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Synthesis, Characterization, and Cellular Uptake of DNA-Binding Rose Bengal Peptidoconjugates

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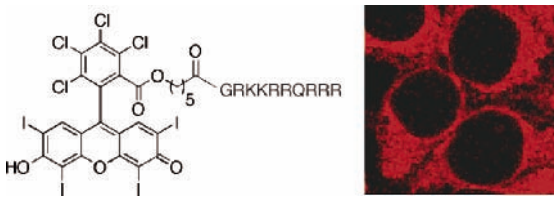
ABSTRACT



Peptide conjugates of the xanthene dye rose bengal (RB) are described featuring sequences that promote DNA binding. The complexation of these conjugates with DNA causes efficient quenching of the fluorophore singlet state and suppresses singlet oxygen production. When incubated with human cells, the RB conjugates pass through the cell membrane but are not visualized in the nucleus. This behavior is in stark contrast to that exhibited by structurally analogous conjugates containing the unhalogenated xanthene dye fluorescein. These results highlight the marked sensitivity of cell permeability characteristics to subtle structural differences.

Rose bengal (RB) is a xanthene dye with a high efficiency of singlet oxygen ($^1O_2$) formation, as it readily populates a triplet state upon photoexcitation.[1] Due to its photoactivity, RB has been explored as a photodynamic sensitizer for cancer chemotherapy[2] and for inactivation of either viruses,[3] gram-positive bacteria, or protazoa[4] and can induce photohemolysis and photothrombosis.[5]

We have prepared a series of novel RB−peptide conjugates in order to explore how the xanthene chromophore can be engineered as a DNA-binding and cellular probe. To promote complexation with polyanionic nucleic acids and cellular uptake, the HIV-1 transactivator of transcription (Tat) peptide was selected as the appended sequence. The Tat peptide is a highly basic region of nine amino acids (Tat_{49-57}: RKKRRQRRR) known to facilitate cellular permeability.[6] Here, we monitor the photophysical properties of RB−Tat conjugates to study the effects of DNA binding and we investigate the cellular localization of these compounds. Our findings indicate that DNA binding strongly influences the photochemical reactivity of RB. Moreover, the derivatization of the Tat peptide with RB alters the cellular permeability of this sequence.

A RB derivative suitable for peptide coupling was synthesized using a procedure originally developed by Neckers *et al.* (Scheme 1).[7] RB 5-carboxypentyl ester (**2**) was prepared by refluxing RB disodium salt (**1**) with 6-bromohexanoic acid in water/acetone (1:1) for 24 h. The crude material was purified via flash chromatography to afford the desired compound in moderate yield.

Six versions of the Tat peptide were incorporated into the RB conjugates. The sequences synthesized, in addition to

[†] These authors contributed equally to this work.

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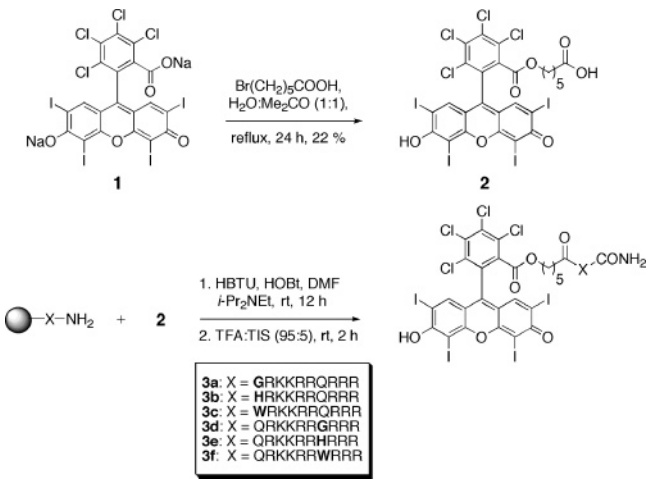
Scheme 1. Synthesis of RB−COOH and RB−Peptidoconjugates[a]



[a] See Supporting Information for synthetic protocols and compound characterization.

the Tat sequence, also incorporated a glycine (G), histidine (H), or tryptophan (W) residue proximal (**3a**−**c**) or distal (**3d**−**f**) to the dye. These variants were prepared to evaluate how single amino acids affected the spectroscopic properties of the conjugates. RB conjugates **3a**−**f** were prepared on Rink amide solid support, and the N-terminus was capped with the RB derivative **2** using standard solid-phase Fmoc chemistry and cleaved from the resin (Scheme 1). Purification by HPLC afforded RB−peptide conjugates **3a**−**f**. The RB conjugates were then characterized using MALDI-TOF mass spectrometry, UV−vis spectroscopy, and fluorescence spectroscopy. The characterization methods and spectral data are described in Supporting Information.

