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Lysine-tagged peptide coupling onto polylactide nanoparticles coated with activated ester-based amphiphilic copolymer: A route to highly peptide-functionalized biodegradable carriers

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ABSTRACT

Efficient biomolecule conjugation to the surface of biodegradable colloidal carriers is crucial for their targeting efficiency in drug/vaccine delivery applications. We here propose a potent strategy to drastically improve peptide immobilization on biodegradable polylactide (PLA) nanoparticles (NPs). Our approach particularly relies on the use of an amphiphilic block copolymer PLA-*b*-poly(*N*-acryloxysuccinimide-*co*-*N*-vinylpyrrolidone) (PLA-*b*-P(NAS-*co*-NVP)) as NP surface modifier, whose the N-succinimidyl (NS) ester functions of the NAS units along the polymer chain ensure N-terminal amine peptide coupling. The well-known immunostimulatory peptide sequence derived from the human interleukin 1β (IL-1β), VQGEESNDK, was coupled on the NPs of 169 nm mean diameter in phosphate buffer (pH 8, 10 mM). A maximum amount of 2 mg immobilized per gram of NPs (i.e. 0.042 peptide nm^{-2}) was obtained. Introduction of a three lysine tag at the peptide N-terminus (KKKVQGEESNDK) resulted in a dramatic improvement of the immobilized peptide amounts (27.5 mg/g NP, i.e. 0.417 peptide nm^{-2}). As a comparison, the density of tagged peptide achievable on surfactant free PLA NPs of similar size (140 nm), through classical EDC or EDC/NHS activation of the surface PLA carboxylic end-groups, was found to be 6 mg/g NP (i.e. 0.075 peptide nm^{-2}), showing the decisive impact of the P(NAS-*co*-NVP)-based hairy corona for high peptide coupling. These results demonstrate that combined use of lysine tag and PLA-*b*-P(NAS-*co*-NVP) surfactant represents a valuable platform to tune and optimize surface bio-functionalization of PLA-based biodegradable carriers.

1. Introduction

Biodegradable polylactide (PLA) and poly(lactide-*co*-glycolide) (PLGA) based nanoparticles (NPs) are attractive candidate carriers for drug [1,2] and vaccine [3–5] delivery. In this field, efficient surface conjugation of selective biomolecules, particularly peptidic ones [6–8], is of crucial importance for the capacity of the carrier to efficiently target cells of interest. However, the ability of PLA NPs for surface modification is rather poor due to the lack of functional groups along the PLA backbone. By far, such conjugation has been typically achieved by two kinds of strategies: (i) the post-coupling of the peptides on the prepared NPs, which requires activation of the PLA carboxylic end-groups of the NP surface through classical EDC/NHS activation, and generally leads to low biomolecule densities [9–11]; (ii) chain-end modification of polyethylene glycol (PEG)-based compounds (PEG-*b*-PLA, PEG-*b*-PCL, Pluronics) used as surfactant [6,12–15]. Regarding this strategy, several teams have showed that (peptidic) ligand density has a significant impact on NP cell uptake, by varying the ratio of peptide modified PEG-based surfactant to non-functionalized one in the NP preparation process [6,14]. However maximal ligand density achievable on the NPs is still limited due to the non-functionalizable polyether nature of PEG backbone (i.e. 1–2 ligands max. per copolymer chain). Moreover, the chemistry for conjugation of the ligand on the PEG chain end can be laborious, often requiring several steps [12]. There is thus a need for versatile alternative strategies affording improved surface bio-functionalization of PLA NPs. In a recent work [16], we described the synthesis of a novel amphiphilic block copolymer PLA-*b*-poly(*N*-acryloxysuccinimide-*co*-*N*-vinylpyrrolidone) (PLA-*b*-P(NAS-*co*-NVP)), whose NAS and NVP units have a strong alternating tendency. Its use as a surface modifier in the PLA nanoprecipitation process (Fig. 1) leads to NPs

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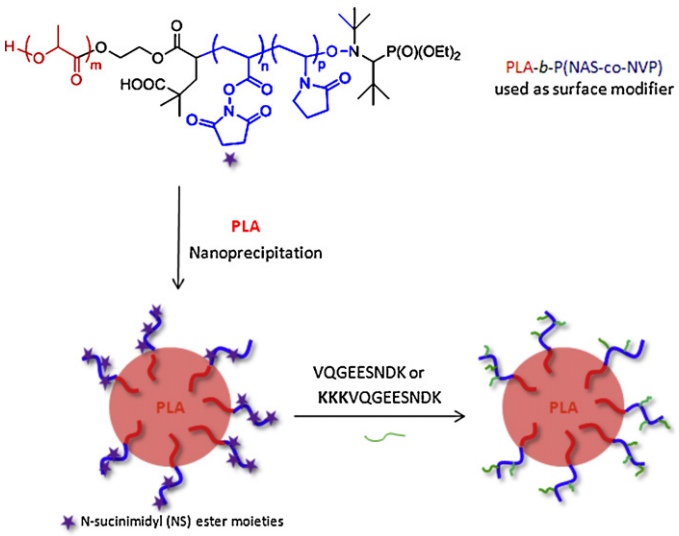

Fig. 1. Strategy of preparation of the PLA-*b*-P(NAS-*co*-NVP) (NS-cop) coated nanoparticles and further peptide functionalized NPs.

presenting at their surface a high density of N-succinimidyl (NS) ester moieties potentially available for coupling of biomolecules (2.4 functions nm^{-2}, omitting the hydrolyzed fraction) while NVP units offer a convenient alternative to PEG [17]. In a vaccine delivery perspective, we further interested to introduce at NP surface the immunostimulatory sequence VQGEESNDK of the IL-1β [18] (so-called iIL-1β). Coupling of such sequence on the negatively charged NPs [16] was expected to be difficult through its α-amine (considering the presence of three carboxylate lateral functions from the two E and D residues, and a C-terminal lysine). We thus studied, as a comparison, a peptide analog in which a three lysine tag was introduced at the peptide N-terminus (i.e. KKKVQGEESNDK). To our knowledge, the lysine tag strategy [19] for favoring peptide coupling has never been applied to polymeric NPs, particularly PLA/PLGA-based carrier systems. We show hereafter that the combined use of lysine tag and P(NAS-*co*-NVP) block represents a promising platform to drastically enhance peptide immobilization on PLA NPs.

