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Multiple antibody conjugation to PEG grafted copolymer improves sensitivity of immunoassays

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Abstract

A polyethylene glycol (PEG) grafted copolymer based on a N-vinyl pyrrolidone (NVP) backbone was designed for conjugating antibodies (Abs) at PEG chain ends, leading to multivalent (“multi-antibody”) bioconjugate for use as a detection system in immunoassays. For this purpose, a poly(N-vinyl pyrrolidone-co-N-acryloxysuccinimide) (P(NVP-co-NAS)) copolymer was prepared by nitroxide mediated polymerization (NMP) and the N-succinimidyl activated esters of the NAS units were used for grafting heterobifunctional amine-PEG-maleimide by amidation. The PEG maleimide end-groups were further conjugated to Abs through thiol-maleimide addition. Enzyme-linked immunosorbent assay (ELISA) performed with the obtained bioconjugate in the detection phase showed significant improvement in antigen detection as compared to standard protocol with free Ab. These results point out the relevance of this straightforward approach to improve antibody accessibility and reduce background noise (thanks to anti-fouling PEG) for enhanced sensitivity in the field of immunoassays.

Keywords: PEG grafted copolymer, bioconjugation, antibodies, immunoassays

1. Introduction

Immunoassay is a leading testing method for detecting analytes/biomarkers in diseases diagnostics, but also in other contexts including food testing, environment monitoring and biosecurity. Immunoassays use a solid phase coated antigen (or antibody) that can specifically bind to the target antibody (or antigen) that is detected and quantified with an enzyme label and related substrate. Improving sensitivity of immunoassays is of prime importance as, for sure, low detection levels will increase success of treatments and healing chances (“the sooner, the better”).

Great efforts have thus been performed to enhance immunoassay sensitivity, either by (i) reducing the background noise or (ii) amplifying the signals [1,2]. The first strategy has largely relied on coatings with natural proteins as blocking agents (*e.g.*, bovine serum albumin) and antifouling hydrophilic polymers based on polyethylene glycol (PEG) [3–5] or zwitterionic moieties such as sulfobetaine [6,7] and phosphorylcholine [8,9], to reduce non-specific protein adsorption. As for signal amplification, it can be achieved by acting on either capture or detection steps. Optimizing the capture of the target requires to increase accessibility, orientation and density of the immobilized capture probes, to favor recognition event with the target analyte. For this purpose, different relevant immobilization strategies

were proposed, involving streptavidin-biotinylated peptide/protein systems [10], protein conjugation onto reactive (co)polymers [11,12], dendrimers [13], micelles [14] and nanofibers [15,16], or immobilization through 3-aminopropyltriethoxysilane (APTES) [17], phenylboronic acid [18], polydopamine [19] or azide-alkyne click [20] chemistries. Regarding detection optimization, many studies have consisted in increasing local concentration of the detectable antibody or labeling agent (enzyme or fluorophore) through immobilization on nano-objects, such as nanospherical poly(acrylic acid) brushes [21], silica-gold nanoshells [22] or block copolymer micelles [23]. Other detection systems based on polymerized horseradish peroxidase (HRP) enzyme [24,25] or novel efficient substrates of HRP as compared to classically used tetramethylbenzidine (TMB) [26] were also developed.

While such developed systems have proven (more or less) effective at improving sensitivity of the tests, they generally require tedious and complex multi-step chemical processes and surface modification, possibly involving costly and toxic compounds. Thus, there is a currently unmet need for more straightforward approaches able to provide cost effective, eco-friendly, improved immunoassay. In this paper, we propose a new strategy relying on an easily achievable PEG grafted copolymer affording antifouling properties and multi-attachment of detectable antibodies (Fig. 1A). While one of the antibodies of the macromolecule can bind to its target analyte (antigen), the other coupled antibodies are expected to be highly accessible for the labelling agent, leading to an improved immunoassay sensitivity as compared to conventional immunoassay with free Ab (Fig. 1B). In addition, multivalent character may be also expected to improve affinity of antibody for the targeted antigen [27], further contributing to improve sensitivity as well, even if chemical immobilization (PEGylation) may alter affinity to some extent.

