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Optical Properties and Application of a Reactive and Bio reducible Thiol-Containing Tetramethylrhodamine Dimer

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Abstract

Thiolated dimeric tetramethylrhodamine (TAMRA) was synthesized in a straightforward procedure utilizing commercially available 5(6)-succinimidyl TAMRA and cystamine hydrochloride. The thiol-containing TAMRA dimer displayed distinct spectral properties in reduced and oxidized forms; covalent dimer formation produced greater effects on the spectral properties than previously reported for non-covalent TAMRA dimers or dimers formed with shorter carbon spacers. The resulting TAMRA disulfide dimer exhibited a hypsochromic shift of 34 nm relative to the reduced monomer species and an isosbestic point at 532 nm between reduced monomeric and oxidized dimeric forms. Molar extinction coefficients of the monomer and dimer relative to moles of TAMRA were similar ($6.61 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ and $6.42 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$, respectively). However, fluorescence emission was altered with >93% of dye fluorescence quenched in phosphate buffered saline upon dimer formation. A 520:554 nm absorbance intensity ratio of 2.64 was observed for the oxidized ssTAMRA dimer, almost twice as high as values reported for non-covalent TAMRA dimers. The resulting disulfide dye was easily reduced using both soluble and agarose gel immobilized tris(2-carboxyethyl) phosphine and fresh cell lysate from cultured RAW 264.7 macrophage cells. Absorbance intensity ratios at 554 and 520 nm were used to determine the oxidation half-life of a $1.2 \times 10^{-5} \text{ M}$ solution of reduced TAMRA stored in ambient atmosphere to be ~50 hr at 22 °C. The free thiol dye was further reacted with maleimide-derivatized poly(hydroxypropyl methacrylamide) to yield the dye-labeled polymer conjugate. This dye derivative should prove useful as a dithiol reduction-sensitive fluorescent probe in cellular tracking systems as well as a thiol-based dye-labeling reagent due to its easy preparation from readily available materials, environmental sensitivity and simple activation to produce distinct spectral states. The enhanced spectral properties of the covalent TAMRA dimer described here could be useful to prepare more advanced reporter molecules and bioconjugates.

Keywords

Thiolated fluorescent dye; dimer fluorescence quenching; dimer absorbance shift; intracellular fluorescence probe; bio reduction reporter

INTRODUCTION

Reactive fluorescent dyes have many biological and biomedical applications as assay reporters and tracking molecules both *in vitro* and *in vivo* (1–4). A number of reactive fluorescent dyes are commercially available containing functional groups such as N-hydroxy succinimide (NHS) esters, isothiocyanates, maleimides, or iodoacetamides to couple with naturally occurring reactive amine and thiol functional groups on peptides, nucleic acids, saccharides,

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proteins and other biomolecules (5). Currently, several thiolated fluorescent dyes are commercially available, produced by modification of fluorescein (Invitrogen and Toronto Research Chemicals Inc.), tetramethylrhodamine (TAMRA), sulforhodamine, dansyl and Texas Red® (Toronto Research Chemicals Inc.). These dyes are available with protective groups (acetyl, methanethiosulfonate) attached to the desired reactive thiol or sold as the free mercaptan form (although a mixture of disulfide and mercaptan dyes is likely). Of the above-mentioned reactive dyes, care must be taken when using fluorescein-based derivatives as they are known to be pH-sensitive, with quantum yield decreasing by ~70–80% upon acidification and formation of the neutral molecule (6,7). Observing such fluorescein-labeled molecules that are trafficked to lysosomes within cells is difficult due to decreased dye fluorescence signal within these acidic subcellular compartments.

The current popularity of reducible disulfide-containing polymeric cellular delivery systems for therapeutic transgenes, tumor drugs, and imaging agents merits development of new disulfide exchange reagents capable of providing intracellular reporting information on cell-based polymer degradation (8–10). Investigation of a non-exotic dimeric thiol-containing, pH-insensitive, reactive fluorescent dye reagent was the focus of this work. Tetramethylrhodamine (TAMRA) was chosen for this study because of its excellent photostability, and quantum yield unaffected at acidic pH (11). In fact, several intracellular pH estimates have been described based on the ratio of fluorescence signal from simultaneous observations of fluorescein and TAMRA (12–14).

In this short work, we reevaluate the optical properties of dimeric TAMRA with the dimer covalently linked via a disulfide bond. This dimer was reversibly converted to monomer by addition of disulfide reducing agent or biological milieu. The observed effect of covalent dimer formation on fluorescence and absorption spectra was significantly higher than previously observed in non-covalent dimer systems.

