Self-Assembly

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When Molecular Probes Meet Self-Assembly: An Enhanced Quenching Effect**

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Abstract: *We demonstrate that the incorporation of one or two amino acids of phenylalanine (F) or 4-fluoro phenylalanine (^{f}F) will greatly lower the background fluorescence intensities of conventional quenched probes with quenchers. This enhanced quenching effect was due to the synergetic effect of the aggregation caused quenching and the presence of a quencher. Such strategy will not greatly affect the enzyme recognition properties to the probes. We also demonstrated that our self-assembled nanoprobe with the enhanced quenching effect showed a better performance in cells for the detection of cell apoptosis than the unassembled probes. Our study demonstrates that using molecular self-assembly can optimize and improve the performance of molecular probes and it provides a simple but very useful strategy to boost the signal-to-noise ratios of fluorescence probes.*

We introduced a novel strategy to generate molecular probes with extremely low backgrounds by using the "optimized self-assembly enhanced quenching effect". The development of optical probes and sensors with good fluorescence turn-on properties has attracted extensive research interests due to their distinct features, such as fast processing time, low cost, technical simplicity (without the need of expensive machines), and signal amplification.[1] There are several strategies to design and achieve molecular probes with fluorescence turn-on properties including aggregation-induced emission (AIE),[2] disaggregation-induced emission (DIE),[3] liberation of fluorescent dyes from quenched probes with quenchers,[4] and use of environment-sensitive fluorescence dyes.[5] Probes designed by these strategies have shown promising applications to recognize and detect environmentally and biologically important species.

One of the prerequisites of molecular probes with good fluorescence turn-on properties is to lower their background fluorescence intensities. To achieve this goal, two strategies have been developed and widely used, one is the integration of a fluorescent dye with its quencher and the other one is based on aggregation-caused quenching (ACQ). The low background fluorescence of probes developed by the former strategy was due to fluorescence resonance energy transfer (FRET) and for those developed by the latter one it was mainly due to π–π interactions. There is a challenge to synergistically combine both strategies to achieve probes with extremely low background fluorescence but still with good analyte recognition properties, because aggregated probes usually possess lower water solubility and diminished analyte recognition properties than conventional quenched probes with quenchers. We envision that, by using molecular self-assembly, one can obtain probes with extremely low background fluorescence and retain their good water solubility and recognition properties (Figure 1). In this study, we reported on such molecular probes through the introduction of single amino acids or short peptides with optimized self-assembly properties compared to conventional quenched probes.

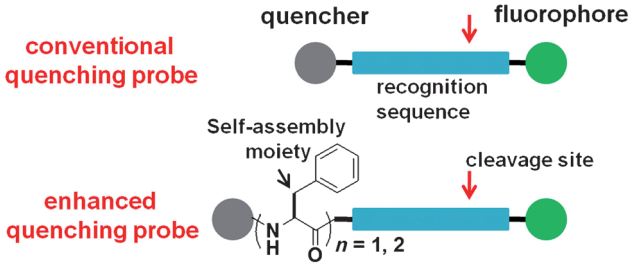

Figure 1. The design principle for probes with lower background fluorescence compared to conventional quenched probes by optimized molecular self-assembly.

Our lab focuses on the development of self-assembled materials based on short peptides.[6,7] To generate short peptide-based self-assembling materials, aromatic capping groups are used for the short peptides, such as pyrene,[8]

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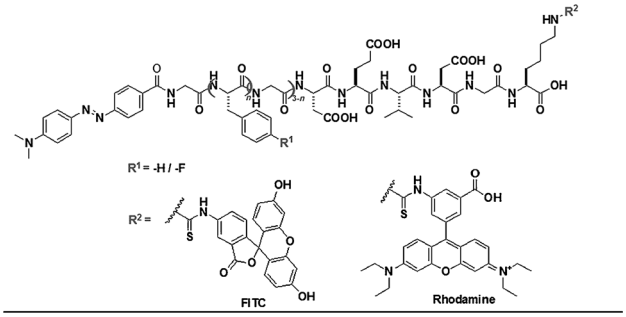
Supporting information for this article (including the synthesis and characterization of all compounds, an optical image of a hydrogel of Dabcyl-GFFY, emission spectra of different solutions, CMC values, TEM images, confocal images, and detailed experimental procedures) is available on the WWW under http://dx.doi.org/10.1002/anie.201411833.