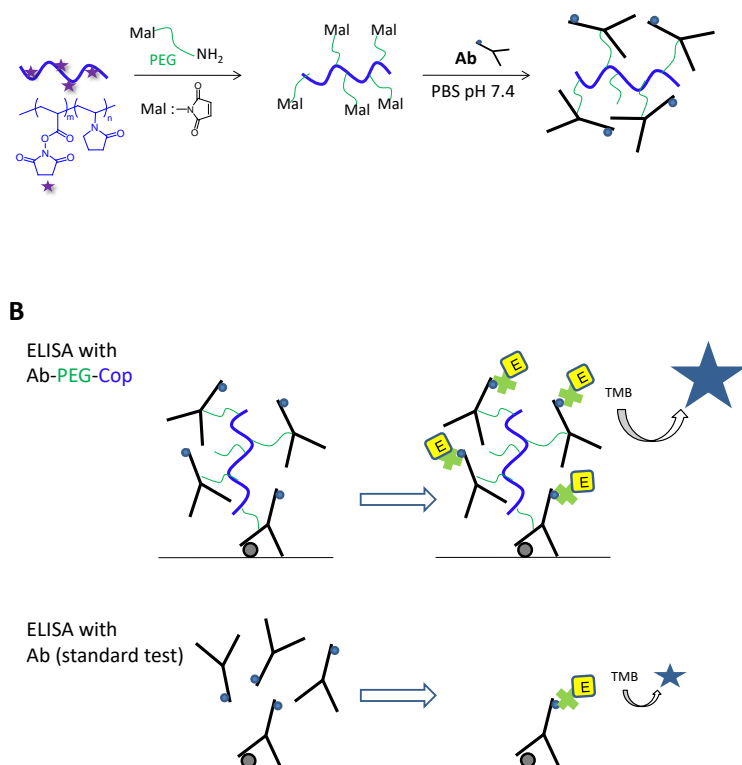


Fig. 1. A. Scheme of preparation of the PEG grafted copolymer for multi-attachment of antibodies (Abs), leading to Ab-PEG-Cop conjugate; **B.** Scheme of enzyme-linked immunosorbent assay (ELISA) performed with the Ab-PEG-Cop conjugate (top) versus free Ab (bottom, standard ELISA).

2. Experimental section

2.1. Materials

The poly(N-vinyl pyrrolidone-co-N-acryloxysuccinimide) (P(NAS-co-NVP)) copolymer ($M_n=45000$ g.mol⁻¹, NAS/NVP molar ratio of 54/46) was synthesized by nitroxide mediated polymerization (NMP) from BlocBuilder alkoxyamine (Arkema, France), as previously described [28]. Mal-PEG-NH₂ (10 kg.mol⁻¹) was purchased from Creative PEGWorks. Solvents were provided by Merck and used as received.

2.2. P(NVP-co-NAS)-g-PEG-Mal copolymer synthesis and characterization

3 mL of P(NAS-co-NVP) copolymer solution (3 mg.mL⁻¹ in DMF), 3 mL NH₂-PEG-Mal solution at various concentrations in DMF (3, 13.5 and 27 mg.mL⁻¹) and 2 eq. of triethylamine per amine of PEG were introduced in a glass vial and stirred over 20 hours at room temperature. Concentrations in the final coupling medium were: [P(NAS-co-NVP)] = 1.5 mg.mL⁻¹, [Mal-PEG-NH₂] = 1.5, 6.75 and 13.5 mg.mL⁻¹, namely 1, 4.5 and 9 mg Mal-PEG-NH₂ per mg of P(NAS-co-NVP) copolymer. Coupling yield of NH₂-PEG-Mal on P(NAS-co-NVP) was determined by quantifying residual amines after coupling by fluorescamine assay. 30 μ L of fluorescamine solution (0.4 mg mL⁻¹ in DMSO) was placed in a 96-well microplate and 90 μ L of crude reaction mixture five-fold pre-diluted in PBS was added (triplicate samples). The reaction was allowed to proceed for 20 min in the dark, at room temperature, and the fluorescence emission intensity was measured at 477 nm for an excitation wavelength of 416 nm (BioTek SynergyMx). It was checked that P(NAS-co-NVP) copolymer under the same conditions, gave rise to negligible fluorescence. The calibration curve of fluorescence intensity (at 477 nm) versus PEG-NH₂ concentration obtained in the same conditions in absence of copolymer allowed determination of coupling yield. The crude coupling solution was used as such for further coupling of proteins/Ab.

For SEC and ¹H NMR and ATR-FTIR analysis, the crude coupling mixture was precipitated in diethylether, followed by drying under vacuum. SEC was performed on a chromatographic equipment composed of a 1260 Infinity Agilent Technologies pump connected to two TSK gel G2500 and G6000 columns (Tosoh Bioscience) in series. Detection system was a multi-angle light scattering (MALS) detector HELEOS II (Wyatt Technology) operating at 664 nm coupled on line to a Wyatt Optilab T-Rex differential refractometer. Sample solutions at 2–5 mg/mL were prepared and eluted in phosphate buffer (pH 7.2). Solutions were previously filtered through 0.45 μ m pore size membranes (Millipore) before injection. ¹H NMR experiments were recorded using a Bruker AVANCE III Spectrometer operating at 400.13 MHz. ATR-FTIR analysis was performed on Spectrum Two spectrometer (Perkin Elmer), equipped with a single reflection diamond ATR accessory.

