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Strain Release in Organic Photonic Nanoparticles for Protease Sensing

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Supporting Information

ABSTRACT: Proteases are overexpressed in most cancers and proteolytic activity has been shown to be a viable marker for cancer imaging in vivo. Herein, we describe the synthesis of luminescence-quenched shell cross-linked nanoparticles as photonic nanoprobes for protease sensing. Protease sensing scheme is based on a "turn-on" mechanism where the protease cleaves peptide cross-linkers of the fluorescence-quenched shell cross-linked NP (OFF state) leading to a highly emissive non-cross linked NP (ON state). The cross-linked particles can be strained by exposure to a good solvent and proteolysis allows for particle expansion (swelling) and a recovery of the luminescence.

The diagnosis of cancer at its earliest stages is critical to obtain optimal treatment. New imaging methods that detect smaller malignant cancer tumors have the potential to dramatically improve our ability to fight this disease. Optical imaging methods with inorganic nanoparticles,[1] organic dye-containing nanoparticles,[2] single wall nanotubes (SWNT),[3] and metal nanoclusters[4] have been developed.

Proteases are involved in cancer,[5] AIDS, neurodegenerative diseases, and inflammation. Proteolytic activity has been recently proven as an effective marker for cancer imaging in vivo.[6] As a result, there is an increasing interest in protease sensing as an alternative to other imaging techniques for cancer detection. There are several examples of nanoprobes for in vivo fluorescence[7] and magnetic resonance[8] monitoring of protease activity. These sensing schemes are based upon the release of a quencher/relaxation agents from the nanoprobe.

We report, herein, fluorescent organic nanoparticles (NPs) as imaging probes for protease activity and demonstrate a new strain-release transduction mechanism. Our NP design (Figure 1a) consists of a highly fluorescent core and an external hydrogel coating displaying reactive functional groups. The core is based on poly(phenylene ethynylene) (PPE) bearing pentiptycene units that minimize interpolymer π-aggregation and a minority (5% or less) far red emitting segment (chromophore) inserted randomly into the PPE backbone. Our choice of pentiptycene is based on their previously demonstrated outstanding performance as sensors for the detection of chemical and biological analytes,[9] and explosives.[10] A critical component of our design is that changes in the local environment of the NPs will be amplified by the mobility of the excitons, which enhances energy transfer (ET) to the chromophores. An advantage of NPs based on organic

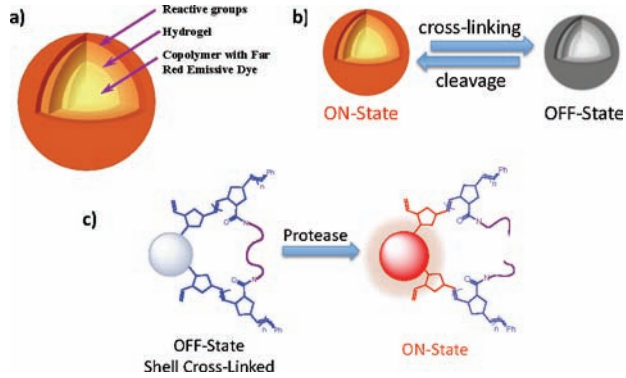

Figure 1. (a) Schematic representation of NPs structure. (b) The ON- and OFF-states and relationship of cross-linking in the other polymer shell of the NPs. (c) Protease triggered "turn on" sensing scheme.

conjugated polymers is the exceptionally large optical absorbance of these structures, coupled with their ability to behave as antennas, effectively collecting the excitations and providing for high signals.[11,12] This sensitivity can ultimately enable imaging technologies using moderate laser powers.

As we detail here, our NP design involves the assembly of an "ABCBA" pentablock polymer structure wherein the central "C" block is a semiconducting PPE. The terminal "A" hydrogel block provides the hydrophilicity needed for in vivo studies and prevents aggregation of the NPs under physiological conditions. The "B" block is designed to be reacted post-NP formation to incorporate functional groups that produce transduction events that affect the particle's emission properties.

