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Efficient solid phase strategy for preparation of modified xanthene dyes for biolabelling†

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An efficient solid phase strategy for the versatile functionalisation of xanthene dyes for conjugation and labelling of biomolecules is presented. The low cost, high purity and excellent spectral properties of the obtained materials provide an attractive alternative for the labelling of a wide range of molecules.

Fluorescent dyes have attracted considerable attention, mainly due to their applications across a wide range of scientific disciplines. The possibility of conjugation of these chemical entities to biomolecules has provided new opportunities for direct visualisation of many biochemical processes.[1] Therefore, fluorescent probes have become an efficient and indispensable tool to track biomolecule locations in living cells and to detect a specific ligand or macromolecule *via* their functional groups,[2,3] for both *in vitro*,[4,5] and more recently *in vivo*.[6,7] Due to particular requirements of different assays, fluorescent dyes have been tailored with a variety of functional groups for further conjugation. Notably, the development of functionalised chemical reporters has enabled their conjugation with biomolecules for dynamic imaging within cells[8] and has played a key role in the rapid expansion of next-gene sequencing platforms based on sequencing by synthesis.[9] Fluorescein and other xanthene dyes such as rhodamine remain some of the most widely used fluorophores, which are used generally for a variety of fluorescence-based diagnostic and imaging applications. Nowadays, commercial available xanthene dyes are widely used such as N-hydroxysuccinimide esters, cadaverin and maleimide derivatives.[10–12] However, many of these current sensors based on xanthene fluorophores have relatively poor water solubility requiring organic co-solvents for aqueous-based assays, being their fluorescence emission wavelength lower than 550 nm. This wavelength emission makes them susceptible to interference from tissue autofluorescence in biological sensing applications. In the last few years a number of analyte sensors have been designed using these scaffolds either *via* synthesis of new xanthene based dyes [13,14] or by chemical modification at either the 3′ or 6′ positions of the dye.[15–17] In particular, napthoxanthene dyes exhibit longer excitation and emission wavelengths due to the introduction of a fused benzo ring into the xanthene structure. A classic example is napthofluorescein that has a far-red behaviour under basic conditions ($\lambda_{max}/\lambda_{em}$ of 595/660 nm) (Fig. 1, compound **1**).[18] Recently some napthofluorescein-based fluorescent sensor for in *vivo* imaging have been developed.[19] Moreover, as far as we are aware, this family of xanthene-based dyes is only commercially available in an electrophilic form (carboxylic derivative, compound **2** in Fig. 1) which means that conjugation is limited to biological cargoes possessing amine functionality. With this in mind, there is a need for modified xanthene dyes containing a range of functional groups that allow straightforward conjugation with a wider variety of biomolecules. Additional properties such as water-solubility and long absorption and emission wavelengths (tissue optical transparency appears from approximately 650 nm)[5] are also desirable. Recently, several synthetic strategies in solution have been described for the preparation of xanthene-based dyes (by introduction of various functional groups in the benzyl fragment to allow their attachment to a carrier molecule).[20,21] Herein, we report an efficient two-steps solid phase synthesis approach for the versatile functionalisation of xanthene-based dyes without requiring further purification steps and their subsequent conjugation to biomolecules. In particular we have successfully applied this methodology to obtain novel water soluble derivatives of the far-red xanthene dye, naphthofluorescein.

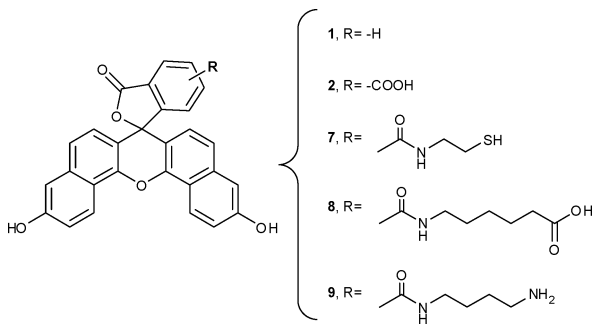

Fig. 1 Structure of naphthofluorescein (**1**, R = H), carboxyl derivative (**2**, R = -COOH) and new derivatives **7–9**.

