Electronic Supplementary Information

Improving bioassay sensitivity through immobilization of bio-probes onto reactive micelles

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Materials

The PLA-*b*-P(NAS-*co*-NVP) block copolymer (19,000 and 22,000 g.mol^{-1} for PLA and P(NAS-co-NVP), respectively; NAS/NVP molar ratio: 53/47, $Đ$=1.6), referred as "cop", was prepared and characterized as previously described.[1] p24 protein was purchased from PX'Therapeutics (France, 2.4 mg.mL^{-1} in PBS, pH 7.4, Mw=24 kDa). NH_2-PEG-biotin (3.4 kDa) was purchased from Creative PEGWorks.

Micelle preparation

Five milliliters of a copolymer solution in acetonitrile (10 mg.mL^{-1}) were added to 10 mL of milli-Q water, under agitation (200 rpm), allowing the formation of micelles. Acetonitrile and a part of water were removed by evaporation under reduced pressure. The micelle concentration was determined by measuring the solid content, after heating the micellar solution to constant weight in an oven at 70°C for 24 h.

p24 protein and NH_2-PEG-biotin coupling on the micelles

p24 protein: the coupling of p24 protein at the micellar surface was performed by adding a volume (typically 300 µL) of micelle suspension (5.2 $mg.mL^{-1}$), to the same volume of p24 in PBS (pH 7.4) at a concentration of 0.6 $mg.mL^{-1}$ (concentrations in the coupling medium: 2.6 $mg.mL^{-1}$ for the micelles and 0.3 $mg.mL^{-1}$ for the protein p24). The samples were allowed to stir at room temperature for 16 h. The coupling sample was referred as "cop-p24". The same procedure was followed in absence of p24 in PBS to afford control micelle sample (referred as "free cop").

NH_2-PEG-biotin: the coupling of p24 protein at the micellar surface was performed by adding a volume (typically 300 µL) of micelle suspension (5.2 $mg.mL^{-1}$), to the same volume of NH_2-PEG-biotin in phosphate buffer (20 mM, pH 8) at a concentration of 6.5 $mg.mL^{-1}$ (concentrations in the coupling medium: 2.6 $mg.mL^{-1}$ for the micelles and 3.25 $mg.mL^{-1}$ for PEG-biotin). The samples were allowed to stir at room temperature for 16 h. The same procedure was followed in absence of NH_2-PEG-biotin to afford control micelle sample.

Protein coupling analysis by SDS-PAGE and UV

SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) was used to discriminate the p24 coupled to micelles from the free p24 using an electrophoresis equipment Bio-Rad. The concentration gel was 4% and the separation gel 15% final enacryl/bisacrylamide. The p24-micelles were mixed with the carrier buffer (LaemmliSample 5× Buffer: 300 mM Tris-Cl pH 6.8, 10% SDS, 40% glycerol, 10 mM dithiothreitol, 0.05% bromophenol-blue) (p24-micelle/carrier buffer: 4/1 v/v). The migration was carried out at 100 V for 10 min and at 200 V for 40 min. Both gels (separation and concentration) were used for revelation. The gels were further stained with Coomassie blue. The free p24 (at the same concentration as in the micellar solution) and the micelles without p24 (free cop) were used as a control.

NHS anion released upon coupling/hydrolysis was analyzed by UV spectroscopy at λ_{max}=260 nm (Infinite M1000, Tecan, UV transparent 96-well microplates star Greiner) for cop-p24 and free cop

samples, after 5-fold dilution in water (micelle concentration: 0.52 $mg.mL^{-1}$). Spectrum of the micelles in pure water (0.52 $mg.mL^{-1}$) was used as reference, and subtracted to sample spectra.

2,4,6-Trinitrobenzene sulfonic acid (TNBS) assay

10 µL of coupling sample (micelles + amine-PEG-biotin in phosphate buffer) , 10 µl of TNBS solution (0.05 % in water) and 80 µL phosphate buffer 20 mM pH 8 were placed in a 96-well plate (Greiner), which was incubated at 37°C for 2 hours in darkness. The absorbance was measured at 404 nm. The typical spectrum from 330 to 420 nm was also typically recorded to check the absence of any side products. The calibration was performed with amine-PEG-biotin in the coupling conditions (phosphate buffer pH 8). Micelles alone were also checked and did not induce interfering absorbance.

