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Paper

An Amplified Fluorescence Emission of Bolaamphiphilic Perylene-Azacrown Ether Derivatives Directed towards Molecular Recognition Events

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Bappaditya Roy,[a] Takao Noguchi,[a,b] Daisuke Yoshihara,[b] Tasuhiro Yamamoto,[b] Junji Sakamoto,[b] and Seiji Shinkai*[a,b,c]

Long-term creative approaches have been considered in the molecular probe design to overcome the quenching effect of the important dyes in aqueous medium. Using the rational donor- acceptor based design principle, we here demonstrate different fluorescence states of an non-conjugated symmetrical perylene-azacrown ether system in solution: from the molecular to the aggregate states. The ethylene-spacer, exceptionally, is capable of fluorescence enhancement even in the aggregated state (organic nanoparticle, ONPs, 44 nm) overcoming the quenching effect on changing the solvent from tetrahydrofuran to water. The ONPs with crown ether receptor at the surface show colloidal stability in aqueous solution. Further, an improved fluorescent state is developed via ONPs-polymer (protamine, Pro) hybridization. Supramolecular interactions between the crown ring and guanidinium group in Pro play an important role in the ONPs-Pro hybrid formation. The decorated fluorescent hybrid state is finally used as a nano-probe for the sensing of heparin via the turn-OFF mechanism. The decoration method is further generalized by recognition of the nucleotides. Herein, we detail the bottom-up approach to the molecular design and development of the different fluorescent states of an useful probe. Most excitingly, this new approach is very general and adaptive to facile detection.

Introduction

The aggregation-induced emission (AIE)[1] and photoinduced electron transfer (PET)[2] approaches have been used to design fluorescent probes unimolecularly as well as in the aggregated states which lead to a fluorescence (FL) turn-ON response in the presence of guest. In contrast, aggregation-caused quenching (ACQ)[3] dyes have their own limitations as they form FL OFF-state while aggregate, and perylene bisimide (PBI) is one of the useful candidate among ACQ series. So far the design of perylene-based probes have been suffered from their extremely low fluorescent backgrounds in aqueous solution.[4] An approach is made, therefore, to realize a wide range of FL intensity: from a completely OFF background to highly intense FL-state by rolling the process through discrete molecular states to achieve the desired targets. In this present study, we have introduced a synergistic approach to overcome this limitation and realize a wide range of FL intensity.

The prerequisite quadrupolar molecular structures (Figure 1) were designed for the molecular recognition by considering: (i) donor-acceptor (D-A), (ii) guest receptor site, and (iii) water dispersity. The 1-aza-18-crown-6-ether (ACE) group fitted well into those criteria, and the PBI was chosen to act as an efficient acceptor. For assembly formation our strategy was based on the well-established "push-pull" mechanism that depicts when a conjugated D-A system forms aggregate or crystal, the FL efficiency is changed compared to its monomeric state.[5] The keen interest towards D-A systems was considered to use the tailored "push-pull" mechanism,[6] and the formation of the desired structure would exhibit different optical properties in the aqueous solution. A suitable solvent composition allows the generation of the fluorescent organic nanoparticles (ONPs) that consent to tune the ground-state electronic structure, and a polarization through electron delocalization along the conjugated ways permitting maximum dipoles with minimum energy gaps to reach 'cyanine-limit'.[7] Instead of a conjugated D-A systems, our approach is more close to the original concept but it allows electron transfer 'through space' (except PyBzACE). Now, these D-A systems were allowed to exhibit optical changes similar to those in the conjugated systems by changing the solvent composition. Motivated by the deficiency, we targeted on the development of FL ON-state in the aggregated microenvironment of ACQ-dyes. The solubility in water and the PET effect were found different for all the synthesized ACE-derivatives.[8] Among all, PyC3ACE and PyC4ACE showed the highest FL intensity with minimum PET efficiencies, whereas PyBzACE and PyC2DMA have the highest PET efficiencies, and showed the minimum FL intensities. Only the other ethylene-spacer derivatives (PyC2ACE and PyC2EG) exhibited moderate PET / FL properties and generated

