

ELECTRONIC SUPPLEMENTARY INFORMATION

Labeling of native proteins with fluorescent RAFT polymer probes: Application to the detection of a cell surface protein using flow cytometry

Damien Duret,^{a,b†} Zofia Haftek-Terreau,^{b†‡} Matthieu Carretier,^{a,b} Thomas Berki,^{a,b}, Catherine Ladavière,^a Karine Monier,^{b,c} Philippe Bouvet,^{b,c} Jacqueline Marvel,^d Yann Leverrier,^d Marie-Thérèse Charreyre^{a,b} and Arnaud Favier^{a,b*}

^a Univ Lyon, Université Lyon 1, INSA de Lyon, CNRS, Laboratoire Ingénierie des Matériaux Polymères, UMR5223, F-69621 Villeurbanne, France.

^b Univ Lyon, ENS de Lyon, CNRS, Laboratoire Joliot-Curie, USR3010, F-69364 Lyon, France.

^c Univ Lyon, Centre de Recherche en Cancérologie de Lyon, Cancer Cell Plasticity Department, UMR INSERM 1052 CNRS 5286, Centre Léon Bérard, F-69008 Lyon, France.

^d Univ Lyon, INSERM, ENS de Lyon, CNRS, Université Lyon 1, Centre International de Recherche en Infectiologie (CIRI), U1111, F-69007 Lyon, France.

¹H NMR spectra of the (fluorescent) polymers obtained from the same poly(NAM-*stat*-NAS) copolymer.

AEM-capped copolymer. The thiol-ene reaction was performed with a reference AEM-capped copolymer without dye (Table 2). As for the PNAM homopolymers, the ¹H NMR spectrum of the AEM-capped copolymer after thiol-ene showed the expected absence of aromatic protons (due to the aminolysis of the dithiobenzoate ω-chain-end upon AEM-capping) and the appearance of a peak corresponding to the succinimidyl protons of the activated ester chain ends at 2.8 ppm (Fig. S1). This confirmed the introduction of the activated ester group at the ω-chain-end of the AEM-capped copolymer. In this case, since the succinimidyl proton peak partially overlapped with the broad peaks of the AEM-modified units of the copolymers, the yield of the chain-end functionalization could however not be determined precisely (although it seemed very high).