2.3. Conjugation of proteins/antibodies

Fab' fragment antibody (anti-S100B), due to its known available thiol groups, was primary used as a model to assess coupling on the copolymer by thiol maleimide reaction. Anti-p24 biotinylated polyclonal antibody was further used for conjugation for purpose of ELISA detection. The number of free thiol groups Ab determined from 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB, Ellman reagent) assay as previously described [29], was ~1-2 per antibody molecule. Fab' and Ab were coupled on the PEG-grafted copolymer at an antibody/maleimide of 1/1 and 2/1 molar ratio, respectively, in PBS pH 7.4 at room temperature. In brief, 15 μ L of the crude PEG grafted copolymer solution (1.5 mg.mL⁻¹ PEG content) was dissolved in PBS (1470 μ L) and stirred at room temperature for 20h to hydrolyse the NS ester functions, for ensuring selective coupling through thiol-maleimide coupling. Then, 15 μ L of Fab' or antibody solution was added, and the coupling solution was stirred overnight. The obtained solution was further 10-fold diluted in PBS for ELISA test (1/1000 diluted Ab). The control solutions, namely free Ab or Ab conjugated to native P(NVP-co-NAS) copolymer without PEG, were prepared in strictly the

same conditions to ensure identical concentration of Ab used in the tests. For Ab conjugated to native P(NVP-co-NAS) (through amine-NS ester reaction), the hydrolysis step was skipped to maintain reactivity of NS activated esters.

2.4. SDS-PAGE analysis

Coupling of proteins/Ab on copolymer was assessed by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) as previously described [14]. In brief, Ab/protein-copolymer crude coupling solutions, were mixed with the carrier buffer (Laemmli Sample 5× Buffer: 300 mM Tris-Cl pH 6.8, 10% SDS, 40% glycerol, 10 mM dithiothreitol, 0.05% bromophenol blue) (sample/carrier buffer: 4/1 v/v). The migration was carried out at 100 V for 10 min and at 200 V for 40 min. The gels were further stained with Coomassie blue staining solution (ThermoFisher Scientific, USA). Free Ab/proteins and free copolymers were analysed in the same conditions, as controls.

2.5. Enzyme-linked immunosorbent assay (ELISA) Immunoassay

Ninety-six-well plates (Nunc MaxiSorp, Thermo Fisher Scientific, USA) were coated overnight at room temperature with 100 μ L of p24 immobilized on micelles or free p24 at concentration of 1 μ g.mL⁻¹ in PBS. After removing the solution, the plates were blocked for 1 h at 37 °C with 250 μ L of PBS containing 10% horse serum. Plates were washed 3 times with PBS-0.05% Tween 20 (PBS-T, 300 μ L) using a Thermo Scientific autoplate washer (also used for the subsequent washings). Then, 100 μ L/well of free or polymer-coupled Ab (biotinylated anti-p24) solutions as previously prepared (or further diluted) were added and the plate incubated for 1 h at 37 °C. Following washings with PBS-T, the plates were reacted with peroxidase conjugated streptavidin at 1:20,000 dilution (from a 1 mg.mL⁻¹ solution, Jackson ImmunoResearch, West Grove, USA) in PBS-T containing 10% horse serum for 30 min at 37 °C. Plates were washed with PBS-T, revealed using 100 μ L/well of tetramethylbenzidine (TMB) substrate (OptEIA™, BD Biosciences) and incubated for 30 min, protected from the light. The reaction was stopped with 100 μ L of 1N sulfuric acid and the optical density at 450 nm with a correction at 620 nm was measured using a microplate reader (Multiskan FC, Thermo Fisher Scientific, France).

3. Results and discussion

3.1. Synthesis of P(NAS-co-NVP)-g-PEG-Mal copolymer (PEG-Cop)

To improve accessibility of antibody molecules to both its target analyte (antigen) and labelling agent for detection, our approach relied on the use of a quite high molecular weight PEG spacer (10 kg.mol⁻¹) (Figures 1A and B). The latter is coupled to the P(NAS-co-NVP) copolymer chain through amidation reaction between amine at PEG chain end and NS ester moieties of the NAS units, while the maleimide PEG chain end will ensure further coupling of the antibodies via their thiol groups.

