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ORIGINAL PAPER

Hydrophobic Interactions in Donor-Disulphide-Acceptor (DSSA) Probes Looking Beyond Fluorescence Resonance Energy Transfer Theory

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Abstract Donor –linker –acceptor (DSSA) is a concept in fluorescence chemistry with acceptor being a fluorescent compound (FRET) or quencher. The DSSA probes used to measure thiol levels in vitro and in vivo. The reduction potential of these dyes are in the range of −0.60 V, much lower than the best thiol reductant reported in literature, the DTT (−0.33 V). DSSA disulphide having an unusually low reduction potential compared to the typical thiol reductants is a puzzle. Secondly, DSSA probes have a cyclized rhodamine ring as acceptor which does not have any spectral overlap with fluorescein, but quenches its absorbance and fluorescence. To understand the structural features of DSSA probes, we have synthesized DSSANa and DSSAOr. The calculated reduction potential of these dyes suggest that DSSA probes have an alternate mechanism from the FRET based quenching, namely hydrophobic interaction or dye to dye quenching. The standard reduction potential change with increasing complexity and steric hindrance of the molecule is small, suggesting that ultra- low Eo' has no contribution from the disulphide linker and is based on structural interactions between fluorescein and cyclized rhodamine. Our results help to understand the DSSA probe quenching mechanism and provide ways to design fluorescent probes.

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Abbreviation

DSSA	Donor–Disulfide linker–acceptor
$DSSA_{Al}$ –DSSA	Probe with cystamine as a linker, $DSSA_{Ar}$ –DSSA probe probe with diaminophenyl disulfide as a linker
$DSSA_{Na}$ – DSSA	Probe with 2,2'-dithiodi (1-naphthylamine) as a linker
$DSSA_{Or}$ – DSSA	Probe with o-diamino diphenyl disulphide as a linker
FRET	Forster *(*Fluorescence) resonance energy transfer, chemiluminescence resonance energy transfer (CRET)

Introduction

Dithiols plays an important role in biology. They form the basis of redox state in cells to give active confirmation for proteins. Cysteine is responsible for forming disulphides in proteins and glutathione (GSH) mediates the redox state inside cells by switching between reduced and oxidized glutathione (GSH/GSSG) [1, 2]. Most aquatic organisms like zebra fish have thiol rich proteins in chorion to protect the embryo's heavy metal contamination [3, 4]. The switch between reduced and oxidised form help the capture and release of heavy metal toxins like Hg, Pb present in water. Considering the quantum of pollutants released in fresh water (aquatic bodies) every year the survival of the aquatic species is due to the in built redox mechanism and switch of SH/S-S of chorion proteins. But the extent of the variability of the thiol redox state within different cellular micro environments is not well understood. To address this we reported a set of fluorescent

probes called DSSA probes comprising of a donor and acceptor separated by a disulphide [5]. The DSSA probe for in vivo fluorescent imaging of cells and organisms has taken a rapid flourishing and there are more than forty different fluorescent probes reported in literature for thiol determination [6–14]. Some of these are now sold commercially for mitochondria imaging systems based on cyclized rhodamine chemistry [15]. FRET probes are the most commonly use in biological application, There are different types of FERT base probes are reported in literature [16, 17]. Qualitative measurement of cellular thiols in cells using the DSSA probes has grown to a separate fluorescence field. The thiol measurement tools offered by DSSA system are unique that it measures the redox state inside cell without affecting the cellular redox state as shown in Fig. 1. The best thiol reductant known in literature is DTT and it has standard reduction potential of −0.33 V [18]. The DSSA probes are disulphides and surprisingly carry a redox potential of −0.6 V and still have no significant effect on the cellular redox state. Even the standard reductions potential of other common reductants like BME (−0.26 V) and TCEP (−0.34 V) [5, 19, 20] are not even in the reach of the redox potential of DSSA probes. Understanding the unusually low redox potential of DSSA probes offers designing in vivo imaging tools for biochemists and cell biologists.

Thiol Reactive Probes

Redox state inside cell is a result of dynamic equilibrium of GSH/GSSG and protein thiols. Hence, static probes like thiol reactive compounds provide only information of redox state at a given time and not the dynamic equilibrium, hence redox sensitive FRET probes were developed for measuring GSH/GSSG inside the cell [21] . The examples of static probes used for thiol quantitation in vitro are DTNB, maleimide substituted compounds, acid chlorides, NBD-F, etc. [22]. Inside cell thiol associated enzymes like thioredoxin, glutathione s-transferase, glutathione oxidase etc. rebalance the GSH/GSSG equilibrium [23, 24]. The total thiol concentration of a cell inclusive of GSH, GSSG, protein thiols constitutes about 100 mM and a thiol reactive compound of 100 mM cannot be introduced inside a living cell. Hence, none of the thiol reactive probes are useful for in vivo imaging of live cells [6–14]. Difference in using dithiol probes and thiol specific reagents for in vivo applications and their reaction mechanism is as discussed in Fig. 1, Q is a static probe and reacts with a thiol RSH and gives RSQ. If probe Q is used to monitor changes in concentration of thiols in real-time, the probe Q is consumed within first few seconds due to large excess of thiols (Le Chatlier's principle). Using static probes provides only minimal information about redox state and has to be done only by dithio probes which participate in the GSH/GSSG oxidation-reduction mechanism. However, the 100 mM concentration of thiols inside cells necessitates use of fluorescent dithiol probes of very low reduction potential like DSSA probes. DSSA probes offer a simple way to measure changes in intra cellular thiol levels by switching between oxidized and reduced forms.

Functioning of DSSA Probes & Lack of Understanding of Mechanism

It is well documented that disulphides inside cells participate in thiol exchange and the DSSA probes are reported to participate in similar exchange process. Due to DSSA probe's low standard reduction potential, they are difficult to reduce and could survive in an extremely thiol rich environment with just 1–10 μM probe. Fig 1 explains the uniqueness of DSSA probes in reaction with thiols. The DSSA probes in general prefer to stay in oxidized form and have very low reduction potential. We reported that a 10 μM DSSA probe can stay partially in oxidized form even in the presence of 10–100 mM DTT [5]. We also reported that two dithiol probes $DSSA_{Ar}$ & $DSSA_{Al}$ the molecule structure as shown in Fig. 2, can the cross cell membrane due to turn on/off mechanism [5] of the cyclized Rhodamine group [25–33]. Hence, DSSA probe in mM concentration of thiol creates a dynamic equilibrium wherein depending on the concentration of free thiols, the DSSA probe stays in either oxidized or reduced form. The mechanism of DSSA probes is still a puzzle and how did DSSA probes possess a reduction potential lower than protein reductants like DTT, TCEP and BME and a 10 μM DSSA probe can stay oxidized in presence of 10 mM thiol need to be understood. The fluorescence based reduction potential determination needs to be understood in the context of structural

