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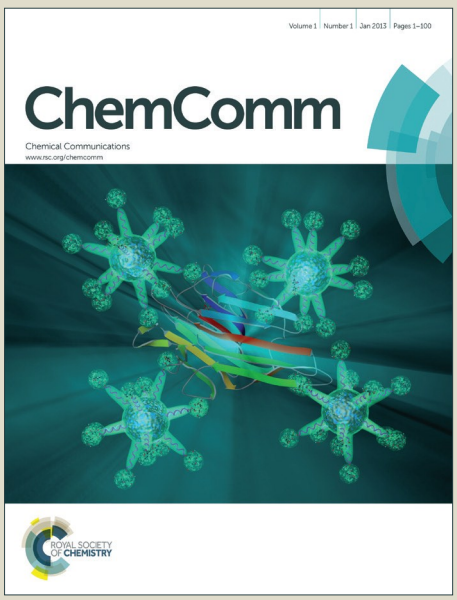

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Colorimetric and Fluorometric Monitoring of the Helix Composition of Collagen-like Peptides at nM level

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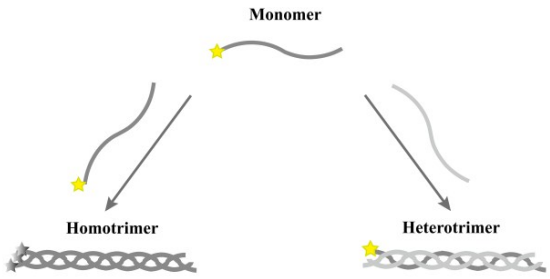
We have demonstrated that the incorporation of a dye-labeled collagen-like peptide in the homotrimeric versus heterotrimeric context results in visible color changes and distinct fluorescence. The unique fluorescence self-quenching assay can unambiguously determine the helix composition of heterotrimers at nM level, far extending our capability to characterize collagen triple helix.

Biophysical characterization of collagen mimic peptides using mainly CD and NMR techniques has greatly enhanced our understanding of the helix control of collagen, the essential structural protein in human body. Type I collagen, the major component of bone, skin, tendon and ligament, is a heterotrimer consisting of two identical α1(I) and one α2(I) chains. CD studies of a mixture of two or three delicately designed triple helical peptides utilizing electrostatic interactions have shown the formation of AAB or ABC- type heterotrimers, since the thermal stability of the heterotrimer is different from that of each composite chain alone.[1-5] NMR studies of one isotopically labeled peptide chain in the mixture with another unlabeled peptide chain modeling complementary natural interruption sequences have indicated different chemical shifts of the same labeled residue in the homotrimeric versus heterotrimeric context.[6, 7] To make the CD or NMR characterization feasible for the heterotrimer system, peptides have to either possess differential thermal stabilities, or to label with expensive isotopes, respectively. A relatively high concentration of model peptides is also a prerequisite for both techniques, particularly NMR.

We herein report the design of a fluorescent method to characterize the helix composition of collagen-like peptides without those prerequisites. Fluorescence self-quenching has been utilized to study protein folding, conformational fluctuations of an unfolded protein and amyloid β (Aβ) peptide aggregation.[8-11] This method requires conjugation of only one type of fluorophore, instead of the two required by FRET, therefore simplifying the labeling procedure. Fluorescence self-quenching has proved a powerful tool for single protein systems and we now adapt this method to investigate a heterogeneous mixture of collagen-like peptides (Scheme 1). We use FAM, a convenient fluorescein-based fluorescent dye, covalently attached to a collagen mimetic peptide. We hypothesize that the FAM-labeled peptide shows strong fluorescence in the monomeric state, while the formation of homotrimer results in the close proximity of the dye, leading to fluorescence self-quenching. In contrast, when the FAM-labeled peptide forms heterotrimer with a complementary unlabeled collagen-like peptide, the strong fluorescence remains. The difference in fluorescence intensity will allow us to distinguish homotrimers from heterotrimers and determine the helix composition of collagen-like peptides.

