How Colloidal Nanoparticles Could Facilitate Multiplexed Measurements of Different Analytes with Analyte-Sensitive Organic Fluorophores

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ABSTRACT Multiplexed measurements of several analytes in parallel using analyte-sensitive organic fluorophores can be hampered by spectral overlap of the different fluorophores. The authors discuss how nanoparticles can help to overcome this problem. First, different organic fluorophores can be separated spatially by confining them to separate containers, each bearing a nanoparticle-based barcode. Second, by coupling different fluorophores to nanoparticles with different fluorescence lifetimes that serve as donors for excitation transfer, the effective fluorescence lifetime of the organic fluorophores as acceptors can be tuned by fluorescence resonance energy transfer (FRET). Thus, the fluorophores can be distinguished by their effective lifetimes. This is an example of how the modification of classical functional materials has already yielded improved and even new functionalities by the integration of nanoparticles with hybrid materials. We outline future opportunities in this area.

Sensing of analytes is important for a large variation of applications. One common detection technique is fluorescence detection of analyte-sensitive fluorophores. Such analyte-sensitive fluorophores are (often organic) fluorescence dyes, of which (in general) the fluorescence emission intensity selectively depends on the presence of a specific analyte. The presence of an analyte can either enhance or quench the fluorescence, depending on the chemical nature of the fluorescence dye. Such fluorophores are available for many different analytes, such as H^+,[1] K^+,[2,3] Na^+,[4,5] and Cl^- ions,[6,7] *etc*. Naturally, one would like to use several of these fluorophores in parallel in order to determine simultaneously the concentration of different analytes.[8] Leaving aside the fact that the selectivity of many fluorophores to the target ligand is sometimes not as high as required, there is one fundamental problem for such multiplexed detection. The response of different fluorophores can (at simultaneous excitation) only be distinguished in the case where they emit at different wavelengths (λ). Although a few fluorophores can be independently detected, the number of fluorophores that can be spectrally distinguished in the optical regime is clearly limited and thus hinders multiplexing (Figure 1).

RESOLVING FLUOROPHORE EMISSIONS WITH HYBRID MATERIALS

Nanotechnology, which enables the design and assembly of new materials,[9] offers an exciting toolkit for this purpose. In the case of sensing, we argue that colloidal nanoparticles, as an integral building block of nanotechnology, have the potential to improve existing materials—classic analyte-sensitive fluorophores—leading to new advanced hybrid material. Hereby the functional part, the emission intensity, which depends on the concentration of a specific analyte, would be provided by traditional analyte-sensitive fluorophores. Organic macromolecules/biological molecules would form the glue with which to assemble colloidal nanoparticles as recognition/transducer elements with analyte-sensitive fluorophores.

For such an improved hybrid material, different resolution mechanisms (besides spectral resolution) could be employed. In fact, emission of different fluorophores cannot only be distinguished spectrally (*i.e.*, in dependence of the wavelength λ_{em}) but also spatially (*i.e.*, in dependence of the location x_{em}). The emission of two fluorophores can be distinguished, even if the emission spectra completely overlap. This could be achieved when the two fluorophores are not at the same position, that is, the distance is bigger than the optical resolution limit. However, in order to distinguish between different fluorophores, each of them would have to be provided with a recognition element. Fluorescent nanoparticles in the form of colloidal quantum dots have been demonstrated to form suitable fluorescent barcodes as recognition elements.[10−12] To combine quantum-dot-based barcodes and analyte-sensitive fluorophores, the fluorophores could be integrated in a carrier matrix[13−18] with the quantum-dot barcode on the surface of the carrier.[19] Polyelectrolyte capsules[20] have proven to be a versatile system for this