Aggregation induced interactions of π-systems of semiconducting polymers often lead to fluorescence self-quenching processes as a result of energy transfer to weakly emitting excimer-type associations between individual chains.[10a] Accordingly, a subtle tuning of the π-associations in the core of the nanoparticle controls the emission intensity of the NPs. Taking advantage of this property, we have developed a luminescence "turn ON"−"turn OFF" system. Our designs involve the formation of NPs with tightly packed PPEs that are trapped in partially fluorescence quenched state by cross-linking the other shells of NPs (OFF state) by reaction of external succinimide groups in the B-polymer block with a short peptide (Figure 1b). For the protease sensing reported herein, we have used the following sequence [H]-Lys-Cys-Arg-Pro-Leu-Ala-Leu-Trp-Arg-Ser-Lys-[NH_2] as a shell cross-linking agent. A protease

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sensing scheme making use of our NPs is illustrated in Figure 1c and is a "turn-ON" mechanism wherein a NP is captured in a nonequilibrium contracted (strained) OFF state, and when the protease cleaves specific positions of the peptide sequence,[13] the particle can expand to an emissive ON state.

The design of the semiconductive polymer NP core is critical and the aggregation induced quenching is required to be readily modulated. To balance the interpolymer forces and also make use of energy transfer, we have made use of a pentiptycene elaborated PPE developed in our group, which is copolymerized with a lower band-gap perylene minority emitter. Although the pentiptycene PPEs are relatively immune from aggregation induced quenching, the presence of the perylene monomer results in segments that are capable of undergoing π-associations leading to reduced (quenched) emission in aggregated states. An appropriate perylene dye that functions as a well-behaved monomer for the Sonogashira–Hagihara polymerizations was used to create PPE. To this end, we developed a functionalized perylene diacetylene **2** (see Supporting Information) that displays a high photoluminescence quantum yield. A predictable amount of this fluorophore can be readily inserted into the polymer backbone to serve as a high efficiency emissive trap for excitons. The PPE-block was synthesized by cross-coupling of the two different acetylenes, namely, **2** and the pentiptycene diacetylene, and a slight excess of a dialkoxy diiodoarene derivative. Quantitative incorporations of the perylene monomer (**2**) were achieved. This allows a control of the amount of the low band gap perylene emitter in the polymer between 0.5 and 5 mol % by easily controlling the stoichiometry of the reaction. The well-behaved nature of this polymerization is also confirmed by the fact that these polymers display polydispersities similar to their non-perylene containing analogues.[10] End-capped materials (Figure 2) were obtained by quenching the polymerization reaction

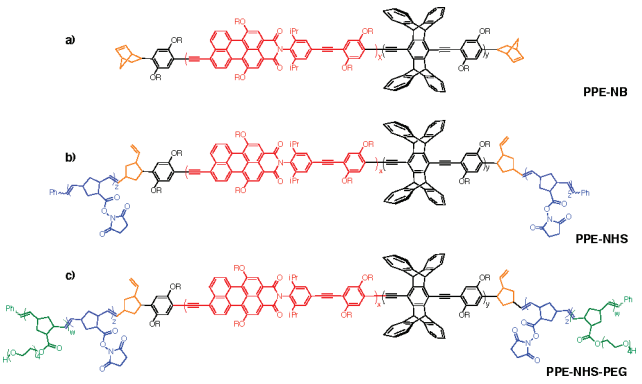

Figure 2. Molecular structures of block copolymers: (a) **PPE-NB**; (b) BCB-triblock **PPE-NHS**; (c) ABCBA-pentablock **PPE-NHS-TEG**.

with an excess of norbornadiene in the presence of formic acid, which acts as a hydride donor.[14] The resultant **PPE-NB** allows for these materials to react with olefin metathesis catalysts. Thus, **PPE-NB** can be reacted with living ring-opening olefin metathesis polymerizations (ROMP) to create BCB-triblock **PPE-NHS** and ABCBA-pentablock **PPE-NHS-TEG** copolymers (Figure 2). All the polymers were characterized by ^{1}H NMR and their relative molecular weights were measured (GPC).

Nanoparticles were fabricated by dropwise precipitation of diluted THF polymer solutions into distilled water under sonication.[15] The size of the nanoparticles was measured by Dynamic Light Scattering (DLS) techniques. Depending upon the initial concentration in the THF solution, it is possible to tune the size of the nanoparticles over a wide range of diameters. For biological applications, we have implemented conditions to obtain dispersions of nanoparticles around 80 nm in diameter with narrow size distributions.

