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Effect of Hofmeister series salts and BSA on fluorescein compounds

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Abstract: The biophysical parameters like pH of the medium, temperature, refractive index, extinction coefficient of fluorescent compound etc. are responsible for fluorescence quantum yield. Even after maintaining these parameters constant in an assay, peculiarities do occur with respect to quantum yield due to presence of salts and proteins present in the medium. Most of the fluorescent compounds have different quantum yields in presence of Hofmeister series salts and their behaviour has never been studied thoroughly. We performed a systematic study of effects of Hofmeister series salts (urea, sodium chloride, ammonium sulphate, and guanidine isothiocyanate) and BSA (0.1 mg/mL) on fluorescence quantum yield for seven different fluorescent compounds (fluorescein, fluorescein isothiocyanate, DSSA, FITC-attached staurosporine, FITC-attached estrogen, FITC-attached NADH and FITC-attached NADPH). Presence of guanidine isothiocyanate and $(NH_4)_2SO_4$) has drastically reduced fluorescence quantum yield of all compounds. In general, presence of salts like urea has increased quantum yield, BSA and other salts have minimum effect on quantum yield. Among the dyes studied, the two groups obtained are fluorescein, FITC and E2-FITC in the first; FITC-staurosporine, FITC-NADH and FITC-NADPH in the second, which have similar fluorescence quantum yield behaviour in the studied buffers. DSSA seems to be unique from all the studied buffers due to the presence of two dyes in its structure.
Keywords: Hofmeister series, Fluorescent probes, Kosmotropes, Chaotropes, Fluorescence quantum yield.

Introduction

Fluorescent compounds are the major class of compounds used in protein-ligand interaction studies, ligand binding assays, cell labelling agents and as protein biomarkers[1-3]. There are a number of recent applications of probes as chemical proteomic tools making them part of the biochemist tool box. The properties of the fluorescent compounds are dependent on pH of the medium, temperature, refractive index, extinction coefficient of compounds etc.[4] The effects of these traditional parameters are widely studied in the photochemistry perspective. Inherently, macromolecules are subjected to Hofmeister series experiments to determine the appropriate protein precipitating conditions[5]. The protein precipitating reagents like ammonium sulphate [6], denaturing agents like urea and guanidine isothiocyanate are widely used and their effects on protein aggregation and aqueous environment distribution are well studied[7-10]. At molecular level, the current understanding of the Hofmeister series effects on proteins is due to either effects of ions on water structure or due to salt protein binding. The water structure hypothesis states that some ions ("kosmotropes") enhance the

structure of the surrounding ions and enhance the hydrophobic effect, thereby stabilizing the proteins and some ions ("chaotropes") disrupt the water structure and denature the proteins[11-13]. The second salt-protein hypothesis states that ions which denature proteins are the ones which bind them and the stabilizing salts are excluded from the protein surface. Recently, Shimizu *et al.* reported Kirkwood-Buff theory based calculations and found evidence in support of salt-protein hypothesis[14]. Though there are theoretical models to explain Hofmeister series, the only experimental protein studied to date is bovine serum albumin (BSA) [15] and often the Hofmeister series results with other proteins are complicated and do not obey Hofmeister series. The Hofmeister series was observed in a wide range of phenomena including colloidal chemistry, membrane effects, surface and polymer chemistry[16-20]. Interestingly, the fluorescent dyes like proteins also have varying hydrophobic characteristics and show aggregation and precipitation in aqueous solutions. It is interesting to understand the effects of Hofmeister salts and BSA on fluorescent dyes considering the similarity in hydrophobicity and aggregation properties. It is common for a biochemist to observe that the assay conditions often change fluorescent behaviour of dyes drastically and these changes could be mistaken for unique results. There is no systematic study of these Hofmeister salts on small molecule fluorescent dyes. Considering the increase in chemical proteomic applications of fluorescent probes in biochemistry, characterization of fluorescent properties in biochemical assay conditions is essential. In this paper we discuss the effect of Hofmeister salts on fluorescent dyes and show that dyes are different from macromolecules, and have unique behaviour with Hofmeister salts and BSA.