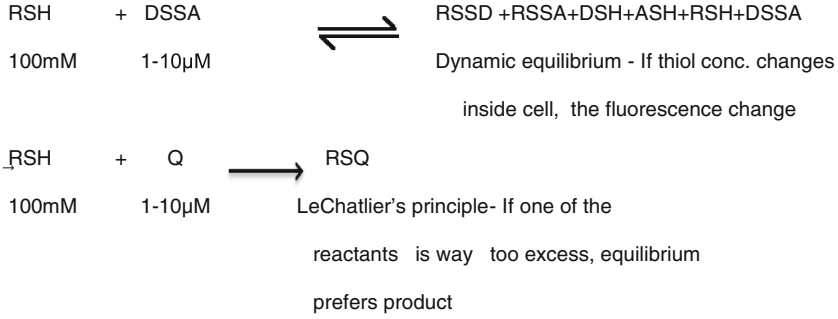
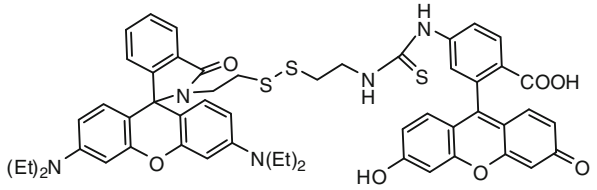


Fig. 1 Difference of using dithiol probes vs thiol specific reagents for in vivo applications



$DSSA_{Al}$ = Al subscript means cystamine linker

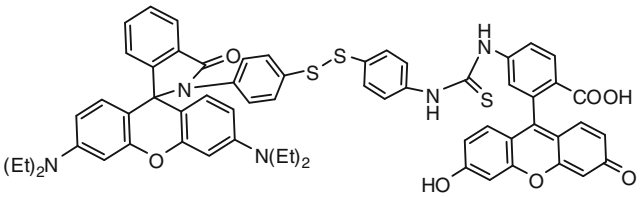


Fig. 2 The structure of disulphides synthesized in Reference- 5 ($DSSA_{Ar}$ & $DSSA_{Al}$)

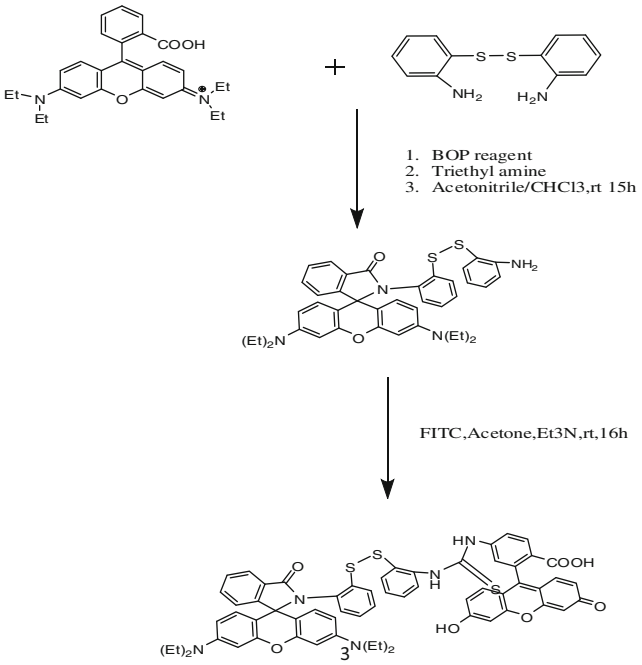
features of these dyes like dye to dye interactions involving hydrophobic effects, steric hindrance, static and dynamic quenching [34]. We attempted to understand these effects using two new DSSA probes which carry the similar structural features of original $DSSA_{Ar}$ and $DSSA_{Al}$, but having an even lower reduction potential.

Materials and Methods

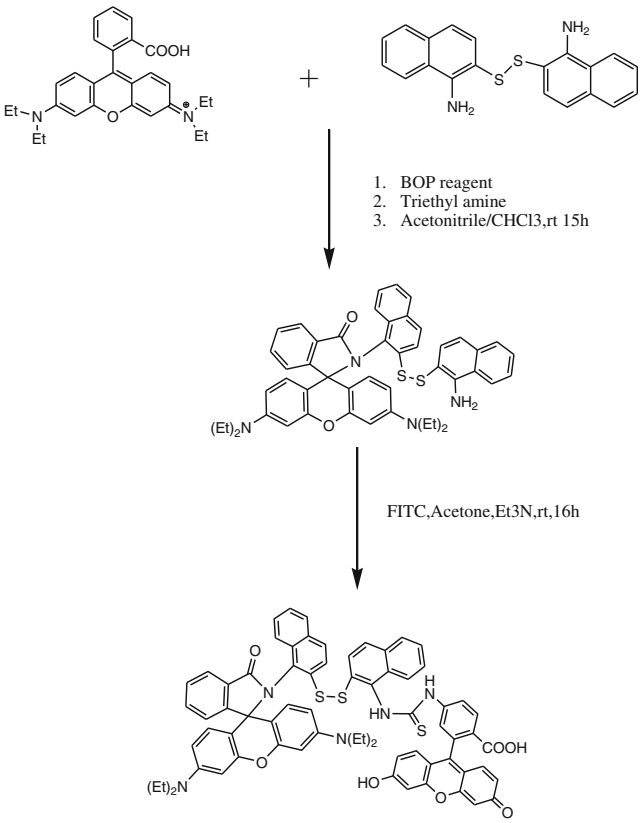
Reagents: 2-amino phenyl disulphide, Rhodamine-B, FITC, Chloroform, acetonitrile, 2, 2'-dithio di (1-naphthyl amine), BOP reagent, DTT, TCEP, BME and HEPES were purchased from Sigma Aldrich. The fluorescence spectra were recorded on a Shimadzu spectro fluorophotometer RF-5301PC. Absorbance readings were done on a Beckman-Coulter DU 700 UV/VIS spectrometer. All spectral readings reported in this document were done in 100 mM Tris buffer, pH 8.2.

Synthesis of DSSA Probes

DSSA probes were synthesized using the protocol reported by our group [5]. In brief, the diamino-diaryl-disulphide is reacted with rhodamine B in 4:1 acetonitrile/chloroform in the presence of triethyl amine and BOP reagent for 15 h at room temperature. The mono substituted rhodamine amide is purified by silica gel (230–400 mesh) using 20 % ethyl acetate in hexane. The purified amide is reacted with FITC in acetone at room temperature for 12 h. The final DSSA probes are purified by silica gel chromatography using 30 % hexane in ethyl acetate. Overall reaction yield of $DSSA_{Or}$ is 10 %, $DSSA_{Na}$ yield is 6 % (Scheme 1 & 2).



Scheme 1 Synthesis of $DSSA_{Or}$. Rhodamine B is reacted with O-amino diphenyl disulphide reacted in presence of BOP reagent and triethyl amine as reported in Ref 5. The resultant non-fluorescent cyclized rhodamine product is reacted with FITC to obtain $DSSA_{Or}$



Scheme 2 Synthesis of the $DSSA_{Na}$ followed by same procedure like $DSSA_{Or}$ (Scheme 1) using 2, 2'-dithio di (1-naphthyl amine) as a starting material

Purification of Probes for Characterization and Fluorescence Studies

DSSA dyes are purified by repeated preparative TLC to obtain necessary purity for CHN analysis. The same product is used for absorbance and fluorescence studies.