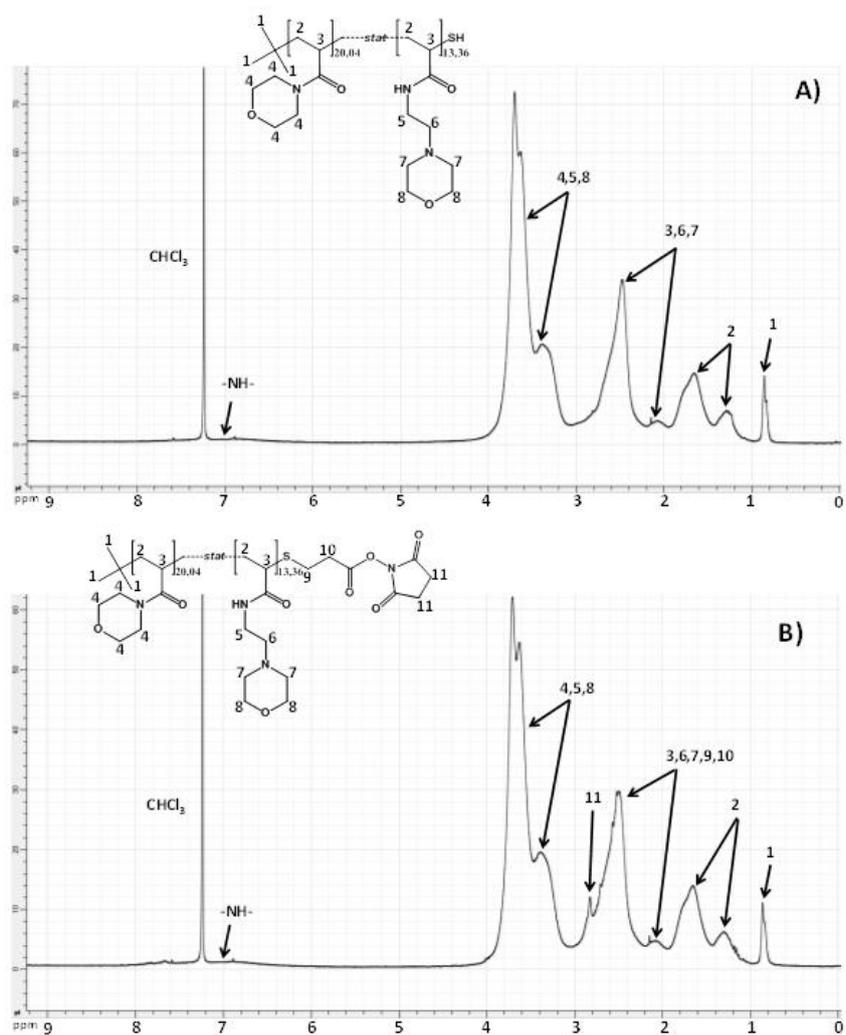
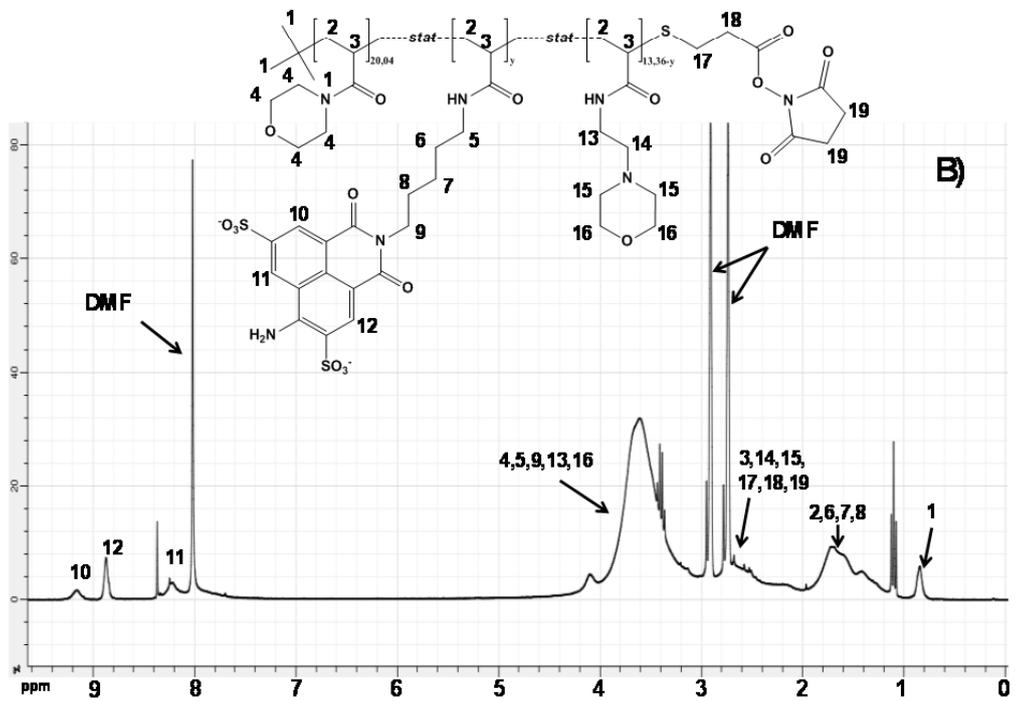
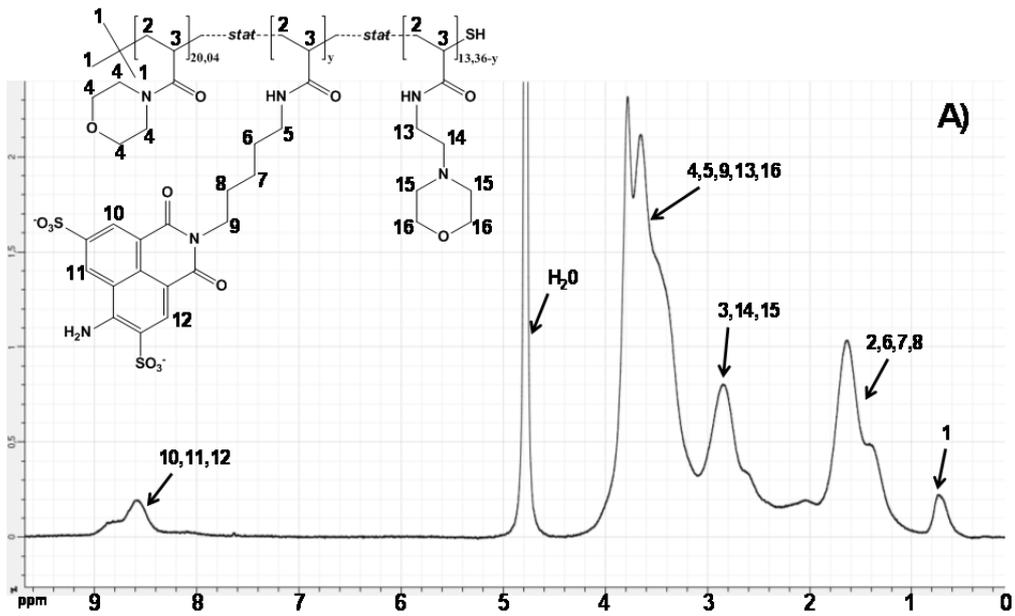


