Site-specific near-infrared fluorescent labelling of proteins on cysteine residues with meso -chloro-substituted heptamethine cyanine dyes

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Site-Specific Near-Infrared Fluorescent Labelling of Proteins on Cysteine Residues with *meso*-Chloro-Substituted Heptamethine Cyanine Dyes

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Near-infrared (NIR) fluorescence imaging is a promising new medical imaging modality. Associated with a targeting molecule, NIR fluorophores can accumulate selectively in tissues of interest and become valuable tools for the diagnosis and therapy of various pathologies. To facilitate the design of targeted NIR imaging agents, it is important to identify simple and affordable fluorescent probes, allowing rapid labelling of biovectors such as proteins, ideally in a site-specific manner. Here, we demonstrate that heptamethine cyanine based fluorophores, such as IR-783, that contain a chloro-cyclohexyl moiety within their polymethine chain can react selectively, at neutral pH, with cysteine residues in proteins to give stable, site-specifically labelled conjugates, that emit in the NIR spectral window. This reaction is exemplified with the labelling of peptides and two protein models: albumin and a Fab' antibody fragment. The resulting fluorescent proteins are stable and suitable for *in vivo* NIR imaging applications, as shown on a mice model. This straightforward one-step procedure, that does not require the prior derivatisation of the fluorophore with a bioconjugatable handle, should facilitate the production and use of near-infrared labelled proteins in life sciences.

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Electronic Supplementary Information (ESI) available: Experimental procedures, characterization data, stability studies and biodistribution data.

Introduction

Near-infrared (NIR) fluorescence imaging is a promising new medical imaging modality that can potentially provide the sensitivity of nuclear medicine techniques, without the limitations associated with the handling and toxicity of radioactive materials.[1] It often relies on the use of organic-based fluorophores that absorb and emit in the 650-900 nm (NIR-I) spectral window, which permits relatively deep photon penetration into tissue, and minimal tissue autofluorescence. The best example of such fluorescent organic dye is probably indocyanine green (ICG), which has been approved by the FDA for about 60 years to measure cardiac output, liver function or to study the anatomy of the retinal vessels.[2] NIR fluorescence imaging has already clearly demonstrated its potential in preclinical research and will probably contribute to revolutionize cancer surgery by allowing image-guided intraoperative surgery.[3,4]

Current efforts focus on the design of targeted NIR-fluorescent agents, which are able to accumulate specifically in tissues of interest. Active targeting can be readily achieved through the covalent attachment of the NIR fluorophore to proteins, such as antibodies, that display excellent affinity and selectivity for their antigens.[5–7] This conventional biolabelling is currently achieved through derivatisation of protein's lysine residues with fluorophores bearing a bioconjugatable moiety reactive towards primary amino groups (i.e., active esters and isothiocyanates).[8] However, this random approach tends to be replaced with site-specific strategies, which aim at attaching the fluorescent organic dye to a specific position of the protein.[9] Indeed, it is now well-documented that the number and positions of fluorescent labels on a protein sequence can have a dramatic impact on the biodistribution properties of the resulting imaging agent.[10,11]

A simple and efficient way to achieve site-specific labelling of proteins consists in introducing, at the intended position, a cysteine residue. Indeed, the greater nucleophilicity of thiols, compared to amines under physiological conditions, allows their selective labelling in the presence of electrophiles.[12] Several NIR dyes able to react selectively with cysteines and related biothiols (*i.e.*, homocysteine and glutathione) have been described in the literature. They usually consist in fluorophore scaffolds functionalised with a bioconjugatable handle such as maleimide, iodoacetamide, sulfone, alkene or 2-cyanobenzothiazole groups.[12,13] Although effective, these thiol-reactive NIR probes are often hard to synthesize, eventually patented, and/or commercially available at prohibitive price (often >100$ per mg), which may prevent their widespread use, in particular in preclinical research.

Here, we show that **IR-783** (Fig. 1), a water-soluble NIR heptamethine cyanine dye carrying a central 4-chlorocyclohexyl ring within its polymethine chain (known as *meso* position), can directly label cysteine residues, under mild conditions.

By simply controlling the pH of labelling mixture, it is possible to achieve selective nucleophilic substitution of its *meso*-chlorine by thiols, as demonstrated on a peptide and two protein models. This post-synthetic derivatisation procedure of a cheap, commercially available, NIR-emitting cyanine dye (< 0.5$/mg) promptly provides stable fluorescent bioconjugates suitable for *in vivo* imaging applications.

