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SYNTHESIS AND FLUORESCENCE PROPERTIES OF OREGON GREEN 514 LABELED PEPTIDES

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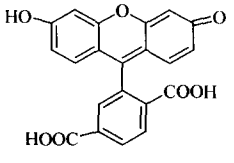
Abstract : Oregon Green 514, with high photostability and a pKa of 4.8, is suitable for imaging applications. Labeling of peptide-bound resin with the carboxylic acid form was optimized. Peptide conjugation resulted in altered fluorescence properties of the dye including a quenching of the intensity compared with that in aqueous buffers with a slight red-shift of the emission maximum.

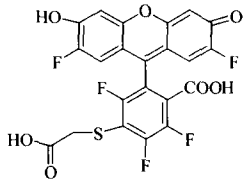
Introduction :

The photobleaching[1] and pH sensitivity[2] of unsubstituted fluorescein make quantitative measurements with this fluorophore problematic. These limitations have encouraged the development of alternative fluorophores, among them the new fluorescein-like dyes[3]. The superior photostability of Oregon Green 514 (OG 514) makes this fluorophore the preferred fluorescein substitute for fluorescence imaging applications[4]. Furthermore, introduction of electron-withdrawing groups onto fluorescein dyes (Scheme 1) lowers the pKa of the phenolic group to 5 or below. The pKa of 4.8 for OG 514, versus 6.4 for fluorescein[4], virtually eliminates the photo sensitivity at neutral pH. These properties prompted us to label immunogenic peptides with OG 514 to follow their cellular uptake by microspectrofluorometry[5].

We describe here the optimization of a solid-phase procedure to introduce the fluorophore onto a side chain-protected resin-bound peptide. Moreover, we have explored the fluorescence properties of OG 514 linked to a peptide prior to our microspectrofluorometric studies. We noticed that the OG 514-bound peptide (OG-peptide) exhibits a different fluorescence behavior compared to the free OG 514 dye (OG-OH).



6-carboxyfluorescein



Oregon Green 514 carboxylic acid 6-isomer

Scheme 1

Results and Discussion :

Our peptide of interest is the TT-NP6 peptide which is consisted of two copies of a promiscuous T-helper epitope (T: residues 288-302 from the fusion protein of the measles virus) linked to a T-cytotoxic epitope (NP6: residues 52-60 from the nucleoprotein of measles virus). A remarkable feature of the TT-NP6

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peptide[6] resides in its capability to induce virus-specific cytotoxic T-lymphocytes (CTL) responses in the absence of any adjuvant and hydrophobic component[7]. It is believed that exogenous peptides work at inducing CTL responses by interacting with the plasma membrane of the antigen presenting cells[8]. This could in turn facilitate the internalization of the T-cytotoxic epitope into the cytoplasm of target cells. To follow the location of the peptide into living cells by *in situ* microspectrofluorometry[5], the TT-NP6 peptide and two of its fragments, *i.e.* the T-NP6 and TT peptides[6], were labeled with Oregon Green 514, a new photostable dye with high quantum yield. To keep the dye far apart from the peptide for potentially reducing the quenching that typically occurs upon conjugation, OG 514 was coupled to the ε–NH_2 of an extra-lysine added at the N-terminal position of the peptide.

Synthesis of OG 514 labeled peptides : Peptide chemists have taken advantage of the supported peptide chemistry to add fluorophore onto side-chain protected resin-bound peptides[8,9] if the dye is inert to trifluoroacetic acid (TFA) treatment. Moreover, Weber[10] *et al.* reported that the use of carboxyfluorescein as being more efficient than its preformed activated analogues. As OG 514 is a fluorescein-like dye, we chose to introduce OG 514 to peptide-bound resin in its carboxylic acid form. The high price of OG 514 urged us to optimize the reaction, in particular, to reduce the excess of the dye in relation to the peptide-bound resin from 10, as described by Weber[10] for carboxyfluorescein, to 2.

The protected peptide-resin was synthesized using standard procedures of Fmoc chemistry with 1-hydroxybenzotriazole (HOBt) / dicyclohexylcarbodiimide (DCC) activation on a p-alkoxybenzyl alcohol polystyrene resin[11]. Boc-Lys(Fmoc)OH was added to the N-terminal position of the peptide. After Fmoc deprotection, OG 514 was coupled on the ε–NH_2 of the extra lysine (Table 1).

Table 1 : Results of the Oregon Green labeling experiments.