Fig. S1. ^1H NMR spectra in CDCl_3 of the AEM-capped copolymer without dyes, before (A) and after (B) thiol-ene reaction.

Fluorescent polymer probes. For each fluorescent polymer probes, the ^1H NMR spectra showed the disappearance of the peaks corresponding to the aromatic protons of the dithiobenzoate chains-ends (7.3-8.0 ppm) and the appearance of peaks corresponding to the aromatic protons of the dye (Fig. S2 and S3).

It was however not possible to quantify precisely the introduction of the activated ester at the ω -chain-end using ^1H NMR due to peak superimposition (see below).

LY- and AF647-probes. LY- and the AF647-probes were only soluble in aqueous media or in polar organic solvents such as DMF or DMSO. In deuterated DMF (Fig. S2), the succinimidyl peak superimposed with the broad peaks of the polymer backbone (note that these peaks are even broader in polar than in low polarity organic solvents) as well as with the peaks of DMF. Conversely, for the Dansyl-probe that was both soluble in a large range of organic solvent and in aqueous media, NMR analysis could be performed in CDCl_3 (Fig. S3). In this case, the succinimidyl peak superimposed with the large singlet peak of the methyl protons of Dansyl dyes. Although the latter clearly exhibited a shoulder on its low ppm side (attributed to the succinimidyl protons), it was not possible to properly quantify the average number of NHS ester chain-ends per polymer chain.



