Surface modification of resorcinarene based self-assembled solid lipid nanoparticles for drug targeting†

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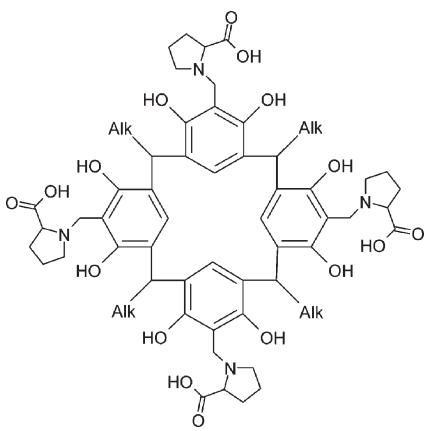
Prolyl-bearing amphiphilic resorcinarenes, *e.g.* tetrakis(*N*-methylprolyl)tetraundecylcalix[4]resorcinarene, self-assemble as stable solid lipid nanoparticles; these fully characterized systems could be further functionalized at their surface with proteins, and interact with specific antibodies bound on a sensor surface.

Nano-scaled non-viral drug delivery systems[1] are attracting increasing attention in biomedical sciences and offer new possibilities for the design of efficient tools for cancer therapies. The toxicity of some drugs such as antineoplastic agents[2] used in these therapies requires the use of carrying and targeting systems so as to bring the active compound to the tumor cells and avoid normal tissue damage. Among the different targeting systems studied, immuno-liposomes using antibodies as targeting ligands and lipid vesicles as carriers represent one of the most promising approaches.[3] Nevertheless, liposomes present some drawbacks like limited stability in biological media as well as sensitivity to external parameters such as temperature or osmotic pressure. In order to circumvent these salient weak points, other drug-carrying systems have been developed. Among them, solid lipid nanoparticles (SLNs) are receiving increasing attention because of their physico-chemical properties.[4] They are generally prepared using natural solid lipids, emulsifiers, water and depending on the preparation method a co-solvent. In addition to these natural solid lipids, macrocyclic synthetic molecules have been shown to be good candidates to replace these amphiphiles. The cyclodextrins have been demonstrated to self-assemble as SLNs when suitably modified;[5] but their intrinsic toxicity limits their use for biomedical applications. SLNs based on calixarenes or resorcinarenes have been studied and it has been demonstrated that in addition to their remarkable physico-chemical properties,[6] they present no intrinsic toxicity.[7] In addition, they have shown remarkable properties as controlling agents of a widely used UV absorber (*trans*-2-ethylhexyl-4-methoxycinnamate) which offers a new range of applications of these systems as carriers for sunscreens.[6d] In spite of these advances in the study of calixarene based SLNs, to the best of our knowledge no example of targeting systems has been published to date.

Herein we report on the preparation of SLNs based on a prolyl-bearing resorcinarene (L-RA-Pro); their characterization using photon correlation spectroscopy (PCS), atomic force microscopy (AFM) and scanning electron microscopy (SEM), their chemical modification with bovine serum albumin and their interactions with surface bound anti-albumin antibodies using surface plasmon resonance (SPR).

The molecular formula of L-RA-Pro is given in Scheme 1. The SLNs were prepared by the solvent displacement method,[6b] and photon correlation spectroscopy measurements‡ revealed that the suspensions produced are composed of particles possessing an average hydrodynamic diameter of 195(±5) nm and a polydispersity index of 0.1. The hydrodynamic diameter of the SLNs prepared in the same conditions with the parent molecule, the tetraundecylcalix[4]resorcinarene, is 200(±5) nm with a polydispersity index of 0.1. It could therefore be assumed that even if there is a slight difference between these two different populations of particles, the effect of the prolyl moiety is fairly weak.

The amphiphilic properties of L-RA-Pro have been previously established by the Langmuir balance method; it was demonstrated that these molecules form monomolecular layers at the air–water interface, with prolyl moieties immersed in the water subphase, and able to interact with copper(II) and to form a ternary supramolecular enantioselective complex with phenylalanine.[8] Therefore, assuming that the complex process of self-organization of amphiphiles to form SLNs is driven by amphiphilic self-assembly, it could be expected that the prolyl moieties of L-RA-Pro are partially accessible at the surface of the SLNs and could then be submitted to further chemical treatments. To confirm this hypothesis, L-RA-Pro-based SLNs were submitted to chemical activation using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) and *N*-hydroxysuccinimide (NHS); and subsequently reacted with bovine serum albumin (BSA) to produce proteo-SLNs, as shown in Scheme 2.§



Scheme 1 Molecular formula of L-RA-Pro (Alk = CH_3–$(CH_2)_{10}$–).

