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A Near-Infrared Luminescent Label Based on Yb^{III} Ions and Its Application in a Fluoroimmunoassay**

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There has been a great deal of interest in luminescent lanthanide complexes since Weissman's discovery that in these complexes "the excitation may be accomplished, under suitable conditions, through light absorption by other constituents of the rare earth compound with subsequent transfer of energy to the rare earth ion".^[1] The energy transfer from organic chromophores to lanthanide ions that he described provides an effective way to excite the long-lived, sharply spiked emission. Direct excitation of lanthanide ions is difficult because of the forbidden nature of the electronic transitions in these ions. One of the most important applications of lanthanide complexes is as luminescent labels in clinical diagnostics where they provide an alternative to radioactive probes.^[2–5] Since lanthanide luminescence decays much more slowly than the autofluorescence from the biological material, the latter is suppressed when using time-gated detection.

Research on luminescent lanthanide complexes has been almost exclusively devoted to europium(III) and terbium(III) complexes.^[6–8] The energetics of sensitized luminescence, however, dictate that those complexes must be excited with ultraviolet light,^[9] which is not desirable when working with vital biosystems, requires special optics, and causes extensive scattering in inhomogeneous media. Although some recent work has demonstrated that the excitation window for Eu^{III} complexes can be extended to the violet edge of the visible region^[10] or even to the blue region,^[11] it would be desirable to use significantly longer wavelengths for excitation. Lanthanide ions that emit in the near-infrared, such as ytterbium(III),^[12] neodymium(III),^[13] and erbium(III), may be excited through organic dyes that absorb at wavelengths extending towards the red region of the spectrum. With their potential use as luminescent labels in mind we designed and studied well-defined, water-soluble near-infrared luminescent lanthanide complexes containing organic dyes as the light-absorbing unit.^[14] Recently, we found that 4',5'-bis[*N,N*-bis(carboxymethyl)aminomethyl]-fluorescein (fluorexon, Fx) is a very efficient sensitizer and a strongly binding ligand for near-infrared-luminescent lanthanide ions.^[15] The thermodynamic stability of the [Yb(Fx)] complex is comparable to that of [Yb(EDTA)] (EDTA = ethylenediaminetetraacetate).

Here we report on the ligand FxITC (**5**; Scheme 1), which is structurally similar to Fx but carries an isothiocyanate group (ITC) that is reactive towards amine groups and so can be coupled to proteins. The iminodiacetic acid groups ensure firm complexation of lanthanide ions (like they do in [Yb(Fx)]), and the dichlorofluorescein chromophore acts as a sensitizer to near-infrared lanthanide luminescence.

The synthesis of FxITC (Scheme 1) reflects the idea of combining the syntheses of Fx^[16] and fluorescein isothiocyanate.^[17] 2',7'-Dichloro-4-nitrofluorescein diacetate was obtained by condensing 4-nitrophthalic acid with 4-chlororesorcinol at 240 °C and was separated from the 5-nitro isomer by fractional crystallization from acetic acid.^[18] The dichloro derivative was chosen to prevent formation of isomers during the introduction of the methyleneiminodiacetic acid groups. Moreover, the "heavy" chlorine atoms might enhance intersystem crossing in the chromophore which is favorable for sensitizing lanthanide luminescence.^[15]

Direct introduction of the methyleneiminodiacetic acid groups, as used in the production of Fx,^[16] was not successful. Reaction of iminodiacetic acid dimethyl ester^[19] with **2**^[20] and subsequent hydrolysis of the ester yielded **3**, the desired nitro precursor to FxITC. Reduction of **3** using H₂/Raney Ni proceeded cleanly, but gave rise to the dinickel complex of **4**, which can be observed with electrospray mass spectrometry. Therefore, **3** was reduced using Na₂S/NaHS in dilute NaOH. After purification by preparative HPLC (C₈ column, eluent: water:acetonitrile:formic acid (155:45:1)), **4** was converted into FxITC using thiophosgene in acetone.

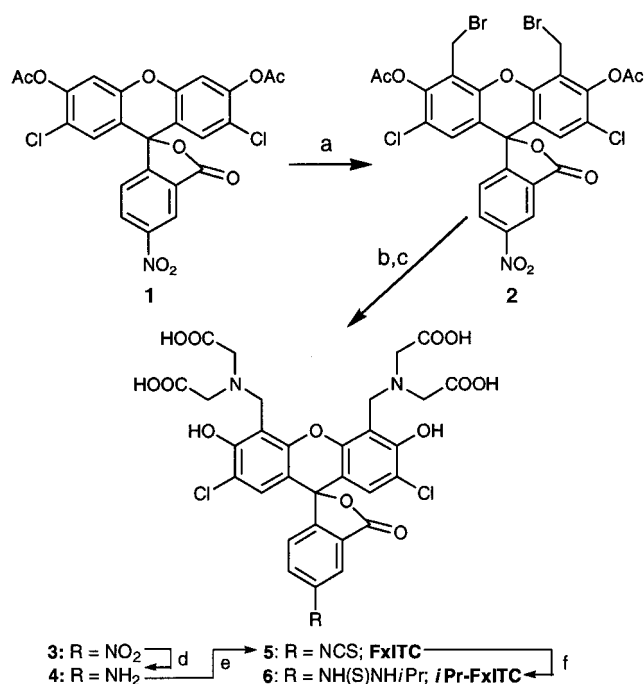
The isopropylamine adduct **6** shows the same complexation behavior towards lanthanide ions as Fx does.^[15] Fluorimetric titrations with Yb^{III} ions indicate formation of a 1:1 complex as long as there is no excess Yb^{III} ions. Excess lanthanide ions leads to the formation of poorly emitting aggregates that so far have escaped further characterization. Such aggregates,