$DSSA_{Na}$: Found (expected): C 71.37 (71.30), H 4.78 (4.77), N 6.05 (6.02), Mass: 1162.3292 (1162.3993)

^{1}H NMR (acetone-d6): 9.4 (s, 1H), 9.2 (s, 1H), 9.1 (s, 1H), 8.2 (1H), 7.9 (2H), 7.6 (2H), 7.5 (6H), 7.4 (2H), 7.0 (2H), 6.8 (2H), 6.6 (2H), 6.5 (8H), 6.4 (2H), 6.2 (2H), 3.3 (8H), 1.3 (12H).

^{13}C NMR (acetone-d6): 207, 186, 166, 165, 161, 156, 154 (two peaks), 146, 140, 135, 134 (four peaks), 132, 130, 129, 127 (six peaks), 125, 124 (four peaks), 116, 112, 110, 109, 107, 105, 101, 98, 47, 13.

$DSSA_{Or}$: Found (expected): C 68.99 (68.97), H 4.87 (4.84), N 6.62 (6.59), Mass: 1062.2985 (1062.2819) ^{1}H NMR (acetone-d6): 9.5 (s, 1H), 9.3 (s, 1H), 9.1 (s, 1H), 8.2 (1H), 7.6 (2H), 7.7 (6H), 7.4 (2H), 7.1 (1H), 7.0 (3H), 6.5 (8H), 6.4 (2H) 6.2 (2H), 3.4 (8H), 1.2 (12H).

^{13}C NMR (acetone-d6): 203, 182, 167, 165, 158, 154, 153 (two peaks), 147, 140, 137, 134, 133 (three peaks), 131, 130, 128 (six peaks), 124 (three peaks), 117, 113, 110, 109, 106, 104, 100, 98, 44, 12.

Results

Absorbance and Fluorescence of DSSA Probes

The absorbance and fluorescence spectra of synthesized molecules are similar to the DSSA probes reported earlier. The ortho phenyl ($DSSA_{Or}$) or naphthyl rings ($DSSA_{Na}$) do not participate in the conjugation of fluorescein ring and the spectra are similar to $DSSA_{Ar}$. This study is to find correlation between the structure of the molecules and their standard reduction potentials. Hence, comparison of the structure of molecules and their redox potentials can be done in the present study only if fluorescence and absorbance spectra are similar [5]. The $DSSA_{Na}$ had a slightly lower intensity when compared to $DSSA_{Or}$ at the same concentration of dye as shown in Fig. 3, but the change is not too significant and hence both $DSSA_{Or}$ and $DSSA_{Na}$ are good model compounds.

Determination of Standard Reduction Potentials

The procedure followed is as reported earlier by our group and a detailed discussion was done in the earlier publication [5] on calculating each of the involved terms. The standard reduction potentials of DTT ($E^{o'}$=−0.33 V) from reference [18] and standard reduction potential of TCEP is −0.32 V from our earlier publication [5]. A reaction between DSSA probe and TCEP can be considered as a E_{cell} reaction.

Hence, $E_{cell}=E_{cathode}-E_{anode}$

$E_{cell}=E_{DSSA}-E_{TCEP}$

$E_{DSSA}=E_{cell}+E_{TCEP}$

The E_{TCEP} reported by our group is −0.32 V and it is used to determine the E_{DSSA} [5]. The standard reduction potentials of disulphides synthesized in Ref 5 ($DSSA_{Ar}$ & $DSSA_{Al}$) and thiol reductants (DTT & BME) are given in Table 1. The standard reduction potentials of disulphides synthesized in this study ($DSSA_{Or}$ & $DSSA_{Na}$) are given in Table 2. The detailed procedure of standard reduction potential calculation including the explanation of each term is given in Table 3A. Table 3B provides the calculation of standard reduction potential of (E_{DSSANa}) and Table 3C provides the calculation of standard reduction potential of (E_{DSSAOr}).

Reactivity of DSSA Probes With Different Reductants

We studied the fluorescence response of DSSA probes in the presence of TCEP and DTT and the results are given in Fig. 4.

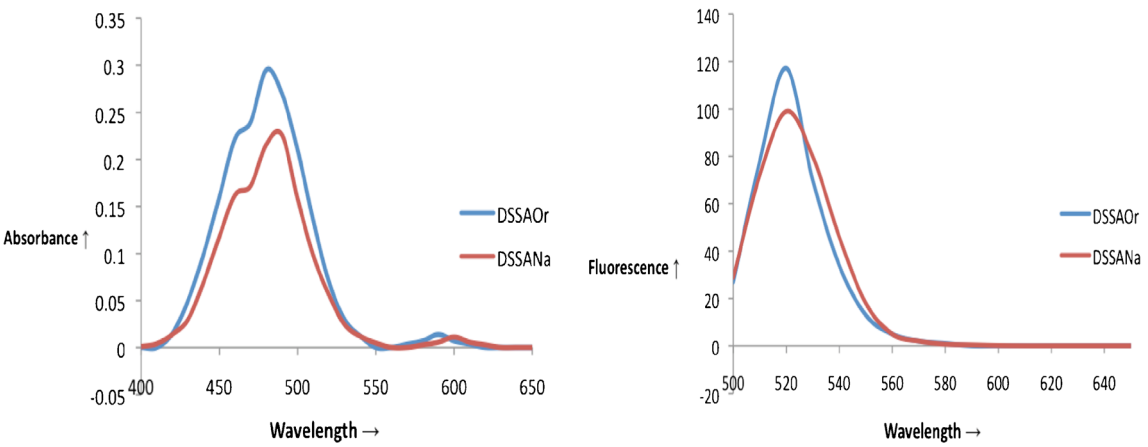


Fig. 3 The absorbance and emission spectra of $DSSA_{Or}$ and $DSSA_{Na}$ respectively. The concentrations used for the study are 125 μM (absorbance) and 2.5 uM (fluorescence)

Table 1 Standard reduction potentials of disulphides developed in this study in Ref 5 ($DSSA_{Ar}$ & $DSSA_{Al}$) and thiol reductants (DTT & BME)

Compound	$E^{o'}$ (V)
DTT	−0.33
TCEP	−0.32
BME	−0.26
$DSSA_{Ar}$	−0.66
$DSSA_{Al}$	−0.604

The probe was used at 2.5 μM concentration and the study was done in 100 mM Tris pH 8.2 buffer. Increase in fluorescence is monitored over time and the fluorescence response at different concentrations is studied.

Purification of DSSA Probes

We purified DSSA compounds using preparative TLC and these rhodamine derivatives switch between cyclized and non-cyclized form in the presence of acidic silicagel as shown in Fig. 5. For obtaining the pure compounds for spectral studies, the DSSA probe from silica gel was extracted with methanol, concentrated and suspended in chloroform.