[a] Institute for Advanced Study, Kyushu University, 744 Moto-oka, Nishi-ku, Fukuoka 819-0395, Japan
[b] Nanotechnology Laboratory, Institute of Systems, Information Technologies and Nanotechnologies (ISIT), 4-1 Kyudai-Shinmachi, Nishi-ku, Fukuoka 819-0388, Japan
[c] Department of Nanoscience, Faculty of Engineering, Sojo University, 4-22-1 Ikeda, Nishi-ku, Kumamoto 860-0082, Japan
E-mail: shinkai_center@mail.cstm.kyushu-u.ac.jp
Fax: (+81)92-805-3814
SI contains the details about the synthesis procedure, NMR data, experimental procedures and supporting data, and details about the molecular recognition processes.

strong fluorescent colors by changing the solvent from THF to water. Again, the concerned FL generation is not the real AIE emission;[1] however relating to both the observations that can be

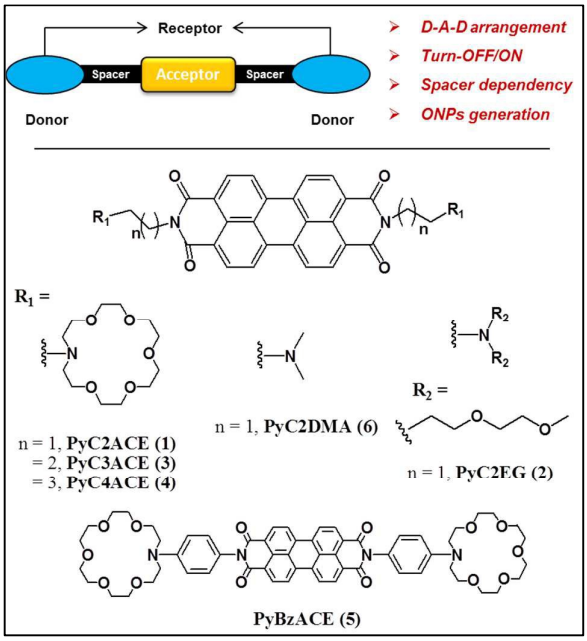

Figure 1. The molecular design principle for the receptor-based donor-acceptor probes with low fluorescent background, and the chemical structures of the dumbbell-shaped perylene derivatives bearing 1-aza-18-crown-6, dimethylamine, and *N,N*-bis(2-(2-methoxyethoxy)ethane)amine appended symmetrically at both the imide positions.

termed as a pseudo-AIE phenomenon. In similar experiments, the other derivatives showed quenching property which is most general. Therefore we concentrated only on the PyC2ACE-derivative. The fluorescent PyC2ACE nano-aggregates or ONPs extend the high surface area and the receptor site on the surface, and are further used as a template to form ONPs-polymer hybrid which showed advanced FL enhancement. In the following segments we have established the cation binding study in unimolecular state, solvent induced aggregated ONPs formation and FL switching, and finally promoted the biomolecular recognition events with the polymer-decorated amplified FL-state.

Results and Discussion

The basic understanding behind the design principle of the azacrown tethered perylene-cored donor-acceptor type derivatives is to enjoy the FL intensity change in the presence of cationic guests. The selected PyC2ACE with its moderate optical properties in THF was considered as molecularly dispersed enough to show FL 'OFF-ON' switching. A turn-ON FL response was observed when a large number of cationic guests, e.g., metal ions (M^{n+}), ammonium ion, and acidic proton were introduced to the fluorophore.[8] Therefore, a prospect lies there to recover the lost FL intensity of the derivatives in the aggregated state. So, we imaged to build a molecular state via induced aggregation where the fluorophore units will remain in a metastable position that could enable the FL property to reach at different states which could further be easily disturbed by the external guests.