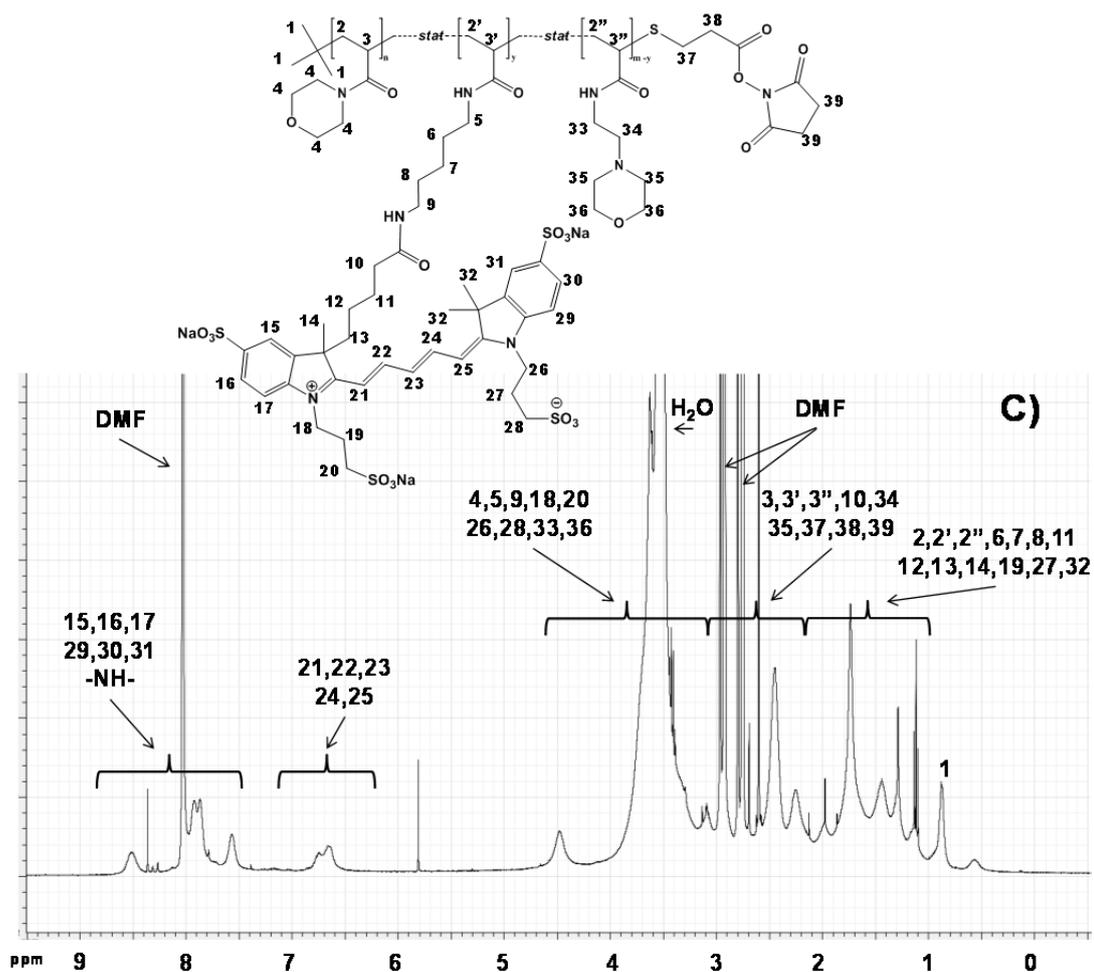


Fig. S2. ¹H NMR spectra of the LY-probe before (A, D₂O) and after (B, DMF-*d*₇) the thiol-ene reaction and of the AF647-probe-NHS ester (C, DMF-*d*₇).