Results and discussion

The cyanine-based fluorophore **IR-783** was first described and synthesised in 2004 by Patonay *et al.*[14] Its spectral properties are suitable for *in vivo* applications, with a high molar absorption coefficient, excitation/emission maxima in the

optical therapeutic window and an acceptable fluorescence quantum yield under physiological conditions.[15] Furthermore, this NIR fluorophore has already been assessed in animal models and showed no systemic toxicity in mice, at doses up to 37.5 mg/kg/day for one month.[16] Owing to these promising properties, **IR-783** has been conjugated to various biomolecules (peptides,[17] glucose derivatives,[18,19] monoclonal antibodies,[20] and even bacteriophages[21]). These fluorescent labellings required the prior reaction of **IR-783** with a bifunctional cross-linker composed of i) a nucleophilic function (primary amine, phenol, or thiol) used to substitute the *meso*-chlorine atom, and ii) a terminal reactive handle, such as an activated ester, an isothiocyanate or a maleimide group. Alternatively, Gorris *et al.* described in 2011 the direct conjugation of **IR-783** to proteins without pre-introducing a bioconjugatable tether onto the core structure of this fluorophore.[22] The labelling was carried out through the substitution of the chlorine atom by protein's amine groups at 37°C and pH 8.4. Interestingly, this reaction was associated with a significant hypsochromic shift in the absorbance of this heptamethine cyanine (colour of solution changed from green to blue) which allowed the monitoring of the reaction. The main limitations of this bioconjugation method were its non-site-specific nature, and the slow kinetics of substitution reaction leading to low conversion rates, even in alkaline conditions (ca. 20% after 60 h).

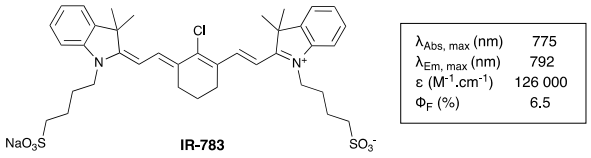

Fig. 1 Structure and main photophysical properties of the NIR-emitting cyanine dye **IR-783**. $\lambda_{Abs,\ max}$, $\lambda_{Em,\ max}$ and ε were measured in PBS pH 7.4, at 25 °C; Φ_F value in PBS pH 7.4, at 25 °C, was obtained from literature.[15]

Since the kinetics of the substitution reaction depends on the nature of the nucleophile and the pH of the reaction, we hypothesised that, by judiciously choosing the bioconjugation conditions, one could achieve the specific labelling of cysteine residues, while keeping valuable spectral features of **IR-783**.

Validation of the strategy on model peptides

This strategy was first tested on a model peptide, Ac-CKYWGRGDS-NH_2, which contains all common nucleophilic functions found in proteins (Fig. 2).[23,24] The reaction was carried out at neutral pH (phosphate buffer, PB, pH 7.1) to favor cysteine reactivity versus that of lysine. In the presence of an excess (25 equiv.) of **IR-783**, the sole product detected after 1 h was the mono-labelled peptide conjugate **1** (Fig. S1). The reaction was scaled up and allowed us to isolate **1** in 86% yield after purification by semi-preparative RP-HPLC. Fluorescent peptide **1** displayed photophysical properties in PBS (pH 7.4) typical of a "stabilised" Cy7 derivative with absorbance and emission maxima at 792 and 825 nm respectively (Fig S4).[21]

It has been reported in the literature that the reaction between *meso*-chloro-substituted heptamethine cyanine dyes and cysteine, or N-terminal cysteinyl residues, can lead to the formation of amine-substituted cyanines.[25] This reaction is caused by the substitution of the chlorine atom by the thiolate group of cysteine, followed by the spontaneous intramolecular displacement of sulphur by the α-amino group of cysteine, through a five-membered cyclic transition state. It could be envisaged that a similar intramolecular reaction could occur in **IR-783**-labelled peptides and proteins, where thiol-conjugated **IR-783** would be transferred to a nearby lysine residue. To assess whether this risk is real, a series of peptides was synthesized, in which the distance between the labelled cysteinyl residue and the lysine residue was varied (Table 1).

