Electronic Supplementary Information

"Reactive nanoprecipitation": a one step route to functionalized polylactide-based nanoparticles

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Materials

Poly(D,L-lactide) (PLA50, M_n=30,000 g/mol, PDI = 1.7) with a carboxylic end group was purchased from Phusis (Grenoble, France). The PLA-*b*-P(NAS-*co*-NVP) block copolymer (19,000 and 13,000 g.mol^{-1} for PLA and P(NAS-co-NVP), respectively; NAS/NVP molar ratio: 53/47, PDI=1.5), prepared and characterized as previously described (*J. Polym. Sci. Part A: Polym. Chem*., **2011**, *49*, 1341) was used for peptide coupling experiments. A PLA-*b*-P(NAS-*co*-NVP) block copolymer analog of 19,000 and 22,000 g.mol^{-1} for PLA and P(NAS-co-NVP), respectively (NAS/NVP molar ratio: 53/47, PDI=1.6), was used for protein coupling experiments. KKKVQGEESNDK peptide sequence of the IL-1β (referred as ILP peptide) was synthesized by solid phase method (Fmoc amide resin) using Fmoc/^{t}Bu chemistry, and characterized by mass spectrometry ([M+H]$^+$=1388.8, M=1387.7) and HPLC, as previously described (*Colloids Surf. B*, **2013**, *103*, 298). Peptide powder used for

experiments was under TFA salt form (M = 1959). p24 protein was purchased from PX'Therapeutics (France, 2.4 $mg.mL^{-1}$ in PBS, pH 7.4, Mw=24 kDa).

One-step preparation of peptide functionalized nanoparticles (NPs)

PLA (40 mg) and PLA-NS copolymer (4 mg) were dissolved in 4.4 mL of acetonitrile (total polymer concentration: 10 $mg.mL^{-1}$). Typically, 1 mL of this organic solution was added in 1 mL of peptide solution at a given concentration (ranging from 0 to 0.48 $mg.mL^{-1}$) in phosphate buffer pH 8, 20 mM. The medium was stirred for 2 h. A small volume of dispersion (200 µL) was kept for peptide coupling analysis by HPLC (see below). On the rest of the dispersion (1.8 mL), the acetonitrile was removed for 20 h under a fume hood. The NPs were further washed from the non-coupled peptide and released NHS by centrifugation (8000 g for 15 min) and pellet redispersion in water. In additional experiments, NPs encapsulating Nile Red were prepared. The same procedure as described above was performed, except Nile Red was dissolved in the acetonitile (0.11 $mg.mL^{-1}$) along with the polymers. In this case, after removal of acetonitrile, the precipitated Nile Red in the medium (i.e. non encapsulated in the NPs) was removed by centrifugation at low speed rate (2000 g for 5 minutes). The NP dispersion was further washed from non coupled peptide and NHS by centrifugation/redispersion.

Peptide coupling analysis by HPLC

Peptide coupling yields on the NPs and immobilized amounts were obtained through determination of the amounts of non-coupled peptide in the supernatants by reverse phase

HPLC. For this purpose, after NP preparation, a small volume (200 µL) of the NP dispersion (in acetontirile:water 1:1) was centrifuged at 15000 g for 10 min and the supernatant collected. The HPLC analysis was performed on a Agilent 1100 series instrument (column: Jupiter, Phenomenex, C18, 5 µm, 250 x 4.6 mm) using a linear water/acetonitrile gradient (0.8 $mL.min^{-1}$, eluent A is 0.1% TFA in water, eluent B is 0.09% TFA in acetonitrile/water 70/30 by vol., 0-40% B in 15 min, then 40-0% B in 2 min, UV detection at 215 nm). The peptide was detected at 12.6 min. The NHS was also detected at 6.2 min. Peptide calibration curve (peak area (PA) vs concentration) was established in the same conditions than for coupling, in the absence of the NPs (phosphate buffer, 20 mM, pH 8/acetonitrile 50/50). The coupling yield was obtained by the relation $CY=100 \times (PA_0\text{-}PA) / PA_0$, where PA_0 is the peak area corresponding to the initially introduced peptide concentration and PA is the peak area relative to the peptide concentration in the supernatant of the dispersions after coupling. The immobilized peptide amount per gram of NP ($N_{mg/g}$) was obtained by the relation: $N_{mg/g}= CY \times N_{0,\ mg/g}$, where $N_{0,\ mg/g}$ is the initially introduced peptide amount per gram of NPs.

One-step preparation of protein (p24) functionalized NPs

PLA-NS copolymer was dissolved in DMSO at a concentration of 52.7 $mg.mL^{-1}$. Typically, 100 µL of this organic solution was added in 1900 µL of solution of the p24 protein at a concentration of 0.316 $mg.mL^{-1}$ in PBS pH 7.4 (p24 and PLA-NS concentrations of 0.3 and 2.635 $mg.mL^{-1}$, respectively). The medium was stirred for 2 h.