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Scheme 1. Synthesis of FxITC. Reagents and conditions: a) paraformaldehyde (90 equiv), 33% HBr in acetic acid, 110 °C, 2 h; acetic anhydride (100 equiv) added dropwise at 20 °C, reflux, 1 h, 81 %; b) iminodiacetic acid dimethyl ester (15 equiv) in dichloromethane, 90 h at 20 °C, 69 %; c) methanol/50% NaOH, 16 h at 20 °C, 99 %; d) Na₂S, NaHS in 0.6 M NaOH, reflux 15 min; e) thiophosgene (200 equiv) in acetone/water, 30 min at 20 °C; f) isopropylamine, 8 h at 20 °C.

however, are not formed when FxITC is bound to avidin. Titrating these protein conjugates with Yb^{III} ions yields a curve typical for the exclusive formation of the 1:1 complex.

Excitation of the avidin conjugate of the Yb^{III} complex leads to a metal-centered near-infrared luminescence with a quantum yield equal to that of [Yb(Fx)]. The corresponding excitation spectrum closely matches the absorption spectrum of the complex (Figure 1). No quenching of the luminescence

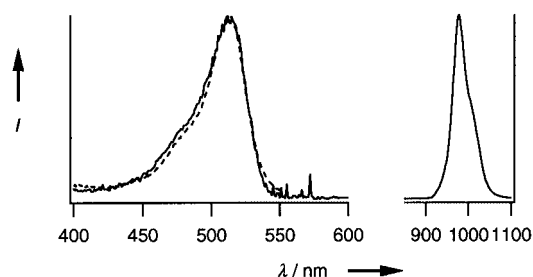


Figure 1. Luminescence spectra of [Yb(FxITC)]-labeled avidin in saline Tris-HCl buffer (pH 8.3). Left: excitation spectrum ($\lambda_{\text{em}} = 980$ nm). The visible absorption profile is shown as a dotted line. Right: emission spectrum ($\lambda_{\text{exc}} = 510$ nm).

by dissolved molecular oxygen was observed, which demonstrates that the transfer of excitation energy from the organic chromophore to the lanthanide ion is a rapid and efficient process. These observations show that the photoluminescence of the label is not adversely affected by conjugation to a protein, which furthermore has the desirable consequence

that addition of excess lanthanide ions does not influence the 1:1 antenna:lanthanide stoichiometry in the complex.

The complexes and the avidin conjugates have appreciable extinction coefficients (Table 1). Their quantum yields and luminescence lifetimes also compare well to those of [Yb(Fx)]. The absolute quantum yields, however, are low.

Table 1. Photophysical data for iPr-FxITC, its Yb^{III} complex, and the protein conjugates of [Yb(FxITC)] in aqueous buffer.

	$\Phi_{\text{rel}}^{[a]}$	$\epsilon_{\text{max}} [\text{M}^{-1} \text{cm}^{-1}]^{[b]}$	$\lambda_{\text{max}} [\text{nm}]^{[c]}$	$\tau_{\text{NIR}} [\mu\text{s}]^{[d]}$
[Yb(Fx)] ^[15]	1 ^[e]	125 000	502	1.9
iPr-FxITC	–	125 000	509	–
[Yb(iPr-FxITC)]	0.6	145 000	516	1.8
avidin-[Yb(FxITC)] (3.2 l/p) ^[f]	1.0	–	514	2.0
avidin-[Yb(FxITC)] (5.2 l/p) ^[f]	0.8	–	514	2.0
anti-hCG-[Yb(FxITC)]	–	–	–	2.2

[a] Φ_{rel} = near-infrared luminescence quantum yield relative to that of [Yb(Fx)]. [b] ϵ_{max} = molar extinction coefficient. [c] λ_{max} = absorption maximum. [d] τ_{NIR} = near-infrared luminescence lifetime monitored at 980 nm. [e] The absolute quantum yield of [Yb(Fx)] in Tris is 7×10^{-4} .^[15] [f] l/p means labels per protein.