fluorenyl (Fmoc),[9] and naphthalene (Nap)[10] groups. Many functional molecules possess aromatic groups, which can be used as capping groups for short peptides to make functional self-assembled materials. For example, therapeutic agents have been integrated with peptides to produce self-delivery systems.[8,11] Fluorescent molecules have also been applied to generate supramolecular hydrogels with distinct fluorescent properties.[12] We realized that many quenchers were aromatic and many probes were based on peptides. We therefore envisioned that the integration of self-assembling peptides in conventional quenched probes might result in self-assembling molecular probes with enhanced quenching effects (Figure 1), because of the synergetic effect of a quencher and the ACQ.

To test our hypothesis, we choose the widely used quencher dabcyl and tested whether it could be used as an aromatic group for peptides to generate self-assembling molecules. Dabcyl has a free carboxylic acid group that can be directly used for solid-phase peptide synthesis (SPPS) to produce N-terminal dabcyl-capped peptides. We designed and synthesized dabcyl-GFFY (Scheme S-1) and it indeed formed a molecular hydrogel (Figure S-22), indicating its high possibility to produce self-assembling quenched molecular probes. We therefore designed compounds of dabcyl-GF$_n$G$_{(3-n)}$DEVDGK(FITC) (Scheme 1, $n = 0$–3) as possible



	FITC-F$_0$	FITC-F$_1$	FITC-F$_2$	FITC-F$_3$	FITC-^{f}F$_1$	Rho-^{f}F$_0$	Rho-^{f}F$_1$	Rho-^{f}F$_2$
R$_1$=	H	H	H	H	F	H	F	F
R$_2$=	FITC	FITC	FITC	FITC	FITC	Rho	Rho	Rho
n =	0	1	2	3	1	0	1	2

Scheme 1. Chemical structures of fluorescent probes (F, ^{f}F, and FITC represent phenylalanine, 4-fluoro phenylalanine, and fluorescein isothiocyanate, respectively).

self-assembled probes for the enzyme caspase 3. We opted to test the effect of the number of phenylalanine (F) moieties on the self-assembling, the background fluorescence intensity before enzymatic cleavage, and the recovered fluorescence intensity after enzymatic cleavage of the compounds.

The designed four compounds were obtained through SPPS in combination with solution synthesis and then purified by high-performance liquid chromatography (HPLC). We first studied the self-assembly of the four compounds by dynamic light scattering (DLS). As shown in Figure S-23, the critical micelle concentration (CMC) for FITC-F$_0$, FITC-F$_1$, FITC-F$_2$, and FITC-F$_3$ was 504, 181, 140, and 267 μg mL^{-1}, respectively. The lower CMC values of peptides with F (FITC-F$_1$, FITC-F$_2$, and FITC-F$_3$) than that without F (FITC-F$_0$) indicated that the introduction of aromatic F could improve the self-assembly properties of peptides. We then used transmission electron microscopy (TEM) and DLS to characterize the self-assembled nanostructures. As shown in Figure S-24, the FITC-F$_0$ exhibited an amorphous morphology. The three peptides with F self-assembled into nanoparticles (Figure S-24 B–D). The size of the nanoparticles was about 150, 550, and 400 nm for FITC-F$_1$, FITC-F$_2$, and FITC-F$_3$, respectively (Figure S-25). These observations also indicated that peptide derivatives with F were excellent self-assembling molecules, which were similar to other self-assembling short peptides containing F.[7,13]