First, P(NAS-co-NVP) (Cop) was synthesized by NMP using the MAMA-SG1 alkoxyamine as previously described [28], targeting a molecular weight of 40000 g.mol⁻¹. As previously studied, NMP of these comonomers is partially controlled, with a strong alternating tendency for NAS and NVP units, thus providing a high density in NS ester groups for coupling [28,30]. The number-averaged molecular weight determined by SEC-MALS (phosphate buffer pH 7.2 as eluent, hydrolysed copolymer) was 45000 g.mol⁻¹, with a dispersity of 1.6 (Fig. 2, blue line chromatogram).

Mal-PEG-NH₂ was coupled to Cop in DMF in presence of triethylamine to obtain PEG grafted copolymer (PEG-Cop). Coupling efficiency was determined on the crude reaction mixture for increasing introduced PEG amounts (with constant Cop amount), by quantifying the residual amines using the fluorescamine assay, as described in experimental section and in Table 1. This assay is based on the

formation of a fluorescent product upon reaction of fluorescamine with primary amines, leading to detectable fluorescence intensity signal proportional to amine concentration in the medium. Fluorescence intensity values were systematically corrected by subtracting the background fluorescence intensity value of sample in absence of PEG-NH₂ (bottom line). To note, Cop presence induced a quite negligible fluorescence background signal. Coupling efficiencies were 98 %, 44 % and 32 % for introduced Mal-PEG-NH₂ concentrations of 1.5, 6.75 and 13.5 mg.mL⁻¹, respectively, for a constant Cop concentration of 1.5 mg.mL⁻¹, namely 1, 4.5 and 9 mg/mg Cop (which corresponds to 5, 22 and 45 PEG chains per Cop chain). This decreasing yield, when increasing PEG amounts, traduced the difficulty for PEG chains to couple on the copolymer once the first PEG macromolecules were immobilized, due to steric hindrance, and although NS esters on the copolymer were always in large excess as compared to PEG amines. From these values, it came that about 5, 10 and 15 PEG chains could be coupled on the copolymer increasing the introduced amounts of PEG (1, 4.5 and 9 mg/mg Cop, respectively). The coupling product of nearly complete yield (5 PEG grafts) was selected for further studies, because large amounts of remaining free Mal-PEG-NH₂ in the two other copolymer samples would be involved in reaction with Abs in the subsequent step, negatively impacting our approach.

Table 1. Coupling efficiency and density of Mal-PEG-NH₂ on P(NAS-co-NVP) copolymer (Cop), determined by the fluorescamine assay.

C(PEG-NH ₂) ₀ (mg.mL ⁻¹)	I(PEG-NH ₂) ₀ ^a (a.u.)	I(reaction) ^a (a.u.)	C(PEG-NH ₂) _{residual} ^b (mg.mL ⁻¹)	Coupling yield (CY) ^c	Number of PEG grafts per Cop
1.5	9550	251	0.02	98	5
6.75	43090	24130	3.80	44	10
13.5	85160	58090	9.18	32	15
<i>0</i>	<i>603</i>	<i>694</i>	-	-	-

^a Fluorescence intensity values obtained after subtraction of the background fluorescence intensity values (no PEG-NH₂, bottom of the table, in italic)

^b Residual concentration obtained from calibration line I(PEG-NH₂)₀ vs. C(PEG-NH₂)₀

^c CY = 100 x [1 - C(PEG-NH₂)_{residual} / C(PEG-NH₂)₀]

SEC-MALS analysis of the selected coupling product (corresponding to 1 mg Mal-PEG-NH₂ per mg copolymer) confirmed PEG coupling with high efficiency, consistent with the results obtained from fluorescamine assay (98 %). Indeed, a clear shift tower towards higher molecular weights was observed for the coupling product as compared to P(NAS-co-NVP) and Mal-PEG- NH₂ precursors, as a result of effective coupling, with no remaining precursor clearly detectable (Fig. 2). The molecular weight of the coupling product was determined to be about 107000 g.mol⁻¹ (Đ = 3.1), compared to 45000 g.mol⁻¹ for P(NAS-co-NVP) copolymer, consistent with about 5 PEG chains coupled on the copolymer, corroborating the fluorescamine experiments. It is to mention that SEC analysis was performed in phosphate buffer pH 7.2 (thus on hydrolysed copolymers) to be closer to the final conditions in which the copolymers will be used (immunoassays). In such conditions, the analysis of the Mal-PEG-NH₂ precursor was not optimal, with a large/bimodal chromatogram observed (continuous green line). The chromatogram of the final coupling product had, as a result, a similar shape compared to this PEG precursor. To note, analysis of PEG precursor in acetate buffer pH 4.8 as eluent which gave a higher quality chromatogram (dotted green line). Slight peak at higher elution time was however still observed, that could be linked to partial adsorption phenomena on the column.