EXPERIMENTAL PROCEDURES

General

All solvents and reagents were purchased from commercial sources and used as received. NMR spectra were recorded on a Magnex Scientific Inova 400 MHz instrument in DMSO- d_6 containing 5% w/v tetramethylsilane as an internal standard. Absorbance measurements were obtained on a Varian Cary 500 Scan and BioTek Synergy 2 plate reader instruments. All samples were weighed to the nearest 0.01 mg using a Mettler Toledo Pro Balance 220/81 GX 0.1/0.01MG analytical balance. Size exclusion chromatography was performed using a HP1050 HPLC system equipped with an HP1040A refractive index detector and Biosil 125 column. Sodium phosphate buffer (0.05 M, pH 7.4) mobile phase, 100 μ L sample injection, and 0.5 mL/min flow rate was used for each analysis. RAW 264.7 murine monocyte-macrophage cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 (Mediatech) supplemented with 10% fetal bovine serum (FBS, HyClone, Inc.) and 1% penicillin-streptomycin (Life Technologies). Cultures were maintained in T-175 TCPS flasks (Nunc™) under standard conditions: incubation at 37 °C, 98% humidity and 5% CO₂ and dissociated from culture flasks by incubation with Ca²⁺- and Mg²⁺- free cell culture grade Hank's balanced salt solution (HBSS, Life Technologies).

Synthesis of disulfide TAMRA dimer

TAMRA disulfide dimer (ssTAMRA) was prepared by reacting 5-(6)-carboxytetramethylrhodamine, succinimidyl ester (5(6) TAMRA-NHS, AnaSpec) with cystamine hydrochloride activated using diisopropylethylamine (DIPEA). In a typical experiment, 9.6 mg cystamine hydrochloride (0.085 mmol, 0.9 eq amines) was activated in 2.5

mL dimethylformamide (DMF) containing 30 μ L DIPEA (0.17 mmol, 1.8 eq). This solution was then added to a solution of 50.0 mg TAMRA-NHS (0.095 mmol, 1.0 eq) in 1.5 mL DMF and stirred at room temperature for 30 minutes. Then 15 μ L (0.086 mmol, 1.0 eq) of DIPEA was added and the reaction was stirred for an additional hour. Product was precipitated by addition of 50 mL cold diethyl ether, washed several times with diethyl ether and dried under vacuum overnight. Solids were further purified by repeated precipitation from methanol into cold ether. Product yield: 75%. Formation of the desired dimer was confirmed by mass spectrometry, MS-FAB $M[H^+]$ calculated = 977.336, found = 977.334. 1H NMR analysis confirmed formation of the new amide bonds, with four relevant peaks observed in an unambiguous region of the spectrum, corresponding to combinations of 5 and 6 TAMRA isomers (8.58, 8.91, 9.00, 9.07 ppm, triplets, total integration = 2H). The structure of ssTAMRA is shown in Figure 1.

Determination of molar extinction coefficients

ssTAMRA was dried under vacuum overnight then weighed to the nearest 0.01 mg inside of a 200 mL ($\pm$ 0.1 mL) volumetric flask and then subsequently dissolved in 200 mL buffer (0.01 M sodium phosphate, 0.15 M NaCl, 10 mM EDTA, pH = 7.2). This solution was then serially diluted using 25 mL ($\pm$ 0.03 mL) volumetric flasks. The absorbance at 520 nm was measured to the nearest 1×10^{-6} absorbance units using a Varian Cary 500 Scan spectrometer. The molar extinction was determined as the slope of the bestfit line of a plot of absorbance vs. concentration ($y = 128358x$, $R^2 = 0.9999$), and further normalized to moles TAMRA by dividing this value by 2. The molar extinction of reduced sTAMRA was determined in a similar fashion, except that ssTAMRA was dissolved in phosphate buffer supplemented with 10 mM TCEP.HCl and then incubated for 30 minutes at room temperature before preparing diluted samples. The resulting bestfit line from a plot of absorbance vs. concentration was found to be $y = 66131x$, $R^2 = 0.9999$. The sample purity was assumed to be 100%, as no starting material was observed in mass spectrometry or NMR measurements.

Oxidation of sTAMRA

Disulfide oxidation was determined following complete reduction of ssTAMRA with agarose gel-immobilized TCEP (Pierce) in buffer (0.01 M sodium phosphate, 0.15 M NaCl, 10 mM EDTA, pH 7.2). Upon removal of reducing agent by filtration, the resulting sTAMRA solution (1.2×10^{-5} M) was capped under ambient atmosphere and maintained in the dark at room temperature (22 $^{\circ}C$), and absorbance spectra were recorded at various times. The rate of disulfide formation was determined by plotting the 554/520 nm absorbance ratios.

