate was suspended in 25 mL of water, heated to 70 °C for 10 min, centrifuged, and then taken up in 100 mL of 2-morpholinoethanesulfonic acid (MES) buffer, pH 7.0, with an ionic strength (IS) of 30 mM (containing 0.5% sodium azide in order to prevent bacterial growth). After sonication for 1 h, the suspension remained clear and stable for months when stored in the dark at 4 °C.

Coupling of Biotin to Carboxy-Modified Nanospheres. To covalently link biomolecules to the carboxy-modified PAN-COOH/OH nanospheres for the avidin assay, a two-step reaction was performed over the one-step coupling reaction due to limitations that may be expected when larger molecules are coupled. A few drops of 1 M hydrochloric acid were added to 50 mL of the nanosphere suspension described above until the nanospheres were precipitated. The precipitate was washed twice with water, taken up in 50 mL of MES buffer of pH 6.5 (IS 30 mM), and then sonicated for 1 h. While stirring, 100 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) was added to the completely suspended nanospheres. They were allowed to react for 20 min at 25 °C, with continuous mixing. After centrifugation, the nanospheres were washed twice with water, taken up in 25 mL of phosphate buffer (pH 8.0, IS 30 mM), and then sonicated for 1 h. Then, 3.4 mg of biotin ethylenediamine was dissolved in 10 mL of phosphate buffer (pH 8.0, IS 30 mM) and combined with the nanosphere suspension. After a reaction time of 2 h at 25 °C under continuous stirring, a few drops of 1 M hydrochloric acid were added and the precipitated nanospheres were centrifuged. They were thoroughly washed with water, sonicated for 1 h, and resuspended in 50 mL of MES buffer (pH 7.0, IS 30 mM, 0.5% sodium azide). The suspension was stored in the dark at 4 °C.

To block remaining free carboxy groups on the surface of the nanospheres, they were converted into hydroxyl groups to prevent aggregation and unspecific binding. The following protocol was used. A 50-mg aliquot of EDC and 125 mL of 2-aminoethanol were added at once to 25 mL of the above suspension of biotin-labeled nanospheres. They were allowed to react for 2 h at 25 °C, centrifuged, washed twice with water, treated under sonication for 1 h, and resuspended in 25 mL of MES buffer (pH 7.0, IS 30 mM, 0.5% sodium azide). The suspension was stored in the dark at 4 °C.

Preparation of Polyelectrolyte Acceptor Solution. The cationic polyelectrolyte C-587 (1.0 g of the solution) and 6.92 mg (10.0 μmol) of the sodium salt of BPB were dissolved in 1 L of water to yield a solution with the following concentrations: \( c_{\text{polyelectrolyte}} = 1.0 \text{ μmol L}^{-1} \); \( c_{\text{BPB}} = 10.0 \text{ μmol L}^{-1} \). This solution with a polyelectrolyte/dye ratio of 1:10 was used undiluted for the polyelectrolyte titration experiments.

Instrumentation and Measurements. Absorption spectra were acquired on a Perkin-Elmer Lambda 14 P spectrophotometer and fluorescence excitation and emission spectra on a Perkin-Elmer LS 50 B spectrofluorometer. All nonfluorescence apparent decay time measurements were performed with a fiber-optic setup described previously. The modulation frequency depends on the ruthenium complex used and is 45 kHz for Ru(dpp). Phosphorescent dyes were excited with sinusoidally modulated light. A dual-phase lock-in amplifier DSP 830 from Stanford Research (Gilching, Germany) was used for modulating the LED and for lock-in measurements of the phase shifts of luminescence. Apparent decay times \( \tau \) were calculated from phase angles \( \theta \) obtained by a single-frequency measurement and assuming single-exponential decay profiles, where \( \nu \) is the modulation frequency, via the following equation:

\[
\tau = \tan \theta / 2\nu
\]

The optical system consists of a blue LED NSPB 500 from Nichia (Nürnberg, Germany) as light source \( (\lambda_{\text{max}} = 470 \text{ nm}) \), combined with a blue band-pass filter (FTICA) from Schott or a HT141 Bright Blue filter from Lee P.V.P. Lighting (Brussels, Belgium), a bifurcated glass fiber bundle (NA 0.46, diameter 2 mm) connected to a thermostated \( (25 \text{ °C}) \) homemade black cuvette holder and a red-sensitive photomultiplier tube (PMT, H5701-02) from Hamamatsu (Herschting, Germany). Emission light was filtered with convenient high-pass filters such as the 135 Deep Golden Amber filter from Lee P.V.P. Lighting or an OG570 filter from Schott. The quartz cuvette containing the sample was fixed in a 90° angle to the excitation light source. With this setup of a 90° detection angle, the highest possible limit of detection is achieved, i.e., a maximum signal with minimal background.