Discussion

Nomenclature & Synthesis of $DSSA_{Or}$ and $DSSA_{Na}$

Dr. Daniel Sem coined the DSSA probe word and it means a fluorescence donor and acceptor are separated by a disulphide bridge. The subscript 'Ar' means the disulphide has an aryl ring and 'Al' means the disulphide bridge has an aliphatic linker. $DSSA_{Ar}$ was a model compound reported by Dr. Sem's group and had higher $E^{o'}$ compared to $E^{o'}$ of molecules of the present study [5, 35]. The shift from aliphatic chain to aromatic chain has caused −0.06 V decrease in $E^{o'}$. With respect to flexibility of disulphide bridge $DSSA_{Al}$ is expected to possess a better structure for hydrophobic interactions compared to $DSSA_{Ar}$. But as it was proven by Timothy (2011) that π- π interaction in the DSSA probes determines the structure of the dye and $DSSA_{Ar}$ has better quenching helped by π interaction of benzene ring in 4-di amino diphenyl disulphide with attached fluorophores [36]. This conclusion was based on two compounds reported in [5] and necessiate further study. If the linkers can quench the fluorescence of fluorescent probes due to π- π interactions, going from benzene ring to naphthalene ring should provide a significantly lower standard reduction potentials. There are two possible π- π interactions in DSSA probes and one is coming from the linker and the other is due to the interaction between fluorescein and cyclized rhodamine.

Table 2 Standard reduction potentials of disulphides developed in this study $DSSA_{Or}$ & $DSSA_{Na}$

Compound	$E^{o'}$ (V)
$DSSA_{Or}$	−0.697
$DSSA_{Na}$	−0.693

We used $DSSA_{Ar}$ as a model compound and synthesized $DSSA_{Or}$ and $DSSA_{Na}$. $DSSA_{Or}$ is the exact same compound as $DSSA_{Ar}$ except the linker is 2, 2'-diamino diphenyl disulphide. The ortho position of amine and sulphur gave a sterically crowded DSSA probe with same fluorescence and absorbance as $DSSA_{Ar}$. $DSSA_{Na}$ is an analogue of $DSSA_{Or}$ except benzene ring is occupied by naphthalene ring in the linker and has increased steric hindrance at the disulphide ring. The synthesis of these molecules was difficult due to the low yields and need for repeated purification on preparative TLC due to the streaky nature of these dyes on silica. Use of a drop of acetic acid has helped in separation of these dyes in mobile phase (but removal of acetic acid became tough) process. Hence, the last two preparative TLC was done in the same mobile phase in the absence of acetic acid. Fig 5A shows that after the reaction of rhodamine with an amine gives a non-fluorescent compound. Fig 5B shows that the rhodamine ring becomes fluorescent within 10 min due to the acidity of the silica gel. The cyclized and open forms in the context of DSSA probes are discussed earlier using buffers with different pH elsewhere [5]. But purification of DSSA probes done using preparative silicagel requires the extraction with methanol to extract both cyclized and non-cyclized DSSA probes. Upon concentration of methanol, the solid was resuspended in chloroform or dichloromethane to convert the DSSA probes to non-cyclized form.

$DSSA_{Na}$ and $DSSA_{Or}$ Reactivity With DTT & TCEP

As designed, these two new DSSA probes absorbance and fluorescence spectra have similar characteristics as $DSSA_{Ar.}$ The reaction with thiols is also similar albeit slower as the complexity and steric hindrance have increased. TCEP was the most reactive reductant followed by DTT [37]. DTT has given less F_{max} compared to TCEP similar to our earlier observations [5]. We found it interesting to see if DSSA probes can be reduced by DTT equivalent to TCEP. $DSSA_{Na}$ and $DSSA_{Or}$ have similar reactivity towards DTT and TCEP. Fig 4 shows the reactivity of different concentrations of DTT and TCEP with the disulphides of this study. Among the tested reductants, TCEP is the best reductant and it affected both absorbance and fluorescence spectra of the dyes.

We heated the DSSA probes along with DTT at 60 °C and observed that the F_{max} obtained using TCEP and DTT at 60 °C is similar. Heating TCEP with DSSA probes did not yield any

Table 3 Reduction potential determination of DSSA probes using TCEP's reduction potential (Using reported protocol from [5]). This table has three divisions namely A, B, C. A describes the redox reaction between TCEP and DSSA probes. B & C explain the reduction potential calculation using fluorescence from DSSA probes as basis. The terms of the equation are TCEP (mM): Concentration of TCEP in mM; [$TCEP_{red}$]: Amount of TCEP remained in reduced form after reduction; [DSSA]: Concentration of DSSA probe participated in the reaction; [DS]/[AS]: Concentration of DS or AS released during reduction of DSSA; [$TCEP_{Ox}$]: Amount of TCEP oxidized; K_{eq}: Equilibrium constant; F_{DSSA}: Fluorescence reading of DSSA probe in the presence of that particular TCEP concentration; E_{cell}: Cell potential; E_{DSSA}: Standard reduction potential of DSSA probe. F_{min}: Background fluorescence of 5 uM DSSA probe in the absence of reductant; F_{max}: It is the maximum fluorescence obtained upon reaction with 200 mM TCEP at reflux temperature for few hours

A

$$TCEP + H_2O + DSSA \rightleftharpoons TCEP = O + DSH + ASH$$

$$E_{cell} = E_{cathode} - E_{anode}$$

$$E_{cell} = E_{DSSA} - E_{TCEP}$$

$$E_{DSSA} = E_{cell} + E_{TCEP}$$

$$TCE\ P_{red} = [TCEP\ in\ M] - [DS]/[AS]$$

$$[DSSA] = initial\ concentration\ of\ dye - [DS]/[AS]$$

$$[DS]/[AS] = 5\ uM * (fluorescence\ reading\ at\ given\ concentration - F_{min})/(F_{max} - F_{min})$$

$[TCEP_{OX}]$ = same as [DS]/[AS], one molar equivalent of TCEP and DSSA are involved in reaction with each other and the $TCEP_{OX}$ formed will be same as [DS]/[AS]

K_{eq}=([DS]/[AS])^3/([$TCEP_{red}$]*[DSSA])

F_{DSSA} = the fluorescence reading obtained for corresponding concentration of TCEP

E_{cell} = ((0.0592/number of electrons in reaction)*log10 (Keq)), number of electrons = 2

B $DSSA_{Na}$ redox potential determined using TCEP's redox potential

TCEP (mM)	[$TCEP_{red}$]	[$DSSA_{Na}$]	[DS]/[AS]	[$TCEP_{ox}$]	K_{eq}	F_{DSSANa}	E_{cell}	E_{DSSANa}
2	1.9999992	4.153E-06	8.47E-07	8.47E-07	7.32E-14	1352	−0.38882	−0.70793
4	3.9999985	3.529E-06	1.47E-06	1.47E-06	2.25E-13	2200	−0.37436	−0.69348
8	7.9999979	2.882E-06	2.12E-06	2.12E-06	4.12E-13	3081	−0.36659	−0.68571
16	15.999997	2.382E-06	2.62E-06	2.62E-06	4.71E-13	3760	−0.36489	−0.68401
							Average	−0.69278
							standard deviation	0.01091