Materials and Methods

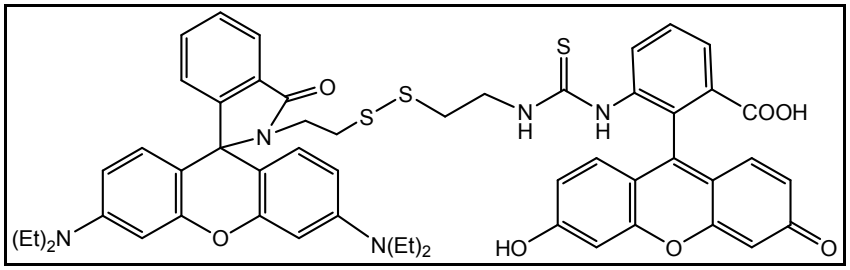
All salts, buffers, and enzymes, except where noted, were from Sigma-Aldrich and were of biochemical reagent grade. They were used without any further treatment or purification. Buffer pH was adjusted using either NaOH or HCl. Standard sample of BSA (1 mg/mL) was obtained from Amersham Biosciences and diluted to 0.1 mg/mL in 100mM HEPES buffer. Fluorescein, FITC, HEPES base, urea, sodium chloride, ammonium sulphate, guanidine isothiocyanate, and sodium hydroxide were purchased from Aldrich. The buffers used in this study are 100mM HEPES and 100mM HEPES containing 1M Hofmeister salt adjusted to pH 7.4. Fluorescence measurements were made on a Thermo Appliskan Multimode Microplate Reader at 25°C, using 96-well plates. The reader was equipped with 485nm excitation and 520-nm emission filters, both with a 10nm band pass. A Beckman-Coulter DU 700 UV/VIS spectrophotometer was used for analysis of column fractions, as well as for the UV/Vis-based kinase assay measurements. All spectroscopic measurements were taken at 25^{o}C in a 1 mL quartz cuvette.

Results and Discussion

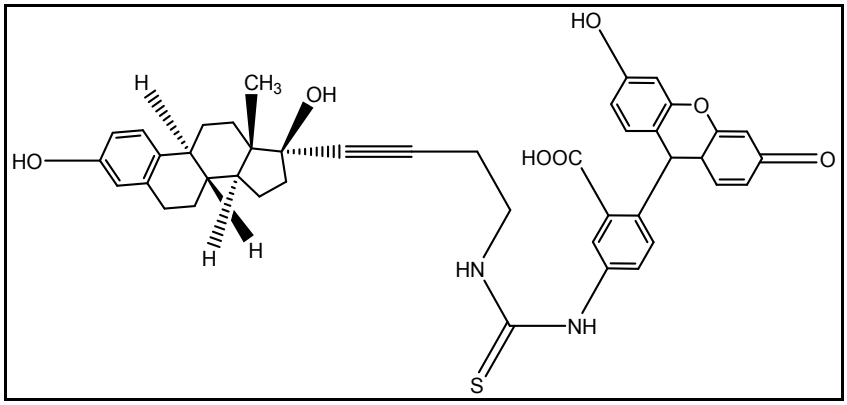
Selection of Fluorescent compounds

Our group is involved in development of fluorescent analogues for different biochemical applications including cell permeable probes for quantitation of changes in the cellular thiols (DSSA) [21], estradiol analogue with a three carbon amine linker at 17 α-position (E2-FITC) as zebrafish estrogen receptor probe[22,23]. As a reference, fluorescein and FITC were used along with NADH-FITC (FITC attached to NADH), NADPH-FITC (FITC attached to NADPH), and staurosporine-FITC (FITC attached to staurosporine). All the compounds selected for this study have common fluorescein as core molecules and allow the comparison between molecules. DSSA has a cyclized rhodamine and FITC attached on either side of cystamine. DSSA probes are proven to cross the bacterial cell membranes, offering molecules with molecular weight close to 1000 and have a hydrophobic core with a protruding acid group providing a micellar type molecule[21, 28]. E2-FITC has an estrogen core attached to FITC and a phenolic group on steroidal aromatic ring. The secondary alcohol on steroidal D-ring and acid group on FITC could act as a hydrophobic sphere with polar groups on the surface. NADH-FITC and NADPH-FITC are in the molecular weight range of 600 and offer low molecular weight mimics of biochemical molecules and on the other hand staurosporine-FITC is a high molecular weight mimic in the molecular weight range of 900. All the probes selected were assumed to form hydrophobic core with polar group on the surface similar to proteins and the concentrations at which this work is performed is far below the possible aggregate-forming concentrations of respective dyes.