(1) Molecular Aggregation and Generation of Fluorescent ONPs

For assembly formation, we first solubilized the fluorophores in THF, homogeneously, and further blended it with water at different fraction ($f_{w,\%}$) varying the solvent polarity and the solvation power. The difference in the solubility in THF and water remained as a critical condition for the ease and rapid production of the aggregate in the simple solvent mixing method. For PyC2ACE, exceptionally,[9] a continuous FL enhancement was observed from the very first added fraction of water in THF (Figure 2a). The continuous enhancement was observed up to $f_{w,70\%}$ by a relative FL enhancement (I_R) of about 50 times; further, a decrease in the intensity was observed (Figure 2b). Interestingly, at the higher fraction ($f_{w,90\%}$) of water the FL intensity was still at the satisfactory level ($I_R \approx 17$ times). The resulting solutions appeared as limpid and stable over a few months, and at the higher fraction the solutions appeared as bright red-color under the normal light (Figure 2a). This apparent behavior in the dispersity and stability is unambiguously ascribed to the role of ACE which helps to stabilize the colloidal dispersions by promoting the solvation. The '*Hydrophile-Lipophile Balance (HLB)*' value,[8] which is a key property index for the amphiphiles, was calculated for envisaging the structure formation by the molecules. With a HLB value of 10.83, PyC2ACE showed the highest colloidal stability, and the corresponding zeta-potential was found as 0·79 mV in the colloidal state of a dialyzed sample in ~100% aqueous solution.[8] The colloidal states at different $f_{w,\%}$ appeared as particle morphology under the FL microscope. At $f_{w,30\%}$, the PyC2ACE particles start to get well-shaped and homogeneously distributed in the solution.[8] The AFM and TEM measurements[8] are close to uniform with an average size of 45-50 nm indicating the formation of well-defined discrete nanoparticles. Further, a single-nanoparticle was characterized,[8] both in solution and in dried state, under the FL microscope. The FL color of the ONPs changed from yellow to red and a 128 nm long spectrum redshift was found in the dried ONPs. The excimer and charge transfer interaction carried a combined contribution to the observed red color and the higher peak generation in the emission spectrum.[7,10]

For added structural studies we measured X-ray scattering and excited state lifetime values in the dry and solution state of the aggregated PBI-ACE systems, correspondingly.[8] It showed more amorphous character for the ethylene-spacers compared to the other derivatives. This assumes to prefer that the ethylene spacer attained local mismatch (state I) during the aggregation, whereas more chromophoric π-stacking was experienced (state II) by the longer and the aromatic spacers (Figure 2d). One may consider, therefore, that the PET effect can also be responsible for the FL enhancement; as in the presence of water, the highly basic tertiary 'N' could be protonated.[11] So we did a pH titration experiment with the dialyzed PyC2ACE nanoparticles at $f_{w\sim100\%}$ (pH = 6.7).[8] The sensitivity was checked in a wide range of pH = 3-11, and the result appeared as a sigmoidal change (Figure 3a). At acidic pH, the particles showed FL enhancement along with the disruption of the particle structures.[8] Whereas, at the basic condition, the structure remained same but a strong quenching was observed. As a result, the PET effect was stopped in the aggregated state by the water molecules, and FL enhancement was observed.[12] The above discussed two results, PET efficiency and the spacer type, perhaps