By systematically monitoring the spectroscopic properties of the parent compound **1**, the modified dye **2**, and the RB conjugates **3a**−**f**, we were able to assess the effects of DNA binding and chemical functionalization. The absorption spectra of the conjugates revealed only small variations for the derivatives in a given solvent.[8] In methanol, the modified dye **2** and the RB conjugates **3a**−**f** show similar absorption spectra with a small red shift in λ_{max}. In aqueous buffer, the absorption of the conjugates is red-shifted more significantly relative to the parent compound, with λ_{max} values ranging from 550 to 565 nm for RB derivatives (Figure 1). For **2** and **3a**−**f**, a shoulder at ∼525 nm indicates that a low level of aggregation occurs (Figure 1),[9] likely because the charge of the ring system is decreased upon esterification of the free carboxyl. Upon addition of calf thymus (CT) DNA, the absorption spectra of the RB conjugates are not significantly altered (Figure 1). Overall, the RB−peptide conjugates exhibit similar absorbance spectra in a given solvent, indicating that the peptide sequence does not exert a strong influence on the electronic properties of the chromophore.

(8) See Supporting Information for more data and information.

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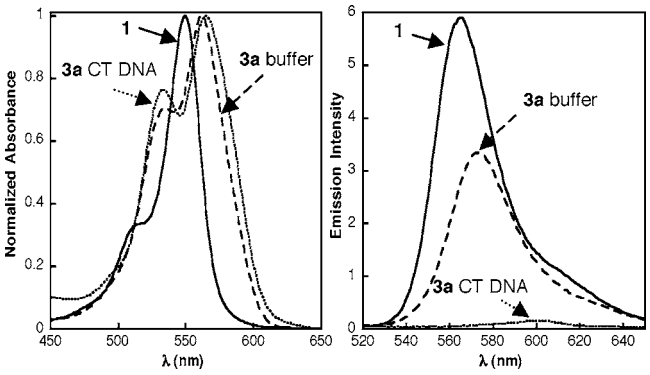

Figure 1. Normalized absorption spectra and unnormalized emission spectra for **1** (solid line) and **3a** (dashed line) in buffer and **3a** in the presence of CT DNA (dotted line).

In contrast, the fluorescence spectra of RB−peptide conjugates show a strong dependence on solvent, the presence of DNA, and the composition of the appended sequence (Figure 1). In methanol, the emission intensities of conjugates **3a**−**f** were similar to **1** (Table 1). The quantum yields of the conjugates are not strongly influenced by the peptide sequence. In aqueous buffer, the quantum yields of all the RB conjugates are reduced relative to that of the parent compound. Self-quenching of RB in aqueous solution has been observed previously and was attributed to aggregation.[9] Furthermore, the tryptophan-containing peptide conjugate, **3c**, is significantly less emissive than the other conjugates; its quantum yield is decreased by >5-fold relative to those containing G or H and >3-fold lower than conjugate **3f**, which contains W at a more distal position within the chain (Table 1). The decrease in emission intensity observed for **3c** likely results from reductive quenching of RB by tryptophan, given the favorable driving force for this reaction.[8] This deactivation pathway would be less efficient when RB and W are separated by intervening amino acids, consistent with the larger quantum yield of **3f** versus **3c**.

In the presence of CT DNA, large decreases in the quantum yields for RB conjugates **3a**−**f** are observed relative to **1** and **2**. The fluorescence of the peptide conjugates is almost completely quenched in the presence of DNA, whereas **1** and **2** are unaffected (Table 1). Given that the large positive charge of the RB conjugates would promote DNA binding, it is reasonable that the quenching results from the interaction of the conjugates with DNA. The loss of emission in the presence of DNA was used to determine DNA binding affinities. Dissociation constants (K_d) of 0.25 ± 0.01, 0.67 ± 0.03, and 0.42 ± 0.02 μM for **3a**−**c**, respectively, were obtained, indicating that the conjugates have high affinities for DNA and that small changes in the peptide sequence did not drastically perturb the interaction with DNA.