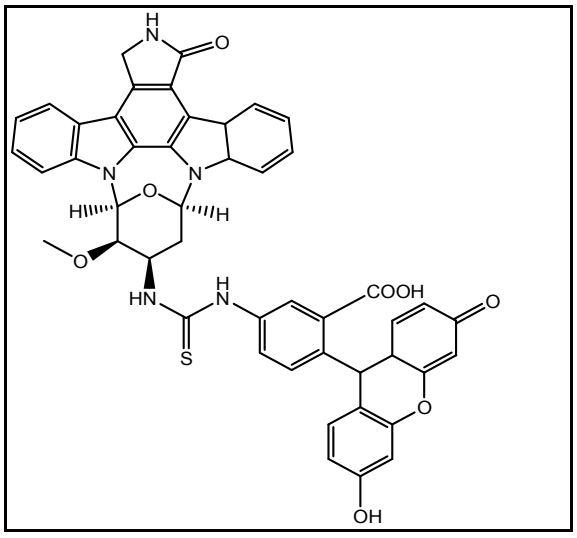
DSSA



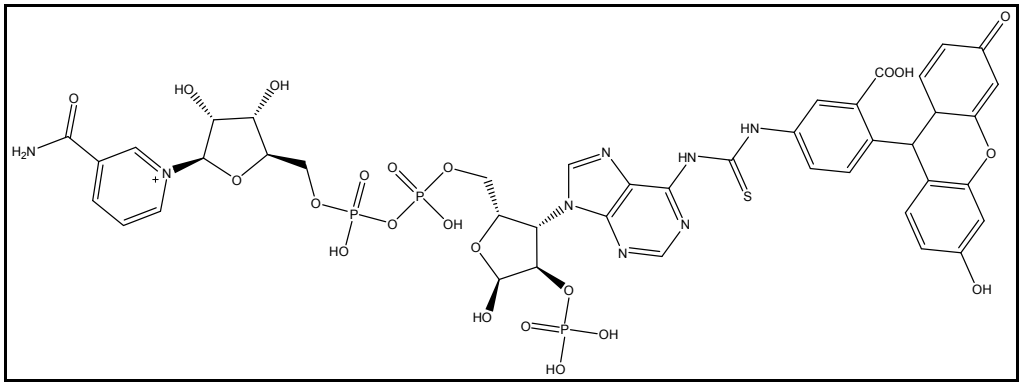
E2-FITC



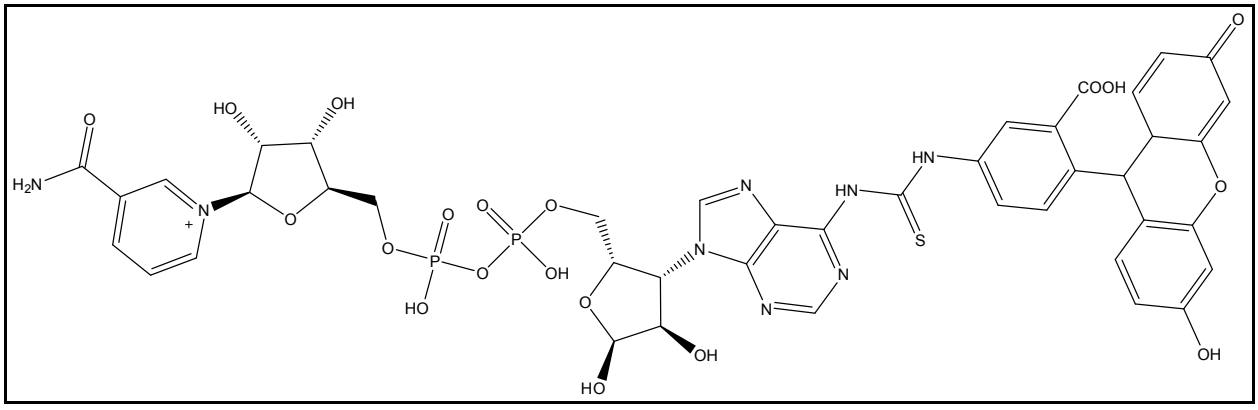
FITC-Staurosporine



FITC-NADPH



FITC-NADH



Aqueous solutions of fluorescent compounds

FITC and fluorescein have solubility in water and the stocks were made accordingly in pH 7.4, 100mM HEPES buffer. Other five fluorescent compound's stocks are prepared either in methanol or DMSO and diluted to working concentrations in pH 7.4, 100mM HEPES buffer. In all the working solutions, the organic solvent concentration was maintained below 0.1%. As a note, pH of the buffer is always measured before and after dissolving fluorescent compound and if needed pH was adjusted to 7.4. Similarly, after addition of 1M Hofmeister series salts to the HEPES buffer, pH was adjusted back to 7.4.

Absorbance readings

Table 1 - A comparison of absorbance data for various fluorescein based dyes.