C $DSSA_{Or}$ redox potential determined using TCEP's redox potential

TCEP (mM)	[$TCEP_{red}$]	[$DSSA_{Or}$]	[DS]/[AS]	[$TCEP_{ox}$]	K_{eq}	F_{DSSAOr}	E_{cell}	E_{DSSAOr}
2	1.9999992	4.234E-06	7.66E-07	7.66E-07	5.3E-14	1352	−0.393	−0.712
4	3.9999987	3.65E-06	1.35E-06	1.35E-06	1.68E-13	2200	−0.378	−0.697
8	7.999998	3.043E-06	1.96E-06	1.96E-06	3.08E-13	3081	−0.370	−0.689
16	15.999998	2.576E-06	2.42E-06	2.42E-06	3.46E-13	3760	−0.369	−0.688
							Average	−0.697
							standard deviation	0.011

F_{min} 200

F_{max} 7000

F_{min} 240

F_{max} 7500

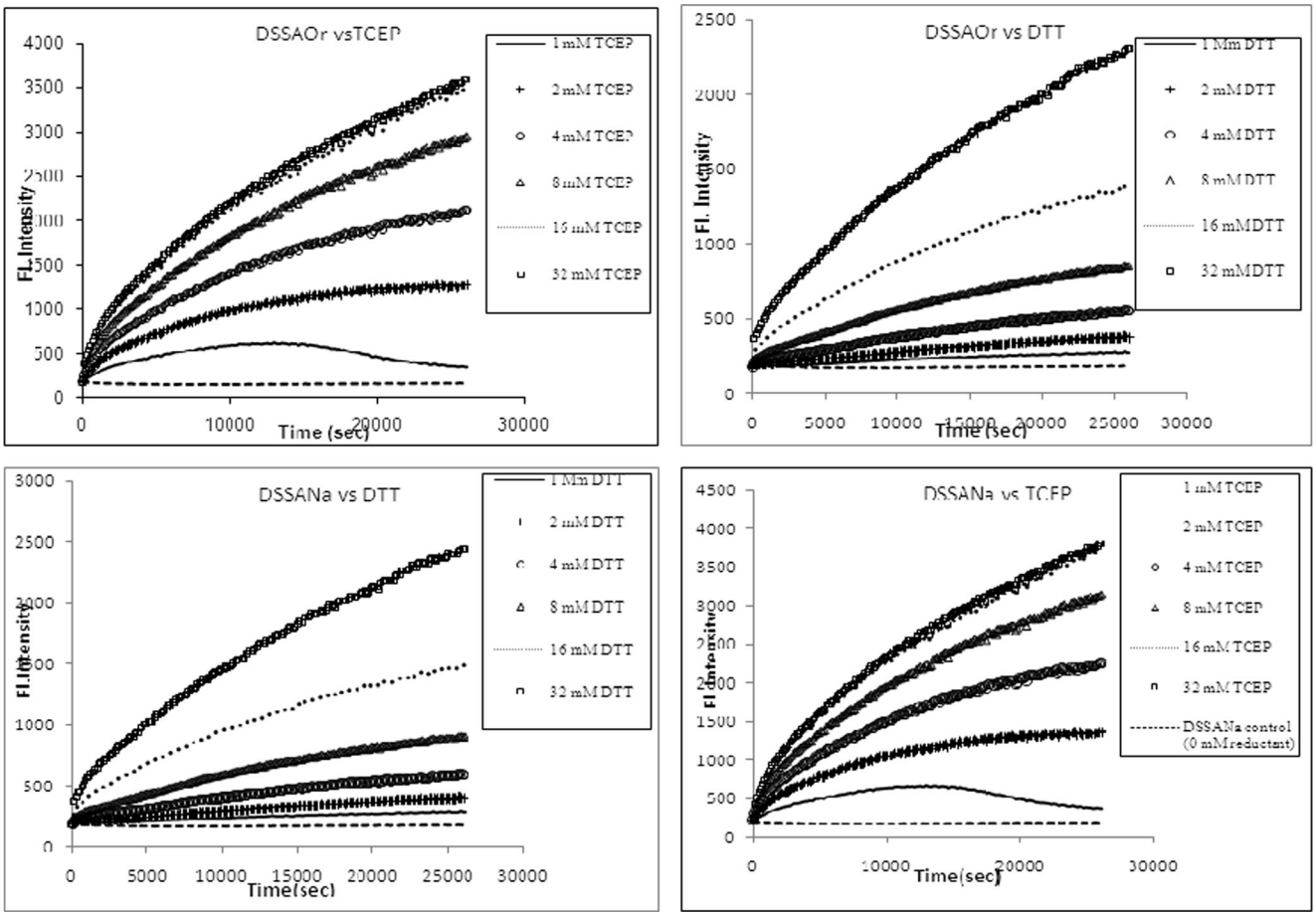


Fig. 4 Shows the reactivity of different concentrations of DTT and TCEP with $DSSA_{Na}$ and $DSSA_{Or}$

improvement in F_{max} indicating that 200 mM TCEP has reduced the DSSA probe completely. Complete reduction of 5 μM DSSA probe requires 200 mM TCEP for few hours' shows the robustness of these dyes for in vivo applications. The DSSA probes were designed to be highly resistant to the reduction in thiol rich cellular environment (about 100 mM of thiols) and they were proven to serve the purpose intended.

Significance of Reduction Potential of DSSA Probes

Increasing complexity and steric hindrance in the DSSA probes has lowered the $E^{o'}$ making them highly resistant to reduction. The change in $E^{o'}$ between $DSSA_{Al}$ and $DSSA_{Ar}$ was significant as reported in Ref [5] and our approach of increasing steric hindrance did not result in significant change in $E^{o'}$. This confirms that the linker has minimal role in $E^{o'}$ and the change observed between $DSSA_{Al}$ and $DSSA_{Ar}$ is due to the π- π interactions between fluorescein and cyclized rhodamine. Even the 'ortho' to 'para' switch in linker ($DSSA_{Ar}$ vs $DSSA_{Or}$) has minimal contribution to $E^{o'}$ as shown in Table 1 & 2. The hydrophobic interactions between the dyes (fluorescein and rhodamine) need to be studied more deeply as the FRET partner rhodamine is in cyclized conformation. Rhodamine B upon reaction with an amine forms the amide which forms a cyclised lactum and is non-fluorescent and the ring opening and closing is reversible in presence of acid as shown in Fig. 5.