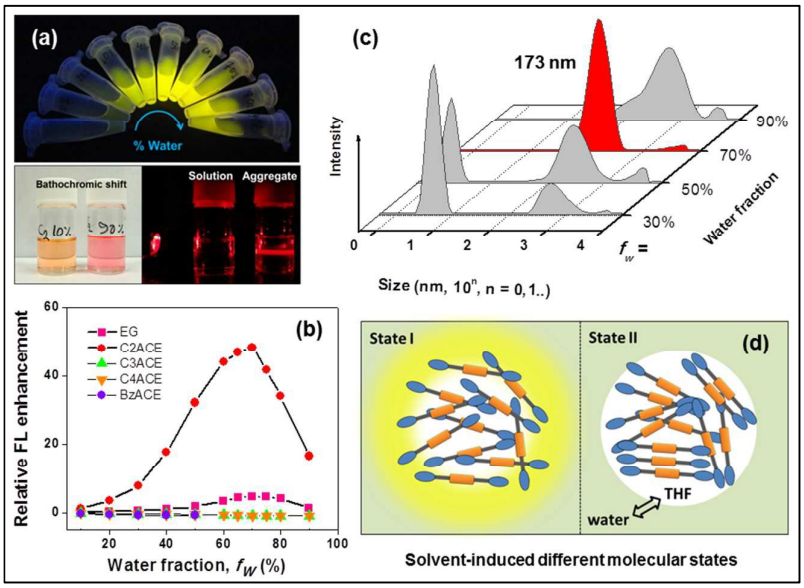

Figure 2. (a) Solvent-induced FL generation from the OFF-state of PyC2ACE derivative in THF: the aggregate formation was confirmed by the visual colorimetric change from orange to red ($f_{w,\%}$ = 10-90), and the corresponding '*Rayleigh Scattering*' experiment (laser, λ = 635 nm); (b) relative intensity changes of the different perylene-derivatives are plotted against the increased $f_{w,\%}$ into the THF solutions at 10·0 μM and 20 °C; (c) the hydrodynamic sizes of PyC2ACE (10·0 μM) at different $f_{w,\%}$ and at 20 °C are plotted to compare the change; and (d) State I (fluorescent) and State II (non-fluorescent) represent the two different arrangements acquired by the ACE-derivatives in water-THF mixture solvent: at higher $f_{w,\%}$, the water molecules are present in numbers surrounding the THF molecules forming a core-shell type solvent arrangement.

pretend in accordance to consider the fluorescent ONPs generation as a result of the critical spacer length.

After considering the fact about molecular constrain we decided to change the environmental rigidity by changing the solvent viscosity. The effect of the viscosity change was measured in FL spectrometer by preparing a stock solution of PyC2ACE in ethylene glycol (EG, η = 1.61 X 10^{-2} Pa.s), and then gradually increasing the fraction of glycerol (a high viscous liquid, η = 1.41 Pa.s) (Figure 3c). Recently, Xu et al.[13] proposed a strong correlation between viscosity and PET of the donor-acceptor systems. The FL change of these systems can be ascribed to a viscosity-dependent conformational change. A similar increase in the FL intensity was observed up to a certain value of viscosity, but a small decrease in the intensity was found at the higher viscosity. So, it can now be proposed that a free molecule can remains in a balanced state between condensed (U-shaped, the crown ring is much closer to PBI moiety) state and extended (N-shaped) state of the D-A-D system (Figure 3d).[8] We can consider, therefore, that the ethylene-spacer is very special from the FL viewpoint in the molecular self-assembly. However, detail studies describing generation of these special FL properties are needed to be considered. Hence, we have successfully established a new fluorescent state in the aggregated form, and now ready to use its functionality towards biomolecular recogntion. The colloidal state of the ONPs was found stable over a month and also under the thermal or photo-conditions.[8] The stability check in the acidic and salt conditions implies that external stimuli can generate a disturbance surrounding the neutral particle surfaces.[8]