The most probable source of the fluorescence quenching observed for the conjugates in the presence of DNA is charge transfer between the excited state of RB and the nucleic acid

Table 1. Fluorescence Quantum Yields (Φ) for RB−Peptide Conjugates[a]

compound	Φ_{rel} (methanol)[b]	Φ_{rel} (buffer)[b]	Φ_{rel} (DNA)[c]
RB (**1**)	0.0800 ± 0.0001	0.0124 ± 0.0002	0.0114 ± 0.0001
RB−COOH (**2**)	0.0820 ± 0.0002	0.0150 ± 0.0001	0.0140 ± 0.0002
RB−**G**RKKRRQRRR (**3a**)	0.0851 ± 0.0006	0.0177 ± 0.0005	0.0009 ± 0.0002
RB−**H**RKKRRQRRR (**3b**)	0.0876 ± 0.0006	0.0135 ± 0.0008	0.0009 ± 0.0001
RB−**W**RKKRRQRRR (**3c**)	0.0711 ± 0.0006	0.0026 ± 0.0005	0.0008 ± 0.0001
RB−QRKKRR**G**RRR (**3d**)	0.0867 ± 0.0004	0.0215 ± 0.0004	0.0007 ± 0.0001
RB−QRKKRR**H**RRR (**3e**)	0.0994 ± 0.0004	0.0201 ± 0.0001	0.0007 ± 0.0002
RB−QRKKRR**W**RRR (**3f**)	0.0814 ± 0.0006	0.0100 ± 0.0005	0.0005 ± 0.0001

[a] See Supporting Information for methods used to obtain values tabulated. Residues shown in bold in decapeptide conjugates represent positions varied. [b] Measured in samples containing 5 μM RB−peptide. [c] Measured in samples containing 5 μM RB−peptide, 50 μM bp CT DNA.

bases.[10] Experiments monitoring the emission of **3a** in the presence of different homopolymeric oligonucleotides indicated that the most easily oxidized DNA base, guanine, produced the highest levels of quenching.[8] The excited-state quenching also appears to disfavor the production of ^{3}RB, as we observed that $^1O_2$ production by the conjugates is reduced in the presence of DNA.[8] Oxidative DNA damage was not observed after photoexcitation of the RB−peptide conjugates as evaluated by denaturing polyacrylamide gel electrophoresis (data not shown). In addition to illustrating that the DNA-bound RB conjugates do not produce $^1O_2$, these results also indicate that charge transfer between G and RB must have a fast recombination rate such that the formation of oxidized base lesions is avoided.

The Tat peptide sequence featured in the RB conjugates described here is known to facilitate uptake by human cells.[6] Previous studies with the Tat sequence have provided evidence for permeation throughout the cell, presumably due to uptake by endocytotic vesicles.[11−13] Wender and coworkers have illustrated that a Tat_{49-57}-derived sequence, conjugated to another xanthene dye, fluorescein (Fl), was able to translocate through the cellular membrane.[14] Unnatural backbones that mimic the Tat sequence such as β-peptides conjugated to Fl have also been shown to translocate across the cellular membrane despite a drastic alteration from the natural peptide structure.[12,15]

To determine if the RB−Tat peptidoconjugates exhibited a similar cellular localization profile as the Fl analogues previously described,[11−13] confocal microscopy was used to visualize RB−**G**RKKRRQRRR within HeLa cells in comparison to the identical compound containing Fl.[16] When cells incubated with both compounds were evaluated, the Fl-peptide **4** was localized throughout the cell, consistent with previous reports of Fl conjugates.[11−14] However, markedly different behavior was observed with the RB−Tat conjugate **3a**. This compound appears to be excluded from the nucleus (Figures 2A and 2B). Other RB conjugates tested displayed