To test whether the self-assembling properties would enhance the quenching effect of dabcyl to the FITC in the probes, we measured their background fluorescence intensity. As shown in Figure 2B, FITC-F$_0$ exhibited the highest

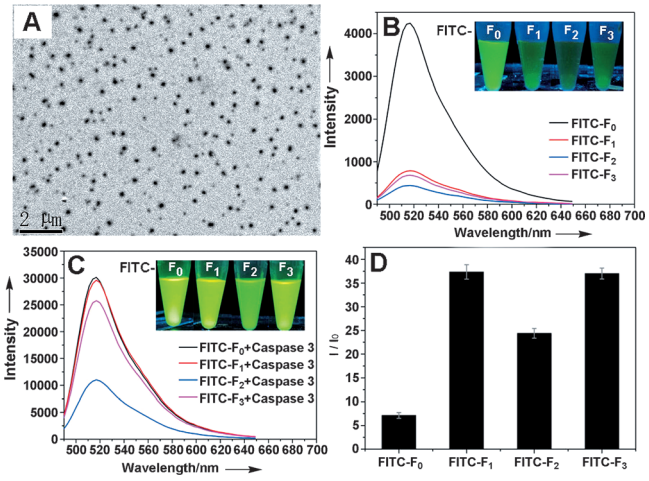


Figure 2. A) The TEM image of FITC-F$_1$ at the concentration of 150 μM. Emission spectra ($\lambda_{exc.} = 465$ nm) and optical images (excited by a UV lamp) of solutions of different compounds at the concentration of 150 μM B) before and C) after enzymatic cleavage. D) I/I_0 values of different compounds before and after enzymatic cleavage.

background fluorescence (about 4200 at 150 μM), whereas FITC-F$_1$, FITC-F$_2$, and FITC-F$_3$ showed much lower background fluorescence at the same concentration (780, 435, and 680, respectively), which was about 4.5-, 9-, and 5.2-fold lower than that of FITC-F$_0$, respectively. These observations indicated that the self-assembling property could indeed enhance the quenching effect of dabcyl to the fluorophores. There was an odd-even effect in the quenching effect of the probes,[14] and the background fluorescence intensity of probes with even numbers of F possessed lower fluorescence intensities than those with odd numbers of F (FITC-F$_4$ was about 7-fold lower than that of FITC-F$_0$ (Figure S-28A).

We therefore measured the fluorescence recovery properties of the compounds after enzymatic cleavage by caspase 3. After being incubated with caspase 3 (10 U mL^{-1}) overnight at 37 °C, more than 99 % of the four compounds was cleaved at the carboxylic terminal of DEVD (Figure S-26). The TEM images showed that most of the nanoparticles had disap-

peared and transformed into short nanofibers after enzymatic cleavage (Figure S-27). The nanofibers were formed by dabcyl-peptides because there were no dabcyl-peptides observed in the filtrate when the solution of FITC-F_2 was passed through a 0.45 μm filter membrane after enzymatic cleavage (Figure S-29). The fluorescence spectra of the resulting solutions were then recorded (Figure 2C). The emission intensity of FITC-F_1 was similar to that of FITC-F_0, indicating its excellent fluorescence recovery property. There was also an odd-even effect in the fluorescence recovery properties of the probes, and probes with odd numbers of F had better recovery properties than those with even numbers of F (Figures 2C and S-28B). The reason for probes FITC-F_2 and FITC-F_4 being unable to fully recover the fluorescence after enzymatic cleavage was probably due to the stronger interactions of the FITC-peptide to the short nanofibers formed by dabcyl-peptides. The calculated I/I_0 values of the four probes were about 7.1, 37.3, 24.4, and 37.0 for FITC-F_0, FITC-F_1, FITC-F_2, and FITC-F_3, respectively (Figure 2D). The highest I/I_0 value and the simplest chemical structure of FITC-F_1 suggested its high potential as a molecular probe with an enhanced quenching effect compared to the other three compounds.