Probe	HEPES	BSA	Urea	$(NH_4)_2SO_4$	NaCl	Guanidine Isothiocyanate
Fluorescein	0.485	0.413	0.391	0.487	0.458	0.408
FITC	0.546	0.511	0.394	0.597	0.592	0.497
FITC-NADH	0.430	0.469	0.669	0.808	0.596	0.578
FITC-NADPH	0.208	0.306	0.331	0.484	0.411	0.306
E2-FITC	0.409	0.420	0.361	0.482	0.434	0.499
FITC-staurosporine	0.143	0.182	0.271	0.350	0.267	0.188
DSSA	0.010	0.034	0.032	0.036	0.027	0.024
DSSA (reduced)	0.030	0.026	0.025	0.044	0.034	0.045

Absorbance readings were recorded for all seven fluorescent compounds at 485nm. The concentrations used for the measurement were 1.28µM, 2.56µM, 5.12µM and 10.24µM in quadruplicates Table 1. These concentrations were chosen in such a way that the absorbance values are within the range of 0.01 to 1.0 absorbance units. The concentrations at which the linear behaviour is observed are taken as appropriate concentrations for fluorescent studies. The concentration- dependent linear behaviour of absorbance is shown in Figure 1. DSSA has random absorbance at 485nm and only above these concentrations, the absorbance is concentration-dependent and is shown in Figure 2. DSSA probes have cyclized rhodamine which was reported by us to possess ability to quench FITC absorbance and fluorescence. It is the reason for requirement of higher concentrations of DSSA probes compared to other FITC probes.

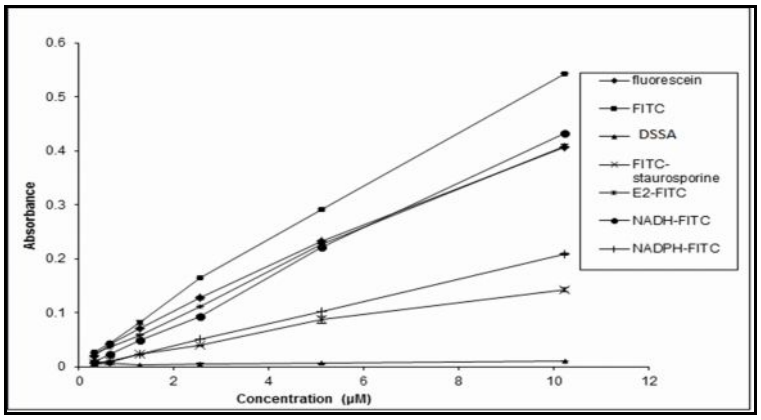


Figure 1 - Absorbance of different fluorescent probes at 485nM measured at different concentrations 1.28µM, 2.56µM, 5.12µM and 10.24µM. The buffer used for experiment is 100Mm HEPES, pH-7.4 and the UV-Visible Spectrophotometer is Beckmann-Coulter DU700.

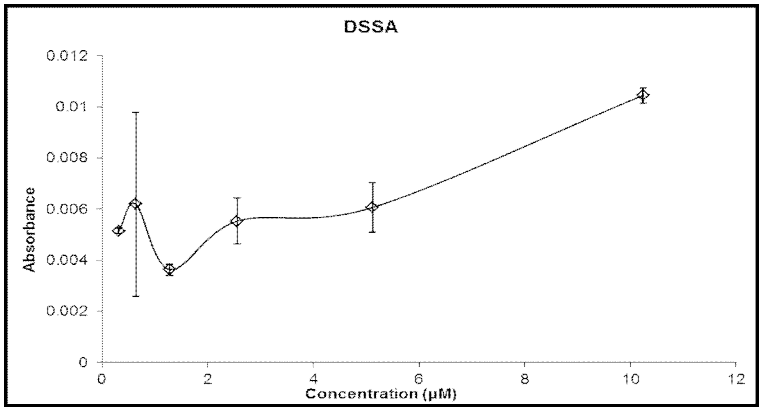


Figure 2 - Absorbance of DSSA measured with increasing concentrations of dye.

Fluorescence readings

Fluorescence readings were recorded for all seven fluorescent compounds at 485nm.The concentration used for the measurement were 1.28µM, 2.56µM, 5.12µM and 10.24µM in triplicates Table 2. The fluorescence values measured from the Appliskan multimode microplate reader and with the appropriate buffer controls are applied in a single 96 well plate. The fluorescent intensity values are referenced to fluorescein's intensity and are not absolute numbers like absorbance values. Considering the fluorescent quantum yields calculated are relative to fluorescein, this referencing to fluorescein gave reproducible and reliable results. The concentration-dependent linear behaviour of fluorescence intensity of all seven compounds is shown in Figure 3. DSSA probe has random fluorescence at 520nM and only above these concentrations; the fluorescence is concentration-dependent and is shown in Figure 4.