During synthesis and purification of DSSA probes the compounds are non-fluorescent on TLC plates but after few minutes, the UV visible spot becomes red in color due to acidity of silica gel. As rhodamine ring is in cyclized form at pH-7.4, it is not expected to participate in FRET quenching of fluorescein ring. As discussed in Fig. 6 the fluorescence quenching of fluorescein is governed by following equation

$$\begin{aligned} &\textit{Total quenching} = \textit{FRET} + \textit{hydrophobic } \pi{-}\pi \textit{ interactions} \\ &\textit{due to linker}(L) + \textit{Hydrophobic } \pi{-}\pi \textit{ interactions of} \\ &\textit{fluorescein and cyclized rhodamine } (P) \end{aligned} \tag{1}$$

The FRET contribution in DSSA probes is zero and hence all the quenching observed is due to the hydrophobic π-π interactions (L+P). This is evident from no drop in $E^{o'}$ for $DSSA_{Na}$ due to shift from benzene based linker to

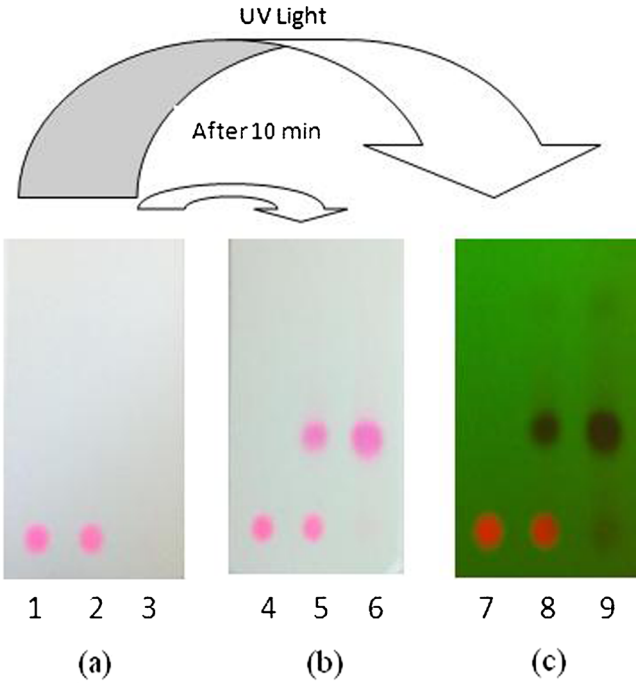


Fig. 5 Visual appearances on silicagel TLC plates and concept of acidic ring closure/opening. Plate A shows the reactant (1), product (3) and reactant+product (2). Plate A shows that after reaction of rhodamine B with diamine, the product loses its characteristic red color on the TLC plate. Plate B is plate A after 10 min. During 10 min, the acidic nature of silica gel on TLC plate opens the ring of rhodamine B. This clearly shows that rhodamine B is cyclized after reaction with diamine and becomes fluorescent only under acidic conditions. Plate C is same as plate A but under UV 365 nm illumination. The spot 7 is fluorescent (rhodamine B) compared to spot 9 which still has brownish tinge indicating that product is not fluorescent

naphthalene. $DSSA_{Na}$ has slightly lower absorbance and fluorescence intensities compared to $DSSA_{Or}$ because the naphthalene ring also participated in hydrophobic π-π interaction and hence quenches the fluorescein fluorescence (Fig. 3). As the π-π interactions are a ground state interactions, the $E^{o'}$ calculation might have a contribution from the π-π interaction terms (L+P), which cannot be measured individually by fluorescence. By synthesizing $DSSA_{Na}$ and $DSSA_{Or}$ we could identify that the contribution of L term in Equation 1 is negligible. The ground state interaction is also established by the fact that heating the DSSA probes with DTT at 60 °C gave F_{max} similar to TCEP. The hydrophobic structures of DSSA probes are flexible and at higher temperature are more accessible to the reductants. Though DSSA probes have initiated plethora of publications for synthesis of FRET based probes [6–14], this study proves that DSSA probes follow the hydrophobic π-π quenching and may not be classified as FRET probes. Surprisingly, these probes can't be classified similar to quencher based Taqman probes either. Though ground state dye to dye quenchings are reported in literature, they never have been used for design of fluorescent probes for specific applications and DSSA probes are the first in that line.

Significance of Change in Absorbance Spectra of DSSA Probes

A change in absorbance spectra of DSSA probes indicates that there is ground state quenching of the fluorescein compound. When a quencher is attached to a fluorescent compound, changes in emission spectra are common. But, as shown in Fig. 5A, the rhodamine exists in cyclized form and in our earlier paper, we have shown that even at pH 5 only a small percentage of rhodamine exists in open form and participates in FRET with fluorescein. In this study we used intentionally 100 mM Tris at pH 8.2 as buffer to eliminate the possibility of FRET contribution from rhodamine in fluorescein quenching. The quenching observed in absorbance spectra has no contribution from absorbance or emission of rhodamine ring in

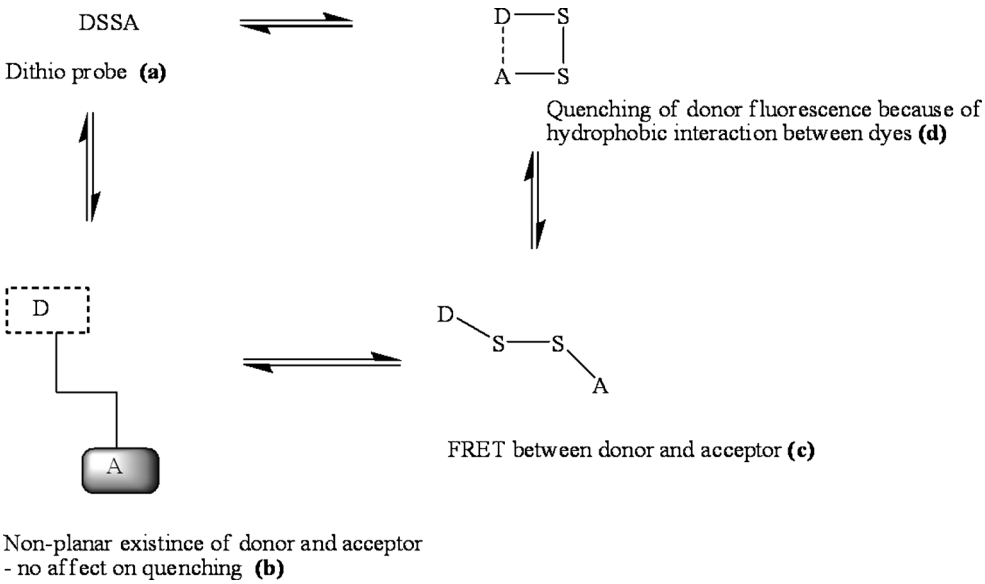


Fig 6 Possible fluorescence quenching mechanism in DSSA probes. The total quenching experience by donor is equal to sum of quenchings contributed by hydrophobic and FRET interactions

DSSA derivatives. Our current understanding of fluorescence chemistry suggests that the spectral overlap is a must for quenching and the quenching offered by hydrophobic interactions are very minimal. This perception probably requires relook due to the reported results from this paper and reference [5]. The hydrophobic interaction is a general term for non-spectral interactions between two molecules and it will be based on chemical or structural interactions. The exact orientation of the fluorescein and cyclized rhodamine groups in DSSA probes can be understood only by crystallographic studies and our efforts as of now in obtaining single crystal of DSSA probes were unsuccessful.