2. Materials and methods

2.1. Materials

N-Hydroxysuccinimide (NHS, 98%) and *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC, >97%) were purchased from Aldrich. Poly(D,L-lactide) (PLA50, M_n = 30,000 g mol^{-1}, PDI = 1.7) with a carboxylic end group was purchased from Phusis (Grenoble, France). PLA-*b*-P(NAS-*co*-NVP) copolymer (referred as "NS-cop", 19,000-*b*-13,000 g mol^{-1}, PDI = 1.5, NAS and NVP molar ratios of 53% and 47%) and PLA nanoparticles coated with NS-cop (NS-cop NPs) or surfactant-free (NP0) were prepared and characterized as previously described [16]. VQGEESNDK peptide sequence of the IL-1β (referred as iIL-1β and "non-tagged" peptide) and KKKVQGEESNDK lysine tagged peptide analog (referred as KKK-iIL-1β and "tagged" peptide) were synthesized by solid phase method (Fmoc amide resin) using Fmoc/^{t}Bu chemistry, and characterized by mass spectrometry ($[M+H]^+$ = 1004.6 for non-tagged peptide (M = 1003.5); $[M+H]^+$ = 1388.8 for tagged peptide (M = 1387.7)) and HPLC. Peptide powder used for experiments was under TFA salt form ($M_{non\text{-}tagged\ peptide}$ = 1232; $M_{tagged\ peptide}$ = 1959).

2.2. Peptide coupling on the particles

Typically, 200 μL of peptide solution at a given concentration (ranging from 0.12 to 0.42 mg mL^{-1}) in phosphate buffer pH 8, 20 mM were briefly added to 200 μL of NS-cop NPs dispersion at 10 mg mL^{-1} in milli-Q water, and the coupling medium stirred for 2 or 24 h (final coupling medium: NPs: 5 mg mL^{-1}, peptide: 0.06–0.21 mg mL^{-1}, phosphate buffer, 10 mM, pH 8). Then, the dispersion was centrifuged at 15,000 × *g* for 10 min and the supernatant analyzed by HPLC for quantifying non-coupled peptide. Peptide coupling on naked PLA NPs (NP0) was performed following the same procedure, except that the carboxylic groups at the surface of the PLA NPs dispersion (200 μL, 10 mg mL^{-1}) were activated by addition of 7 μL of EDC solution (8.77 mg mL^{-1} in water, freshly prepared) and (eventually) 4.7 μL of NHS solution (7.9 mg mL^{-1} in water) prior to addition of the 200 μL peptide solution. Coupling was studied at pH 8 and 6.1 for EDC/NHS activation, and pH 6.1 for single EDC activation.

The number of moles of the surface COOH groups per gram of NP0 activable by EDC was determined by HPLC on supernatants by the relation $N = N_{EDC,0} \times (PA_{EDC,0} - PA_{EDC})/PA_{EDC,0}$, where $N_{EDC,0}$ is the initially introduced number of moles of EDC per gram of NP0, $PA_{EDC,0}$ is the EDC peak area (at 8.1 min) corresponding the initially introduced concentration, and PA_{EDC} is the EDC peak area corresponding to the remaining concentration in the supernatant after 2 h. The number of COOH functions per nm^2 was calculated using the specific surface of the NP0 nanoparticles.

2.3. 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 (recovered as mentioned above) by reverse phase (RP) HPLC. The HPLC analysis was performed on a Agilent 1100 series instrument (column: Jupiter, Phenomenex, C18, 5 μm, 250 × 4.6 mm; injected volume: 20 μL) 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, injected volume: 20 μL). The non-tagged peptide was detected at 12.1 min retention time and the tagged one at 12.6 min. The NHS was also detected at 6.2 min. Peptide and NHS calibration curves (peak area (PA) vs concentration) were established in the same conditions than for coupling, in the absence of the NPs (phosphate buffer, 10 mM, pH 8). The coupling yield (CY) was obtained by the relation $CY = 100 \times (PA_0 - PA)/PA_0$, 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: $N_{mg/g} = CY \times N_{0,mg/g}/100$ where $N_{0,mg/g}$ is the initially introduced peptide amount per gram of NPs. The number of immobilized peptide per nm^2 was calculated using the specific surface of the nanoparticles.

2.4. Peptide-polymer conjugate analysis by RP-HPLC and GPC after NP hydrolysis

NVP-based block with grafted peptide (or without peptide, as a reference) was analyzed by HPLC and GPC after destroying NPs through PLA hydrolysis. The procedure was as follows: the copolymer coated NPs functionalized (or not) with the tagged peptide (27.5 mg/g NP, 10 mL, 5 mg mL^{-1} NP) was washed from NHS and non-coupled peptide by centrifugation (15,000 × *g*, 10 min) and redispersion in water. After one more centrifugation (15,000 × *g*, 10 min) and supernatant removal, the pellet was washed one last