Implementation of the Polyelectrolyte Binding Study. In the polyelectrolyte binding study, RET from the phosphorescent donor nanospheres to the polyelectrolyte acceptor solution was investigated. The six samples for the titration experiment contained each 100 μL of Ru(dpp) donor suspension (PAN-COOH nanospheres \( \lambda_{\text{max}} = 678 \text{ nm} \)) and 0, 10, 20, 40, 65, and 115 μL of the polyelectrolyte acceptor solution, respectively. All samples were filled with phosphate buffer \( (\rho_{\text{phosphate}} = 10 \text{ mM}, \text{ pH } 7.0, 
IS 20 \text{ mM}) \) to a total volume of 2 mL, and the solution was mixed and incubated for 1 h. Then, phase angles were recorded.
For all titrations, the concentration of the Ru(dpp) donor nanospheres was kept constant at 33.9 mg L$^{-1}$ ($C_{\text{Ru(dpp)}}$ 0.87 $\mu$mol L$^{-1}$).

Implementation of Assay. In the avidin assay, applying the biotinylated phosphorescent donor nanospheres described above, 10 samples containing 100 $\mu$L of Ru(dpp) donor suspension (PAN-COOH/OH nanospheres labeled with biotin, $c_{\text{nanospheres}}$ 0.662 g L$^{-1}$) were mixed with 0, 10, 50, 100, 250, 500, 750, 1000, 1250, and 1500 $\mu$L of avidin ($C_{\text{avidin}}$ 9.47 $\mu$mol L$^{-1}$), respectively. MES buffer ($C_{\text{buffer}}$ 10 mM, pH 7.0, IS 30 mM) was added to a total volume of 1650 $\mu$L, and the solutions were mixed and incubated for 1 h. Afterward, 350 $\mu$L of the solution of the streptavidin (SA) labeled with AF633 ($C_{A_{\text{F633}}}$ 4.0 $\mu$mol L$^{-1}$; $M_{W_{\text{AF633}}}$ $\gg M_{W_{\text{SA}}}$) was added to each of the 10 samples and the solutions were incubated for another 1 h. Then, luminescence emission spectra were recorded and phase angles were measured.

In this assay, the concentrations of both the Ru(dpp) donor nanospheres and the AF633-labeled SA acceptor were kept constant at 33.1 mg L$^{-1}$ ($C_{\text{Ru(dpp)}}$ 0.85 $\mu$mol L$^{-1}$) and 0.7 $\mu$mol L$^{-1}$ ($C_{\text{AF633}}$ 2.41 $\mu$mol L$^{-1}$; AF633/SA ratio 3.5), respectively, so that the ratio of acceptor to donor (2.8) is constant during the whole assay as well.

RESULTS AND DISCUSSION

Choice of Luminescent Donor and Acceptor Dyes. The donor dye used in both the polyelectrolyte binding study and the avidin assay is the phosphorescent ruthenium(II) complex Ru(dpp). It has an apparent luminescence decay time in the order of 6 $\mu$s and yields bright luminescent nanospheres; its luminescence has a Stokes' shift as large as 145 nm ($\lambda_{\text{em}}$ 465 nm, $\lambda_{\text{ex}}$ 610 nm).6,16 Due to its positive charge, it strongly interacts with polymers containing negatively charged groups such as poly(acrylonitrile-co-acrylic acid). It is extracted quantitatively into the nanospheres during the preparation process. Even in a lipophilic environment, e.g., if proteins are present in the sample, no dye leaching occurs in aqueous solutions. The quantum yield is 0.38 in PAN, and the relatively large molar absorbance ($\varepsilon$ $\approx$ 28,000 L mol$^{-1}$ cm$^{-1}$) of the ruthenium complex is of further advantage. We assume that the encapsulation of the dye into the polyelectrolyte matrix is the reason for the high quantum yield. The use of phosphorescent nanospheres eliminates background fluorescence, and this leads to a higher limit of detection. The luminescence detection limit can be determined by measuring the apparent decay times of nanosphere suspensions at various dilutions. The detection limit was found to be 0.2 mg L$^{-1}$ in cuvettes and 12 mg L$^{-1}$ in microtiter plates, which correspond to a Ru(dpp) concentration of 2.8 nmol L$^{-1}$ in cuvettes and 173 nmol L$^{-1}$ in microtiter plates.