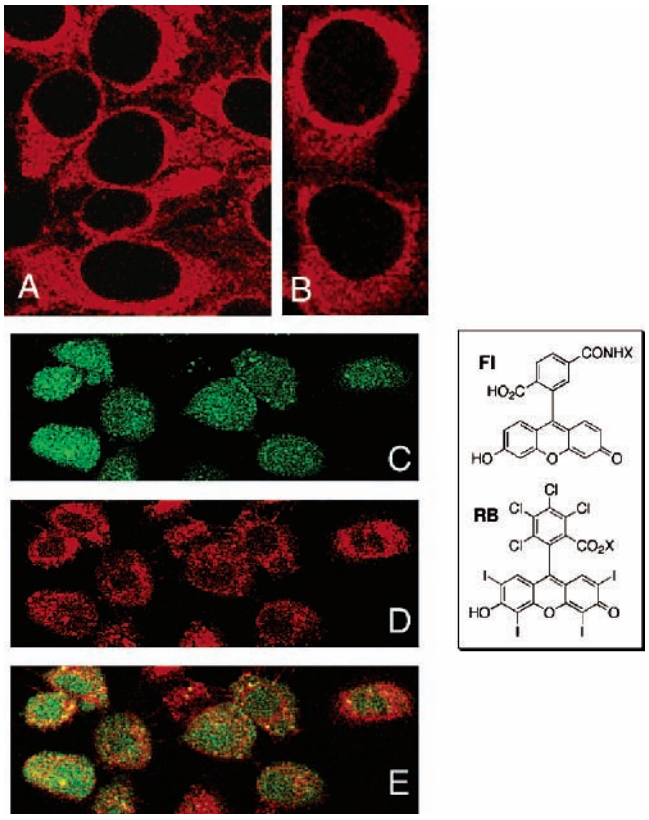

Figure 2. Confocal microscopy images of unfixed HeLa cells incubated for 1.5 h at 37 °C with 10 μM **3a** and **4**. (A,B) HeLa cells incubated with only **3a** show fluorescence throughout the cytoplasm, but nuclear uptake is not observed. (C−E) HeLa cells incubated with both **3a** and **4**. (C) Green fluorescent image shows the uptake of **4**. (D) Red fluorescent image illustrates internalization of **3a**. (E) Merged image of green and red fluorescent images indicate cytoplasmic uptake for **3a** and punctuated cytoplasmic and nuclear fluorescence for **4**. For reference, the structures of RB and Fl are shown at right where X = G-Tat peptide sequence.

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(16) Synthesis and purification of **4** is discussed in Supporting Information.

the same patterns of localization (data not shown). To confirm the different localization profiles of the Fl and RB−Tat conjugates, the two compounds were coincubated with HeLa cells and visualized. The emission of the RB and Fl fluorophores are distinguishable, and therefore the location of each dye could be assessed individually. As shown in Figure 2E, the Fl-Tat conjugate appears to have penetrated the nucleus, while the RB−Tat conjugate appears to be excluded from this cellular compartment. The covisualization of these compounds clearly illustrates that they are segregated differently despite the presence of the same peptide sequence. The quenching of fluorescence for the DNA-bound RB conjugates could in principle obscure visualization of the nucleoli, but the fact that no fluorescence whatsoever is apparent within the interior of the cell indicates that the concentration of the compound within the nucleus is extremely low.[17]

The microscopy results suggest that the structures of the two xanthene dyes, Fl and RB, significantly perturb the cellular uptake properties of the appended peptide. These two dyes have the same heterocyclic scaffold, but the RB ring system is heavily halogenated. The observations described here may indicate that cellular permeability is strongly affected by heteroatoms and that localization is dictated by the cargo appended to a peptide, not necessarily by the sequence itself.

(17) We would expect that if the localizations of the Fl-Tat conjugate (Fl-Tat is not quenched when bound to DNA) and RB−Tat conjugates were identical, the quenching observed for RB would simply produce images with dark regions around the nucleoli, rather than within the entire nucleus.

In conclusion, a series of novel RB−peptide conjugates have been synthesized that bind DNA and enter human cells. By monitoring the spectroscopic properties of these compounds, we were able to investigate the effects of peptide sequence and cell permeability. The presence of the peptide sequence influences the emissive properties of RB and facilitates delivery to DNA, which promotes emission quenching. When incubated with human cells, the RB conjugates pass through the cell membrane but are excluded from the nucleus. The behavior observed with the new compounds described here is strikingly different from that of analogues with similar structures, indicating that cellular permeability is sensitive to subtle structural differences of appended species.

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Supporting Information Available: Experimental procedures and full characterization of compounds **2**, **3a**−**f**, and **4**. This material is available free of charge via the Internet at http://pubs.acs.org.

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