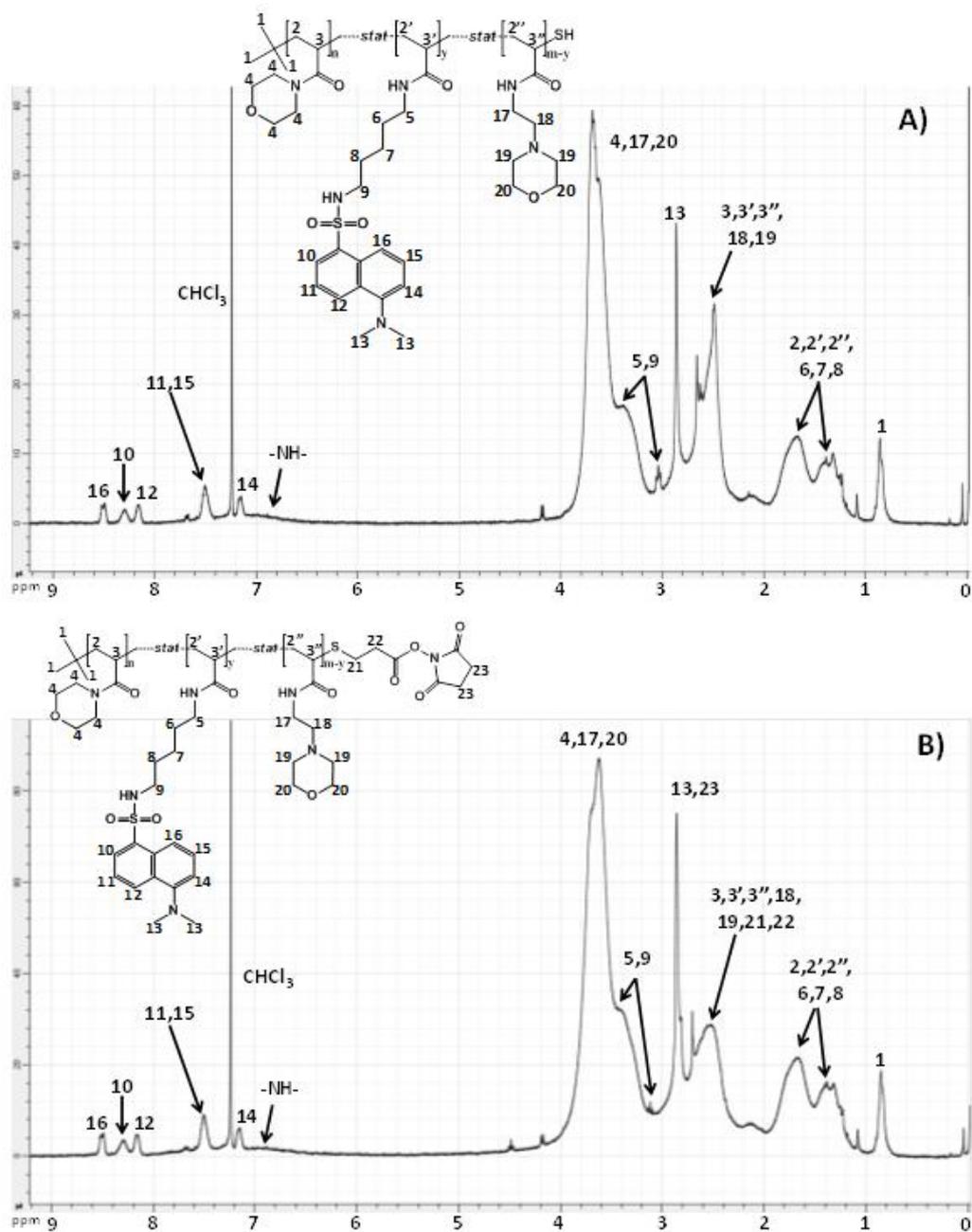


Fig. S3. ^1H NMR spectra in CDCl_3 of the Dansyl-probe before (A) and after (B) the thiol-ene reaction

Evaluation polymer-protein conjugation by SDS-PAGE.

Influence of the protein loading on SDS-PAGE analysis. As shown in Fig. S4, when using 5 μg protein per well (G,H,I), a slight saturation of the gel impaired the separation resolution. However, the good sensitivity allowed observing secondary populations characterized by a relatively low proportion. On the opposite, when using 1 μg protein per well (A,B,C), the main populations were observed with a good resolution whereas the secondary populations might not be witnessed.

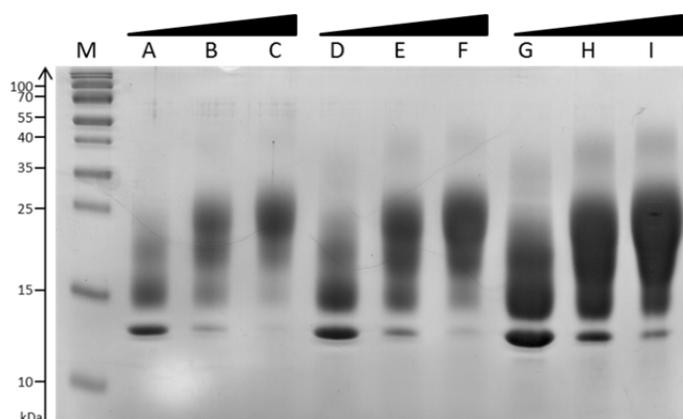


Fig. S4. SDS-PAGE analysis obtained after the conjugation of the tBu-PNAM-NHS ester sample with $DP_n = 22$ with lysozyme at pH 7.4 for 4h. Different gel loading were compared (A,B,C) 1 μg of protein; (D,E,F) 2 μg of protein; (G,H,I) 5 μg of protein. Conjugation reactions were performed at 3 different polymer/lysozyme P/L molar ratios: 10 (A,D,G); 30 (B,E,H); 50 (C,F,I). Marker of protein standards (M). Staining: coomassie blue.

Conjugation of tBu-PNAM-NHS ester samples with $DP_n = 93$ with lysozyme. The conjugation with lysozyme of two different tBu-PNAM-NHS ester samples with $DP_n = 93$ prepared from the same tBu-PNAM-SH batch (amount of free lysozyme and average number of polymer chains per protein) was compared (Fig. S5). The experimental variability that was noticed could be attributed either to a slight decrease of the thiol-ene efficiency when increasing the polymer MW (which is expected for reactions on chain-ends), or to some variability in the polymer/lysozyme conjugation which can be affected by steric hindrance. Indeed, once bound to the protein, polymer chains with an increased MW may reduce the accessibility of other potential conjugation sites.