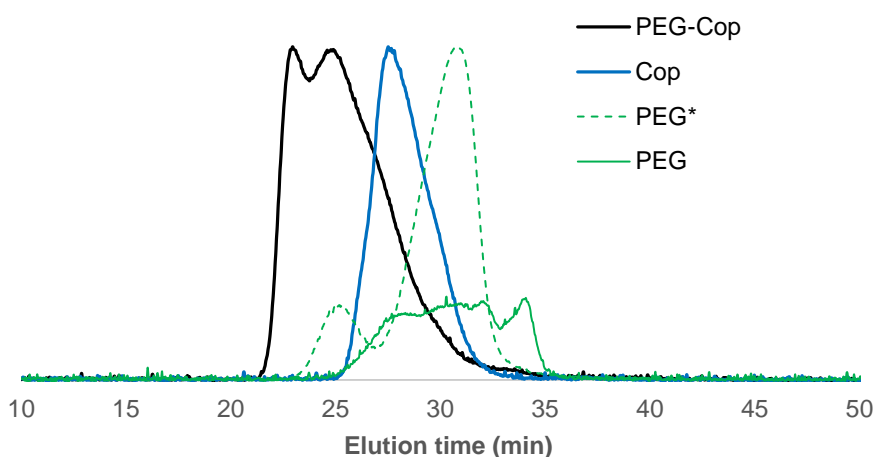


Fig. 2. SEC-MALS chromatograms of the PEG-Cop grafted copolymer, and both Cop and PEG precursors. Analysis was performed with phosphate buffer eluent pH 7.2 except for PEG* (dashed line), for which analysis was performed with acetate buffer pH 4.8.

ATR-FTIR analysis of the PEG-Cop coupling product (non-hydrolysed) showed characteristic bands of both P(NAS-co-NVP) copolymer backbone (C=O elongation bands of NVP and NAS units at 1655 and 1740 cm^{-1} , respectively) and PEG (C-O elongation band in the region of 1100 cm^{-1} , -CH₂- elongation band at 2880 cm^{-1}), as shown in Fig. 3. Interestingly, for the coupling product, elongation band appeared in region of 3400 cm^{-1} (amide N-H elongation) as well as increase of the band close to 1650 cm^{-1} (amide C=O elongation), evidencing the formation of amide bond upon coupling of PEG.

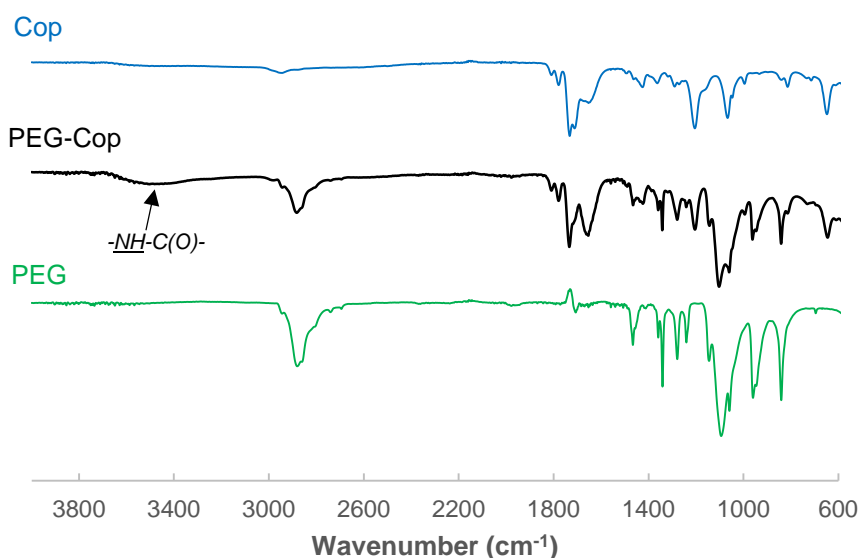


Fig. 3. ATR-FTIR analysis of Cop (top), PEG-Cop (middle) and PEG (bottom).

The ¹H NMR spectrum of the coupling product also showed typical peaks from both initial polymers (Fig. 4). Importantly, the maleimide groups at PEG chain end were conserved on the final copolymer,

as assessed by the presence of the maleimide -CH₂- peak at 7.0 ppm, thus available for further coupling with thiol containing biomolecules.