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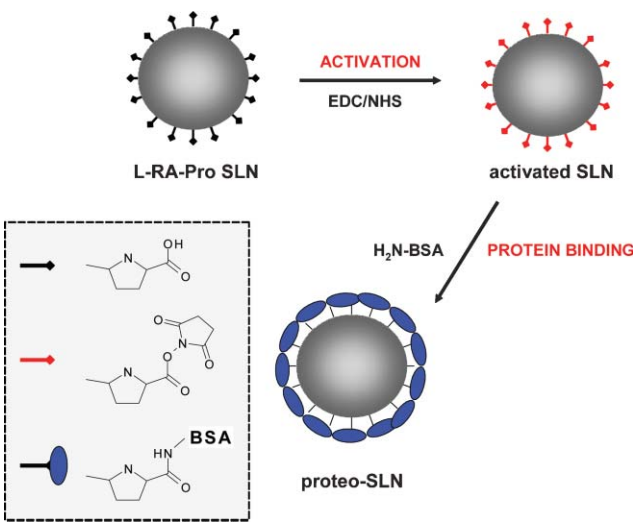
† Electronic supplementary information (ESI) available: Experimental details, AFM and SEM images of L-RA-Pro-based SLNs. See DOI: 10.1039/b703106h

Briefly, suspensions of L-RA-Pro-based SLNs in water were reacted with a mixture of EDC–NHS for 1 h, so as to activate the carboxylate functions at the surface of the SLNs *via* the formation of succinimidyl esters. After removing excess reactants by dialysis, the activated SLNs were incubated with a buffered solution of BSA (0.1 mg mL^{-1}) for 1 h. Ethanolamine was added to stop the reaction by deactivating succinimidyl esters. Reference samples were prepared incubating the SLNs with BSA but skipping the EDC–NHS activation step. The resulting suspensions were centrifuged to spin-down the particles and to remove unreacted BSA. During these consecutive steps, no aggregation of the SLNs was observed; the resuspension of the particles after centrifugation was performed submitting the pellet to a quick vortex treatment, showing the lack of aggregation tendency of these systems even after their chemical modification. PCS experiments confirmed this observation; both the average hydrodynamic diameter and the polydispersity index show no significant changes after chemical treatments, with values of respectively 200($\pm$5) nm and 0.1. These results also demonstrate that the mild conditions used in the reaction of surface modification do not destabilize the particles.

The evidence of the successful grafting of BSA at the surface of L-RA-Pro SLNs arises from their capability of interacting with surface bound polyclonal specific antibodies. A carboxymethyl-dextran surface plasmon resonance chip was modified using EDC–NHS activation.¶ Interactions with proteo-SLN were investigated by means of SPR using varying concentrations of SLNs. The sensograms obtained for concentrations of 12.5 and 25 mg L^{-1} (of L-RA-Pro) are presented in Fig. 1.

It could be seen that for both concentrations, at the beginning of the injection, there is a fast take-off of the signal with a nearly linear increase without a clear flattening before the end of the injection (*i.e.* no saturation of the chip); the intensity of the response depends on the concentration of proteo-SLNs injected. These results demonstrate that there is an interaction between the BSA modified L-RA-Pro SLNs and surface-bound anti-BSA antibodies. This interaction could be destabilized using NaOH solution (10 mM) which is commonly used to weaken antibody–antigen interactions in SPR experiments. It has been previously demonstrated that calixarene based SLNs could interact with



Scheme 2 Synthetic route to proteo-SLNs.

