Improved flow cytometry analysis of antibody bioconjugated nanoparticles (NPs) by primary amines quantification on NPs surface

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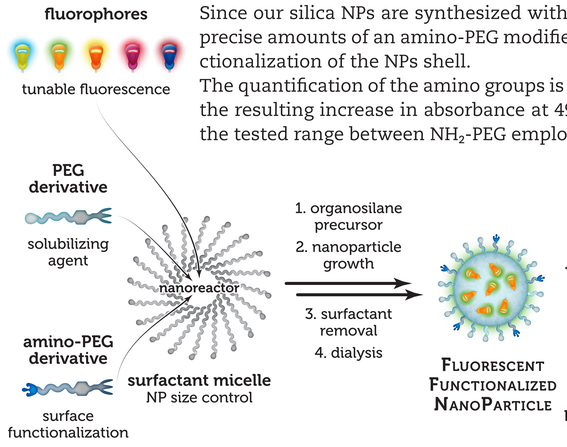
Introduction

Antibody conjugated fluorescent silica nanoparticles are emerging as a promising tool in immunofluorescence assays, thanks to the improved features compared to molecular fluorophore-based probes, such as better photostability and enhanced emission.
As might be expected, for an optimal immunofluorescence performance, a crucial point is the site-specific bioconjugation between the nanoparticle (NP) and the antibody, via the primary amino groups located on the surface of the NP. In order to optimize this process, a complete control and characterization of the NPs surface has proven to be essential.

Surface Characterization



Since our silica NPs are synthesized with an outer layer of PEG to improve stability in water, by adding precise amounts of an amino-PEG modified reactant (NH_2-PEG), it is possible to fine-tune the degree functionalization of the NPs shell.
The quantification of the amino groups is performed by means of coupling with FITC and then analyzing the resulting increase in absorbance at 495 nm (fig. 1). Data correlation shows a linear behavior within the tested range between NH_2-PEG employed in synthesis and the NH_2 groups detected (fig. 2).

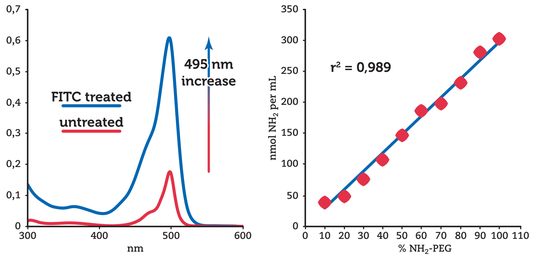


Fig. 1 Absorbance spectra of FITC treated and untreated 70% NH_2-PEG NPs

Fig. 2 % NH_2-PEG vs nmol NH_2

Bioconjugation

Thanks to the fine tuning of primary amines on the NPs surface and its relative quantification, it is now possible to evaluate and conveniently modulate the molar ratio between:

- NH_2 on NPs surface and cross-linker used for NPs activation;
- NH_2 on NPs surface and antibody used for bioconjugation.

By changing these parameters, it is possible to identify the optimal conditions for the bioconjugation of activated silica NPs with antibodies and highlight the differences between conjugates obtained in different ways.

One of the main parameters for monitoring the degree of substitution and the good quality of our products is the F/P ratio (nmol fluorophore/nmol of protein): a higher F/P is commonly a distinctive feature of better performing conjugates, as many immunofluorescent assays reveal.

According to the analysis of the two conjugates in fig. 4 and 6, CD4-NF_B520 $[NH_2]_{LOW}$ has a final F/P of 3.2, while the CD4-NF_B520 $[NH_2]_{HIGH}$ has F/P of 5.8, showing that a higher degree of amines yields a greater F/P value and thus a better quality of the bioconjugate, as flow citometry tests below confirm.

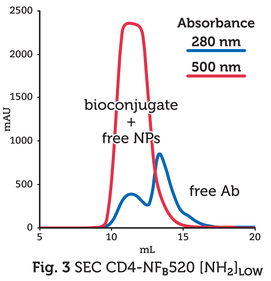


Fig. 3 SEC CD4-NF_B520 $[NH_2]_{LOW}$

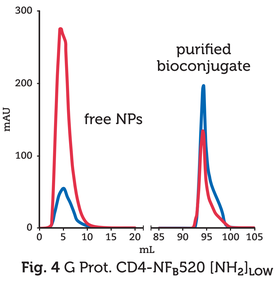


Fig. 4 G Prot. CD4-NF_B520 $[NH_2]_{LOW}$

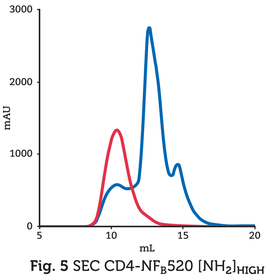


Fig. 5 SEC CD4-NF_B520 $[NH_2]_{HIGH}$

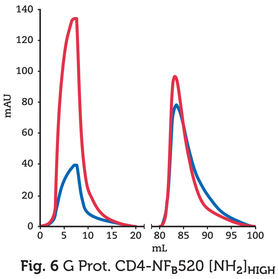
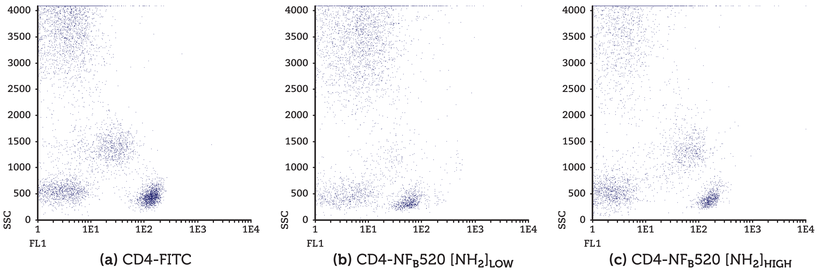


Fig. 6 G Prot. CD4-NF_B520 $[NH_2]_{HIGH}$

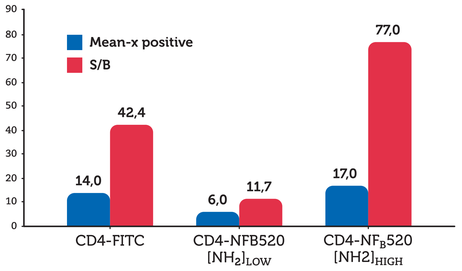
Flow Citometry

The flow cytometry results show that a higher concentration of NH_2 on the NPs surface leads to an improvement in the bioconjugated antibody performance.
Dot plots compare anti-CD4 bioconjugated with a classical fluorophore (fig. 7a) and with NPs NF_B520 with low (fig. 7b) and high concentration of NH_2 on the surface (fig. 7c).



(a) CD4-FITC (b) CD4-NF_B520 $[NH_2]_{LOW}$ (c) CD4-NF_B520 $[NH_2]_{HIGH}$

Fig. 7 Dot Plots SSC vs FL1 of human peripheral whole blood sample. The images are elaborated using Infinicyt™ software, v. 1.7 (Cytognos SL, Salamanca, Spain).



The histogram in figure 8 analyzes the flow cytometry statistics of the three bioconjugated antibodies showed in the dot plots: high concentration of NH_2 yields a higher fluorescence (mean-x positive) and a better discrimination (S/B) between the different cellular populations.

Fig. 8 Histogram comparing FC statistics:
- blue mean-x positive cells - fluorescence intensity of T lymphocyte positive CD4 cells;
- red signal to background on a T lymphocyte positive CD4 cells.