(2) Surface Modification and Guest-induced Fluorescence Amplification

An ONP-surface with large surface-to-volume-ratio and charge density close to zero provided a convenient platform for surface modification and bioconjugation.[4d] The highly stable ONPs-state of this receptor-based donor-acceptor system was further applied for FL enhancement in the presence of cationic guests. However, it is also well established that solvents compete during the intermolecular bond formation that results in lowering the binding constants; the binding ability of receptor is strongly affected by the polarity of solvents. In water, the ether lone pairs of the crown ring are oriented toward exterior, and the guest coordination becomes energetically less favorable.[7a,14] However at $f_{w,70\%}$, they showed significant FL enhancement.[8] So, other important cationic guests of interest, e.g., protamine **(Pro)**, arginine, poly-lysine, and simple poly-allylamine were used to form ONPs-guest hybrids. The advantage of having a receptor-based neutral surface is that one can functionalize the surface by using simple supramolecular concept, and can select the functionality according to the desired target. During this work, it was found that the signaling contrast becomes more recognizable with **Pro**, an arginine-rich nuclear protein, as a cationic guest instead of the metal-ions. **Pro,** an important biomedical component was introduced onto the ONPs surface to form a functionalized cationic ONPs-polymer hybrid which showed highest FL intensity compared to the preceding FL-states. The stepwise FL change appeared as steady enhancement along with the increased **Pro** concentration, and finally reached a plateau (Figure 4b). A co-assembled network structure of the ONPs-polymer hybrid was found in the TEM image where the less electron-dense polymer chains form a network around darker PBI NPs (marked as arrows, Figure 5e). There might be a chance of partial dissolution of the probe molecules within the networked structure, but not in the solution. The absorbance change[8] at the same chromophoric concentration indicates that a hyperchromic effect is operating, which is mainly caused by the disturbance in the aggregated ONPs structure via ONPs-polymer interactions (state I*).

(3) Molecular Recognition Using ONPs-polymer Hybrid

The interaction of proteins tends to depend on the specific functionality of the ONPs surface. A comparative study by *Shea et al.*[15] revealed that the guanidinium functionalized NPs, compared to primary amino and quaternary ammonium functional groups, showed significantly higher binding towards fibrinogen that is attributed to strong and specific interactions with carboxylate groups on the protein surface. Heparin (**Hep**), known as the most negatively charged biological macromolecule and a clinically important biopolymer.[16a,b] The charge neutralization of **Hep** with **Pro** via the ionic interaction is a very interesting field of research, and a number of reports have been added in this context.[16c] In addition to **Hep**, many other poly-anions were also found potentially competitors for binding. A series of important neutral or

anionic molecules and biopolymers (1·0 mM / 1·0 mg/ml) were tested to evaluate the selectivity of these modified cationic ONPs.

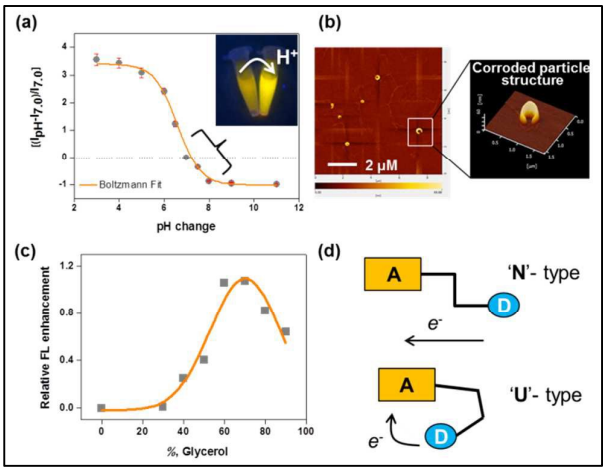

Figure 3. (a) Relative intensity changes are plotted against the pH change [inset: photograph of dialyzed ($f_{w,\ 100\%}$) ONPs solution at different conditions]; (b) structural characterization of ONPs in the presence of K^+-ions under the AFM; (c) The relative intensity change of PyC2ACE (10·0 μM, EG) is plotted against the increasing solvent viscosity by the addition of glycerol; and (d) schematic representation of the possible 'N' and 'U'-type arrangements is sketched according to the free rotation of the alkyl-bridged perylene-ACE systems.