The Nilsson group had demonstrated that peptides with halogenated F exhibited better self-assembling properties than those with F.[15] We therefore replaced F on FITC-F_1 with the 4-fluoro phenylalanine (^{f}F) to test whether it would further decrease the background fluorescence intensity of the probe. The FITC-^{f}F$_1$ exhibited a similar self-assembling ability to FITC-F_1, as indicated by their similar CMC values (Figure S-32A) and the similar morphology of the self-assembled nanoparticles (Figure 3A). The background fluorescence intensity of FITC-^{f}F$_1$ was lowered further compared with that of FITC-F_1 at the same concentration (Figure 3B),

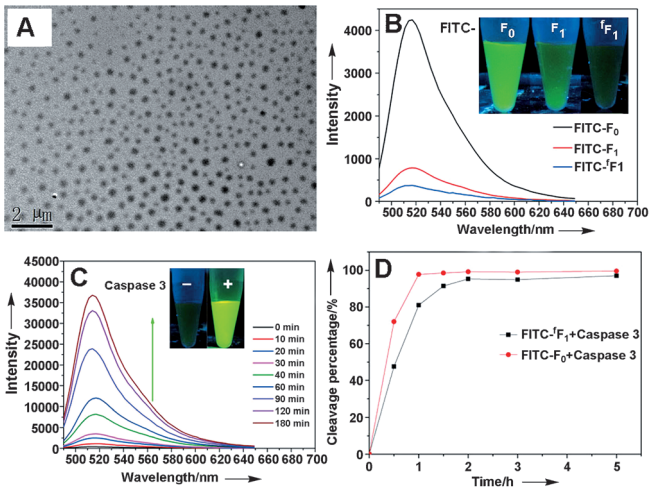


Figure 3. A) The TEM image of FITC-^{f}F$_1$ at the concentration of 150 μM. B) Emission spectra ($\lambda_{exc.}$ = 465 nm) and optical images (excited by a UV lamp) of solutions of different compounds at the concentration of 150 μM. C) Emission spectra ($\lambda_{exc.}$ = 465 nm) of the solution of FITC-^{f}F$_1$ with caspase 3 at different time points and optical images of solutions of FITC-^{f}F$_1$ before and after enzymatic cleavage. D) Kinetics of enzymatic cleavage of FITC-F_0 and FITC-^{f}F$_1$.

and was about 11-fold lower than that of FITC-F_0, whereas it was 4.5-fold lower for FITC-F_1. The FITC-^{f}F$_1$ exhibited a similar fluorescence recovery property than that of FITC-F_0 and FITC-F_1 after enzymatic cleavage (Figure 3C). The I/I_0 values for FITC-^{f}F$_1$ were about 100 at high concentrations of 150 and 75 μM and were about 65 at low concentrations of 38, 10, and 2.5 μM (Figures S-33 and S-34). These values were lower than 10 for FITC-F_0 at both high and low concentrations of 2.5 to 150 μM (Figures S-31 and S-34). The observations suggested the high potential of FITC-^{f}F$_1$ as a molecular probe for the detection of caspase 3. We also compared the kinetics of enzymatic cleavage of the compounds (Figures 3D and S-35). The results indicated that the formation of NPs would cause steric effects, thus decreasing the enzymatic reaction kinetics. As shown in Figure S-35, the speed of enzymatic cleavage of FITC-F_2 and FITC-F_3 was much lower than that for FITC-F_0. However, the speed of enzymatic cleavage of FITC-^{f}F$_1$ and FITC-F_1 with only one hydrophobic amino acid was only slightly lower than that for FITC-F_0. These observations suggested that the high speed of enzymatic cleavage could be retained in the optimized self-assembled probes.