Three different functional groups were considered as modifications of the target xanthene dye: (i) amino, (ii) carboxyl and (iii) sulfydryl, which could act as nucleophile, electrophile and as groups capable of reversible reaction respectively. This

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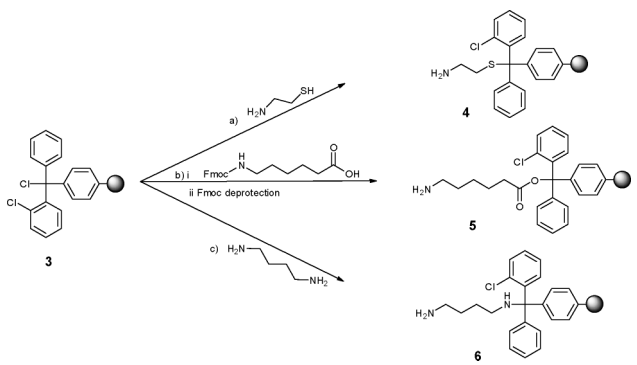
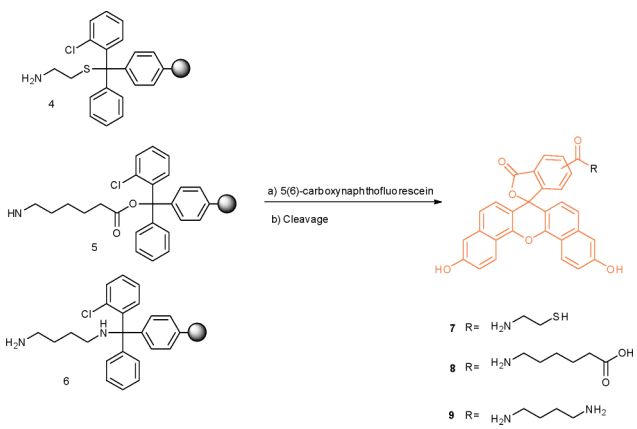
strategy ensures that a wide range of biomolecule reactive sites can be targeted. Moreover, the use of spacer units solves two of the main drawbacks for biolabelling. Firstly, if the aim is to label a biomolecule for microscopy or fluorescence analysis then incorporation of a spacer will decrease steric repulsion between the dye and some residues embedded within the complex biomolecule structure. Additionally, when labelling is done for intracellular tracking of a biomolecule, the use of some spacer units will result in enhanced biocompatibility and can aid the interactions between labelled biomolecules and cell surfaces.[22]

Scheme 1 shows the synthetic strategy used to attach the different spacers to the solid support. This will allow further functionalisation of dyes with either sulfydryl, carboxyl or amino-containing groups. The first step is the reaction of each spacer with the 2-chlorotrityl polystyrene resin **3** (step a, b and c) *via* a nucleophilic substitution (S_N1) mechanism under strong basic conditions using a polar aprotic and resin-friendly solvent as DMF, giving rise to resins **4**, **5** and **6**. The inexpensive and commercial available dye 5(6)-carboxynapthofluorescein (**2**) was then functionalised as shown in Scheme 2. Following preactivation using standard solid phase coupling reagents (oxyma/DIC), dye **2** was reacted with resins **4**, **5** and **6**. After cleavage from the resin using acidic conditions and precipitation using cold ethyl ether the desired compounds **7**, **8** and **9** were obtained in good yield (70–93%) and relatively high purity (77–99%) without the need of further purification. The crude compounds were fully characterized through ^{1}H-NMR, ^{13}C-NMR, high-resolution mass



Scheme 1 Reaction conditions. a) DIPEA/DMF, b) i. DIPEA/DMF, ii. Piperidine/DMF, c) TEA/DMF.