Method	Activation reagent	Reaction time	Purity[a] [%]	Identified peak[b]	Yield after purification
A	DCC/HOBt	24 h	main product : 45% other product :30%	Oregon Green labeled peptide[9] unlabeled peptide	10%
B	TBTU/HOBt	24 h	main product : 90%	Oregon Green labeled peptide	35%
C	TBTU/HOBt	6 h	main product : 90%	Oregon Green labeled peptide	35%

[a] according to the HPLC (214 nm); [b] according to Mass Spectrometry.

Three coupling methods[12] were set up to test coupling reagents (A and B) and reaction time (B and C). Except these parameters, a general procedure was followed. Oregon Green 514 carboxylic acid (OG-OH) (10 μmol, 5 mg) was treated during 1 h with DCC (20 μmol) / HOBt (20 μmol) in N-methyl-2-pyrrolidone (NMP) (400 μL) for the method A or O-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) (10 μmol) / HOBt (10 μmol) and N-ethyldiisopropylamine (DIEA) (20 μmol) in NMP (200 μL) for the methods B and C. The Oregon Green benzotriazolyl ester (10 μmol) was then added to the resin-linked side chain-protected peptide (5 μmol) at room temperature over 24 h (methods A and B) or 6 h (method C). The reagents

were washed from the support. The peptide was cleaved from the resin by TFA containing 5% triisopropylsilane as scavenger and then precipitated into cold diethylether. It was purified by RP-HPLC on a Vidac C8 column 7 μm, 300 Å (250*10mm) using linear gradient of water containing 0.1% TFA and acetonitrile containing 0.1% TFA at a flow rate of 3 mL/min. The HPLC peaks were identified by Electrospray Mass Spectrometry. Table 1 shows that complete conversion of the unlabeled peptide was obtained under TBTU/HOBt activation while classical DCC/HOBt activation allowed only a conversion of 45%. The activation via DCC/HOBt was incompleted likely due to partial conversion of O-acylisourea intermediate into N-acylurea[13]. Uronium salt-based coupling reactions have been reported to be useful for the coupling of sterically hindered amino acids and for the syntheses where coupling times are to be reduced[14], in agreement with our results.

Fluorescence properties of OG-peptide compared to OG-OH[15] : Before microspectrofluorometric studies were to be done, it was necessary to obtain more information about spectral fluorescence properties of the Oregon Green 514 conjugates at different pH and in media with different dielectric constant. Moreover, we investigated whether the coupling to a peptide could influence the fluorescence properties of the dye. Whatever the peptide tested among the three peptides mentioned before[6], similar results were obtained. As an example, we describe here the data obtained with the OG-T-NP6 peptide, so-called OG-peptide.