Reduction of ssTAMRA with cell lysate

Reduction of ssTAMRA was monitored in RAW 264.7 murine monocyte-macrophage cell lysate over time. In this experiment, 1 mL of M-PER $^{\circ}$ cell lysate solution (Pierce, Rockford, IL) was added per 1 g of cell pellet and the resulting solution was processed per manufacturer recommendations and stored at $-20^{\circ}C$ prior to use. Cell lysate solution was diluted 1:1 with ssTAMRA solution to yield a final TAMRA concentration of 1.15×10^{-5} M TAMRA and then incubated at room temperature in a microwell plate. TAMRA absorbance at both 554 and 520 nm was measured at various times using a BioTek Synergy 2 plate reader.

Conjugation of sTAMRA to poly[N-(2-hydroxypropyl)methacrylamide]

Poly N-(2-hydroxypropyl)methacrylamide] (HPMA) copolymer containing reactive esters, further converted to hydrazide groups, was prepared as previously described (15). Prior to hydrazide modification, a portion of reactive esters were used to conjugate Oregon Green 488 fluorescent dye to the polymer backbone (~ 0.7 dyes/polymer) by reaction with amine-containing Oregon Green 488 cadaverine in the presence of DIPEA in DMF. Finally,

hydrazides were converted to maleimide groups by reaction with the heterobifunctional crosslinker *N*-4-(acetylphenyl) maleimide (APM). Preparation of the reactive HPMA copolymer is outlined in the Supporting Information section.

Dimeric ssTAMRA (7.0 mg, 1.6 μ mol) was reduced using TCEP reducing gel (2.5 mL slurry/ μ mol ssTAMRA dimer) for 45 minutes, followed by removal of the reducing agent by filtration through a 0.2 μ m filter. Reduced sTAMRA was then reacted with maleimide-derivatized poly (HPMA) (40.45 mg, 1.60×10^{-6} mol maleimide groups) for 1.5 hr in 0.01 M sodium phosphate, 0.15 M NaCl, 10 mM EDTA, pH 7.2, then remaining maleimide groups were quenched by addition of 2 μ L β -mercaptoethanol. The dual dye-labeled poly(HPMA) product was separated from free sTAMRA dye and desalted using size exclusion chromatography (SEC) (Sephadex G-25, 15 $\times$ 1 cm, H₂O mobile phase) then isolated using a speed-vac. TAMRA content was determined by absorbance measurements at 554 nm using $\epsilon = 6.4 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$. The polymer-TAMRA conjugate was analyzed by SEC; BioSilect 125 column, 0.5 mL/min 0.01 M sodium phosphate buffer containing 0.15 M NaCl and 10 mM EDTA, pH 7.2.

RESULTS AND DISCUSSION

Synthesis of the disulfide-containing ssTAMRA probe was readily achieved by nucleophilic displacement of NHS esters contained on TAMRA by cysteamine's two terminal amines. Cysteamine was selected as the starting material because it contains an internal disulfide bond, thus only amide formation between cysteamine and TAMRA facilitates formation of the disulfide-containing dimer. Preparation of dimeric fluorescent reporter molecules has received much attention due to the different spectral properties of these molecules in monomeric and dimeric forms (16–18). Spectral properties of the reduced monomeric TAMRA (sTAMRA) and oxidized dimeric thiolated TAMRA (ssTAMRA) are summarized in Table 1. Reduction of the disulfide was readily achieved after incubation in 10 mM tris(2-carboxyethyl) phosphine hydrochloride (TCEP-HCl), with no increase in 554 nm sTAMRA monomer absorbance observed after 30 minutes, indicating complete reduction. Oxidized ssTAMRA exhibited a hypsochromic shift of 34 nm relative to the reduced sTAMRA absorption maximum, close to native TAMRA (Fig. 2a, Table 1). This phenomenon has been observed for concentrated aqueous solutions of rhodamine B with the formation of non-covalent dimers (dissociation constant of 6.8×10^{-4} M) observed at mM concentrations (19,20). This hypsochromic shift has also been used to study conformation and subunit exchange of *E. coli* ribosomal protein L7/L12, and conformation of the muscle protein, titin, with cysteine thiol groups labeled with tetramethylrhodamine iodoacetamide (21,22). These studies utilized the ratio of non-covalent dimer absorbance at 518 nm to monomer absorbance at 555 nm to determine proximity of TAMRA labels on the protein, with a 518/554 absorbance ratio of 1–1.3 reported for non-covalent dimeric TAMRA (21). Additionally, a 519/550 ratio for an analogous covalent TAMRA dimer linked by thiourea bonds and an ethylene spacer has been reported to be 1.5 (23). Results for ssTAMRA in this study show a 520/554 ratio of 2.64 for the covalent disulfide dimer, exceeding all spectral ratios previously described and indicating significantly that the longer disulfide 4-carbon spacer used in this study allows for increased dye interaction and enhanced spectral properties. This ratio might also be further increased by using pure starting material, instead of mixed 5 and 6 TAMRA-NHS isomers as reported here.