Protein coupling analysis by SDS-PAGE and fluorescamine assay

SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) was used to discriminate the p24 coupled to NPs 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-NPs 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-NP/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 NP dispersion) and the NPs without p24 were used as a control.

The amino group analysis was determined by the fluorescamine method, as previously described (*Bioconjugate Chem.*, **2004**, *15*, 458).

Enzyme-linked immunosorbent assay (ELISA)

For the detection of p24 antigen, 96-well plates (Maxisorp, Nunc) were coated overnight with free p24 or p24 immobilized on NPs at 10 µg/mL or 1 µg/mL in PBS. The plates were then blocked for 1 h at 37 °C with 200 µl of PBS containing 10% non-fat dry milk and washed 3 times with PBS-0.05% Tween 20 (PBS-T). After 3 washes with PBS-T, 100 µl/well of biotinylated rabbit anti-p24 polyclonal antibody (bioMérieux) was added at various dilutions in PBS-T-10% horse serum and incubated for 1h at 37 °C. Following washes, the plates were reacted with peroxidase conjugated streptavidin (Jackson Immunoresearch, West Grove, PA) for 30 min and developed with BD OptEIA™ substrate (BD Pharmingen, Le Pont de Claix, France) for 30min in the dark. The reaction was stopped with 100µl of 1M sulfuric acid and absorption at 450nm was measured.

Size and zeta potential measurements

Size of the NPs 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. Zeta potentials were measured with Zeta Sizer NanoZS. The measurements of the electrophoretic mobility were carried out at 25°C on dispersion samples highly diluted in phosphate buffer pH 6.1, 10 mM, and the data were converted to the zeta potentials using Smoluchowski equation. The values were the average of five measurements.

Tables and Figures

Table S1. Characteristics of the ILP peptide functionalized NP dispersions (introduced ILP peptide amounts: 48 mg/g NP) in absence or presence of Nile Red (0.11 $mg.mL^{-1}$ in the organic phase during the nanoprecipitation process).

NP	Immobilized peptide amount (mg/g NP)	Mean diameter (nm)	PI	Zeta potential
ILP-NP	26.4	320	0.13	-40.2
Nile Red ILP-NP	26.0	335	0.12	-41.5

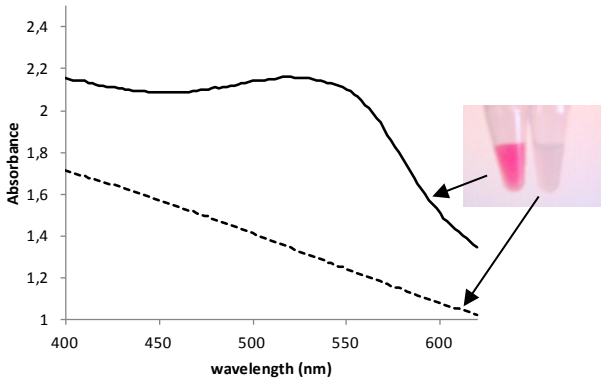

Figure S1. UV spectrum of the ILP peptide functionalized NP dispersions in presence of Nile Red (blank ILP peptide functionalized NPs as a control in dotted line), showing successful Nile Red encapsulation in the NPs.

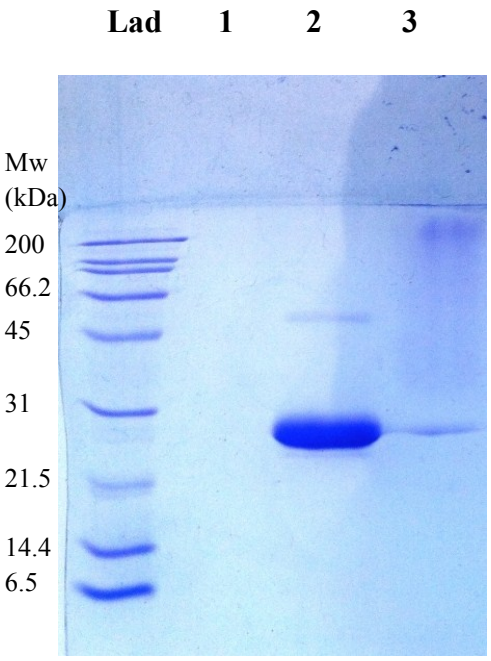

Figure S2. SDS PAGE analysis of p24 protein coupling on NPs; Lad: molecular weight marker, 1. Control NPs without p24 ([NP]=2.635 mg.mL^{-1}); 2. Free p24 ([p24]=0.3 mg.mL^{-1}); 3. NPs conjugated to p24 ([NP]=2.635 mg.mL^{-1}, [p24]= 0.3 mg.mL^{-1})