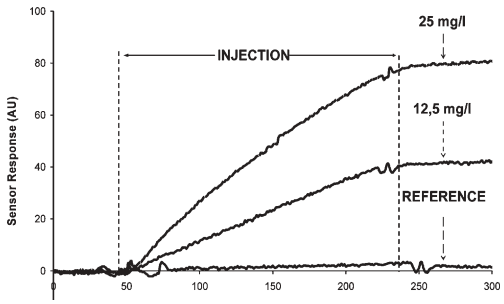


Fig. 1 SPR sensograms measured for SLNs concentrations of 25 and 12.5 mg L^{-1} (of L-RA-Pro). The reference is a suspension of SLNs which had been submitted to all the chemical treatments except the EDC–NHS activation step [AU: arbitrary unit].

BSA, which form a thin layer at the surface of the SLNs.[9] The absence of SPR response with the reference SLNs, exposed to albumin but without chemical activation, shows that albumin is not physically adsorbed on the surface of these SLNs. This also confirms the covalent nature of the bond between chemically activated SLNs and BSA.

Non-contact mode atomic force microscopy (nc-AFM)‖ was carried out on L-RA-Pro-based SLNs and proteo-SLNs, but no good quality images were obtained. This could be explained by a certain aggregation propensity of the SLNs during the drying process. To overcome this, we decided to immobilize the proteo-SLNs on a gold surface on the basis of their recognition properties *vs.* antibodies. Therefore, using DTSP (3,3′-dithiodipropionic acid di(*N*-hydroxysuccinimide) ester), we immobilized anti-BSA antibodies on a gold surface and incubated a proteo-SLN suspension with this surface. The samples were imaged after a thorough rinsing of the sample and drying (experimental details are given in the ESI†); the image obtained is presented in Fig. 2 (a reference sample was prepared under the same conditions, without EDC–NHS activation; no SLNs were detected on the surface with AFM imaging).

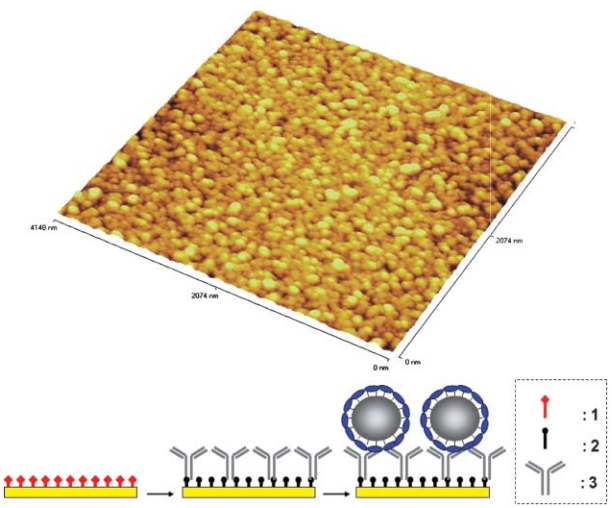


Fig. 2 Non-contact mode AFM of proteo-SLNs immobilized on a gold surface *via* antibody–antigen interactions and schematic representation of the strategy used to attach the SLNs on the surface (**1**: activated ester; **2**: deactivated amide functions, **3**: antibody anti-BSA).

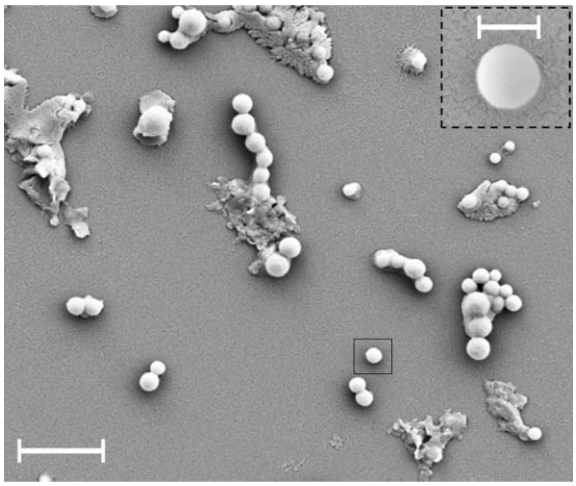


Fig. 3 Scanning electron microscopy image of proteo-SLNs spread on a glass substrate (scale bars 1 μm and 200 nm (inset)).