This is caused by radiationless deactivation of excited lanthanide ions by molecular vibrations (especially C–H and O–H oscillators) in the surrounding matrix,^[21] a process which is especially effective with near-infrared-luminescent lanthanide ions.^[22–25] Very recently, Hasegawa et al. succeeded in pushing the luminescence quantum yield of Nd^{III} ions in organic complexes up as high as 0.03 by careful design of the ligands,^[26] but such (relatively) high quantum yields have thus far only been achieved in organic solvents. In this respect, it should be noted that closely diffusing O–H oscillators (such as water) form a particularly important contribution to the nonradiative deactivation of the luminescent state of Yb^{III} complexes.^[27]

Although the luminescence quantum yield Φ is an important parameter it is surely not the only one determining the detectability of a luminescent label. The molar extinction coefficient ϵ of the label is equally important, and when these two are combined it is apparent that [Yb(FxITC)] performs quite well in comparison to, for example, the Eu^{III} cryptate that is used in a commercial diagnostic test.^[2] The product $\epsilon\Phi$ for the cryptate in water amounts to approximately $100 \text{ M}^{-1} \text{cm}^{-1}$ when light from a N₂ laser with a wavelength of 337 nm is used for excitation.^[28] The avidin conjugate of [Yb(FxITC)] achieves the same $\epsilon\Phi$ value for light with a wavelength of 514 nm.

The robustness of the label was tested in an archetypal medical diagnostic test. The format chosen was that of a simple heterogeneous noncompetitive immunoassay,^[5] in which the analyte to be detected was “sandwiched” between an immobilized and a labeled antibody. Antibodies to hCG (anti-hCG 147b) were immobilized on a small region (diameter 3 mm) of a nitrocellulose membrane by impregnation of 0.5 μL of a 2.7 g L^{-1} solution. After drying the membrane, a mixture of solutions of the [Yb(FxITC)]-labeled antibodies (anti-hCG 293a, 2.7 g L^{-1}) and of the sample to be assayed for hCG (total 20 μL) was spotted onto a different place on the membrane. This spot was eluted with buffer (100 μL) so that

eventually the labeled antibodies and the sample passed the immobilized anti-hCG 147b. The experiment was done both with a positive sample containing 5000 mIU of hCG plus labeled antibodies and a blank that only contained the labeled antibodies. The traces generated by a home-built scanning near-infrared luminescence microscope clearly showed that a luminescence signal is generated in the case of a "positive" test result (Figure 2).

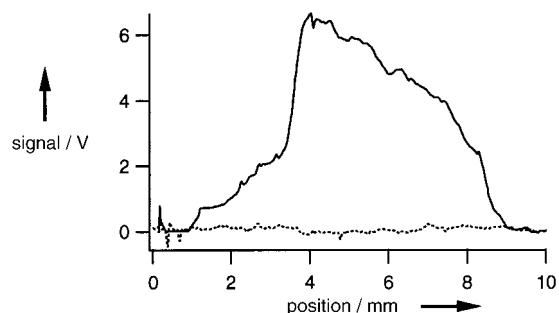


Figure 2. One-dimensional near-infrared fluorescence microscopy scan of the region of the nitrocellulose membrane where anti-hCG is immobilized. The solid line is the near-infrared luminescence signal generated after elution of a solution of labeled anti-hCG and hCG. The dotted line is the signal generated by a solution containing only the labeled anti-hCG.

It has been shown that the protein conjugates of [Yb-(FxITC)] retain photophysical characteristics that are similar to [Yb(Fx)], and that they can be applied in a model medical diagnostic test. Their luminescence is long-lived (microseconds) compared to the optical interferences encountered in biological matrices, such as fluorescence and scatter, and resides in the near-infrared region where biological material is essentially transparent. Most CCD detectors can readily detect the luminescence of Nd^{III} ions at 880 nm and that of Yb^{III} ions at 980 nm. These factors, together with the possibility of exciting these labels with visible light, make them an attractive new class of luminescent labels. However, they would benefit from an improvement of their overall luminescence quantum yield by the suppression^[24] of the nonradiative deactivation^[27] of their luminescent states.

Experimental Section

Conjugation to avidin: A 2 g L⁻¹ solution of FxITC in dry DMSO (40 µL or 80 µL) was added to a 2 g L⁻¹ solution of avidin (250 µL, Sigma) in 0.1 M carbonate buffer (pH 9.5). The mixture was incubated for 3 h at room temperature, with shaking. The solution was diluted to 2.5 mL with a saline 0.05 M Tris-HCl buffer (pH 8.3, 0.15 M NaCl; Tris = tris(hydroxymethyl)aminomethane) and purified by gel chromatography on a PD-10 column (Amersham Pharmacia Biotech) using the saline Tris buffer as an eluent. The number of labels per protein was determined by means of UV/Vis spectrometry using the extinction coefficients of the species at 280 and 514 nm.

Optical spectroscopy: The equipment and experimental procedures have been described in detail previously.^[15] Fluorimetry was carried out using a modified PTI Alphascan apparatus with a Ge detector for the near-infrared and using a modified Spex Fluorolog 3 with a CCD detector for the near-infrared measurements.

Fluorescence microscopy: The excitation light from an Ar⁺ laser (488 nm) was modulated using a mechanical chopper (40 Hz) and delivered to the sample via a dichroic mirror and the microscope objective. The emitted light was detected through the objective by a Ge detector (North Coast

817L) coupled to a lock-in amplifier (Stanford Research SRS530) after passing it through the dichroic mirror and optical filters to remove the scattered excitation light. The sample was moved with respect to the excitation/detection spot using a computer-controlled stage.

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