As a proof-of-concept application, we have chosen two collagen-like peptides $(PRGPOG)_5$ and $(POG)_{10}$. CD studies of a 1:1 mixture of the two peptides indicated the formation of heterotrimer species, while the exact composition (AAB or ABC) was not determined.[12] We thus created a FAM-labeled peptide FAM-$G(PRGPOG)_5$ and an unlabeled peptide $G(POG)_{10}$ for the development of fluorescence self-quenching assay to determine the helix composition of collagen-like peptides.



Scheme 1. Schematic Illustration of the fluorescence self-quenching assay to detect the helix composition of collagen mimetic peptides.

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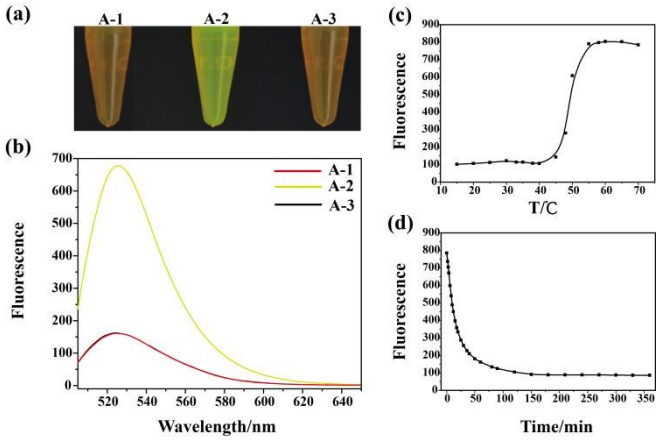

Figure 1. Colorimetric and fluorometric characterization of peptide FAM-G(PRGPOG)$_5$. Visual changes (a) and fluorescent profiles (b) of the peptide solution incubated at 4 °C (A-1), heated to 70 °C for 20 min (A-2), and re-equilibrated at 4 °C (A-3); fluorescence monitoring of the thermal transitions (c) and refolding (d) of the peptide at the wavelength of 524 nm. The emission spectra were taken at an excitation wavelength of 497 nm.

First, the properties of peptide FAM-G(PRGPOG)$_5$ as a homotrimer were characterized (Figure 1). Peptide solution in 20 mM PBS buffer (pH 7.4) was incubated at 4 °C to allow the formation of stable homotrimeric triple-helical molecules. Heating the peptide solution from 4 °C to 70 °C resulted in obvious color changes from peru to yellow, indicating that the peptide in the monomer and homotrimer states displayed different colors. Re-equilibration of the peptide back at 4 °C allowed it to reform the homotrimer conformation, as indicated by the color changing back to peru (Figure 1a). Consistent with the color changes, the fluorescence intensity of the peptide incubated at 4 °C (A-1) was much smaller than that at 70 °C (A-2), indicating that the homotrimer conformation at 4 °C resulted in the close proximity of the FAM dye, thus leading to the fluorescence self-quenching, while the monomer conformation at 70 °C recovered the fluorescence (Figure 1b). Re-equilibration of the peptide at 4 °C (A-3) resulted in the fluorescence quenching again due to the reformation of the homotrimer (Figure 1b). The completely overlapped fluorescence spectra of the peptide incubated at 4 °C and that re-equilibrated at 4 °C (A-1 and A-3) suggested that the refolding process of the peptide from monomer to homotrimer is nicely reversible.

The peptide FAM-G(PRGPOG)$_5$ clearly showed distinct colorimetric and fluorometric profiles for the monomer and homotrimer states. The transition from the homotrimer to monomer was further monitored real-time by fluorescence. The fluorescence remained ~140 till the temperature at 45 °C, jumped to a much higher value, and then reached an equilibrium of ~800 after 55 °C (Figure 1c). The refolding process could also be monitored by fluorescence, indicating that the unfolded peptide could completely refold into homotrimer within ~200 min (Figure 1d). CD measurement showed a similar thermal unfolding and refolding profile for the peptide (Figure S1). The melting temperature of the peptide was estimated as 52 °C, which was comparable to that of a similar peptide without the FAM label, indicating that the FAM dye probably would not significantly affect the stability of the peptide.[12] These results indicated that fluorescence self-quenching assay not only allowed the identification of the final monomer and homotrimer conformation, but also facilitated the real-time monitoring of the thermal unfolding and refolding process.