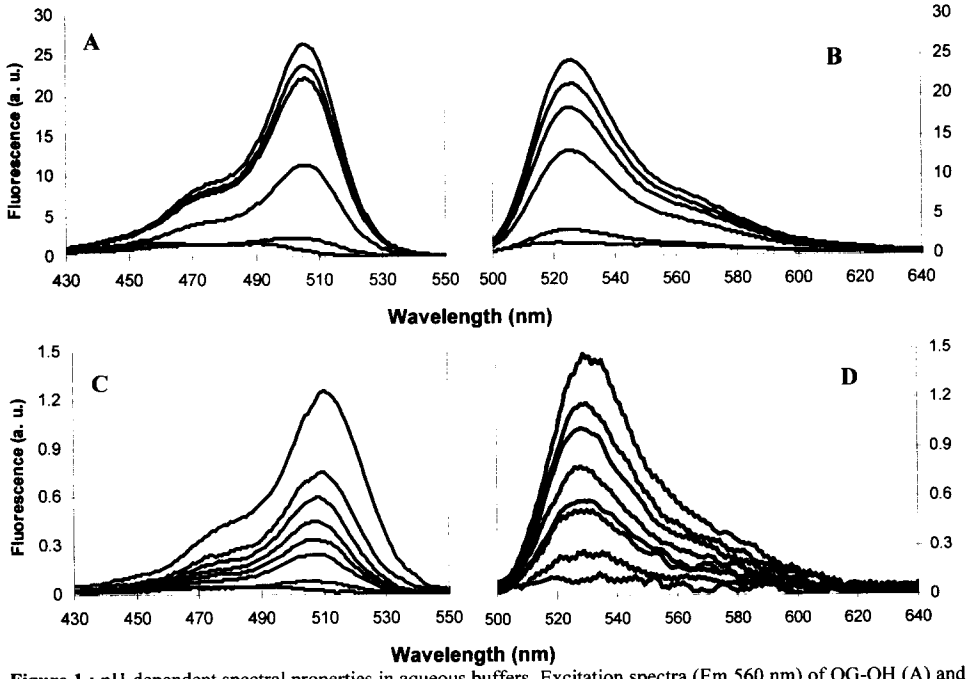

Figure 1 : pH-dependent spectral properties in aqueous buffers. Excitation spectra (Em 560 nm) of OG-OH (A) and OG-peptide (C) at 0.1 μM and emission spectra (Ex 488 nm) of OG-OH (B) and OG-peptide (D) at 0.1 μM. A and B : bottom to top, pH 2 ; 4 ; 5 ; 5.5 ; 6 ; 7.5, a plateau was reached for 7.5. C and D : bottom to top, pH 2 ; 4 ; 5 ; 5.5 ; 6 ; 7.5 ; 8 ; 9.

pH titration in aqueous buffers : pH titrations of OG-OH and OG-peptide were done *in vitro* at a concentration of 0.1 µM in aqueous buffers at pH values ranged from 2 to 9 (Figure 1). Excitation spectra were collected using emission wavelengths of 515, 560 and 580 nm. Like the absorption spectra, the excitation spectra of the dye exhibited prominent features, such as a maximum intensity at 506 nm for OG-OH (Figure 1A) or 510 nm for OG-peptide (Figure 1C), and a shoulder at 470 nm, both of which increased with increasing pH. Fluorescence emission pH titrations were performed using excitation wavelengths of 466, 488 and 502 nm. Emission spectra showed a maximum at about 525 nm for OG-OH (Figure 1B) or 530 nm for OG-peptide (Figure 1D), and a shoulder at 560 nm, both of which increased with increasing pH. The excitation and emission spectra of OG-peptide revealed a red-shift of 4 nm and 5 nm, respectively, compared to the corresponding spectra of the free dye. A similar red-shift in emission maximum has been reported for isothiocyanate fluorescein conjugates[16]. We also observed a quenching on peptide conjugation of about 15-fold. Decrease in fluorescence intensity is almost always reported for other dyes[4] usually due to dye-biomolecule interaction. However, that result was unexpected as OG 514 has been claimed to yield fluorescent conjugates resistant to quenching[3], although no data are yet available about its fluorescence efficiency after conjugation. In addition, it has been reported that the nature of the link between dye and biomolecule affects the fluorescence intensity, *e.g.* the amide link leads to fluorescein or rhodhamine conjugates with higher quantum yield than the thiocarbamyl link[4]. More experiments will be needed to determine whether the thioether link, which is scarcely used, could influence the fluorescence intensity of the OG-conjugates.

pH titration in Triton X100 micelle environment : Figure 2 shows pH titration of OG-OH and OG-peptide in the presence of 0.1% Triton X100 whose non-ionic micelles mimic membrane environment.

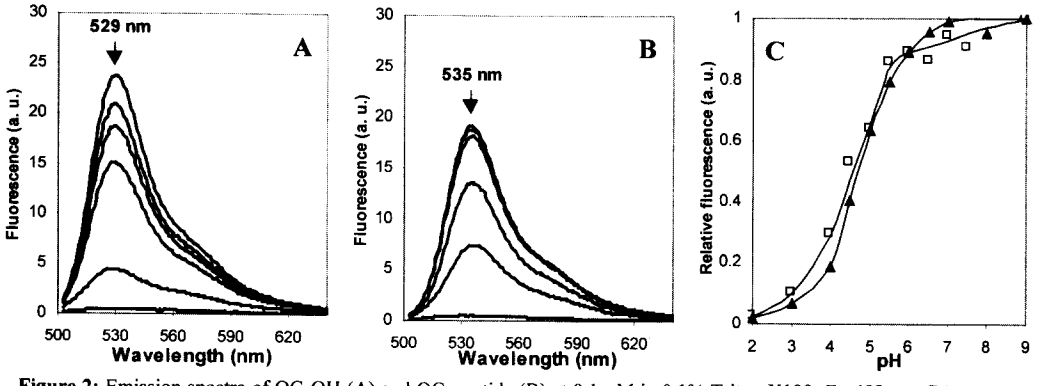

Figure 2: Emission spectra of OG-OH (A) and OG-peptide (B) at 0.1 µM in 0.1% Triton X100; Ex 488 nm. Bottom to top: pH 2; 4; 5; 5.5; 6; 7.5. A plateau was reached for 5. (C) pH titrations of OG-OH (▲) and OG-peptide (□); fluorescence intensities were normalized.