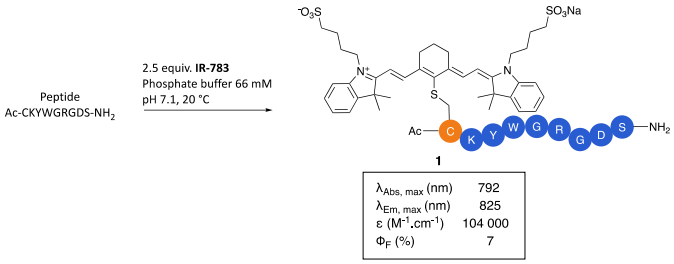

Fig. 2 Cysteine-selective labelling of a model peptide with **IR-783**. The spectral properties of **1** were recorded in PBS pH 7.4 at 25 °C. The fluorescence quantum yield was determined using **IR-783** as reference (Φ_F = 6.5% in PBS, pH 7.4).[15]

Table 1. Assessment of the selectivity of the labelling reaction on model peptides

Peptide substrate	Degree of labelling	Product R_t (RP-HPLC)	Product $\lambda_{(Abs,\ max)}$
Ac-CKYWGRGDS-NH_2	1	4.09 min	791 nm
CKYWGRGDS-NH_2	2	4.33 min	706 nm
Ac-KCYWGRGDS-NH_2	1	4.04 min	788 nm
Ac-KYWGCRGDS-NH_2	1	4.02 min	789 nm
Ac-KYWGRGDSC-NH_2	1	4.05 min	788 nm

The degree of labelling corresponds to the number of **IR-783** dyes covalently bound to each peptide, as determined by RP-HPLC-MS analysis.

Each peptide was mixed with **IR-783** (25 equivalents) in PB pH 7.4, and the reaction mixture was analyzed by RP-HPLC after 1 h and 15 h of incubation at 25°C.

All *N*-acetylated peptides were completely converted, after 1h, into a product of monosubstitution, showing a maximum of absorption at *ca*. 790 nm, characteristic of thiol-substituted heptamethine cyanine dyes. This result was independent of the distance between the cysteine and lysine residues and no changes were observed over the next 15 h. In contrast, the peptide with a free N-terminal cysteine residue afforded a disubstituted product, in which both the amine and thiol groups of cysteine were modified. This product exhibited a distinct UV-Vis absorption spectrum, with a maximum of absorption at 706 nm, which is attributed to the formation of an amine-substituted **IR-783** adduct.

Although these results cannot definitively rule out the possibility of intramolecular transfer of **IR-783** from cysteine residues to the side chain of lysine residues in a specific peptide or protein, this reaction is not favoured. More importantly, the eventual occurrence of such a reaction may be readily detected because it is associated with a large hypsochromic shift in Cy7

UV-vis spectrum. Such evolution has not been observed in the protein models that will be presented below.

Next, we evaluated the *in vitro* stability of **1**. Indeed, previous studies[26–28] have shown that the alkenyl-thioether moiety may undergone further substitution reactions by other nucleophiles found in biological media at high concentrations, such as reduced glutathione (GSH).[29] The fluorescent peptide conjugate **1** was found to be stable in foetal bovine serum at 37 °C with 98% intact peptide detected after 24 h and more than 92% after 55 h (Fig. S5). Degradation was slightly faster in the presence of 0.3 mM GSH at 37 °C with 83% of intact fluorescent bioconjugate persisting after 53 h. Nevertheless, the thioether linkage appeared as sufficiently stable to withstand the biological environment for the time generally assumed to perform biodistribution studies.

Cysteine-specific NIR labelling of a Fab' antibody fragment

Encouraged by these first results, we sought to apply this method to a more relevant protein model. Fab' are monoclonal antibodies fragments that have been used as targeting agents for *in vivo* imaging applications.[30] They can be prepared through the enzymatic digestion of the crystallisable fragment Fc of an IgG, followed by the selective reduction of the disulfide bridges of the hinge region.[33] Fab' fragments retain an affinity comparable to their intact antibody counterparts but with faster pharmacokinetics *in vivo*. The reduced cysteines, in the hinge region of the Fab', are often regarded as ideal bioconjugation sites for the regiospecific labelling of the fragments without risks of interference with the antigen recognition site.[32,33]

Fab' fragments derived from pertuzumab, an anti-HER2 therapeutic monoclonal antibody, were prepared with a purity of 77% at 280 nm (the Fab' fragment was partially contaminated with light chain and heavy chain fragments; Fig S8).[34] **IR-783** (25 equiv.) was added to the Fab' in PB, at 37 °C (Fig. 3A), and the reaction was monitored by RP-HPLC-MS analysis (Fig. S10). It is important to note that other thiol-based nucleophiles (such as reducing agents) should be removed from the reaction medium before the conjugation step. After 1 h, a new peak was detected, assigned to the Fab' covalently labelled with two **IR-783** molecules (fluorescent Fab' conjugate **2**), as indicated by the deconvoluted ESI mass spectrum (Fig. 3B). The labelling of Fab' further increased over time to reach 70% conversion after 26 h. The specificity of the conjugation was confirmed by performing a control experiment on a Fab', whose cysteines had been previously capped by mild alkylation with iodoacetamide. In this case, no labelling was observed when the same bioconjugation conditions were used (25 equiv. of **IR-783**, 37 °C, 24 h; Fig. S11).