From the AFM picture, it could be clearly seen that the nanoparticles form a uniform monolayer on the surface, with the absence of aggregates or multilayers. This phenomenon could be attributed to the specific interaction of the proteo-SLNs and the antibodies immobilized on the surface. The stability of these SLNs which are resistant to drying on the glass surface had already been observed for *p*-acylcalixarene SLNs and was attributed to the solid structure of these particles.[6a] The evaluated volume for these particles observed by AFM is consistent with those calculated from PCS experiments. The calculated size show that these particles are slightly flattened on the surface with a height of 170 ($\pm$20) nm and a diameter of 210 ($\pm$22) nm, these results are consistent with those previously published for *p*-octanoylcalix[4]-arene based SLNs.

SEM experiments‖ were carried out on SLNs spread on a glass surface, the result obtained for proteo-SLNs is presented in Fig. 3.

It could be seen that the self-assembled systems are present on the surface as round particles; the average diameter measured is 220 ($\pm$34) nm and is in good agreement with those obtained from AFM and PCS experiments. They also confirm the stable nature of these particles which resist vacuum treatments during the surface coating and the SEM imaging (10^{-4} Pa). The relative polydispersity observed could be attributed to the sample preparation treatment.

This new approach for modifying the surface of resorcinarene-based SLNs may open new routes for the use of these systems in drug targeting. The work is under way to assess the possibility of targeting these proteo-SLNs in biological systems.

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Notes and references

‡ PCS experiments were carried out using a goniometer (ALV) with a frequency doubled Nd:YAG (yttrium aluminium garnet) laser (532 nm). Scattering was performed at 90° and the photon intensity autocorrelation function $g^2(t)$ was determined with an ALV-5000E correlator. SLNs were prepared mixing a solution of L-RA-Pro (5 mg mL^{-1}) in tetrahydrofuran with water (*cf.* ESI†).

§ L-RA-Pro was synthesized as described elsewhere.[8] Proteo-SLNs were prepared by reacting L-RA-Pro SLNs with EDC-NHS (5 : 2.5 mM) in water during 1 hour at room temperature. The suspensions were then transferred in cellulose dialysis tubes (Snake Skin® MWCO 10K-Pierce Biotechnology) and dialyzed against PBS buffer (10 mM, pH 7, 100 mM NaCl) for 30 min. A reference suspension was prepared in parallel skipping the EDC–NHS activation step. The suspensions were then reacted with a BSA solution (0.1 mg mL^{-1}) in the same PBS buffer, for 1 h. The reaction was then stopped by adding ethanolamine (10 mM). The suspensions were centrifuged at 13500 rpm for 10 min, the supernatant eliminated and the pellet re-suspended in HBS-EP® (BIAcore) buffer (0.01 M HEPES pH 7.4, 0.15 M NaCl, filtered and degassed). This step was repeated twice to eliminate all the unreacted BSA; suspensions were stored at 4 °C.

¶ Binding assays were performed using a BIAcore X® apparatus. CMD5 sensor chips were equilibrated using a BIAcore HBS-EP® buffer using the continuous flow procedure overnight (5 μl min^{-1}). The chip surface modification was carried out on a single channel activating the surface using EDC–NHS (5 : 2 mM) in H_2O, reacting rabbit anti-BSA affinity purified antibodies (Bethyl Lab) in acetate buffer (0.1 mg mL^{-1} in acetate buffer 10 mM pH 4) and inactivating unreacted NHS ester with ethanolamine–HCl (1 M in H_2O, pH 7); the second channel was kept unmodified and used as a reference. The chemical modification of the SPR chip was checked by injecting BSA solutions. Proteo-SLN binding experiments were carried out by injecting 15 μL of proteo-SLN suspensions. Each experiment was repeated at least twice to ensure the reproducibility of the results. The sensor chip was regenerated after each proteo-SLN injection using 5 μL NaOH 10 mM.

‖ AFM experiments were carried out in non-contact amplitude detection mode with a Thermomicroscope Explorer (Santa Clara, USA) system using low resonance frequency silicon nitride probes at a scanning speed of 1 Hz. The image was processed with the SPMLab 5.01 software and is presented unfiltered. SEM experiments were carried at accelerating voltages varying from 0.20 to 10 kV using a Supra 40 V system (Carl Zeiss, Switzerland), on samples prepared by spreading suspensions of SLNs on freshly cleaned glass substrates (cover-slip) and sputter-coated with a Au–Pd alloy.

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