Peptides FAM-G(PRGPOG)$_5$ (denoted as peptide A) and G(POG)$_{10}$ (denoted as peptide B) were mixed in solution at a molar ratio of 1A:2B to look for the formation of heterotrimeric molecules. Just like peptide A alone, the mixture 1A:2B at 4 °C (AB-1) was peru and showed weak fluorescence (Figure 2a-b). Heating the mixture from 4 °C to 70 °C (AB-2) resulted in a change of color from peru to yellow as well as recovered fluorescence, indicating that the peptide experienced transition from homotrimer to monomer (Figure 2a-b). Re-equilibration of the mixture at 4 °C (AB-3) turned the mixture to light yellow instead of peru, indicating that the mixture did not reform the homotrimer of peptide A. The fluorescence of the mixture also largely remained instead of self-quenching to the original value at 4 °C, supporting the formation of heterotrimer species. The real-time fluorescence monitoring of the thermal unfolding and refolding of the peptide mixture 1A:2B clearly indicated the transition of homotrimer into monomer, and then heterotrimer (Figure 2c-d). CD characterization of the peptide mixture 1A:2B maintained at 4 °C after the heating at 70 °C confirmed a similar thermal unfolding and refolding profile as the fluorescence assay demonstrated (Figure S2).

The change in color and fluorescence of the 1A:2B mixture may result from some nonspecific interference of B on FAM-labeled peptide A. Therefore, another control peptide (GPO)$_6$GERSEQ(GPO)$_6$ (denoted as peptide C) was mixed with

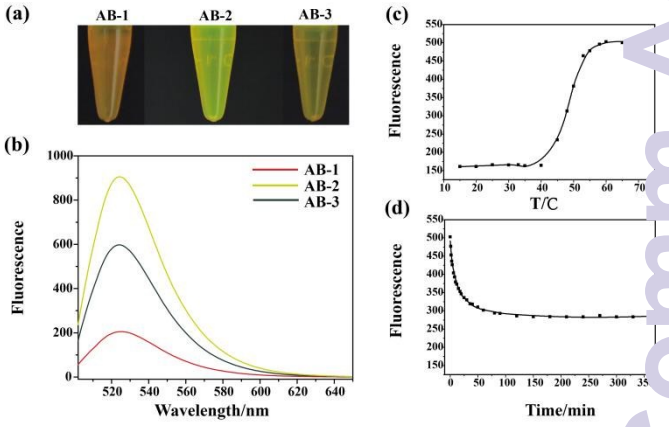

Figure 2. Colorimetric and fluorometric characterization of the mixture of peptide FAM-G(PRGPOG)$_5$ (denoted as peptide A) and G(POG)$_{10}$ (denoted as peptide B) at a molar ratio of 1:2. Visual changes (a) and fluorescent profiles of the peptide mixture 1A:2B incubated at 4 °C (AB-1), heated to 70 °C for 20 min (AB-2), and re-equilibrated at 4 °C (AB-3). Fluorescence monitoring of the thermal transitions (c) and refolding (d) of the peptide mixture 1A:2B at the wavelength of 524 nm. The emission spectra were taken at an excitation wavelength of 497 nm.

peptide A to prepare a solution of 1A:2C following totally the same procedures as 1A:2B. The 1A:2C mixture behaved just like peptide A, rather than the 1A:2B mixture, as the color of 1A:2C returned to peru and the fluorescence self-quenching occurred when the 1A:2C solution was re-equilibrated at 4 °C following the heating at 70 °C (Figure S3a-b). The real-time fluorescence monitoring of the thermal unfolding and refolding of the peptide mixture 1A:2C clearly indicated the transition of homotrimer into monomer, and then homotrimer (Figure S3c-d). The results suggested that the presence of a non-interacting peptide C did not affect the behavior of FAM-labeled peptide A. The huge contrast between peptide mixture 1A:2B and 1A:2C indicated that peptide A and B formed heterotrimer structure, which resulted in color changes and fluorescence recovery. It demonstrated convincingly that colorimetric and fluorometric measurements can distinguish heterotrimer from homotrimer.

To investigate the exact helix composition of the mixture of peptides A and B, the fluorescence spectra were recorded for the A:B at different molar ratios (Figure 3). While the concentration of A was kept constant, the increase of the molar ratio of B:A led to an increase of the fluorescence intensity, and reached a plateau after the ratio was above 2. These results indicated that the composition of the heterotrimer was ABB, suggesting that the fluorescence measurement could conveniently probe the helix composition.