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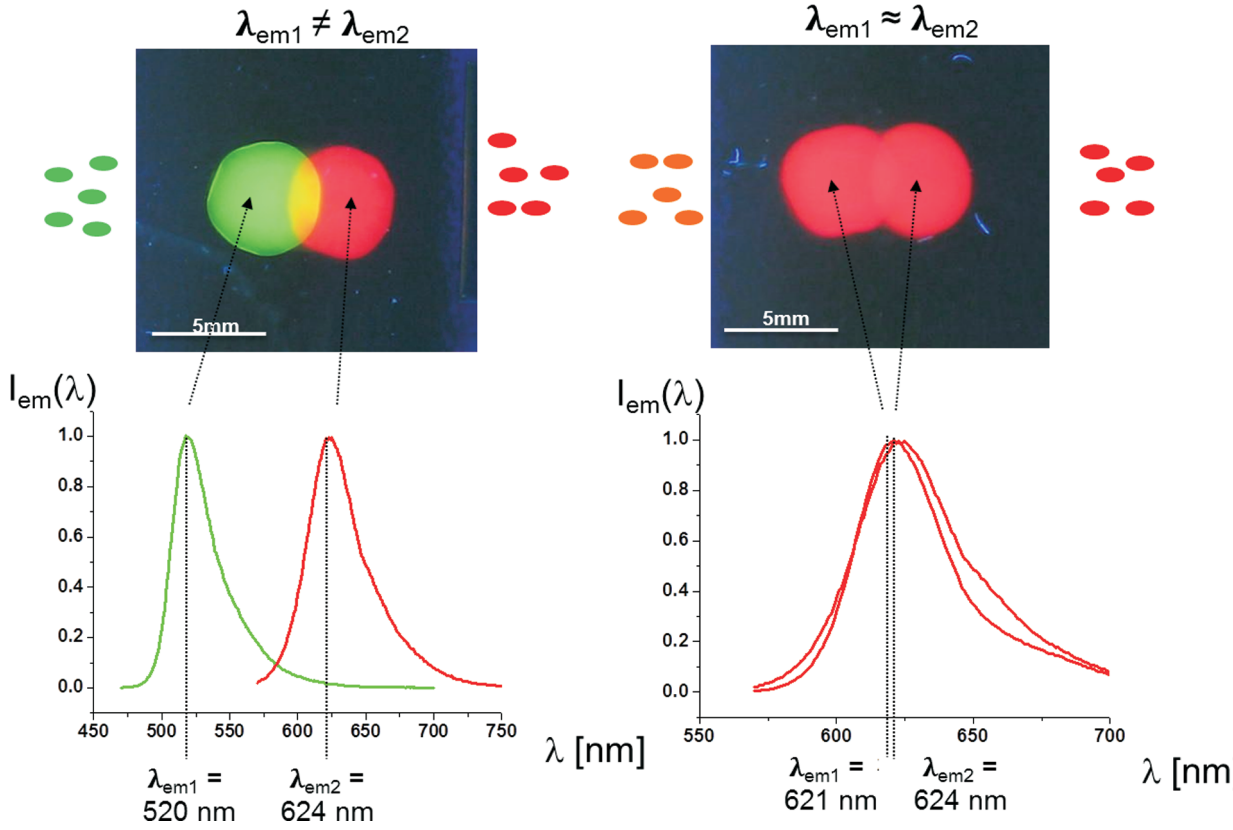

Figure 1. Spectral separation of two different fluorophores. (Left side) Two drops of water mixed with either fluorescein isothiocyanate (FITC) or cresyl violet perchlorate (CV) have been put on two glass coverslips placed on top of each other. The glass coverslips were put onto an UV illumination table and the fluorescence of the drops was imaged with a digital camera. Corresponding normalized fluorescence emission spectra $I_{em}(\lambda)$ have been recorded with a fluorescence spectrometer. As both fluorophores emit at different wavelengths (FITC, λ_{em} = 520 nm; CV, λ_{em} = 623 nm) they both can be distinguished by their color, as can be seen in the regime where the drops overlap with each other. (Right side) The same experiment was performed, but now with two drops containing either CV or ATTO-590 NHS-ester (ATTO-590). As both dyes emit at very similar wavelengths (CV, λ_{em} = 621 nm; ATTO-590, λ_{em} = 624 nm) they cannot be optically resolved. In the contact zone of the two drops, the contribution of both dyes cannot be spectrally distinguished as $\lambda_{em1} \approx \lambda_{em2}$.

purpose[17,18] (Figure 2). If such capsules have diameters ≥0.5 μm, which are larger than the optical resolution limit, fluorescence from their walls can be distinguished by the fluorescence from their cavity. In this way, individual capsules could be read out in a single-particle manner. First, observation of the barcode would provide information about which analyte-sensitive fluorophore is embedded in the cavity of the respective capsule. Second, evaluation of the fluorescence emission originating from the capsule cavity would lead to the concentration of the analyte to which this capsule is sensitive. These carriers could be spatially resolved and simultaneously read out on a single-particle basis. Thus, by confining different analyte-sensitive fluorophores to different carriers, multiplexed measurements of several analytes would be possible, even if the emission spectra of the different analyte-sensitive fluorophores overlap. A drawback is that this method would be limited to particles larger than the optical resolution limit.