The deprotonated (blue) form of BPB served as the acceptor dye in the first experiments because its maximum absorbance (592 nm) is close to the emission of the Ru(dpp) donor and there is almost no absorbance at the excitation wavelength of the donor dye at 470 nm. BPB also has a high molar absorbance (73 000 L mol$^{-1}$ cm$^{-1}$). The deprotonated dye is double negatively charged and can thus easily form stained macromolecules with a cationic polyelectrolyte (such as C-587).

AF633 was chosen as the acceptor for the avidin assay for several reasons. First, its absorption spectrum strongly overlaps the Ru(dpp) emission spectrum and thus makes it attractive for RET applications. Its absorption and emission maximums are at 633 and 650 nm, respectively. Most importantly, it has virtually no intrinsic absorbance at the excitation wavelength of the Ru(dpp) donor (470 nm). Therefore, the phosphorescent donor is selectively excited by the blue LED. The emission of AF633 can be clearly distinguished from the emission of the Ru(dpp), which is at 610 nm. The SA conjugate of AF633 has a dye/protein ratio of 3.5.

Choice of Polyelectrolyte Matrix for Donor Encapsulation. PAN and its derivatives are attractive polymeric matrices for the encapsulation of phosphorescent dyes in micro- and nanospheres.6,15,17,18 They display an extraordinarily poor permeability for gases and ionic as well as uncharged chemical species. Hence, they can protect luminescent dyes against potential luminescence quenchers such as oxygen, with the percentage of quenching $\Delta r$ of only 3–5% (change of $r$ air/N$_2$) and a quantum yield of up to 0.40. The apparent decay time of free Ru(dpp) in water varies between 1.2 $\mu$s when saturated with air and 4.7 $\mu$s when saturated with nitrogen but can be as high as 6.0 $\mu$s in apolar organic solvents.

The nanospheres have a porous structure with a large surface-to-bulk ratio. This opens the way to create a two-sided RET assay with donor and acceptor dyes located in two different phases: Any luminescent donor dye incorporated into the nanospheres during the preparation process is well accessible to an acceptor dye bound to the surface of the nanospheres.

Transmission electron microscopic pictures (not shown) of the nanospheres before the EDC coupling with biotin showed a nearly circular shape and a diameter of roughly 10–50 nm. Static and dynamic light scattering along with laser Doppler anemometry experiments revealed a polydisperse coil with a diameter of the nanospheres of $\sim$50 nm.6

Choice of Polyelectrolyte Matrix for Acceptor Encapsulation. The C-587 poly(diallyldimethylammonium chloride) (DADMAC) coagulant is a highly effective amber liquid cationic polymer of medium molecular weight (20% polymer concentration in water, $M_{W}$ 200 000). It is not affected by the pH of the system, is effective over a wide pH range, and is instantly soluble at all concentrations.

This polyelectrolyte was used as a matrix to form macromolecules stained with BPB for the following reasons: there is a strong interaction with the double negatively charged dye to prevent dissociation, a macromolecule with a high molecular weight can be simulated for the subsequent competitive binding assay, and the polyelectrolyte/BPB acceptor solution can be easily prepared by simply mixing both matrix and dye.

Polyelectrolyte Binding Study. From the characterization of the phosphorescent nanospheres with their size in the range of 10–50 nm and their fractal structure, we conceived that a surface-located acceptor should quench the luminescence of an incorporated donor dye. To prove this, we created a novel and simple tool to test this hypothesis.6


Since the nanospheres contain negatively charged carboxy groups on their surface (due to the acrylic acid comonomer), they attract and electrostatically interact with a cationic polyelectrolyte by building a layer around the nanosphere. In case the polyelectrolyte is labeled with an acceptor dye, the resulting spatial proximity should enable RET from the donor to the acceptor, which can be detected by a decrease in the apparent decay time. This is a generally applicable tool for studying charged nanospheres by applying an acceptor of the opposite charge. The preparation of polyelectrolyte/acceptor dye aggregates can be performed simply by adding an acceptor dye of opposite charge to a polyelectrolyte solution. Such polyelectrolyte aggregates can represent a simple model of acceptor-labeled biomolecules.