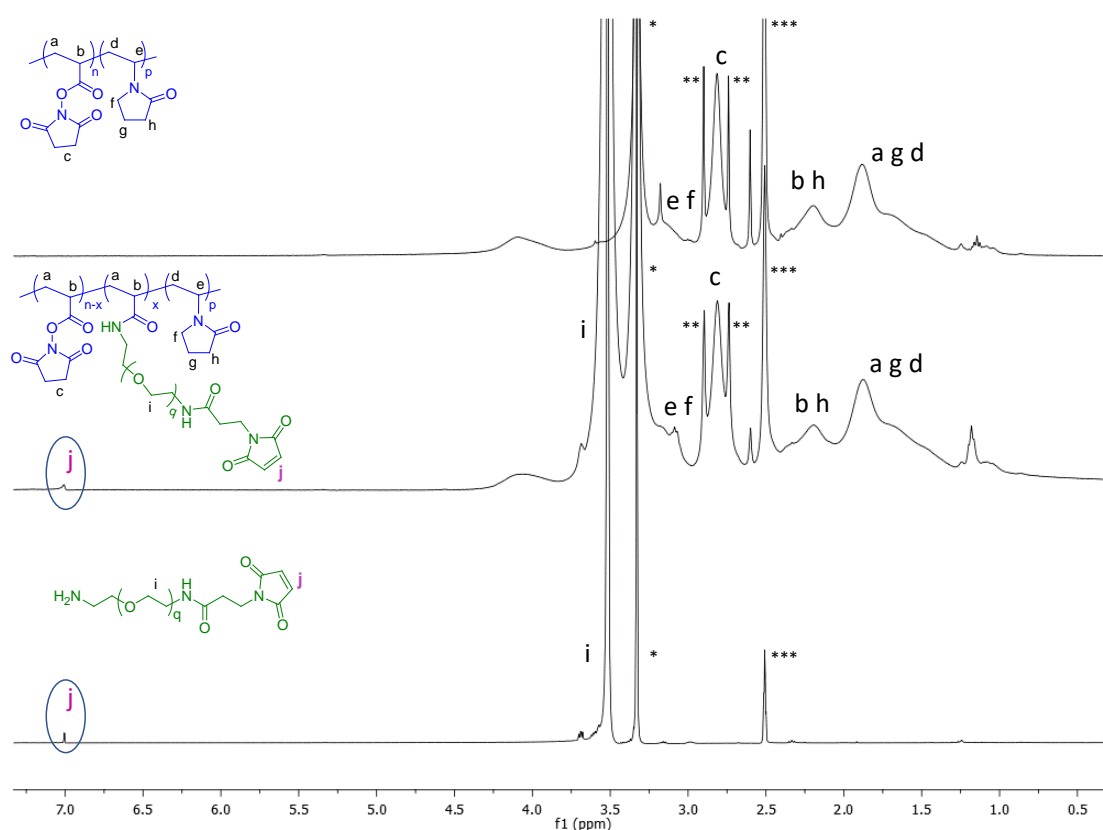


Fig. 4. ¹H-NMR spectra of Cop (top), PEG-Cop (middle) and PEG (bottom) in DMSO-d₆ (*: H₂O; **: DMF; ***: DMSO)

3.2. Coupling proteins/Abs on copolymers

The conjugation of proteins/Abs on the previously prepared Mal-PEG grafted copolymer (crude product solution) was performed through thiol-maleimide reaction (Fig. 1A, right). We had first to ensure that coupling is only mediated by such reaction, through a preliminary step of hydrolysis of the remaining NS activated esters of the P(NVP-co-NAS) backbone in PBS. Indeed, if not hydrolysed, these remaining NS esters could easily react with the amines of Abs, hampering the specificity of the thiol-maleimide reaction and interest of the PEG spacer. We showed that our hydrolysis conditions completely prevented such side reaction. This was clearly assessed by SDS-PAGE (Fig. 5) analysis using well-defined and relatively small molecular weight protein as model, p24 (24 kDa): when P(NVP-co-NAS) copolymer was not hydrolysed, coupling of p24 occurred efficiently with the copolymer, as indicated by disappearance of free p24 band and shifting towards high molecular weight conjugate, unable to migrate through the gel (lane 5). On the contrary, no coupling was observed on the hydrolysed copolymer, as shown by intact p24 free band (lane 4). Such absence of coupling was also confirmed using other model proteins with different structures and molecular weights, namely streptavidin (~66 kDa) and Fab' fragment (~50 kDa) for which intact free bands were observed (lane 7). Interestingly, this also clearly ensures that no undesired side covalent coupling of biomolecules (particularly p24 protein, further used as the capture molecule in ELISA test) could occur through their amines during immunoassay. This could be indeed detrimental for the specificity of the test.