Fluorescence emission of different fluorophores can also be resolved in time. If the decay times τ_{em} of different fluorophores are distinct, the contribution of the respective fluorophores could, in principle, be determined from time-resolved fluorescence measurements.[21,22] This is the basis of time-gated/fluorescence lifetime imaging (FLIM).[23,24] High-quality core−shell quantum dots typically have decay times longer than those of organic fluorophores and thus can be resolved in time-gated measurements.[25] However, to find analyte-sensitive fluorophores where all possess different emission lifetimes, τ_{em}, is unlikely. Here again, nanoparticles in the form of quantum dots could help. If the emission of a quantum dot overlaps with the absorption of an adjacent fluorophore, the excitation of the quantum dot (donor) can lead to a transfer of energy to the fluorophore (acceptor). The resulting effect is the emission of the fluorophore,[26] a phenomenon called fluorescence resonance energy transfer (FRET).[27] One method of creating such an assembly is embedding fluorophores in a polymer shell around quantum dots.[28,29] Importantly, the effective lifetime of the acceptor will be determined by the lifetime of the donor[30] (Figure 3). Donor lifetimes can even be

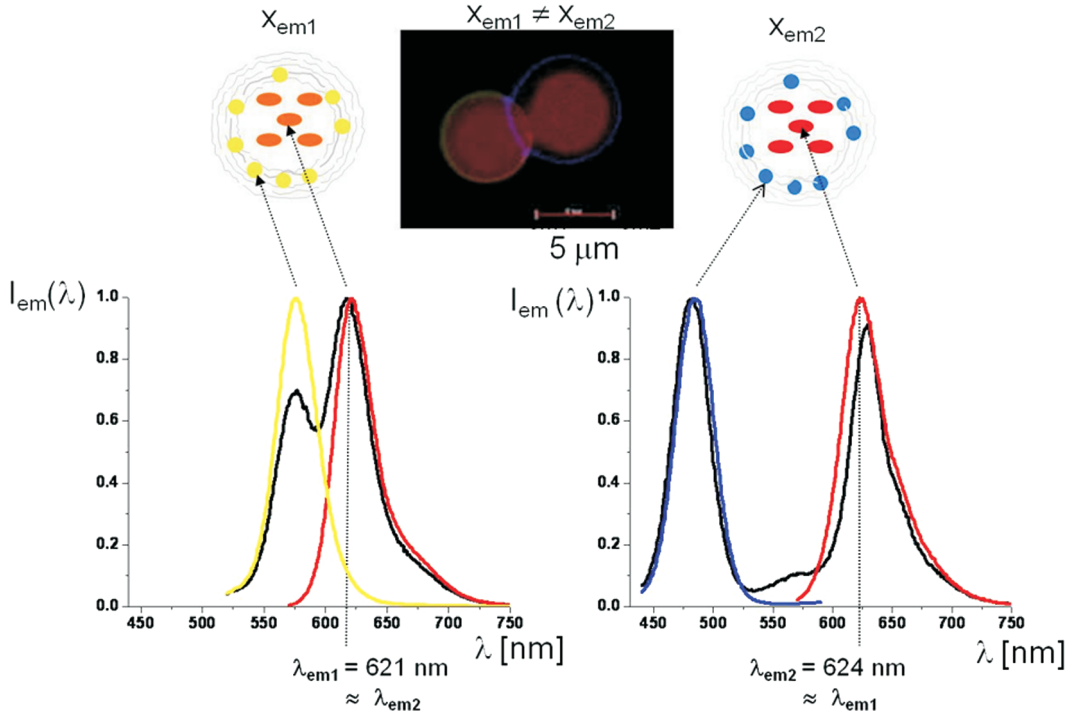

Figure 2. Polyelectrolyte capsules were assembled with layer-by-layer deposition around $CaCO_3$ template cores that had been loaded either with CV conjugated to dextran or ATTO590 conjugated to dextran, followed by dissolution of the template cores, according to previously published protocols.[17,18] Yellow and blue polymer-coated CdSe/ZnS quantum dots[29] were integrated in the polyelectrolyte walls according to earlier reports.[19] This led to two different types of capsules. The first type had CV fluorophore conjugated to dextran in their cavity, and their wall was labeled with yellow fluorescent quantum dots. The second type had ATTO590 fluorophore conjugated to dextran in their cavity, and their wall was labeled with blue fluorescent quantum dots. The capsule walls were permeable to ions.[18] The image shows an overlay of fluorescence images recorded with a blue, yellow, and red filter-set. Though CV and ATTO590 cannot be well resolved spectrally ($\lambda_{em1} \approx \lambda_{em2}$) the respective types of each capsule can be recognized on a single capsule level by the color of fluorescence of the wall when the capsules are located at different positions ($x_{em1} \neq x_{em2}$). Because the type of each capsule can be identified, it is known which fluorophore is inside each capsule and thus one can spatially distinguish the emission of the different fluorophores.