We finally sought to assess whether the efficiency and specificity of our approach were comparable to the conventional maleimide chemistry.[13] Thus, we studied the labelling of pertuzumab Fab' with a commercially available Cy7-maleimide derivative (Fig. S12). Preliminary tests indicated that the maleimide moiety of this Cy7 dye is more reactive than **IR-783**, with a greater proportion of Fab' converted in 1 h (90% conversion in the presence of 15 equiv. of maleimide at pH 7.1). However, the reaction was less specific, as shown by the presence of a significant amount of Fab' labelled with three cyanine molecules instead of two, which suggests the modification of some lysine residues.

These different experiments showed the feasibility of our strategy on complex proteins such as antibody fragments. Unfortunately, our inability to isolate the pertuzumab Fab' fragment with sufficient purity prevented us from evaluating the Fab'-$(IR\text{-}783)_2$ conjugate *in vivo*. It prompted us to perform the labelling by **IR-783** on an alternative protein model.

NIR labelling of albumin and *in vivo* fluorescence imaging

We undertook the conjugation of **IR-783** to bovine serum albumin (BSA), a protein which contains a single free cysteine residue in its protein sequence (Cys-34).

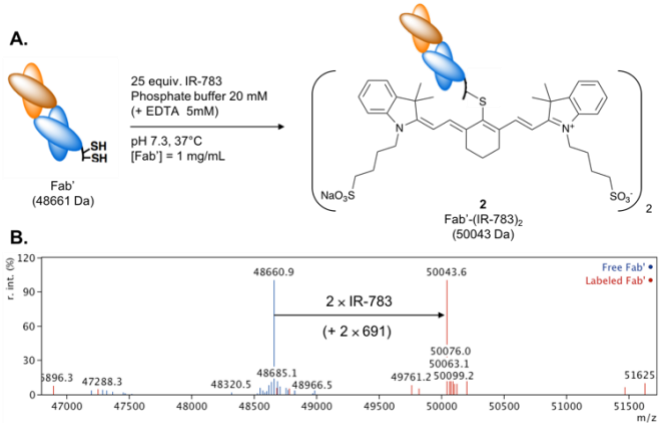

Fig. 3 (A) Labelling reaction of Fab' with **IR-783**. (B) Deconvoluted ESI-MS spectra (recorded in the positive mode) of free Fab' and Fab' labelled with two **IR-783**.

NIR labelling of albumin and *in vivo* fluorescence imaging

We undertook the conjugation of **IR-783** to bovine serum albumin (BSA), a protein which contains a single free cysteine residue in its protein sequence (Cys-34). 10 equiv. of **IR-783** were added to a solution of BSA at 2 mg/mL in PB (pH 7.1) and at room temperature. The reaction was monitored by RP-HPLC-MS analysis. After 1 h, the conversion already reached 95%. The excess of cyanine dye was removed after 5 h, by size-exclusion chromatography, to afford the fluorescently-labelled albumin **3** with > 90% of purity (Fig. 4A). As before, the cysteine-specificity of the reaction was verified by ESI mass spectrometry (Fig. 4B) and UV-Vis absorbance spectroscopy (Fig. S17). Finally, the covalent nature of the bond between the fluorophore and albumin was confirmed by gel electrophoresis performed under denaturing conditions (Fig. 4C). Incubation of the conjugate **3** in human plasma at 37 °C for 48 h did not lead to any detectable release of the fluorophore (Fig. S18).