The experiment was done *in vitro* at a specific physiological condition of pH 7·2 maintained by using 1·0 mM HEPES buffer in $f_{w,70\%}$ solution of the 6·8 μM PyC2ACE ONPs-polymer hybrid. It was found that **Hep** exhibits highest response towards the hybrid state by quenching the FL intensity via a turn-OFF mechanism. As shown in Figure 4b, most of these anions and biomolecules cause interferences; although chondroitin sulfate C (CS-C) and hyaluronic acid (HA), which are analogous to **Hep**, showed almost negligible response. The selectivity of the hybrid state was found as in the order of **Hep**>TSC>>CS-C>HA. This suggests that the electrostatic interactions (high charge density of **Hep**) and the conformation of the sugar dimer played a dominant role in the binding event. All these results indicate that the decorated and amplified FL-state of ONPs can now meet the specific requirements for **Hep** detection. The experimental design and the step-wise FL change lead to a final conclusion that a strategic combination of the molecular design and the self-assembly principle could facilitate the requirement for profiling the FL-states of a material as desired.

A concentration dependent study of the present FL-state was further carried out in a fixed ONPs-polymer hybrid concentration ($f_{w,70\%}$, 6·8 μM PyC2ACE, and 0·75 mg/ml of **Pro**) and by varying the **Hep** fraction. The FL intensity gradually decreased with the increasing **Hep** concentration, and a complete turn-OFF plateau was obtained at a minimum concentration of 0·75 mg/ml (75·0 IU/ml). Commonly, a fixed dose of 1·0-1·3 mg of **Pro** is used for neutralization of 1·0 mg (or 100·0 IU) of **Hep**. In the clinical practice, 1·0 IU of **Pro** is referred for the neutralization of 1·0 IU of **Hep** (i.e., 1·0 mg of **Pro** corresponds to 100·0 IU).[17] Our results indeed pointed out the possibility of 1:1 binding of **Pro** with **Hep** in the hybrid state. This implies that the decorated hybrid system could be useful for the determination of the **Hep** concentration in the clinical sample solution. For this, we further prepared different ONPs-polymer hybrid solutions varying the **Pro** concentration (10·0 -100·0 IU). Addition of **Hep** into those hybrid solutions caused a gradual FL quenching, and we found a saturation point for each. The quenched states reached the plateau quantitatively depending on the initially used **Pro** concentrations during the sample preparation.[8] If we compare the spectral changes and TEM images of the ONPs-**Pro** hybrid state in the absence and presence of the added **Hep** as guest, it is very clear that the metastable hybrid state was disturbed in the presence of **Hep**.[8] However, addition of **Hep** removes **Pro** from the system, and the distorted arrangement in the microenvironment starts to form the aggregated particle structure (State II, Figure 5).[8] However, this time, the aggregation mode[8] is not in favor of forming fluorescent ONPs; instead, they form a turn-OFF state. To establish more usefulness of this present system in bioassay, the detection system was rechecked in the presence of human fetal bovine serum (FBS) in a 1·0 mM HEPES buffered solution ($f_{w,70\%}$, pH 7·2). The statistical comparison (Figure 4c) of the