Enzyme-linked immunosorbent assay (ELISA)

p24 antigen: 96-well plates (Maxisorp, Nunc) were coated overnight (room temperature) with p24 immobilized on micelles or free p24, at varying concentrations (0 to 1 $\mu g.mL^{-1}$) or fixed concentration (1 or 10 $\mu g.mL^{-1}$) in PBS. The plates were then blocked for 1 h at 37 °C with 250 µl of PBS containing 10% horse serum and washed 3 times with PBS-0.05% Tween 20 (PBS-T). The washings were performed with a microplate washer WellWash Versa (Thermo Fisher Scientific) according to a program in three steps: (1) 3 washing cycles with 300 µL PBS-T (2) soaking during 20 s, (3) aspiration (normal mode; aspiration height: 2.6 mm and high speed). The same procedure was applied for the subsequent washings. 100 µl/well of biotinylated rabbit anti-p24 polyclonal antibody (bioMérieux) in PBS-T containing 10% horse serum (constant amount, or 3-fold serial dilutions) was added and the plate incubated for 1h at 37 °C. Following washes with PBS-T, the plates were reacted with peroxidase conjugated streptavidin at 1:20000 dilution (from a 1 $mg.mL^{-1}$ solution, Jackson Immunoresearch, West Grove, PA) in PBS-T containing 10% horse serum for 30 min at 37°C. After washing with PBS-T, 100 µL of TMB substrate (OptEIA™, BD Pharmingen, Le Pont de Claix, France) was added and the plate incubated for 30 min in the dark. The reaction was stopped with 100µl of 1M sulfuric acid and absorbance at 450 nm was measured.

Limit of detection (LOD) and sensitivity were defined as previously described.[2] LOD was obtained by adding three standard deviation (SD) to the mean absorbance of zero standard (i.e. blank, no Ab) (five replicates for SD measurement) and calculating the corresponding Ab dilution ratio from the absorbance signal vs. Ab dilution ratio curves. Sensitivity was defined as the slope of this curve (in the linear domain).

NH_2-PEG-biotin: 96-well plates (Maxisorp, Nunc) were coated overnight with PEG-biotin immobilized on micelles or free PEG-biotin, at varying concentrations (0.001 to 1 ng.mL^{-1}). The plates were then blocked for 1 h at 37 °C with 250 µl of PBS containing 10% horse serum and washed 3 times with PBS-0.05% Tween 20 (PBS-T). The plates were reacted with peroxidase conjugated streptavidin at 1:20000 dilution in PBS-T containing 10% horse serum, for 30 min at 37°C. After washing with PBS-T, 100 µL of TMB substrate and incubated for 30 min in the dark. The reaction was stopped with 100µl of 1M sulfuric acid and absorbance at 450 nm was measured.

Size and CMC measurements

Size of the micelles was determined by dynamic light scattering (DLS) at 25°C, using a Zeta Sizer NanoZS (Malvern instruments, UK). Highly diluted colloidal dispersions in 1 mM NaCl solution were used, and each value is at least the average of three measurements. Critical micellar concentration (CMC) was determined by the DLS method[3] using samples of copolymer concentrations ranging from 0.08 to 100 µg.mL^{-1} in PBS.

FIGURES

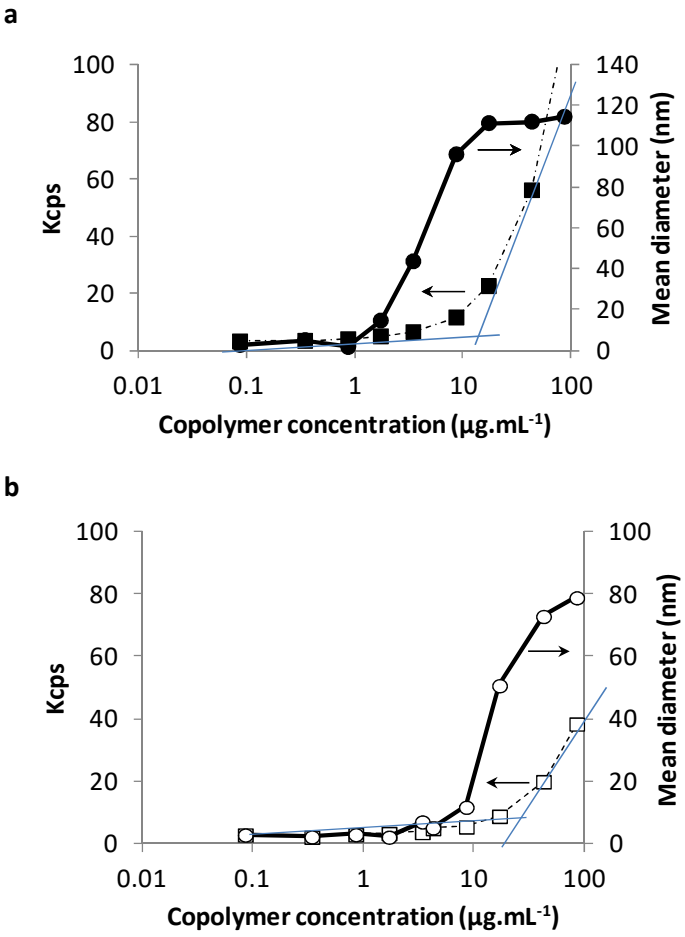
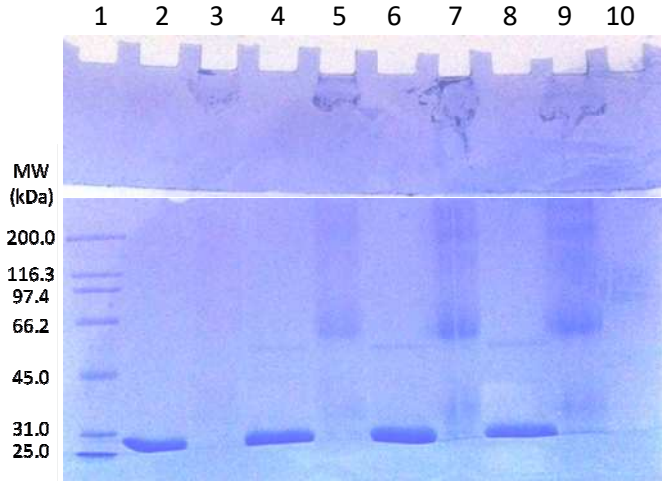