In the explicit study (Figure 1), Ru(dpp)-loaded nanospheres are negatively charged due to the presence of carboxy groups. Therefore, a cationic polyelectrolyte of high molecular weight (C587, MW 200,000) was selected and stained with BPB as acceptor dye. BPB is deep blue ($\lambda_{\text{max}} = 595$ nm) and negatively charged at pH 7.0.

As can be seen in Figure 2, the emission spectrum of Ru(dpp) and the absorbance of BPB overlap significantly, which would allow RET. From Figure 3 it becomes evident that this indeed happens. With no polyelectrolyte acceptor present (and thus at 100% luminescence intensity from the donor), the apparent decay time (4.9 $\mu$s) is close to that of Ru(dpp). While increasing the concentration of the polyelectrolyte/BPB acceptor solution to a saturation limit of 54.4 nmol of polyelectrolyte L$^{-1}$, both the relative luminescence intensity (19%) and the apparent decay time (3.1 $\mu$s) drop due to RET from Ru(dpp) to BPB. Thus, the maximum percentage of intensity quenching that can be reached is 81%.

In Figure 3, it can be seen that the percentage change in the apparent decay time (37%) is rather small compared to the drastic decrease (81%) in luminescence intensity ($\lambda / \tau_0 = 1 / 1.0$). There exist two potential explanations: (1) One has to differentiate between core regions and shell regions of the nanosphere. Any Ru(dpp) donor dye located in the core is less susceptible to quenching by BPB than Ru(dpp) in the shell. (2) It is also possible to have other quenching processes involved such as charge transfer, which causes a drop in the luminescence intensity but not in the apparent decay time. Presently, it is not possible to attribute either explanation. It is very likely that both processes are operative.

The Förster distance $R_0$ (~5 nm) for this Ru(dpp)/BPB system was calculated from experimental data using the following equation (for $R_0$ in nm):

$$R_0 = \frac{0.0211 k \chi^{1/3} \Phi_D^{1/6} J(\lambda)^{1/6}}{n^{2/3}}$$

where $\chi$ is a factor describing the relative orientation in space of the transition dipoles of the donor and the acceptor, $\Phi_D$ is the quantum yield of the donor in the absence of acceptor, $J(\lambda)$ is the overlap integral between the donor’s emission and the acceptor’s absorbance, and $n$ is the refractive index of the medium. The energy rate $k_T$ from the Ru(dpp) donor to the BPB acceptor can be calculated at different donor-to-acceptor distances $r$, using the following equation, where $\tau_0$ is the apparent decay time of the donor in the absence of acceptor ($\tau_0 = 6 \mu$s):

$$k_T = \frac{1}{\tau_0} \left( \frac{R_0}{r} \right)^6$$
Even though the donor—acceptor distance is smaller here than in the final avidin assay (since there is an additional biotin—avidin bridge between the donor and acceptor dyes) and the acceptor dye is not a fluorophore as it is in the avidin assay, this polyelectrolyte study is a fast way to prove that RET can occur from nanospheres to a dye bound to the surface of the spheres. It also serves to quantify the maximum extent of quenching by BPB that is feasible ($\Delta r_{\text{q}} \approx 37\%$ between 0 and 54.4 nmol of polyelectrolyte L$^{-1}$).

**Prototype Assay for Avidin.** Based on the polyelectrolyte binding study and immunoassays described in the literature using phycoerythrin as the energy acceptor dye, we have established a RET avidin binding assay with an acceptor fluorophore—AF633—labeled SA instead of the nonluminescent acceptor dye BPB.20,21 This leads to increased luminescence signals and constant luminescence intensities since a new luminescence—that of the acceptor fluorophore at 650 nm—is stimulated in addition to the one of the donor at 610 nm. The Ru(dpp) dye is long-lived with an apparent decay time in the microsecond time region (6 $\mu$s). This enables the elimination of short-lived nanosecond background fluorescence.

The avidin assay presented is based on the scheme illustrated in Figure 4. In essence, avidin and labeled SA bind competitively to biotin on the surface of the Ru(dpp) donor nanospheres. If labeled SA binds to the nanospheres, RET occurs from Ru(dpp) (excited at 470 nm) to the label of SA, which does not measurably absorb at 470 nm. As a result, the apparent decay time of the emission of the donor decreases and the luminescence intensity of the AF633 label increases.