We further used Fab' molecule, due to its well identified thiol groups, to evidence the coupling on the Mal-PEG grafted copolymer after ester hydrolysis (1 eq. per PEG-maleimide moiety of the grafted copolymer). Covalent coupling through thiol-maleimide reaction was clearly observed, as attested by the formation of high molecular weight bioconjugate far above the 200 kDa marker band, namely at the top of the separation gel, as well as in the concentration gel (lane 9). This was highly consistent with several Fab' molecules immobilized on the PEG grafted copolymer chain, initially featuring a molar mass of about 100 kDa, as previously mentioned. In addition, remaining free Fab' band observed on the gel had a lower intensity compared to that corresponding to coupled Fab' on the bioconjugate, which was consistent with good coupling efficiency. Overall, considering 1 eq. of Fab' introduced per PEG-maleimide and in average 5 PEG grafts per Cop chain, it could be reasonably assumed that at least 3 to 4 Fab' were attached to PEG-Cop copolymer.

With a view to show the versatility of the approach, we then coupled "whole" antibody, typically used in ELISA tests. Again, coupling on the copolymer was observed by SDS-PAGE, through the formation of high molecular weight species far above the 200 kDa marker band (and in concentration gel), with at least two Ab per chain (lane 12). To note, Ab was here used in 2 molar eq. per PEG maleimide moiety, because of a presumably lower reactivity than Fab' molecule considering higher molecular weight (IgG~150 kDa) and potentially less accessible thiol groups. It was thus not surprising to observe more pronounced band corresponding to unreacted free Ab.

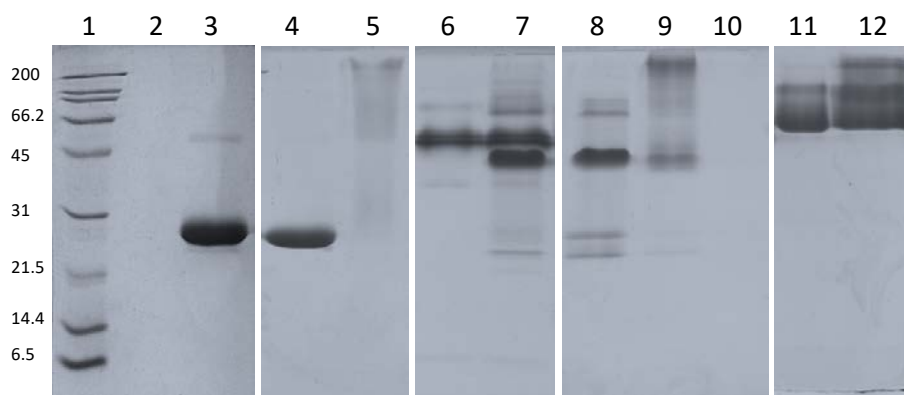


Fig. 5. SDS-PAGE analysis of protein coupling on copolymer, 1: Mass marker (values in kDa), 2: Cop, 3: p24, 4: p24 + Cop (hydrolysed), 5: p24 + Cop, 6: streptavidin, 7: streptavidin + Fab' + cop (hydrolysed), 8: Fab', 9: Fab' + PEG-cop, 10: PEG-Cop, 11: Ab, 12: Ab + PEG-Cop.

3.3. Evaluation in immunoassay

The final Ab-PEG-Cop conjugate previously obtained was evaluated as a detection system in ELISA format (Fig. 6d, left) in comparison with free Ab (Fig. 6d, right), as well as a control conjugate without PEG spacer (Ab-Cop) obtained from coupling of the Ab on the P(NAS-co-NVP) (non-hydrolysed) through classical amidation reaction between amines of Abs and NS esters of the NAS units (Fig. 6d, middle). To note, the coupling solutions were used as such (no purification of the conjugates, presence of free remaining Ab) to ensure strictly same conditions for Ab amounts used for all the tested samples (see experimental section).

We used as a model, coated on the microplate, the p24 antigen which is a well-known capsid protein from human immunodeficiency virus (HIV). As shown in Fig. 6a, the PEG grafted copolymer greatly improved the ELISA test sensitivity as compared to both Ab-Cop and free Ab controls, indicating the beneficial impact of the PEG-grafted copolymer on the detection by favouring accessibility of the

coupled Ab through PEG spacer and maleimide chemistry. Furthermore, the globally lower background values obtained in absence of p24 for the Ab-PEG-Cop showed the effect of anti-fouling PEG for reducing non-specific adsorption (Fig. 6b). To note, copolymer conjugate without PEG (Ab-Cop) had also, in a less extent, a beneficial impact on the background signal, which could be attributed to the NVP based backbone. Indeed, it has been well documented for its repulsive properties toward proteins [31,32]. Nevertheless, this conjugate was less efficient at improving absorbance signal, most probably due to the absence of PEG spacer.

Overall, these enhanced detection properties for Ab-PEG-Cop clearly appeared when plotting the signal-to-background ratio, corresponding to ratio of absorbance in presence of p24 (a) on absorbance in absence of p24 (b), as shown in Fig. 6c. These results are even more interesting considering that this bioconjugate for immunoassay is easy to prepare, relying on two functionalization steps without any need of purification (Fig. 1A), namely PEG amine coupling on the copolymer and simple further incubation of the PEG grafted copolymer with the Ab for maleimide-thiol reaction to occur.