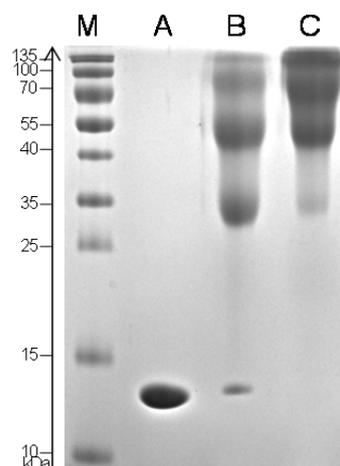


Fig. S5. SDS-PAGE analysis after the conjugation of two different tBu-PNAM-NHS ester samples with $DP_n = 93$ with lysozyme (P/L=50) at pH 7.4 for 4h (B,C). Native lysozyme (A). Gel loading: 2 μ g of protein. Reference: Marker of protein standards (M). Staining: coomassie blue.

Conjugation of the AEM-capped copolymer with lysozyme. Control assays were performed with the AEM-capped copolymer without dye to study its ability to covalently bind lysozyme, in comparison with a PNAM homopolymer sample ($DP_n=31$) used as reference (Fig. S5).

The results with the thiol-ended polymers gave two indications. First, they confirmed the absence of protein conjugation without thiol-ene ω -chain-end functionalization. Second, they indicated that the AEM-capped copolymers, conversely to the PNAM homopolymers, are stained by coomassie blue (used for gel revelation), due to an interaction between the latter and the tertiary amine of the AEM-modified units of the copolymer.¹ Thiol-ended copolymer led to a spread smear in the bottom part of the gel, but nonetheless the darker spot corresponding to the free unbound lysozyme was clearly seen superimposed to it.

For the polymers after thiol-ene reaction, the gels indicated a very efficient conjugation with lysozyme. The results with the tBu-PNAM-NHS ester sample were reproducible (comparison between Fig. S6 and Fig. 3, left) and similar results were obtained with the AEM-capped copolymer. The amount of free lysozyme decreased with the P/L ratio increase. Free lysozyme was almost not detected for P/L=50. A smear corresponding to the conjugates (with a higher MW) was observed in the upper part of the gel, above the smear of the free copolymer. The average number of polymer chains per protein tended to increase with the P/L ratio.

Consequently, these results confirmed the high efficiency of the thiol-ene reaction on the AEM-capped copolymer and the ability of the resulting copolymer to covalently bind native proteins.

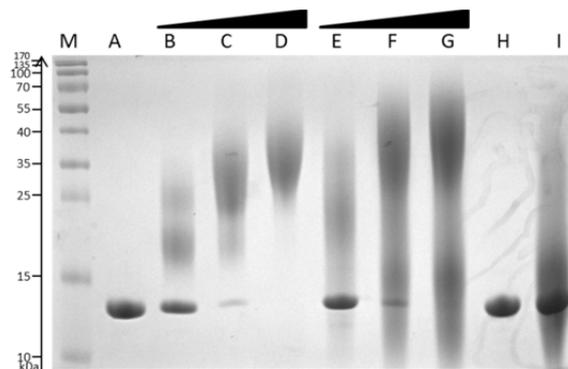


Fig. S6. SDS-PAGE analysis after conjugation with lysozyme at pH 7.4 for 4h. AEM-capped copolymer without dye (E,F,G after thiol-ene; I: before thiol-ene); tBu-PNAM homopolymer with $DP_n = 31$ used as reference (B,C,D: after thiol-ene; H: before thiol-ene). Three polymer/lysozyme P/L molar ratios were tested: 10 (B,E), 30 (C,F) and 50 (D,G,H,I). Gel loading: 2 μ g of protein. References: Marker of protein standards (M); Native lysozyme (A). Staining: coomassie blue.

SDS-PAGE analysis after incubation of LY-probe-NHS ester with lysozyme.