Table 2 - Fluorescence measurements

Probe	HEPES	BSA	Urea	$(NH_4)_2SO_4$	NaCl	Guanidine Isothiocyanate
Fluorescein	46859	36442	49904	52221	59839	6709
FITC	27728	29658	35723	33588	40738	3773
FITC-NADH	27376	32449	38183	29305	39115	6641
FITC-NADPH	16865	18895	22780	22951	28124	3115
E2-FITC	7505	8842	8335	6993	9507	3190
FITC-staurosporine	7133	7880	9780	9141	11427	1569
DSSA	242	250	301	290	348	153
DSSA (reduced)	936	996	1327	1288	1557	353

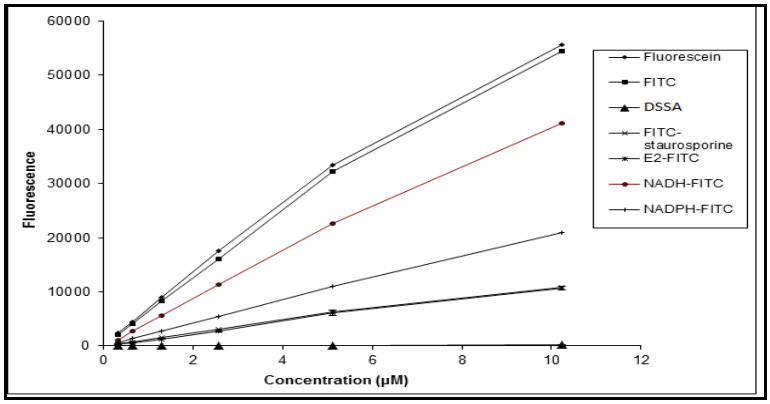


Figure 3 - The variation of fluorescence yield with absorbance for dyes in the micro molar range of concentration.

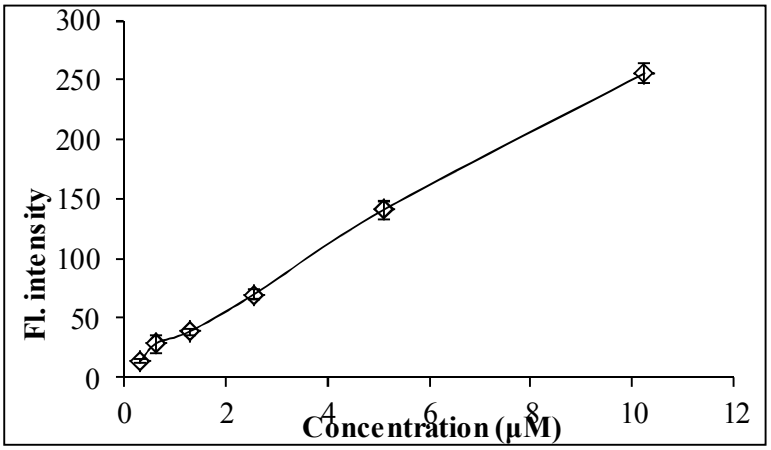


Figure 4 - The change in fluorescence with increasing DSSA concentration.

Fluorescence quantum yield measurement

The literature reported quantum yield for fluorescein in 0.1 N NaOH solution is 0.92 [25]. We used this reference value and calculated fluorescence quantum yield for fluorescein at pH 7.4, 100mM HEPES buffer, for the same buffer containing 1 M Hofmeister series salts and also 0.1 mg/mL BSA Table 3. Using these fluorescein quantum yields, we calculated the fluorescent quantum yields of other six compounds under exact same conditions Table 4. All the fluorescence readings are referenced to fluorescein (standard) and this way the quantum yields calculated are more reliable and accurate. The following formula is used to calculate the fluorescence quantum yields and each reported quantum yield is the average of twelve individual readings (triplicates of four different concentrations). The relative quantum yield [4] is generally determined by comparing the wavelength-integrated intensity of an unknown sample to that of a standard. The quantum yield of the unknown sample is calculated using:

$$Q = Q_R \frac{I}{I_R} \frac{OD_R}{OD} \frac{\eta^2}{\eta_R^2}$$

Where Q is the quantum yield, I is the integrated intensity, η is the refractive index, and OD is the optical density. The subscript R refers to the reference fluorophore of known quantum yield.

Table 3 - Quantum yields of fluorescein under different buffer conditions

	HEPES	BSA	Urea	$(NH_4)_2SO_4$	NaCl	Guanidine Isothiocyanate
Quantum yield	0.730	0.667	1.024	0.762	0.887	0.097
Standard deviation	0.020	0.020	0.034	0.024	0.028	0.003

Table 4 - A summary of the calculated quantum yields for all the fluorescein based dyes.