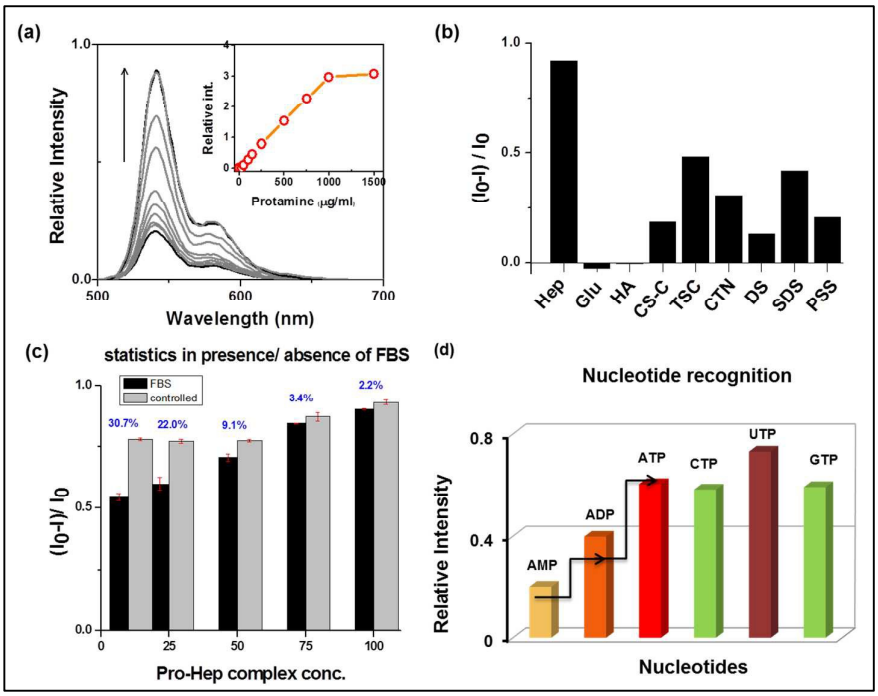

Figure 4. (a) FL intensity of the ONPs (8·0 μM) increases with the addition of the guest, **pro** in 1·0 mM pH 7·4 HEPES buffer at 20 °C [inset: relative intensity, $((I-I_0)/I_0)$ vs. **Pro** concentration (μg/ml)]; (b) a histogram is prepared to compare the effect of different anionic/ neutral guests into the ONPs-**Pro** hybrid (6.4 μM) to quench the enhanced intensity; (c) **Hep** sensing is statistically compared in the presence and absence of FBS (error limit is expressed in percent unit); (d) A histogram is prepared to compare the quenching efficiencies of different nucleotides (as anionic guests, below) in the ONPs-polymer hybrid solution. The sparkline among the adenosine-nucleotides is drawn to show the change in the response, and is clearly depend on the number of anionic charges (AMP-ATP).

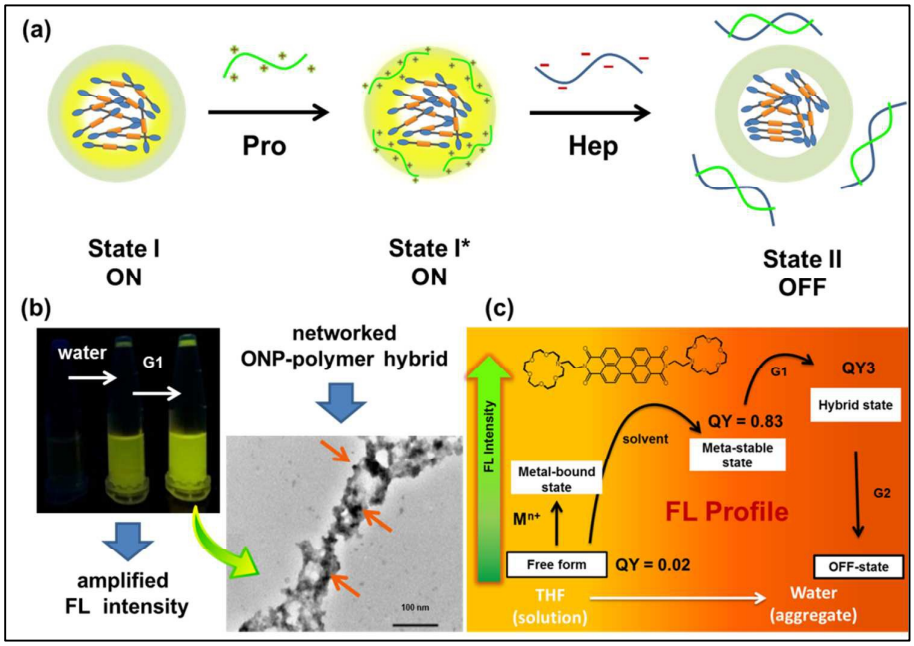

Figure 5. Fluorescence Profiling: (a) schematic presentation of the different FL-states generated by the PyC2ACE derivative under the different conditions; (b) photographs of the PyC2ACE solutions at different conditions (in THF, $f_{w,70\%}$, and in the presence of cationic guest (G1)), and the TEM image of the ONPs-**Pro** hybrid state; and (c) schematic presentation of the step-wise generation of the different FL-states (QY, quantum yield) by changing the solvent polarity and by adding the guests (G).