The fluorescent BSA conjugate **3** was evaluated in BALB/c mice, healthy (n=4) or bearing CT26 colon cancer syngeneic tumor cells on their flanks (n=5).[‡] Fluorescence images were recorded 1 h, 4 h and 24 h after the injection of a dose of 30 µg/mouse (Fig. 5 and S20). The images at 1 h and 4 h p.i. showed an uptake of the fluorescence signal in the tumor, which can be explained

by the enhanced permeability and retention (EPR) effect. After 24 h, part of **3** had been eliminated from the circulation, leading to a higher contrast between the tumor region and surrounding normal tissues. The mice were euthanized and dissected, and the biodistribution of the fluorescent BSA conjugate in major organs was evaluated (Fig. S21-S22). We found a strong retention in the lung and liver of mice, which has been already observed in literature.[35,36] The tumor/muscle and tumor/blood ratio were of 1.5 and 1.9 respectively.

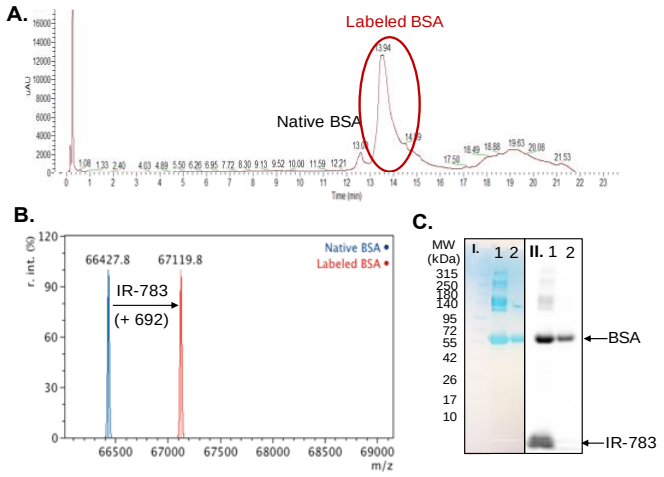

Fig. 4 (A) RP-HPLC elution profile of the purified fluorescent BSA conjugate 3 (detection at 280 nm). (B) Deconvoluted ESI-MS spectra (positive mode) of free BSA and fluorescently labelled BSA. (C) SDS-PAGE analysis of the reaction mixture (line 1) and purified fluorescent BSA conjugate 3 (line 2) after Coomassie blue staining (left panel, I.) and by fluorescence (right panel, II.) with an excitation at 740 nm and emission at 790 nm (bandwidth of 20 and 40 nm respectively).

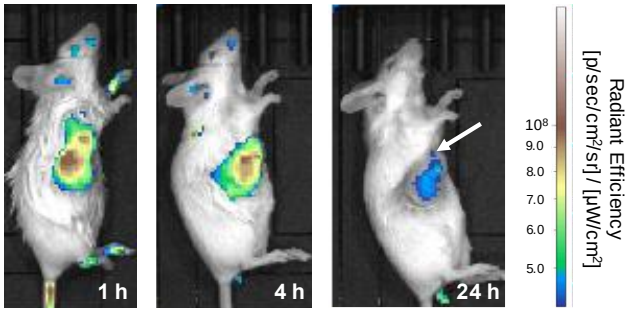

Fig. 5 Representative images of the biodistribution of the fluorescent BSA conjugate **3** by the measurement of fluorescence (excitation at 760 nm and emission at 845 nm (bandwidth of 20 and 40 nm respectively) in a living mouse bearing a CT26 tumor at 1 h, 4 h and 24 h post-injection (p.i.). Fluorescence signal, displayed as radiant efficiency, is superimposed to white light images.

Conclusions

In summary, we have developed a novel straightforward one-step labelling strategy for proteins, based on the use of commercially available NIR-emitting cyanide dye **IR-783**. The conjugation reaction occurs selectively on cysteine residues at neutral pH, through the selective substitution of the *meso*-chlorine atom of this cyanine by thiol group of a cysteine residue. The resulting fluorescent bioconjugates are sufficiently stable in biological media to study their biodistribution *in vivo*.

We expect that this method will facilitate the design of well-defined immunoconjugates for NIR fluorescence imaging. Furthermore, we feel it is obvious that this innovative and easy to implement bioconjugate chemistry should be applicable to other families of organic-based fluorophores bearing a good leaving group within their core structure (*e.g.*, xanthene dyes such as 9-substituted pyronins). Future work will focus on the evaluation of this strategy for the labelling of disulfide-reduced, full-length IgG antibodies.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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Notes

‡ The animal study was conducted in accordance with the legislation on the use of laboratory animals (directive 2010/63/EU) and was approved by accredited Ethical committee (C2ea Grand Campus n°105) and the French Ministries of Research (project #9617) and Agriculture (A 21 231 016 EA).

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