To understand the mechanism of the enhanced quenching effect of our self-assembled probes, we synthesized the compound Ada-G^{f}FGGDEVDGK(FITC) by replacing the quencher dabcyl with the adamantine (Ada) group (Scheme S-6). Similar to our previous results that Ada-peptides could self-assemble to nanoparticles,[16] this compound could also self-assemble to nanoparticles (Figure S-36C) with a CMC value of about 140 μM (Figure S-36A). As shown in Figures S-37, S-30A, and S-36D, the fluorescence intensity of FITC, FITC-F_0, and Ada-^{f}F$_1$-FITC at the concentration of 38 μM was about 82000, 2750, and 50000, respectively. This indicated that the FRET pair quenched 96.7% of the fluorescence of free FITC molecule and the ACQ effect quenched about 39.0% of the fluorescence. The fluorescence intensity of our FITC-^{f}F$_1$ probe at this concentration was only about 180, which meant that the synergistic effect quenched 99.8% of the fluorescence of FITC. Though the construction of the FRET pair could already efficiently quench most of the FITC fluorescence, our strategy could indeed further quench its fluorescence by the synergistic effect of both FRET and ACQ.

One shortcoming of self-assembled probes with DIE properties is that its background fluorescence intensity might be dramatically affected by environment changes such as pH and temperature changes as well as the presence of proteins. We therefore tested whether the background fluorescence of our probes would change upon environmental changes. The results in Figure S-37 show that the temperature change (from 25 to 37 °C) and the presence of up to 10% of bovine serum albumin (BSA) does not dramatically change the background fluorescence intensity of both the unassembled probe of FITC-F_0 and our self-assembled probes of FITC-F_1 and FITC-^{f}F$_1$. A drop of pH from 7.4 to 6.5 slightly decreases the background fluorescence intensity of both unassembled and self-assembled probes. These observations suggested the potential application of our self-assembled probes in complex environments such as in cells.

We also tested the generality of our strategy to generate molecular probes with enhanced quenching effects by replacing the FITC with rhodamine (Rho) to make Rho-F_0 and Rho-$^fF_1$ (Scheme 1). The Rho-$^fF_1$ could also self-assemble to nanoparticles at the concentration of 150 μM (Figure S-39B). However, its background fluorescence intensity was only two times lower than that of Rho-F_0 (Figure S-40A), suggesting that its quenching effect was not as good as that of FITC-F_1. The reason was probably due to the greater hydrophilicity of Rho than FITC (Rho exhibits a free carboxylic acid after being conjugated with peptides). We therefore synthesized Rho-$^fF_2$ with more ^{f}Fs that would possess better self-assembly properties. The Rho-$^fF_2$ has better self-assembly properties because it possesses a smaller critical micelle concentration (CMC) value than Rho-$^fF_1$ (Figure S-40B). It could also self-assemble to nanoparticles at the concentration of 150 μM (Figure S-40C). The probe Rho-$^fF_2$ indeed exhibited a lower background fluorescence intensity than Rho-$^fF_1$ (Figure S-40 A). Upon the addition of caspase 3 to totally cleave the peptides, the fluorescence intensity (Figure S-40D) and enzymatic kinetics (Figure S-41) of both Rho-$^fF_1$ and Rho-$^fF_2$ was similar to that of Rho-F_0, suggesting their excellent fluorescence recovery properties. The less affected steric effect of Rho-$^fF_2$ with two ^{f}Fs than FITC-F_2 was also probably due to the higher hydrophilicity of Rho compared to FITC. The I/I_0 value of Rho-F_0, Rho-$^fF_1$, and Rho-$^fF_2$ was about 4.5, 11.4, and 50.4, respectively (Figure S-44). These results further confirmed that our strategy could be applied for developing fluorescent molecular probes with high signal-to-noise ratios.