FL quenching at the equivalence point at different concentrations supports the applicability of the sensor system at the higher concentration range of either the sensory system or the **Pro**-**Hep** complex. Hence, we can justify our results to be useful for a quantitative measurement of **Hep** concentration in solution.

The surface modification method has been generalized by applying the cationic ONPs-polymer hybrid system for the detection of nucleotides. It is well-known that the nucleotides are the structural components of DNA and RNA. The phosphate groups in the nucleotides carry anionic charges, which encourages us to confirm the diversity of our designed recognition system. So, we selected more common nucleotides, monophosphate to triphosphate, which carry different number of anionic charges. The experiment was done *in vitro* with 1·0 mM nucleotides at a specific physiological condition of pH 7·2 and 1·0 mM HEPES buffer in $f_{w,70\%}$ solution of the ONPs-polymer hybrid containing 6·8 µM ONPs-polymer hybrid. It is already been discussed that the charge density on the anionic guest has the important contribution in the signal generation. Eventually, the monophosphate showed lowest quenching efficiency (Figure 4d). The potential signal contrast was found maximum for triphosphates (I_R of AMP:ADP:ATP = 3·4:6·5:1·0), and among other, UTP showed maximum quenching ability[18]. This establishes that a careful selection of the polymer matrix and fine-tuning of the surface charge could lead to a targeted molecular recognition.

Conclusions

In summary, we have successfully designed and synthesized receptor-based D-A systems to overcome ACQ of PBI dyes via the solvent induced self-assembly mechanism. The variable spacer types establish a requirement for the metastable state formation and so as to generate the aggregated fluorescent state, i.e., ONPs. The ethylene-spacered PBI derivative exhibited impressive dual FL enhancement; first, in the addition of K^+-ion to the molecularly dispersed state in solution, and next, in the solvent induced aggregated state. The formation of ONPs in the aggregated state offers a neutral surface with the crown ether receptor as active site (state I). The FL property was further amplified in the ONPs-polymer hybrid form. A cationic biopolymer, **Pro**, was selected to modify the ONPs surface, which further lifts the FL-state to a higher level (state I*). The selection process of **Pro** confirmed the targeted molecular recognition of **Hep** anticoagulant. The experimental set-ups and results established the quantitative (1:1) measurement of **Hep** in the sample solution. Further, the cationic ONPs surface offers a facile detection process which was established by the recognition of different nucleotides. The assumptions and conclusions are fully supported by the experimental results. In this process we have established different stable fluorescent states that reflect resemblance to the energy profile in a 'Jablonski diagram' (Figure 5c). The schematic approach to a directed molecular recognition and the subsequent FL changes have been successfully established in this study via an artificial platform for the visualization of the different stable FL-states and their utilization for the practical recognition events, such as, protein activity in the living cell, dynamicity in supramolecular chemistry (ATP hydrolysis),[19] monitoring the non-equilibrium processes in vitro, etc.

Acknowledgements

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12 To clarify this assumption, we further synthesized ClPyC3ACE that has significant PET effect compared to its counter molecule PyC3ACE (both have propylene spacer) where PET is absent or negligible. The presence of four chlorine atoms at the bay positions advances more acceptor character into the perylene core. When the solvent polarity was changed from THF to water, a quenching in the FL intensity was observed alike PyC3ACE derivative. For further details, see SI.

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