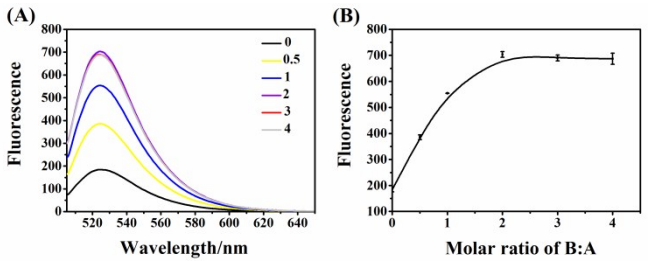

Figure 3. Fluorescence characterization of peptide A and B at different molar ratios. Fluorescence spectra were recorded for peptide A and B at different molar ratios (B:A=0, 0.5, 1, 2, 3, 4) while the concentration of A was kept constant (A). The fluorescence intensity monitored at 524 nm was plotted against the molar ratio of B:A (B).

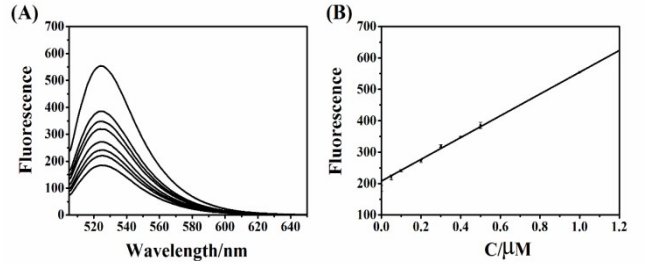

Figure 4. The linear fluorescence restoration of FAM-labeled peptide A by the hybridization with peptide B. The fluorescence emission spectra were measured for peptide A in the presence of various concentrations of B (0、50、100、200、300、400、500、1000 nM) (A). The fluorescence intensity monitored at 524 nm was plotted as a function of the concentration of B (B).

To investigate the potential of this assay to detect heterotrimers, the FAM-labeled peptide A was hybridized with various concentrations of the target peptide B (Figure 4). A linear relationship between the concentration of B and the fluorescence intensity of the platform was observed (R^2=0.99) (Figure 4B). As the concentration of B was increased, the percentage of heterotrimer was increased, therefore leading to an increase of the fluorescence intensity. This fluorescence assay showed a broad linear range from 50 to 1000 nM, with an accurate determination of the target peptide B as low as 36 nM (Figure 4B). These results indicated that this assay can detect the heterotrimer formation at nM level. Studies have shown that aromatics at the ends of collagen-like peptides can promote aggregation, which may interfere biophysical characterization of the peptide.[13-15] The condition of such a low concentration suggests that aggregation is unlikely to affect the fluorescence assay.

The characterization of helix composition of hybrid collagen mimetic peptides is critical for modeling heterotrimeric collagen and their associated diseases where mutations occur in only one or two chains. We have developed a unique fluorescent assay that can distinguish the heterotrimer from homotrimer and determine the exact helix composition of the heterotrimer. We have demonstrated that the incorporation of a dye-labeled collagen-like peptide in the homotrimeric versus heterotrimeric context results in visible color changes and significantly different fluorescence. This simple and robust assay can unambiguously detect heterotrimers which possess similar thermal stabilities as their composite chains, an unconquerable challenge for CD characterization. It also allows us to measure the thermal unfolding and refolding dynamics of collagen-like peptides in real time.

More importantly, we have demonstrated that the fluorescence self-quenching assay can detect the heterotrimer formation at nM level, far extending the capability to characterize collagen triple helix by current techniques. It is still technically challenging to design heterotrimer models of collagen. By utilizing a dye-labeled collagen-like probe peptide, this assay can be applied to screen potential target sequences suitable for the heterotrimer construction for the probe peptide. The assay only needs a tiny amount of samples without isotope labeling, which will greatly expedite the studies of heterotrimeric collagen-like peptides. This highly sensitive approach presented here can be performed on any hybrid system and unambiguously determines the chain composition of a heterotrimer triple-helix.

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