Impact of This Study on Future Design of Fluorescent Compounds Based on π-π Interactions

Though FRET and static quenching are known for a long time, design of fluorescent probes specially utilizing static quenching is rare. This is partly due to the requirement of ratiometric determination of two fluorophores to quantitate the analyte in vivo. But the FRET probe efficiency is related to quite few parameters like overlap integral of donor fluorescence and acceptor absorbance J (λ). In Fig. 7 we show two hypothetical fluorophores with spectral overlap integral J (λ)= 0 (example Rhodamine B & an Infrared dye). By conventional wisdom of FRET these two dyes can never be used as a FRET pair, but the results presented in this paper suggest a relook at possibility of using fluorophores with no spectral overlap as FRET pairs. The DSSA probe has only one fluorophore and the cyclized rhodamine is a non-fluorescent compound, suggesting that even a non-fluorescent compound can quench fluorescence. Though Taqman probes use similar quencher based systems, the quenchers share a spectral overlap with fluorescent compound (eg: FAM and BHQ-1) [38]. The DSSA probes have a new mechanism for quenching with interactions purely hydrophobic in nature. Foster radius between donor and acceptor, quantum efficiency of donor, orientation of donor and acceptor etc. often make the choice of fluorescent molecules difficult [39].

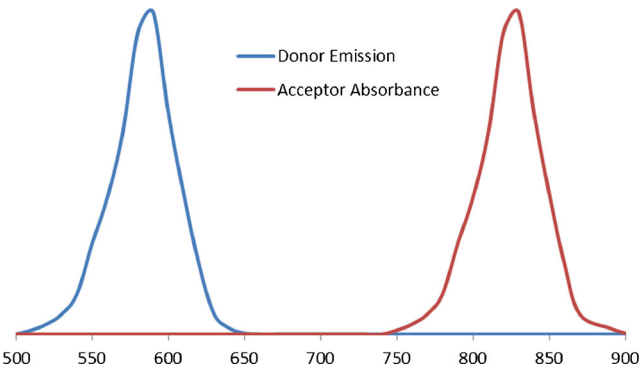


Fig 7 Schematic representation of the two different fluorophores spectral overlap integral J (λ)=0 (example Rhodamine B & an Infrared dye). By convention wisdom of FRET these two dyes can never be used a FRET pair, but the results presented in this paper suggests a relook at possibility of using fluorophores with no spectral overlap as FRET pairs. The DSSA probe has only one fluorescence compound (fluorescein) and the second molecule in structure is a non-fluorescent compound (cyclized rhodamine) suggesting that even a non-fluorescent compound can quench fluoresce. Though Taqman probes use similar quencher based systems, the quenchers share a spectral overlap with fluorescent compound (FAM and BHQ-1 are example for it). The DSSA probes have a third mechanism for quenching with is purely hydrophobic in nature

Most of the parameters discussed above are dependent on the biomolecule used, or dependent too much on the inherent properties of donors and acceptors and less to do with the skill of the researcher. But this study proves that static quenching can help design similar FRET probes with virtually no spectral overlap [40]. It is because the quenching is caused by π-π interaction and hence a near IR probe having absorbance maximum of 1,000 nm can still quench fluorescence of a compound with λ_{em} of 600 nm by hydrophobic interaction having J (λ)=0. This removes the current limitation on the selection of fluorescent probes for FRET applications. As the hydrophobic interactions are known to work over an area of 1,000 nm, designing fluorescent application becomes simple and offers more versatility for biochemist.

Conclusion

This study has given four important informations for fluorescence chemistry

a) The synthesis of $DSSA_{Or}$ and $DSSA_{Na}$ and their respective $E^{o'}$ has suggested that the ultra-low reduction potentials observed in DSSA probes is independent of steric hindrance.
b) The standard reduction potential of DSSA probes is minimally influenced by the functional groups indicating the ultra-low $E^{o'}$ is mostly coming from the interaction between fluorescein and cyclized rhodamine.
c) The rhodamine ring in DSSA probes is in cyclized form and do not possess any excited state interactions with fluorescein. Quenching in DSSA probes is based on hydrophobic interactions and not FRET based.
d) This study suggests that FRET probe design need not be based on J (λ), and the biochemist could select any fluorescent probe if the donor-acceptor pair is based on hydrophobic π-π interactions, making the typical parameters like Forster radius in fluorescence chemistry irrelevant. This conclusion is based on a single group of compounds (DSSA) and needs further study to validate this hypothesis.

