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The influence of systematic structure alterations on the photophysical properties and conjugation characteristics of asymmetric cyanine 5 dyes

S.J. Spa[a,b], A.W. Hensbergen[a], S. van der Wal[a], J. Kuil[c], F.W.B. van Leeuwen[a,b,*]

[a] Interventional Molecular Imaging Laboratory, Department of Radiology, Leiden University Medical Center, The Netherlands
[b] Laboratory of BioNanoTechnology, Wageningen, The Netherlands
[c] Department of Clinical Pharmacy and Toxicology, Leiden University Medical Center, The Netherlands

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ABSTRACT

The light spectrum above 650 nm allows for good tissue penetration depths, therefore far-red and near-infrared fluorescent dyes are popular fluorophores applied in (bio)medical diagnostics, including image-guided surgery. Unfortunately, near-infrared fluorescent dyes often suffer from instability and limited brightness, two important features that, together with the labelling efficiency (*e.g.*, non- one- or di-conjugated products) and serum-dye interactions are key elements that drive *in vivo* characteristics. Due to the fact that stability and brightness of far-red fluorophores are often superior over near-infrared dyes, interest in the use of dyes such as Cy5 is increasing. As there are clear indications that the chemical structure of a dye influences the (photo)physical properties, these properties of ten structural variants of asymmetrical Cy5-(R_1)R_2-(R_3)COOH (R representing the varied substituents) dyes were extensively studied. While stacking in solution was not induced in most of the Cy5 far-red dyes, multimers and stacking characteristics were observed in protein conjugates. Although all dye variants were shown to be stable towards photobleaching, clear differences in brightness and serum interactions were found. Combined, these findings indicate that there is a direct relation between chemical substituents and the properties of Cy5 dyes, and that this feature should be considered when using fluorescent dyes in future tracer development.

1. Introduction

Fluorescent dye-based guidance during surgical interventions is being recognised as an improvement in the accuracy of clinical care [1–3]. In clinical trials, fluorescence imaging has been used as a sole modality or in a bimodal/hybrid form, wherein it extends the field of nuclear medicine [4]. While fluorescence emissions across the light spectrum have been used for image-guided surgery [2], emphasis lies on the use of dyes emitting in the far-red (650 nm < λ_{em} < 750 nm) or near-infrared (NIR; $\lambda_{em} \geq 750$ nm) region [5]. This theoretical preference can be attributed to the enhanced penetration depths and limited autofluorescence at these wavelengths.

Unfortunately, the dye chemistry, stability and/or photophysical properties of near-infrared dyes are limited compared to dyes emitting at lower wavelengths. For example, the most commonly applied near-infrared dye indocyanine green (**ICG**) is prone to stack/aggregate from aqueous solutions and has a low quantum yield (Q_F = 0.3% in H_2O) [6]. More experimental dyes such as IRdye 800CW also have a low quantum yield (Q_F = 3.4% in H_2O) [7] and have been shown to be chemically unstable with respect to endogenous nucleophiles [8]. These limitations have boosted the interest in far-red dyes. For instance, methylene blue (**MB**), a clinically applied dye with a weak far-red fluorescence emission (Q_F = 3% in H_2O) has been applied in humans to image ureters [9], parathyroid glands [10], and bile ducts [11], despite the FDA warning against its use [12]. As an alternative, the Cyanine 5 (Cy5) family provides relatively bright ($\sim 3{\cdot}10^4\,M^{-1}\,cm^{-1}$; $Q_F \approx 20\%$ in H_2O) [13] far-red fluorophores and encompasses many structural variations. A prime example of a Cy5-based imaging agent in clinical use is found in GE-137 (now EM-137), a Cy5 labelled c-Met-targeting peptide that was effectively used for identification of colorectal polyps in humans [14]. Furthermore, the Cy5-containing nanoparticles ^{124}I-cRGDY-PEG-C have been used to target metastatic melanoma [15].