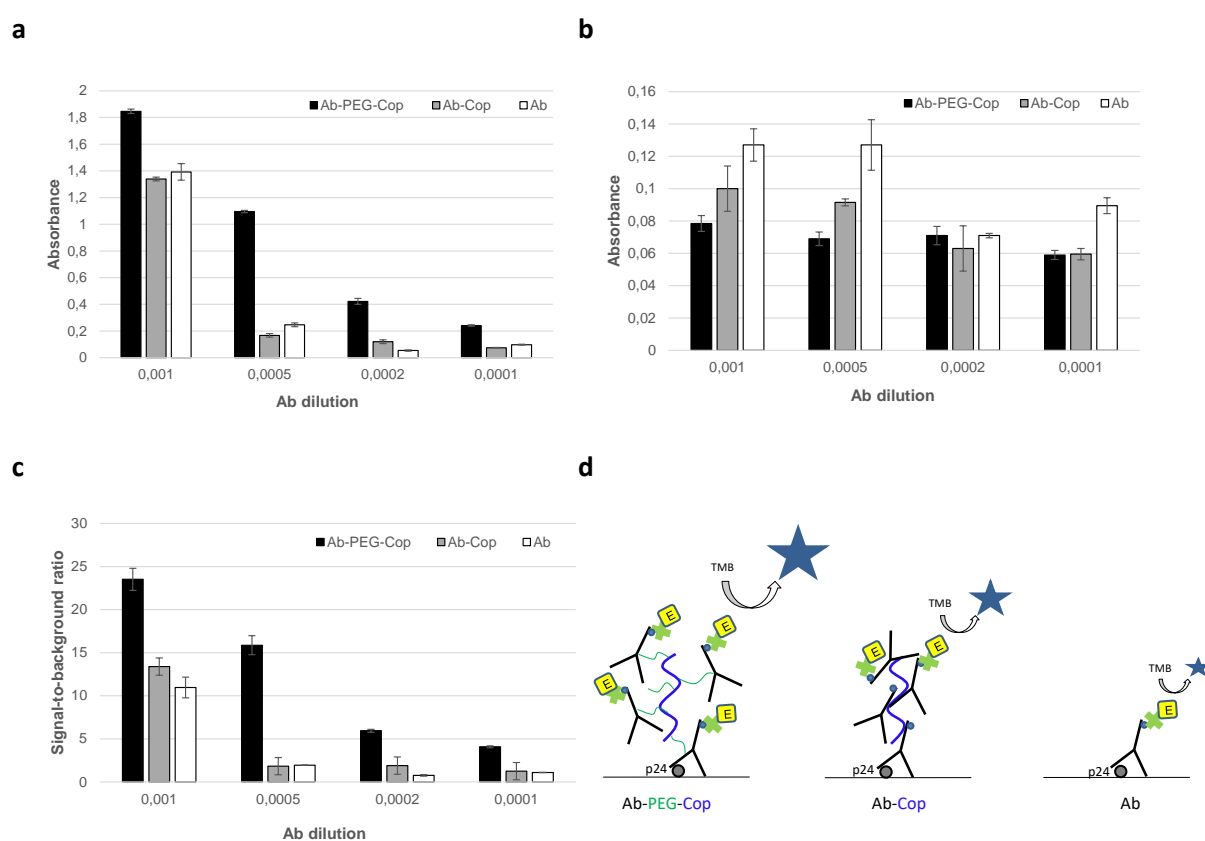


Fig. 6. Enzyme-linked immunosorbent assay (ELISA) with (a) and without (b) coated p24, using Ab-PEG-Cop, Ab-Cop and free Ab as detection systems. (c) representation plotting signal-to-background ratio (ratio of absorbance in presence of p24 on absorbance in absence of p24); (d) Schematized ELISA with the different detection systems: Ab-PEG-Cop, Ab-Cop and free Ab.

4. Conclusion

In this paper, we proposed a new simple and versatile detection tool for immunoassay, based on multi-conjugation of antibodies on the reactive polymer backbone through a PEG spacer and chemoselective maleimide thiol conjugation. Such bioconjugate was easily produced in a straightforward two-step

synthesis and, without any need of purification (*i.e.* remaining free Abs), allowed improved detection sensitivity. We think that this new concept of multi-antibody pegylated conjugate can be applied to a wide variety of antibodies, potentially requiring simple reduction of disulfide bonds to provide thiol groups, when needed, which is well mastered for antibodies, in order to improve immunoassay sensitivity.

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