Probe	HEPES	BSA	Urea	$(NH_4)_2SO_4$	NaCl	Guanidine Isothiocyanate
Fluorescein	0.73	0.68	1.02	0.76	0.89	0.10
FITC	0.38	0.44	0.73	0.40	0.47	0.04
FITC-NADH	0.48	0.53	0.49	0.24	0.42	0.07
FITC-NADPH	0.61	0.46	0.58	0.38	0.45	0.07
E2-FITC	0.14	0.16	0.19	0.10	0.15	0.03
FITC-staurosporine	0.38	0.33	0.29	0.19	0.29	0.05
DSSA	0.18	0.06	0.08	0.06	0.09	0.04
DSSA (reduced)	0.24	0.29	0.42	0.21	0.31	0.05

Refractive index

We used Bausch & Lomb Abbe-3L refractometer at 25°C using sodium D line at 589 nm (η_D^{25}) to obtain refractive index of buffers as shown in Table 5. To determine the accuracy of our measurement and proper functioning of the refractometer, a standard compound's refractive index is determined and compared with the literature published value.

Table 5 - Refractive index for buffers used in this study.

Buffer conditions	HEPES	BSA	Urea	$(NH_4)_2SO_4$	NaCl	Guanidine Isothiocyanate
Refractive index	1.336	1.334	1.352	1.370	1.354	1.397

Discussion

The relationship between fluorescence and absorbance

The relationship between fluorescence and absorbance at increasing concentrations is expected to be a linear one. Non-linearity may point to the existence of significant concentration dependent physico-chemical interactions such as pi-pi interactions, self-aggregation or dye plastic adhesion. Depending on the specific nature, magnitude of interaction, fluorescence, absorbance or both will be affected. The various plots Figure 5 of fluorescence against absorbance are almost linear. This shows that there is no evidence of any significant amount of the above mentioned concentration-dependent effects.

Fluorescence yield as a function of dye concentration

The fluorescence yields were calculated using the equation

$$Q = Q_R \frac{I}{I_R} \frac{OD_R}{OD} \frac{\eta^2}{\eta_R^2}$$

Q - Quantum yield, I - integrated intensity, η - refractive index, OD - optical density. The subscript R refers to the reference.

The yield appears to be steady beyond 2μM, possibly emphasizing the existence of a concentration threshold beyond which dye-plastic or in general dye-container interactions can be neglected. In qualitative terms, the variation shown by the fluorescence yield on the higher concentration end follows hydrophobicity trends. It is noted that the order of fluorescence yields is: FITC, NADPH-FITC, NADH-FITC > staurosporine-FITC > DSSA, E2-FITC.

Earlier discussions have already pointed to the existence of some hydrophobic interactions in DSSA probes [28]. The same can be said of E2-FITC given the relatively large size and hence largely hydrophobic nature of the E2 steroidal core. Staurosporine-FITC has an intermediate fluorescence yield despite the large size of the staurosporine molecule. A closer look at its structure suggests this sugar-based protein kinase inhibitor is capable of hydrogen bonding, lessening the extent of hydrophobic interactions. The balance of hydrogen bonding to solvent vs. hydrophobic interactions dictates the differences shown in the behaviour of staurosporine and E2-containing FITC dyes. Lastly, FITC, NADPH-FITC and NADH-FITC being the least hydrophobic, exhibit the highest fluorescence yields.

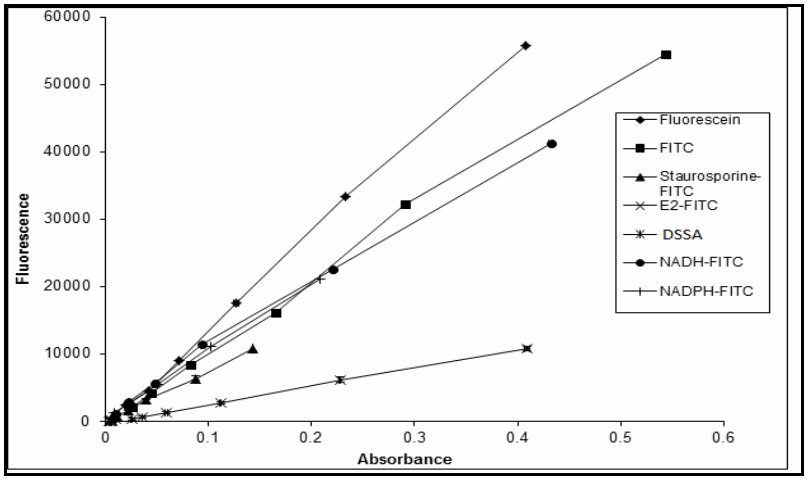


Figure 5 - The variation of fluorescence with absorbance for various FITC-containing dyes.