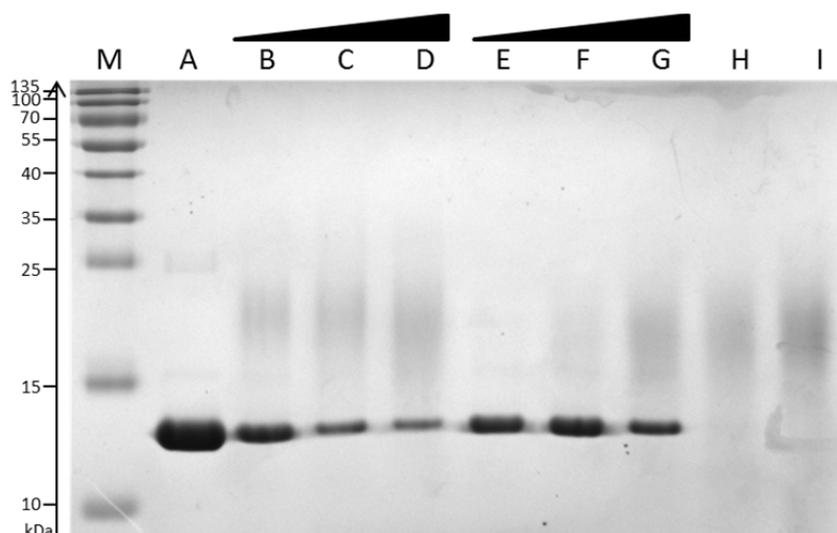


Fig. S7. SDS-PAGE analysis after conjugation of the LY-probes after (B,C,D) or before (E,F,G) thiol-ene with lysozyme at pH 7.4 for 4h. LY-probes alone (H,I). Three P/SA ratios

were tested: 10 (B,E), 30 (C,F) and 50 (D,G,H,I). Gel loading: 2 μg of protein. References: Marker of protein standards (M); Native lysozyme (A). Staining: coomassie blue.

SDS-PAGE analysis after conjugation of Dansyl-probe-NHS ester with streptavidin.

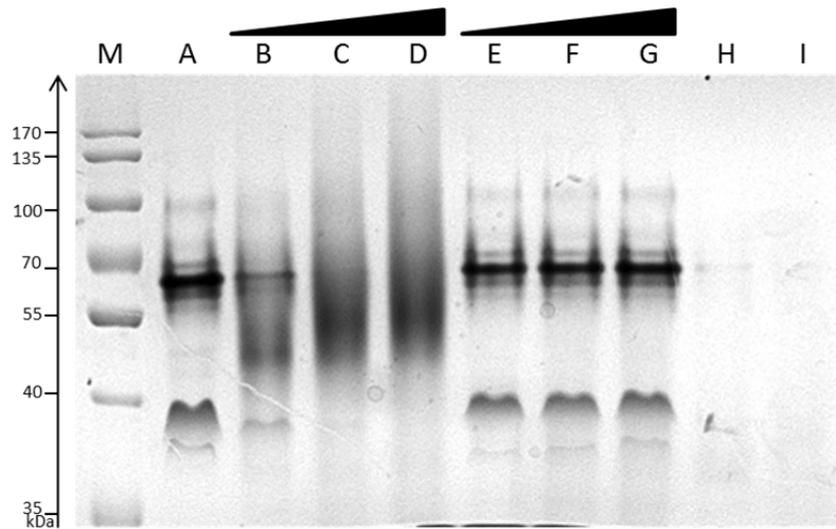


Fig. S8 SDS-PAGE analysis after conjugation of Dansyl-probe-NHS ester (after thiol-ene) (B,C,D) or Dansyl-probe-SH (before thiol-ene) (E,F,G) with native streptavidin at pH 7.4 for 4h. Dansyl-probes alone (H,I). Three P/SA molar ratios were tested: 10 (B,E), 30 (C,F) and 50 (D,G,H,I). Gel loading: 4 μg of protein. References: Marker of protein standards (M); Native streptavidin (A) for which two spots are observed, respectively corresponding to the main tetrameric protein (~ 70 kDa according to reference ladder) and to traces of dimeric protein (~ 38 kDa according to reference ladder). Staining: coomassie blue.

SDS-PAGE analysis after conjugation of LY-probe-NHS ester with streptavidin.