To convert Cy5-dyes into imaging agents of value for fluorescence-guided surgery, these dyes have to be conjugated to targeting vectors. When a targeting vector has multiple conjugation sites, *e.g.* a protein, labelling may not be straightforward. A ratio of one dye per targeting vector is generally aimed at, but the final product often consists of, *e.g.*, a mixture of none-, one-, di-, and/or tri-dye-conjugated imaging agents. In case multiple dyes are located on a single targeting vector, the occurrence of dye-stacking or Förster Resonance Energy Transfer (FRET)

* Corresponding author. Albinusdreef 2, 2333 ZA Leiden, The Netherlands.
E-mail address: f.w.b.van_Leeuwen@lumc.nl (F.W.B. van Leeuwen).

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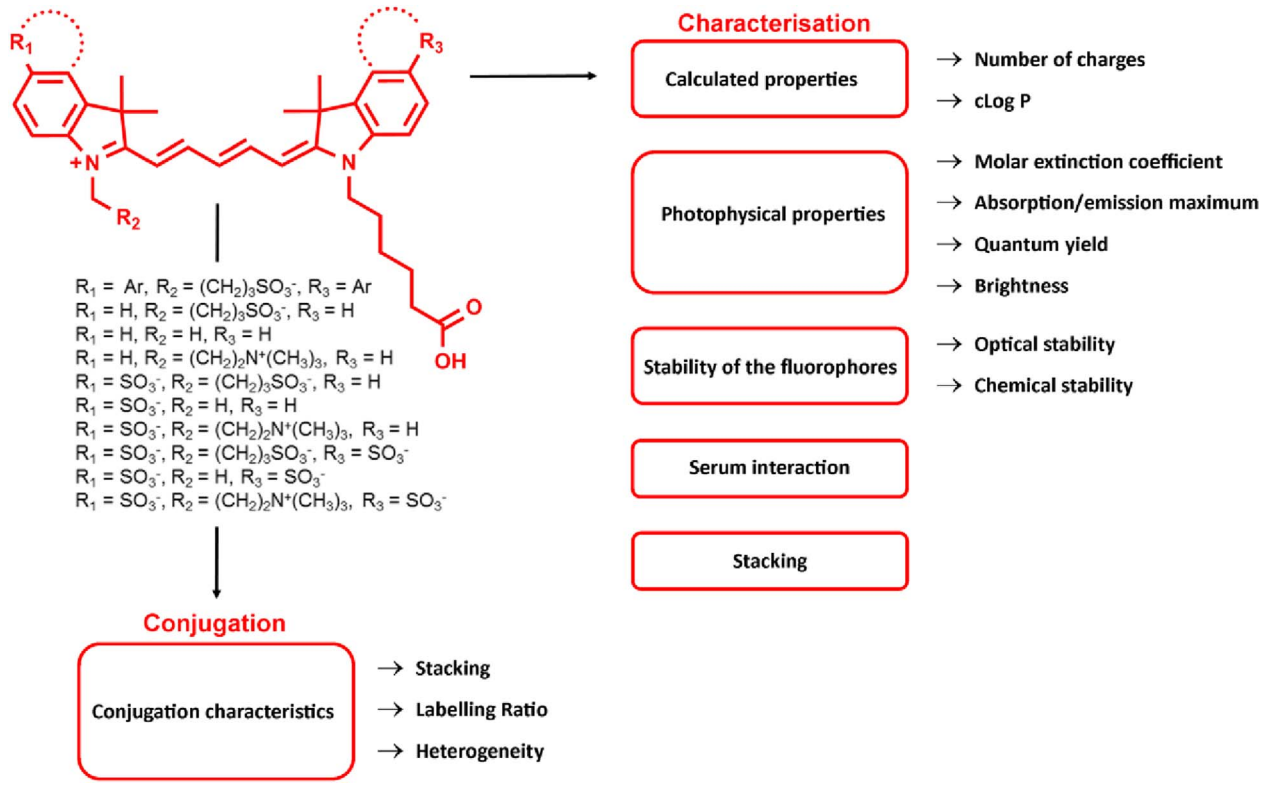

Fig. 1. Overview of the subjects and fluorophore properties investigated and discussed throughout the article.

between the dyes can cause luminescence quenching, a feature that reduces the brightness of the imaging agent [8].