Under these conditions, the emission maximum of OG-peptide (535 nm) was red-shifted by 6 nm in comparison with that of OG-OH (529 nm). Moreover, the emission maximum of OG-peptide as well as that of OG-OH were red-shifted in the presence of Triton as compared with spectra recorded in the absence of Triton (Figure 1B, D). Except this point, the pH-dependent fluorescence of OG-OH in Triton X100 (Figure 2A) was

similar to that in the absence of Triton (Figure 1B), with the same intensity at any pH value from 2 to 9. In contrast, the fluorescence intensity of OG-peptide was increased in Triton X100 (compared Figure 1D and Figure 2B) reaching that for OG-OH. This suggests that no quenching on peptide conjugation occurs in the presence of Triton X100. When the fluorescence intensities were plotted versus pH, superimposable sigmoidal curves were obtained for OG-OH and OG-peptide (Figure 2C) as well as for OG-OH when spectra were recorded in aqueous buffer. A pKa of 5 was evaluated which is in good agreement with the literature[3-4]. However, when plotting the fluorescence intensities of OG-peptide recorded in aqueous buffer versus pH, no inflexion point was obtained but rather a straight line from pH 4 to pH 9. Similar titration curves were obtained from excitation spectra (not shown). Together these data suggest that with the three OG-conjugates[6] tested here, the low efficiency of fluorescence observed in aqueous buffer probably arises from both quenching on conjugation and quenching on pH, with the quenching on conjugation varying with the pH.

Influence of the dielectric constant : We have further investigated the influence of dielectric constant on the fluorescence spectra by using dioxane, a nonpolar solvent which can be mixed with water in all proportions. Figure 3 shows the emission spectra obtained in 0, 10, 30 and 50% dioxane in 50 mM Tris(hydroxymethyl) aminomethane/HCl buffer (Tris, HCl), pH 7. The fluorescence intensity of OG-OH decreased slightly with increasing the percentage of dioxane whereas the fluorescence intensity of OG-peptide in 50% dioxane reached the intensity of OG-OH, 10-fold higher than the one recorded in Tris, HCl. Of interest, the emission maximum is shifted from 525 to 534 nm for OG-OH and from 529 to 535 nm for OG-peptide on increasing the percentage of dioxane in the solvent. This is consistent with the data obtained in the presence of Triton showing that the fluorescence of OG-conjugates increases with more hydrophobic media. The recovery of fluorescence efficiency could be due to the increase in α-helical content of the peptides in hydrophobic media[7]. To rule out this hypothesis, studies with a simple conjugate such as NH_2-Lys(OG 514)-OH will be necessary.

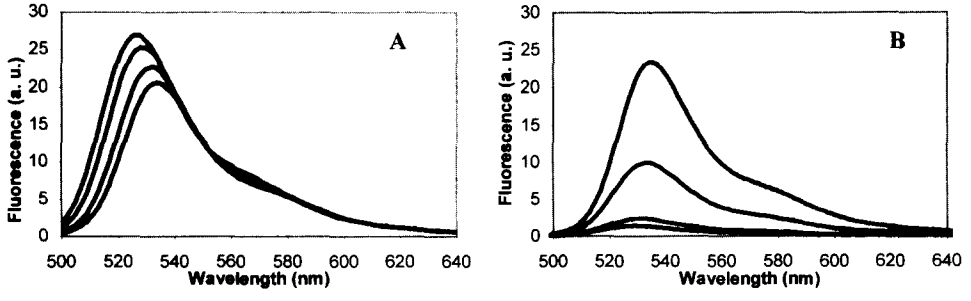

Figure 3 : Emission spectra of OG-OH (A) and OG-peptide (B) (0.1 μM) in various percentages of dioxane in 50 mM Tris, HCl, pH 7. Ex 488 nm. (A) Left to right, (B) Bottom to top: 0, 10, 30, 50 % dioxane.