The non-cross-linked NPs are highly emissive and their emission spectra (Figure 3) show two peaks ascribed to the

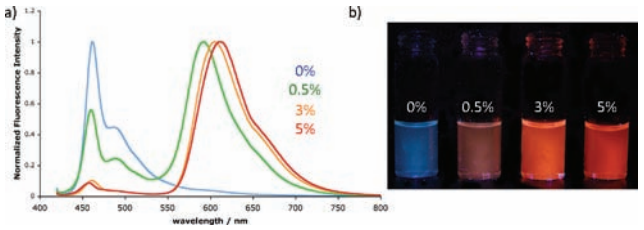

Figure 3. (a) Photoluminescence spectra of aqueous non-cross linked **PPE-NHS** nanoparticle suspensions containing different amounts of dye (λ_{ex}: 410 nm). (b) Aqueous non-cross linked **PPE-NHS** nanoparticle suspensions under irradiation at 365 nm at different dye loading (mol %).

conjugated polymer backbone (454 nm) and the emissive dye (604 nm) maxima (Figure 3). NPs derived from copolymers bearing perylene moieties show higher quantum yields than those lacking this dye.[16] We observe efficient energy transfer (ET) from the PPE backbone to the perylene and the excitation of the nanoparticle at the absorption maximum of the PPE backbone (410 nm) results in a dominant perylene-based emission at around 604 nm. The efficiency of this energy transfer was quantified by measuring the ratio between the fluorescence intensity emitted from the perylene traps (I_{AD}) and the total emission intensity (I) of the nanoparticle. Additionally, the enhancement in the 604 nm emission resulting from the antenna effect of the PPE was measured by the comparison between the perylene emission intensity obtained with excitation of the PPE backbone at 410 nm (I_{AD}) and the emission intensity observed by direct excitation of the perylene centers at 519 nm (I_A) (see Table 1).

Table 1. ET Efficiency of Non-Cross Linked PPE-NB Nanoparticles in Water at Different Perylene Loadings (mol %), Quantum Yields (Calculated against Coumarin 6), and Hydrodynamic Diameter Measured by DLS

% dye	% dye emission I_{AD}/I	amplification I_{AD}/I_A	Φ_f	D_h(DLS)/nm
0.5	79	91-fold	0.21	140 ± 10
3	93	20-fold	0.18	80 ± 10
5	97	24-fold	0.12	132 ± 1

After cross-linking the **PPE-NHS** nanoparticles with [H]-Lys-Cys-Arg-Pro-Leu-Ala-Leu-Trp-Arg-Ser-Lys-[NH_2], we observe a decrease in the emission intensity with quantum yields up to 10-fold lower than the quantum yields of the parent non-cross-linked NP suspensions.

Sensing experiments were performed using a double read-out of the fluorescence emission at the aforementioned two emission maxima (i.e., 454 and 604 nm). We first investigated assays in PBS buffered solutions with suspensions of shell cross-linked NPs fabricated from the BCB-triblock **PPE-NHS** with a

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perylene dye loading of 3 mol %. A 3.3-fold increase at the main chain emission maximum (454 nm) and a 2.3-fold increase at the perylene emission maximum (604 nm) were observed after incubation with trypsin (Figure 4a). These first assays proved

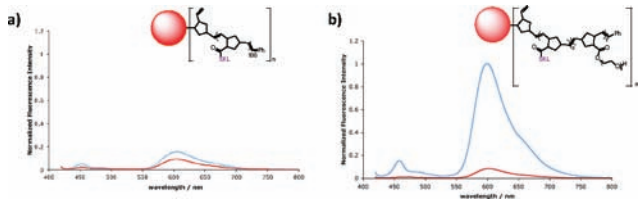

Figure 4. Trypsin sensing assays. Fluorescence spectra of shell cross-linked NP suspensions before (red) and after (blue) incubation with trypsin for different polymers at a core dye loading of 3 mol %: (a) **PPE-NHS** and (b) **PPE-NHS-TEG**.

the feasibility of the proposed model but improvements were needed. The main drawback is the large numbers of linkages that need to be cleaved by the protease to provoke a significant luminescent response. For this reason, a modification on the polymer design was made to lower the number of succinimidyl ester groups (**NHS** block) without lowering the hydrophobic/hydrophilic ratio of the polymer. The resulting ABCBA-pentablock **PPE-NHS-TEG** copolymer has two polynorbornene blocks with different functionalities: the "B" block contains succinimidyl esters and the terminal "A" block is functionalized with tetraethylene glycol (TEG). Because of the spatial proximity of the reactive succinimidyl esters to the luminescent core of the NP, this approach allows an effective quenching of the luminescence of the NPs with a lower cross-linking degree and therefore a faster cleaving process. The assays performed with **PPE-NHS-TEG** at a core perylene dye loading of 3 mol % under identical experimental conditions confirmed the merits of this new design and showed a 15-fold increase at the main chain emission maximum (454 nm) and a 12-fold increase at the perylene emission maximum (604 nm).