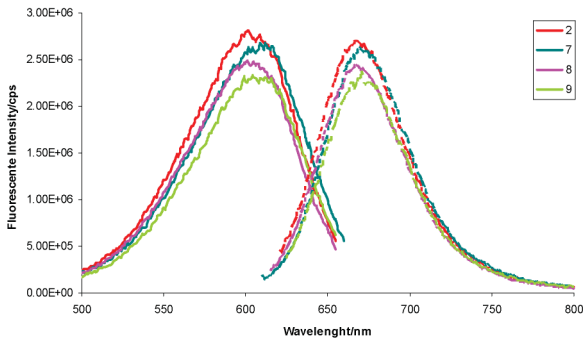
Scheme 2 Reaction conditions. a) compound **1**, Oxyma, DIC, DMF. b) TFA/TIS/DCM.

spectrometry (HRMS) and UV-visible spectroscopy (see ESI for details†).

To further prove the validity of this methodology, 5(6)-carboxyfluorescein and 5(6)-carboxytetramethylrhodamine were also modified successfully obtaining in this way a set of dyes derivatives (compounds **10–15**) with excellent fluorescence properties (see ESI†).

The optical properties of these novel derivatives **7–9** were then determined using UV-visible spectroscopy and spectrofluorimetric analysis. The obtained data of maximum absorbance and extinction coefficients for these compounds are summarised in Fig. 2. These results confirmed that these derivatives behaved in similar manner to carboxynaphthofluorescein **2**. Only small differences of 2–7 nm in the absorption maxima can be observed but all of them retain their fluorescence profile. Moreover it can be observed that their extinction coefficients are higher than the reference.

(a)



(b)

Dye	λmax (exc.)	λmax (em.)	ε (M cm^{-1})	Φrel(%)
2	598	668	43,000	100%
7	609	669	58,600	114%
8	608	667	53,700	85%
9	604	671	50,100	78%

Fig. 2 (a) Spectral properties of **2**, **7**, **8** and **9**. The excitation spectra are represented in the right side (λ_{em} = 668 nm) and the emission spectra in the left side (λ_{exc} = 598 nm) of the diagram. (b) Table summarizing optical properties of **2**, **7**, **8** and **9**. Relative quantum yields were determined as described in ESI.† Measurements performed in basic PBS (pH = 9).

In order to assess the usefulness and applicability of these derivatives, the labelling of biological macromolecules was performed. In particular two proteins found in human blood, myoglobin and cytochrome C were labelled with dye **9**. The spectrofluorimetric analysis of the resulting proteins shows that both proteins were successfully conjugated to fluorophore **9** (Fig. 3) (see ESI for a detailed protein labelling protocol†). Additionally the degree of labelling (DOL) was determined, being 2.82 for cytochrome C and 3.00 for myoglobin (details about calculation of mol dye: mol protein ratios can be found in the ESI†). Importantly, the values of dye : protein ratio on the conjugated proteins are inside of the range of optimal degree of labelling.[23] These results reinforce the potential application of these new far- red derivatives for *in vivo* imaging.

In conclusion, we have reported an easy and efficient method for the versatile modification of inexpensive commercial available naphthoxanthene dyes into a series of derivatives to allow the conjugation to a broader range of biomolecules. The method

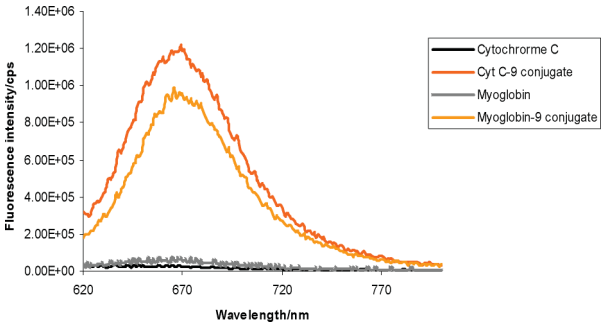


Fig. 3 Fluorescence emission spectra of proteins (cytochrome C and myoglobin) conjugated with **9** (Excitation at 608 nm).

proposed is straightforward and enables, in two steps, the synthesis of functionalized xanthene-based dyes in high yield and purity using a solid-phase strategy. As far as we aware, this is the first time that a substituted solid support is applied for the preparation of xanthenes dyes. This method based on the solid phase strategy has a big advantage - purification steps are performed by simple washings. Even more this method can be applied to functionalise any carboxylic functionalised dye sensor or chemical entity. The low cost, high purity and excellent spectral properties of these materials provide attractive alternatives for the labelling of a wide range of biomolecules.

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