We then tested the application of our probe in live cells for the detection of caspase 3. We selected probes with rhodamine (Rho-F_0 and Rho-$^fF_2$) for the test because of their red emission after the enzymatic cleavage by caspase 3. As shown in Figures 4 and S-45, HeLa cells treated with Rho-F_0 exhibited higher background red fluorescence than those treated with Rho-$^fF_2$ at the concentration of 50 μM probably due to the better quenching effect of Rho-$^fF_2$ than Rho-F_0. Pioneering works had demonstrated that the expression level of caspase 3 could be boosted by the addition of staurosporine (STS) because STS would induce cell apoptosis.[17] We therefore used 2 μM of STS to treat cells to induce cell apoptosis. The results showed that apoptotic cells treated with both Rho-F_0 and Rho-$^fF_2$ exhibited strong red fluorescence, and those treated with Rho-$^fF_2$ had a much stronger red emission under the same conditions. The stronger red emission in Rho-$^fF_2$-treated apoptotic cells was possibly due to the relatively higher uptake of the Rho-$^fF_2$ by the cells (Figure S-46), which was consistent with our previous results that molecules in nanomaterials would show a higher uptake by cells due to the endocytosis than free molecules in unassembled stages.[18] There were no big difference in cellular uptake of both compounds at the lower temperature (4 °C, Figure S-46), also suggesting that the nanoparticles of Rho-$^fF_2$ were taken up by cells through endocytosis. We also stained the cells with both our probe and LysoTracker green (Figure S-47). The results demonstrated that most of the red fluorescent dots from Rho-$^fF_2$ in cells co-localized with green dots from LysoTracker green. This observation also suggested that our probe was taken up by cells through endocytosis. After the addition of STS to induce apoptosis, the cells changed their morphology and our probe was evenly distributed in the whole cells, suggesting that the lysosome membrane was broken and our probe was released from the lysosome to the cytosome.[19] The probe in the cytosome could therefore encounter the enzyme caspase 3. The big difference in red emission between normal cells and apoptotic cells treated with Rho-$^fF_2$ suggested the big potential of Rho-$^fF_2$ in imaging cell apoptosis.

In summary, with the help of molecular self-assembly we can greatly enhance the quenching effect of the quencher of dabcyl in molecular probes by the synergistic effects of FRET and ACQ. Such self-assembled molecular probes with enhanced quenching effects can be easily generated through the incorporation of different numbers of the amino acid ^{f}F. By varying the amount of ^{f}F, we can optimize the properties of the resulting probes including the self-assembly, fluorescence recovery, and the kinetics of enzymatic cleavage. We also demonstrated that our probe could be used to detect caspase 3 in complex environments such as in apoptotic cells with much better performance than the free probe. Our study offers a simple strategy to design fluorescent molecular probes with large signal-to-noise ratios, which will be very useful for the detection of biologically and environmentally important analytes.

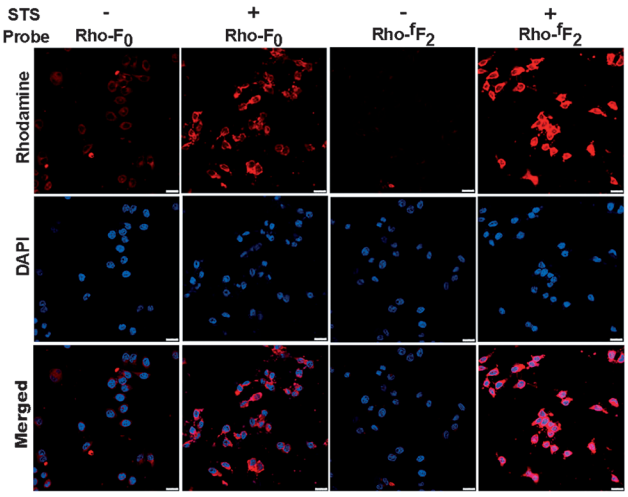


Figure 4. Confocal fluorescence microscopy images of STS-induced apoptotic HeLa cells treated with 50 μM of Rho-F_0 or Rho-$^fF_2$ (cells were stained with nuclear dye DAPI in blue). Scale bars represent 25 μm, 40×).

Keywords: fluorescence quenching · molecular probes · peptide · self-assembly

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