Figure 5 shows the absorbance and emission spectra of both the Ru(dpp) donor and the AF633 acceptor. The hatched area indicates the large overlap integral between the donor emission and the acceptor absorbance which is a prerequisite for efficient RET.

Figure 6 summarizes the apparent decay times and relative luminescence intensities of the overall luminescence signal, i.e., of the emissions of both Ru(dpp) and AF633. If no avidin analyte is present in the solution, the apparent decay time of the assay (2.5 $\mu$s) is reduced due to RET from the Ru(dpp) donor to the AF633 acceptor via the biotin—SA bridge. On the other hand, in the presence of a high avidin concentration (7.1 $\mu$mol L$^{-1}$), the apparent decay time remains that of the donor nanospheres (4.4 $\mu$s) since the biotin binding sites on the nanosphere surface are occupied by avidin.

The relatively high donor concentration ($c_{\text{Ru(dpp)}} = 0.85 \mu$mol L$^{-1}$) was chosen in order to obtain intense luminescence signals and to prove the feasibility of the assay. Naturally, a high donor concentration goes along with a high biotin concentration on the surface of the nanosphere and, thus, a poor limit of detection of the assay. However, this may be overcome by performing a time-resolved measurement. Then, much smaller concentrations can be detected.

Because constant donor and acceptor concentrations were applied, and since quenching of the donor was negligible, the relative luminescence intensities remain virtually the same, varying only between 97% and nearly 100% in contrast to the polyelectrolyte binding study (with the nonfluorescent acceptor BPB) where the relative luminescence intensities decrease sharply with increasing polyelectrolyte concentration, the overall luminescence signals remain nearly constant in the avidin assay since the acceptor AF633 is a fluorescent dye. While the Ru(dpp) emission decreases,

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the AF633 emission increases, leading to almost constant overall luminescence intensities.

Figure 7 pictures selected normalized luminescence emission spectra of that assay. Note that the emissions are normalized to 1 at the emission of the Ru(dpp) donor at 610 nm. With decreasing avidin concentration, the luminescence emission peak of the AF633 acceptor at 650 nm increases relative to the Ru(dpp) emission at 610 nm.

To prove that there was indeed specific binding of AF633-labeled SA to the biotin-labeled donor nanospheres, yet no binding of AF633-labeled SA to simply carboxylated nanospheres, and to show that any increase of the apparent decay time thus came from RET via the biotin-SA bridge, the following negative experiment was carried out. Apparent decay times and luminescence emission spectra were recorded of the following solutions: (i) a solution containing biotin-labeled Ru(dpp) donor nanospheres and AF633-labeled SA, (ii) a solution containing carboxylated Ru(dpp) donor nanospheres and AF633-labeled SA, (iii) a solution containing carboxylated Ru(dpp) donor nanospheres, and (iv) a solution containing biotin-labeled Ru(dpp) donor nanospheres (compare Figure 8). In the case of applying only the carboxylated donor nanospheres, no change in the emission spectra was observed and the apparent decay time stayed nearly constant at ~4.4 μs. No RET occurred since the SA acceptor molecules were prevented from accumulating on the nanosphere surface by the negatively charged carboxy groups. Only when the biotin-labeled donor nanospheres were used, was the AF633 peak clearly observed in the emission spectra at ~650 nm and the apparent decay time decreased from 4.4 to ~3.0 μs upon addition of acceptor.

CONCLUSION

The results presented here prove the feasibility of a novel scheme for homogeneous bioassays with RET from a donor dye incorporated into nanospheres to a surface-bound biomolecule labeled with an acceptor dye. This was demonstrated by a prototype assay for the determination of avidin. Biotin was coupled to nanospheres dyed with Ru(dpp). The assay is based on RET from such nanospheres to fluorescently labeled SA, which causes a drop in the apparent decay time of Ru(dpp) with decreasing concentration of avidin. Due to the long-luminescent apparent decay times, such assays can be performed with low-cost solid-state instrumentation. This assay concept is suitable for the detection of small biomolecules such as avidin. The determination of larger biomolecules, bacteria, or viruses may, however, turn out to be difficult due to exceeding the Förster distance, which prevents RET.

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