The effects of lyotropic salts on dye fluorescence yields

The Figure 6 shows concentration ranges for which quantum yield is derived. At low dye concentrations, the effect of dye container interactions becomes significant and hence the lower concentrations have variation in quantum yields. Towards higher concentrations, this trend is not observed and the quantum yield is independent of concentration of dye. Figure 7 shows the effects of NaCl, $(NH_4)_2SO_4$, urea, BSA and guanidine isothiocyanate on the quantum yield of various FITC-containing dyes. Irrespective of the dye, the trend in fluorescence characteristics of each Hofmeister salt on the dyes (relative to one another) is same indicating that the fluorescein core responds same way to each salt. This suggests that different compounds could have differential fluorescence in different media but for the same fluorescence core, the effects remain same. Conjugation of FITC on a small molecule changes the fluorescence intensity, quantum yield etc. of that compound, but in qualitative terms fluorescein ring, which is responsible for fluorescence from the molecule feels same effect from Hofmeister salts. It is noteworthy that in the dye series investigated, by monitoring the fluorescein core fluorescence, DSSA is the only one with a donor-quencher pair [28]. It should be noted that DSSA will generally not be in alignment with most of the discussed trends. This molecule has significantly lower fluorescence yield compared to other molecules, but the affect of Hofmeister series salts is inline with other compounds in qualitative terms. The cyclized Rh-B in DSSA [28] is known to affect both absorbance and fluorescence of FITC and the same is reflected in presence of the lyotropic salts.

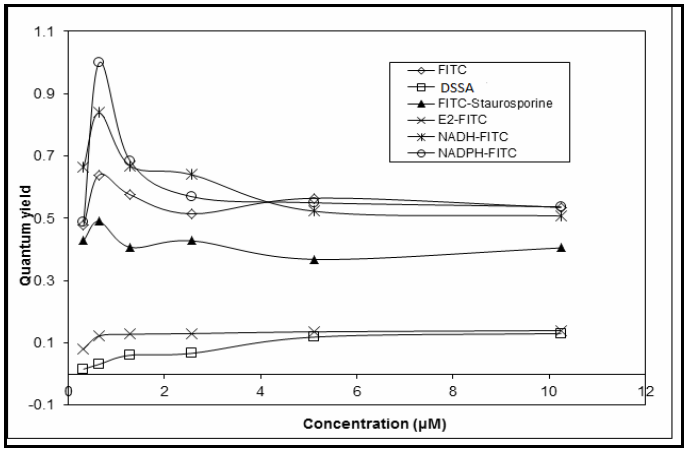


Figure 6 - The variation of quantum yield with dye concentration at pH 8.