Dimer formation had no effect on optical absorption of attached TAMRA, as molar extinction coefficients for reduced and oxidized species were nearly identical. However, fluorescence emission was nearly extinguished upon dimer formation, with fluorescence intensity reduced by > 93% in ssTAMRA when excited at the isosbestic point (532 nm). This is likely due to self-quenching from stacked xanthene rings as previously described for analogous rhodamine B, rhodamine 6G, and TAMRA covalent dimers in aqueous solution (20,24,25). Stacking proximity of a folded covalent TAMRA dimer containing an ethylenediamine spacer has been

estimated as 0.9 nm (23). Fluorescence quenching of rhodamine dimers appears to be solvent dependent, with no quenching observed in mixed water/organic solvent systems but observed in aqueous buffered systems as also described here (22–25).

The reversibility of disulfide reduction/oxidation was determined following complete reduction of ssTAMRA and removal of reducing agent. Reduced dye is susceptible to oxidation by divalent metals such as Zn^{2+} , Cu^{2+} and Mg^{2+} , or oxygen in aqueous media resulting in formation of the dye disulfide dimer. However, in the conditions used in this experiment the major cause of sTAMRA oxidation is likely dissolved oxygen, as deionized water and EDTA were used to reduce ion effects and the solution was not degassed or stored under inert atmosphere. Absorbance spectra at various times are shown in Figure 2a with an isosbestic point apparent at 532 nm. The rate of ssTAMRA disulfide formation was determined by plotting the 554/520 nm absorbance ratios as shown in Figure 2b. The absorbance spectra shown in Figure 2a confirm sTAMRA oxidation and reformation of disulfide bonds by the increasing 520 nm absorbance value over time. In this experiment, the absorbance value for oxidized ssTAMRA never reached the same value as the initial absorbance value shown for reduced sTAMRA, despite very similar extinction coefficients reported in Table 1. This is likely due to the fact that reformation of the ssTAMRA dimer does not proceed to 100% conversion in this time. The reduced dye thiol is relatively stable at μM concentrations in this buffer system for several hours ($t_{1/2} \sim 50$ hr), but stability could likely be enhanced by degassing the storage solution and storing it under inert atmosphere. This type of experiment could be useful for preparation of bioconjugates when confirmation of disulfide reduction or sulfhydryl stability information for specific reaction or storage conditions is required.

The utility of ssTAMRA as a disulfide reduction reporter molecule *in vitro* was demonstrated using fresh lysate from cultured harvested RAW 264.7 macrophage cells. These cells were selected since they are common models for phagocytosis and processing of drugs, toxins, polymers and particles in the endosomal/lysosomal cell trafficking pathway (26). The ssTAMRA dye was effectively reduced in lysed RAW 264.7 cells over time, with ~80% reduction observed after 4 hrs (Fig. 3). Recently, a disulfide-reducible FRET reporter molecule containing folate was used to evaluate disulfide reduction during endocytosis (27). In this approach, the FRET pair is prepared by attachment of dyes to the reporter molecule in different synthetic steps, however, reporter systems based on self-quenching of TAMRA molecules covalently bound in close proximity by disulfide groups may reduce synthetic steps and cost by reaction of the reporter molecule with an excess of relatively inexpensive TAMRA derivative.

Further application of sTAMRA as a traditional reactive probe was realized by covalent reaction with Oregon Green®-labeled, maleimide-derivatized, poly[N-(2-hydroxypropyl) methacrylamide] (polyHPMA), a polymer often selected as a model for polymer-mediated uptake and processing of drugs and other agents to cells both *in vitro* and *in vivo* and much is known about its endosomal and lysosomal trafficking within cells (28,29). This particular fluorescent labeled HPMA copolymer was designed for reversible attachment of thiol-containing peptides via hydrazone and maleimide chemistries and the thiolated ssTAMRA described here served as a model reporter molecule to confirm thiol coupling to the polymer backbone. This dual-labeled water-soluble polymer allowed simultaneous observation of the Oregon Green® backbone and reacted sTAMRA using diode array detection at absorption wavelengths for Oregon Green® 488 (496 nm) and sTAMRA (554 nm) during SEC analysis. It should be noted that once conjugated to the polymer backbone in this fashion, the dye loses its environmental sensitivity, so this experiment was performed to confirm the utility of ssTAMRA as a reactive dye probe analogous to other thiolated dyes. Conjugation of reduced sTAMRA to the polymer backbone was confirmed, as TAMRA eluted in the polymer fraction at 12–22 min as shown in Figure 4 (free dye elutes from this column after 30 minutes under