Conjugation of imaging labels, and especially an excess thereof, may also negatively influence the binding specificity and pharmacokinetics of a targeting vector. Dependent on the size of the targeting vector, the scale of these effects varies [16], being most prominent when relatively small peptides are used [14,17]. Nevertheless, this effect is also reported for larger proteins such as mAb conjugates [18]. When dyes express an affinity for serum proteins such as human serum albumin, *e.g.*, **ICG** and Cy5-(Ar)SO_3-(Ar)SO_3 [19–21], this may further effect the tracer pharmacokinetics.

In order to determine the influence of the structure of a fluorescent dye on its utility as an imaging label, ten Cy5 analogues were synthesised and compared with the reference compound **MB**. By alternating the aromatic (R_1 and R_3) and alkyl substituents (R_2), molecular variations on Cy5-(R_1)R_2-(R_3)COOH were systematically evaluated for their photophysical properties, chemical- and photo-stability, serum protein interaction, dye–dye stacking tendencies, and conjugation efficiency (Fig. 1).

2. Experimental

2.1. Materials and reagents

For the synthesis of the fluorophores (Compound **1–21**), cLog P calculations, and information on the materials used, please refer to the Supporting information (SI). The electron density modelling is reported in Ref. [22].

2.2. Ubiquitin labelling (compound 22–30)

Stock solutions of the NHS-activated fluorophores (**12–21,** see SI) were prepared in DMSO and the percentage of activated dye was determined by HPLC (see also SI 'NHS activation'). Subsequently, Ubiquitin (16 nmol) was dissolved in 500 µL of phosphate buffer (0.1 M, pH 8.4; 2.67 g HNa_2PO_4 + 0.14 g H_2NaPO_4 in 200 µL H_2O). Appropriate amounts of the fluorophore stock solution were added, ensuring that each sample contained 3 equivalents activated dye (50 nmol, 100 µM final concentration) and that the DMSO content in the final solution was < 10%. The mixtures were shaken at room temperature for 6.5 h and the labelled Ubiquitin was washed with PBS by filtration over a 3 K Amicon® filter subsequently. When the filtrate was no longer blue, the residue was collected in 100 µL PBS.

Dye–Ubiquitin conjugates were analysed by mass spectrometry (MALDI-TOF) and absorption measurements (NanoDrop). To determine the average labelling ratio, the dye concentration was calculated from absorption measurements in DMSO around 650 nm (Table 1) and the obtained values were then divided by the known protein concentration (0.16 mM). For compound **30** significant precipitation was observed after the reaction, therefore the protein content in this sample also was determined by absorption (ε_{280} = 1490 M^{-1} cm^{-1}; calculated from the amino acid sequence) [23]. Since Cy5 also shows absorbance at this wavelength, a correction was made by measuring the absorbance of free dye at this concentration and subtracting it from the absorbance value measure for the dye-containing Ubiquitin.

2.3. Photophysical properties

2.3.1. Molar extinction coefficient (ε) of compound 1–11

To obtain a 4 mM stock solution of **MB** (**1**), 3.2 mg Methylene blue hydrate (Fisher Scientific) was dissolved in 4 mM ethylene carbonate in DMSO-d_6 (1500 µL) and the exact concentration was determined by NMR using ethylene carbonate as internal standard [8].

To allow for absorption measurements, the 4 mM stock solutions of the dyes in DMSO-d_6 (**1–11**, for details, see SI) were diluted to 100 µM in DMSO, H_2O or PBS. From the 100 µM concentration, 50 µM and 5 µM concentrations were made from which further two-fold dilution in the same medium followed to obtain a final concentration range of 100, 50, 25, 12.5, 5, 2.5, 1.2, 0.6, and 0.3 µM, respectively. Absorption spectra were measured using 1 mL disposable plastic cuvettes (l = 1 cm; Brand,