To confirm that the changes are the result of the cleavage of the peptide chains, we performed control experiments wherein the ABCBA-pentablock **PPE-NHS-TEG** copolymer was cross-linked with 1,2-bis(2-aminoethoxy)ethane. In this case, exposure to trypsin resulted in no changes in the emission (see the Supporting Information).

The expansion of the particles upon cleaving of the constraining cross-linking peptide chains by the protease can be mimicked by the addition of THF to a suspension of non-cross-linked NPs of **PPE-NB**. The addition of THF produces a reversible swelling of the nanoparticles with an increase in diameter from 200 to 450 nm and a concomitant change of their emission properties. These changes are visually observable upon irradiation at 365 nm (Figure 5a). Addition of other water miscible solvents (acetone, methanol, ethanol) in which the copolymer is insoluble did not produce a similar effect. Furthermore, addition of THF to the shell cross-linked NPs also produced a negligible effect on the size and emission properties of the NPs, confirming that the cross-links constrain the particles in a collapsed state. Figure 5b represents the emission spectra of a 3 mL aqueous suspension of non-cross linked NPs of **PPE-NB** with different amounts of THF. Swelling of the NPs increases the intensity of both the main chain and dye maxima. Nevertheless, the enhancement of both maxima is different so the I_{AD}/I ratio decreases as the NPs swell. This can be explained by considering that swelling with a

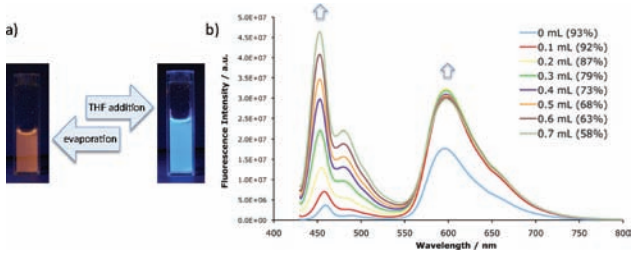

Figure 5. (a) Reversible swelling effect in non-cross-linked NP suspensions of **PPE-NB** (0.5% dye loading) before (left) and after (right) addition of THF. The figure shows a red emissive collapsed NPs suspension (D_h: 200 nm) and a blue emitting swollen NP suspension (D_h: 450 nm) under irradiation at 365 nm. (b) Fluorescence spectra of a 3 mL aqueous NP suspension of **PPE-NB** (3% dye loading) with increasing amount of THF. I_{AD}/I is represented in parentheses (λ_{ex}: 410 nm).

good solvent like THF has two opposite effects on the emission properties. It enhances the total emission intensity by solvating the chains and reducing interpolymer $\pi-\pi$ interactions that produce self-quenching. However, it also reduces the energy transfer to the perylenes by separating the polymer chains. We have previously shown that energy transfer to low energy emissive sites is enhanced by polymer aggregation.[17] The balance of these opposite effects determines the ratio of both maxima and explains the observed behavior.

In summary, tunable highly emissive organic NPs have been developed as selective nanoprobes for protease sensing. The design takes advantage of the amplification of the fluorescence emission of the dye by ET of the light harvested by the backbone of the conjugated polymers and provides a double read-out of the fluorescence emission. An exquisite tuning of the fluorescent emission intensity of NPs was performed by means of aggregation induced quenching by cross-linking using a peptide as a shell cross-linking agent. Our results suggest that the polymer cross-linking effectively strains the NP into a more tightly aggregated and quenched state. The sensory response is ascribed to a swelling-like (strain-release) mechanism in response to the protease cleavage of the cross-links, which results in a "turn-on" fluorescence response. Sensing assays under physiological conditions provide a 15-fold increase of the luminescence intensity after incubation with the protease.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental details including synthetic procedures, characterization of all compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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