these conditions). The small amount of free dye observed after the polymer peak in Figure 4b is a characteristic of the degradable crosslinker used to incorporate maleimide groups into poly (HPMA), designed for intracellular delivery and release of peptides. The amount of dye conjugated to the polymer backbone was 25.9 $\mu\text{mol/g}$, corresponding to ~10% conjugation under the conditions described here. This coupling efficiency could likely be increased by longer reaction time and reaction with more stable precursors, as polymer degradation is evidenced as the tailing peak in Figure 4b.

CONCLUSION

In summary, a reactive thiolated dimeric TAMRA-based fluorescent reporter is described that is inexpensive and convenient to produce. The covalent ssTAMRA disulfide dimer exhibits unique spectral properties useful in the study of disulfide exchange or other disulfide redox processes by either absorbance shifts or increases in fluorescence emission after generation of monomeric sTAMRA. Reduction of ssTAMRA dimers is readily achieved using TCEP, with reduced sTAMRA being reactive to maleimide functionality. Conjugation of this reduced sTAMRA to nucleic acids, enzymes, peptides, polymers, proteins, saccharides or other relevant targets useful for cell trafficking studies is shown to be feasible for application as a reactive fluorescent probe. The sensitivity of ssTAMRA to reduction by biological milieu (i.e., cell lysate) shows its potential as a reporter molecule in cell or organism-based studies where local oxidizing or reducing environmental changes would produce probe spectral shifts *in situ*.

The enhanced spectral properties of the covalent TAMRA dimer described here could be useful to prepare more advanced reporter molecules and bioconjugates. It would be rather simple to build specialty probes containing dimeric TAMRA that are attached to moieties that affect and track activity in biological systems. For example, many heterobifunctional PEGs and multifunctional crosslinkers are available that could be used to build probes for specific applications. The utility of dimeric TAMRA is not limited to disulfide dimers, TAMRA dimers formed using an acetal as the anchor point would result in a pH-sensitive probe. With TAMRA dimers, environment-specific information can be obtained using a single fluorophore rather than two as needed in FRET experiments. This would simplify preparation of probes, eliminating the need for extensive purification and complex reaction schemes. Covalent TAMRA dimers could serve as powerful yet simple tools to interrogate biological systems.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Structure of ssTAMRA dimer.

Figure 2. Oxidation of sTAMRA monomer in 0.1 M sodium phosphate, 0.15 M NaCl, 10 mM EDTA, pH 7.2. a) absorbance spectra of 1.2×10^{-5} M sTAMRA at various times. b) Ratio of 554/520 nm absorbance spectra showing disulfide formation rate.

Figure 3. Reduction of ssTAMRA (1.15×10^{-5} M TAMRA) in fresh cell lysate from cultured RAW 264.7 cells at room temperature. Cell lysate background absorbance has been subtracted at both wavelengths for ratios calculated for each data point.

Figure 4.

Size exclusion chromatography trace of dual-labeled poly(HPMA) in 0.01 M sodium phosphate buffer containing 0.15 M NaCl and 10 mM EDTA, pH 7.2 before (a) and after (b) sTAMRA conjugation. Detection at 496 nm (solid) corresponds to polymer backbone (Oregon Green®), and 554 nm detection (dashed) corresponds to sTAMRA.

Table 1
Spectral properties of oxidized and reduced thiolated TAMRA.

	λ_{max} absorbance (nm)	ϵ ($\text{M}^{-1}\text{cm}^{-1}$) ^a	554/520 ratio	520/554 ratio	λ_{max} emission (nm)	Relative fluorescence ^b
Reduced sTAMRA	554	6.61×10^4 $\pm 0.18 \times 10^4$	2.37	0.42	578	1.00
Oxidized ssTAMRA	520	6.42×10^4 $\pm 0.17 \times 10^4$	0.38	2.64	578	.061

^a expressed relative to moles TAMRA, determined at λ_{max}

^b excitation at isosbestic point, 532 nm