References

1. Schafer FQ, Buettner GR (2001) Redox environment of the cell as viewed through the redox state of the glutathione disulphide/glutathione couple. Free Radical Bio Med 30:1191–1212
2. Reed MC, Thomas RL, Pavisic J, James SJ, Ulrich CM, Nijhout HF et al (2008) A mathematical model of glutathione metabolism. Theor. Biol Med Model 5:3–16. doi:10.1186/1742-4682-5-8
3. Embry MR, Belanger SE, Braunbeck TA, Burgos MG, Halder M, Hinton DE, Léonard MA, Lillicrap A, King TN, Whalej G et al (2010) The fish embryo toxicity test as an animal alternative method in hazard and risk assessment and scientific research. Aquat Toxicol 97:79–87. doi:10.1016/j.aquatox.2009.12.008
4. Kirsten H (2011) Limits of the fish embryo toxicity test with Danio rerio as an alternative to the acute fish toxicity test. Dissertation. Ruperto – Carola University of Heidelberg.
5. Pullela PK, Chiku T, Carvan MJ, Sem DS (2006) Fluorescence-based detection of thiols in vitro and in vivo using dithiol probes. Anal Biochem 352:265–273
6. Yin LL, Chen ZZ, Tong LL, Xu KH, Tang B (2009) Progress on Fluorescent Probes for Thiols. Chinese J Analyt Chem 37:1073–1081. doi:10.1016/S1872-2040(08)60117-6
7. Bouffard J, Kim Y, Swager TM, Weissleder R, Hilderbrand SA (2008) A Highly Selective Fluorescent Probe for Thiol Bioimaging. Org Lett 10:37–40. doi:10.1021/ol702539v
8. Maeda H, Matsuno H, Ushida M, Katayama K, Saeki K, Itoh N (2005) 2,4-Dinitrobenzenesulfonyl Fluoresceins as Fluorescent Alternatives to Ellman's Reagent in Thiol-Quantification Enzyme Assays. Angew Chem Int Ed 44:2922–2925. doi:10.1002/anie.200500114
9. Yi L, Li H, Sun L, Liu L, Zhang C, Xi Z (2009) A Highly Sensitive Fluorescence Probe for Fast Thiol-Quantification Assay of Glutathione Reductase. Angew Chem Int Ed 48:4034–4037. doi:10.1002/anie.200805693
10. Ahn YH, Lee JS, Chang YT (2007) Combinatorial Rosamine Library and Application to in Vivo Glutathione Probe. J Am Chem Soc 129:4510–4511. doi:10.1021/ja068230m
11. Lim CS, Masanta G, Kim HJ, Han JH, Kim HM, Cho BR (2011) Ratiometric Detection of Mitochondrial Thiols with a Two-Photon Fluorescent Probe. J Am Chem Soc 133:11132–11135. doi:10.1021/ja205081s
12. Lee MH, Han JH, Lee JH, Choi HG, Kang C, Kim JS (2012) Mitochondrial Thioredoxin-Responding Off−On Fluorescent Probe. J Am Chem Soc 134:17314–17319. doi:10.1021/ja308446y
13. Zhu B, Zhang X, Li Y, Wang P, Zhang H, Zhuang X (2010) A colorimetric and ratiometric fluorescent probe for thiols and its bioimaging applications. Chem Commun 46:5710–5712. doi:10.1039/c0cc00477d
14. Chen X, Zhou Y, Peng X, Yoon J (2010) Fluorescent and colorimetric probes for detection of thiols. Chem Soc Rev 39:2120–2135. doi:10.1039/b925092a
15. Chazotte B (2011) Labeling Mitochondria with TMRM or TMRE. CSH Protoc 7:895–897. doi:10.1101/pdb.prot5641
16. Zhang S, Yan Y, Bi S (2009) Design of Molecular Beacons as Signaling Probes for Adenosine Triphosphate Detection in Cancer Cells Based on Chemiluminescence Resonance Energy Transfer. Anal Chem 81:8695–8701
17. Habeeb Muhammed M A, Shaw AK, Pal SK, Pradeep T (2008). Quantum Clusters of Gold Exhibiting FRET, J. Phys. Chem. C,112 (37), 14324–14330
18. Cleland WW (1964) Dithiothreitol, a New Protective Reagent for SH Groups. Biochemistry 3:480–482
19. Mickey B, Howard J (1995) Rigidity of Microtubules Is Increased by Stabilizing Agents. J Cell Biol 130:909–917
20. Aitken CE, Marshall RA, Puglisi JD (2008) An Oxygen Scavenging System for Improvement of Dye Stability in Single-Molecule Fluorescence Experiments. Biophys J 94:1826–1835. doi:10.1529/biophysj.107.117689
21. Kolossov VL, Spring BQ, Clegg RM, Henry JJ, Sokolowski A, Kenis PJA, Gaskins HR (2011) Development of a high-dynamic range, GFP-based FRET probe sensitive to oxidative microenvironments. Exp Biol Med (Maywood) 236:681–691
22. Peng H, Chen W, Cheng Y, Hakuna L, Strongin R, Wang B (2012) Thiol Reactive Probes and Chemosensors. Sensors 12:15907–15946. doi:10.3390/s121115907
23. Akif M, Khare G, Tyagi AK, Mande SC, Sardesai AA et al (2008) Functional Studies of Multiple Thioredoxins from Mycobacterium tuberculosis. J Bacteriol 190:7087–7095. doi:10.1128/JB.00159-08
24. Herzog CA (2011) Glutathione and non-glutathione-based oxidant control in the endoplasmic reticulum. J Cell Sci 124:847–855. doi:10.1242/jcs.080895
25. Tang B, Xing Y, Li P, Zhang N, Yu F, Yang G (2007) A Rhodamine-Based Fluorescent Probe Containing a Se-N Bond for Detecting Thiols and Its Application in Living Cells. J Am Chem Soc 129:11666–11667. doi:10.1021/ja072572q
26. Abe H, Ito Y, Shibata A, Ito M (2012) Thiol detection method US 8187825 B2
27. Shibata A, Furukawa K, Abe H, Tsuneda S, Ito Y (2008) Rhodamine-based fluorogenic probe for imaging biological thiol. Bioorg Med Chem Lett 18:2246–2249. doi:10.1016/j.bmcl.2008.03.014
28. Li H, Fan J, Wang J, Tian M, Du J, Sun S, Sun P, Peng X (2009) A fluorescent chemodosimeter specific for cysteine: effective discrimination of cysteine from homocysteine. Chem Commun 39:5904–5906. doi:10.1039/b907511a
29. Cai HH, Wang H, Wang J, Wei W, Yang PH, Cai J (2011) Naked eye detection of glutathione in living cells using rhodamine B-functionalized gold nanoparticles coupled with FRET. Dyes Pigments 92:778–782. doi:10.1016/j.dyepig.2011.06.016
30. Jun ME, Roy B, Ahn KH (2011) Turn-on fluorescent sensing with reactive probes. Chem Commun 47:7583–7601. doi:10.1039/C1CC00014D
31. Long L, Lin W, Chen B, Gao W, Yuan L (2011) Construction of a FRET-based ratiometric fluorescent thiol probe. Chem Commun 47:893–895. doi:10.1039/c0cc03806g
32. Monostori P, Wittmann G, Karg E, Turi S (2009) Determination of glutathione and glutathione disulfide in biological samples: An in-depth review. J Chromatogr B 877:3331–3346. doi:10.1016/j.jchromb.2009.06.016
33. Duan YL, Shi YG, Chen JH, Wu XH, Wang GK, Zhou Y, Zhang JF (2012) 1,8-Naphthyridine modified rhodamine B derivative and Cu2+ complex: colorimetric sensing of thiols in aqueous media. Tetrahedron Lett 53:6544–6547
34. Lakowicz JR (2006) Handbook of principle of fluorescence spectroscopy book. University of Maryland School of. Medicine, USA
35. Sem DS (2009) Method for detecting kinase activity with thiol reactive fluorescent reagents-US 7585643, B2
36. Timothy J (2011) Fluorescence Probes for Cellular Thiol and Disulfide Detection: Sythesis and Biophysical Characterization. Marquette University, Dissertation
37. Getz EB, Xiao M, Chakrabarty T, Cooke R, Selvin PR et al (1999) A Comparison between the Sulfhydryl Reductants Tris (2 carboxyethyl) phosphine and Dithiothreitol for Use in Protein Biochemistry. Anal Biochem 273:73–80
38. Johansson MK, Fidder H, Dick D, Cook RM (2002) Intramolecular dimers: a new strategy to fluorescence quenching in dual-labeled oligonucleotide probes. J Am Chem Soc 124:6950–6956. doi:10.1021/ja025678o
39. Marras SAE (2006) Handbook of Fluorescent Energy Transfer Nucleic Acid Probes: Designs and Protocols. Methods Mol Biol 335:3–16
40. Johansson MK (2006) Handbook of Fluorescent Energy Transfer Nucleic Acid Probes: Designs and Protocols. Methods Mol Biol 335:17–29