further increased by doping of the quantum dots with transition metal elements, such as Mn^{2+}.[31−33] Effective donor lifetimes lasting hundreds of microseconds can be achieved because the internal Mn^{2+} $3d^5$ transitions are dipole-forbidden by spin and parity selection rules. The selection rules are partly relaxed by spin−orbit coupling and p−d hybridization.[34−36] In this way, the effective lifetimes τ_{em} of different analyte-sensitive fluorophores could be tuned by linking them to different donor nanoparticles, each of which possess significantly different lifetimes.

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CONCLUSIONS AND PROSPECTS

In this way, the functionality of traditional analyte-sensitive fluorophores could be improved with the help of nanotechnology. We have outlined the perspective of making hybrid materials of analyte-sensitive fluorophores and colloidal quantum dots, and of making use of the fact that fluorescence emission $I_{em} = I_{em}(\lambda_{em}, x_{em}, \tau_{em})$ can be resolved spectrally (λ_{em}), spatially (x_{em}), and temporally (τ_{em}). This demonstrates that combining nanotechnological compounds such as colloidal nanoparticles with classical molecular compounds could make novel hybrid materials with advanced properties. Naturally, the given example covers only one small area of research and development. Indeed, we believe that by adding nanotechnologically created compounds to traditional materials, hybrid materials with improved properties in a wide context can be created in the future.

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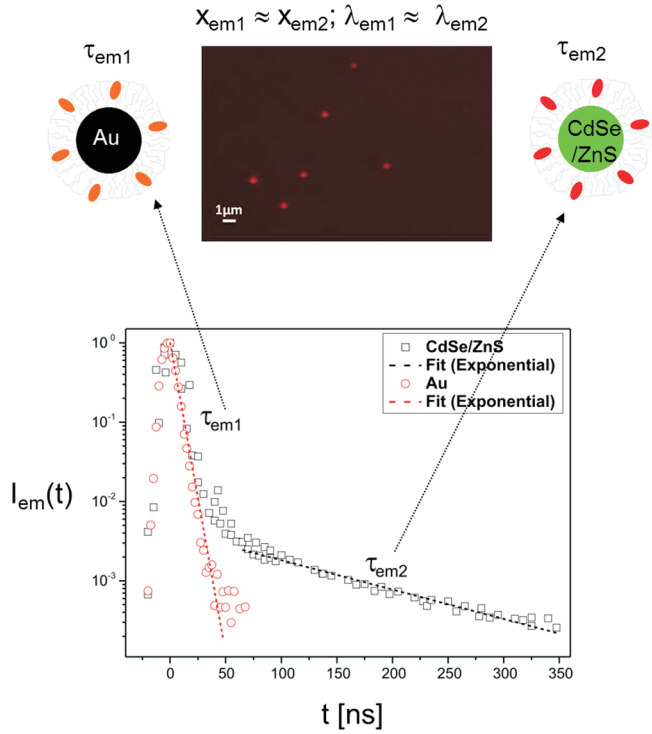

Figure 3. The time-dependent fluorescence emission $I_{em}(t)$ is shown for two different fluorophores: CV and ATTO590, which have similar fluorescence lifetimes. CV fluorophores and ATTO590 fluorophores were integrated in the polymer shell of colloidal Au and CdSe/ZnS nanoparticles, respectively, following previously published protocols.[28,29] Coupling CV to the surface of Au nanoparticles did not significantly affect the fluorescence lifetime τ_{em1} of CV (red circles).[30] On the contrary, the effective lifetime τ_{em2} of ATTO590 (black squares) was increased by FRET with the underlying CdSe/ZnS nanoparticle. In this way, the effective lifetimes of two fluorophores have been put in a different regime by coupling them to different types of nanoparticles.

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