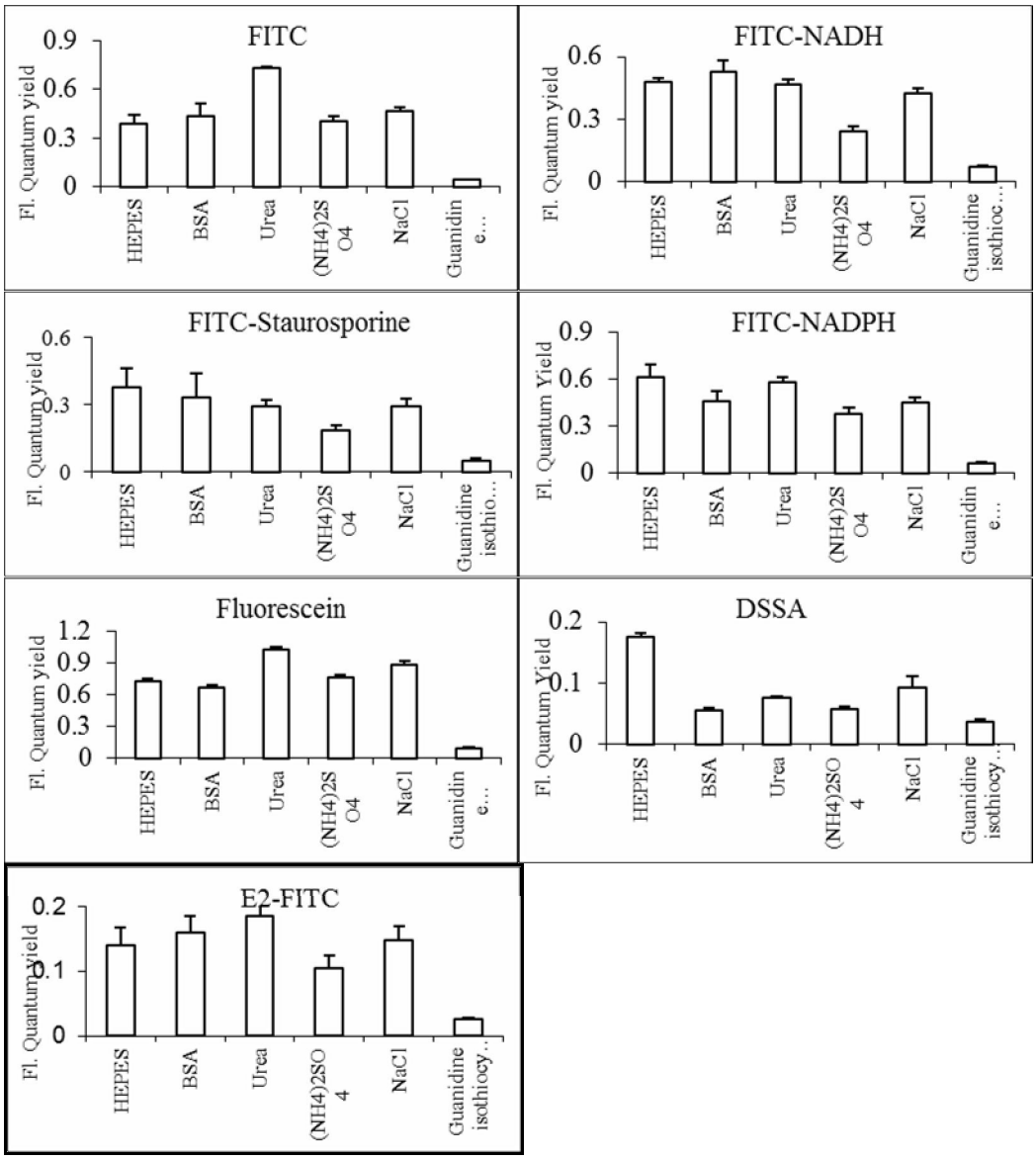


Figure 7: Effects of various additives on the fluorescence yield of FITC-based dye.

Hofmeister series salts & fluorescein compounds

The effects of NaCl and $(NH_4)_2SO_4$, both of which give relatively high quantum yield, follow some consistent pattern whereby the latter diminishes the quantum yield to a greater extent. According to the Hofmeister series theory[25] where $NH_4^+ > Na^+$ and $SO_4^{2-} > Cl^-$, a distinct difference should be observed in quantum yield, instead only minimal differences are observed. However, this is consistent with the fact that in equimolar amounts, $(NH_4)_2SO_4$ has a higher ionic strength, a physical property that tends to force aggregation of hydrophobic substances. The generally high quantum yield in the presence of both salts clearly shows that at 1M concentration, the fluorescein dye conjugates do not cause any appreciable salting out. The quantum yields in the presence of urea and BSA generally leads to high quantum yields while the solubilisation effects of urea and BSA are noted. BSA is effective at solubilizing higher molecular weight hydrophobic molecules, like proteins, while urea is effective for both large and small molecules. This may explain the small difference in quantum yields observed, which are generally in favour of urea. Guanidine isothiocyanate and urea both being strong denaturants [26] however exhibit opposite effects on the quantum yield of dyes. Guanidine isothiocyanate, a strong, charged denaturant capable of very strong associations with the dye molecules, causes dramatic reductions in all the FITC-based dyes studied. This marked difference, which sets it apart from even urea, another denaturant could be due to its positive charge. Studies involving the effects of denaturants on hydrophobic aromatic compounds have proved the existence of cation-pi electron system interactions [27]. These interactions in the case of guanidinium ion could be responsible for the decreased quantum yields.

Conclusions

The conclusions from this study are

a) The two strong protein denaturants, guanidine isothiocyanate and urea, have opposite effects on the quantum yield indicating these dyes are completely different from macromolecules like proteins which often obey Hofmeister series.
b) These dyes does not obey Hofmeister series, the effects on quantum yield may not be due to hydrophobic effect or change in water structure around dyes. The non- obeying of Hofmeister series by dyes and their unique effects on quantum yield based on the structure of the fluorescent dyes makes them a special class to study. This information will be invaluable for biochemists and performing the simple comparative fluorescent experiments in presence of salts of interest could help to address the common anomalies in the experiments like unusual increase or decay in fluorescence, differences in quantum yields due to presence of proteins/salts, inconsistency and differences in *in vitro* and *in vivo* assay results etc.

Acknowledgement

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Abbreviation:

FITC - Fluorescein-5-isothiocyanate, DMSO - Dimethyl sulfoxide, NADH - Nicotinamide adenine dinucleotide, NADPH - Nicotinamide adenine dinucleotide phosphate-oxidase, DSSA – Donor-disulfide-acceptor, E2-FITC - FITC attached estrogen, FITC-NADH - FITC attached NADH, FITC-NADPH - FITC attached NADPH, BSA - Bovine serum albumin, FITC-Staurosporine - FITC attached staurosporine.

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