Figure S1. Critical micellar concentration (CMC) of cop-p24 (a) and free cop (b).



1. Mass marker
2. p24 at 0.3 mg.mL^{-1} in PBS
3. p24 at 0.3 mg.mL^{-1} / 2.6 mg.mL^{-1} cop (0.115 mg.mg^{-1}) in PBS
4. p24 at 0.6 mg.mL^{-1} in PBS
5. p24 at 0.6 mg.mL^{-1} / 2.6 mg.mL^{-1} cop (0.23 mg.mg^{-1}) in PBS
6. p24 at 0.9 mg.mL^{-1} in PBS
7. p24 at 0.9 mg.mL^{-1} / 2.6 mg.mL^{-1} cop (0.345 mg.mg^{-1}) in PBS
8. p24 at 1.2 mg.mL^{-1} in PBS
9. p24 at 1.2 mg.mL^{-1} / 2.6 mg.mL^{-1} cop (0.46 mg.mg^{-1}) in PBS
10. free copolymer (free cop)

Figure S2. SDS PAGE analysis of coupling of p24 protein (at various concentrations) on copolymer micelles (2.6 mg.mL^{-1}) in PBS for 16 h.

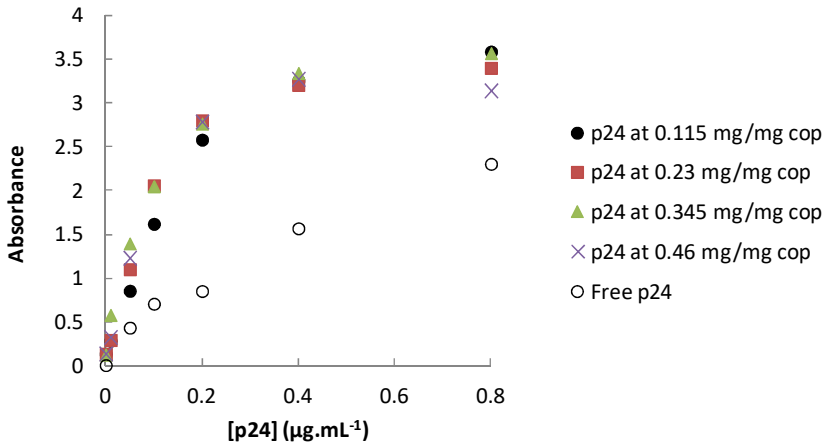


Figure S3. Enzyme-linked immunosorbent assay (ELISA) of free p24 or immobilized p24 on micelles; p24 (free or immobilized) was coated at various concentrations (0 to 0.8 µg.mL^{-1}) and a fixed antibody dilution (1:600) was used for the test.

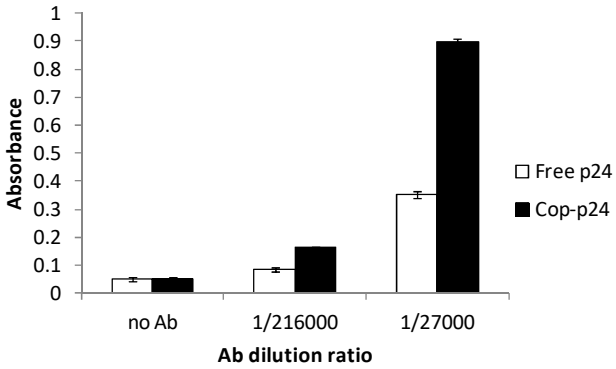


Figure S4. Enzyme-linked immunosorbent assay (ELISA) of free or copolymer micelle immobilized p24 (coating of free or immobilized p24 at 1 µg.mL^{-1}), after 1 month of storage in PBS at 4°C at p24 concentration of 1 µg.mL^{-1}.

References

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2. H. J. Han, R. M. Kannan, S. Wang, G. Mao, J. P. Kusanovic and R. Romero, *Adv. Funct. Mater.*, 2010, **20**, 409–421.

3. a) Y. Wang, A. M. Alb, J. He and S. M. Grayson, *Polym. Chem.*, 2014, ***5***, 622-629; b) W. Cheng, R. Rajendran, W. Ren, L. Gu, Y. Zhang, K. H. Chuang and Y. Liu, *J. Mater. Chem. B*, 2014, **2**, 5295-5301.