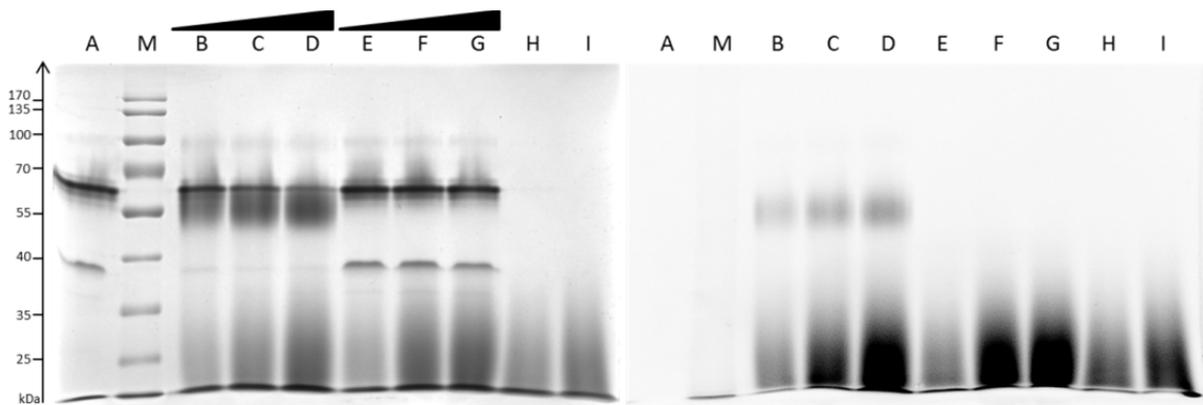


Fig. S9 SDS-PAGE analysis after conjugation of LY-probe-NHS ester (after thiol-ene) (B,C,D) or LY-probe-SH (before thiol-ene) (E,F,G) with native streptavidin at pH 7.4 for 4h. LY-probes alone (H,I). Three P/SA ratios were tested: 10 (B,E), 30 (C,F) and 50 (D,G,H,I). Gel loading: 4 μ g of protein. References: Marker of protein standards (M); Native streptavidin (A) for which two spots are observed, respectively corresponding to the main tetrameric protein (~70 kDa according to reference ladder) and to traces of dimeric protein (~38 kDa according to reference ladder). The same gel was revealed both by coomassie blue staining (Left) and by UV irradiation at 365 nm (Right).

SDS-PAGE analysis after conjugation of AF647-probe-NHS ester with streptavidin.

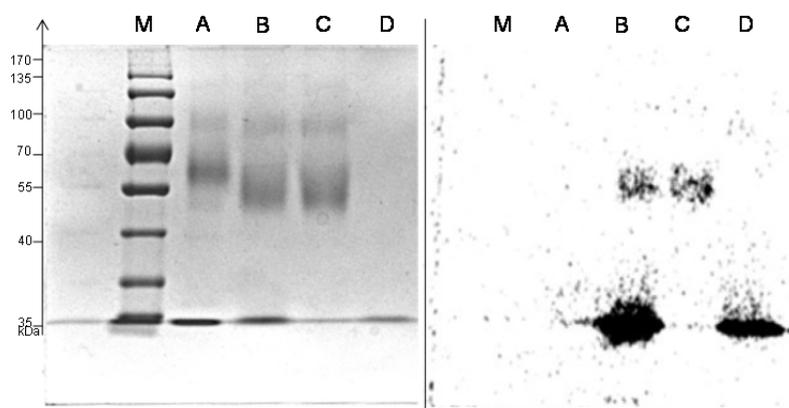


Fig. S10 SDS-PAGE analysis after conjugation of AF647-probe-NHS ester with native streptavidin at pH 7.4 for 4h at P/SA=50 ratio. Gel loading: 4 µg of protein. References: Marker of protein standards (M); Native streptavidin (A). Raw conjugation product (B), Purified conjugate (C) and filtrate of purification by ultrafiltration (D). The same gel was revealed both by coomassie blue staining (Left) and by a FLA-5100 imager (Fujifilm, Japan) with a laser irradiation at 635 nm (Right).

REFERENCES

- 1 P. Religio, M. Bathfield, Z. Haftek-Terreau, M. Beija, A. Favier, M.-J. Giraud-Panis, F. D'Agosto, B. Mandrand, J. P. S. Farinha, M.-T. Charreyre and J. M. G. Martinho, *Polym. Chem.*, 2013, **4**, 2968-2981.