Conclusion :

We have described an optimized solid-phase synthesis that enabled the efficient introduction on peptide-bound resin of Oregon Green 514 in its carboxylic acid form. Moreover, this study reveals that the fluorescence of the free Oregon Green 514 is slightly red-shifted in hydrophobic environment. Three peptides were coupled

to OG 514. Spectral properties of Oregon Green conjugates indicate that the fluorescence was quenched on peptide conjugation in aqueous medium and was totally recovered in more hydrophobic environments. In addition, the emission maximum of conjugates exhibited a slight red-shift with increasing the dielectric constant of the medium. Although these observations have to be extended to some other peptides for general application, the properties described here associated with the resistance of the OG 514 to photobleaching make this dye very attractive for spectral imaging of our peptides. Microspectrofluorometric analyses, which are in progress in our laboratory, will make use of the results described here. Moreover, these observations will be of interest for the choice of the dye for other various fluorometric applications.

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References and Notes :

1. Song, L. ; Hennink, E. J. ; Young, I. T. ; Tanke, H. J. *Biophys. J.* **1995**, 68(6), 2588-2600
2. Sjöback, R. ; Nygren, J. : Kubista, M. *Spectrochim. Acta. A.* **1995**, 51, 7
3. Sun, W. C. ; Gee, K. R. ; Klaubert, D. H. ; Haughland, R. P. *J. Org. Chem.* **1997**, 62, 6469-6475.
4. Haugland, R. P. In *Handbook of Fluorescent Probes and Research Chemicals* ; Spence, M. T. Z. ; Molecular Probes : USA, 1996 ; Sixth Edition, pp 22-28, 562-564
5. Favard, C. ; Valisa, P. ; Egret-Charlier, M. ; Sharonov, S. ; Herben, C. ; Manfait, M. ; Da Silva, E. ; Vigny, P. *Biospectroscopy* **1999**, 5(2), 101-115
6. Sequence of the peptides used: TT-NP6: LSEIKGVIVHRLEGVLSEIKGVIVHR-LEGVLDRLVRLIG, TT: LSEIKGVIVHRLEGVLSEIKGVIVHRLEGV, T-NP6: LSEIKGVIVHRLEGVLDRLVRLIG;
7. Partidos, C.D.; Delmas, A.; and Steward, M.W., *Mol Immunol.* **1996,** 33, 1223-1229.
8 Lelièvre, D. ; Hsu, S. C. ; Daubos, P. ; Favard, C. ; Vigny, P. ; Trudelle, Y. ; Steward, M. W. ; Delmas, A. *Eur. J. Biochem.* **1997**, 249, 895-904
9. (a) Faure, M. P. ; Gaudreau, P. ; Shaw, I. ; Cashman, N. R. ; Beaudet, A. *J. Histochem. Cytochem.* **1994**, 42, 755-763 ; (b) Kropshofer, H. ; Bohlinger, I. ; Max, H. ; Kalbacher, H. *Biochemistry* **1991**, 30, 9177-9187.
10. Weber, P. J. A. ; Bader, J. E. ; Folkers, G. ; Beck-Sickinger, G. B. *Bioorg. Med. Chem. Lett.* **1998**, 8, 597-600
11. Reagents: Oregon Green 514 carboxylic acid was obtained from Molecular Probes (Leiden, The Netherlands). Protected amino acids and resin were obtained from Senn Chemicals (Les Ulis, France) and Neosystem (Strasbourg, France) respectively. The chain side protections were as followed: *tert*-butyl (E, D, S), trityl (H), N-2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (R), *tert*-butoxycarbonyl (K). Peptides were synthesized on an ABI 431 A Applied Biosystems peptide Synthesizer. The mass spectrometer was a VG Quattro II type (Micromass) equipped with an electrospray ion source.
12. The methods of coupling had been tested on three different peptides: method A on the T-NP6 sequence[6], method B on the TT-NP6 sequence[6], and method C on the TT sequence[6].
13. Lloyd-Williams, P. ; Albericio, F. ; Giralt, E. In *Chemical Approches to the Synthesis of Peptides and Proteins*, Rees, C. W. Ed ; CRC Press LLC : Boca Raton, Florida, 1997 ; pp 48-56
14. Schnölzer, M. ; Alewood, P. ; Jones, A. ; Alewood, D. ; Kent, S. B. H. *Int. J. Peptide Protein Res.* **1992**, 40, 180-193
15. Fluorescence spectroscopy was carried out at 20°C on a Fluoromax 2 (Jobin Yvon) fluorometer with a correction for excitation and emission spectra, using 5 mm pathlength quartz cuvettes, a Xenon lamp and 1 nm slits for excitation and emission.
16. Chen, R., F. ; *Arch. Biochem. Biophys.* **1969**, 133, 263-276