Experimental report

19/09/2022

Proposal: 9-13-1028 **Council:** 4/2021

Title: Fate of Enkephalin¿based nanomedicines and stability in protein solutions: different surface charges to investigate the protein adsorption.

Research area: Soft condensed matter

This proposal is a continuation of 9-13-781

Main proposer: **Fabienne TESTARD**

Experimental team: Fabienne TESTARD
Frederic GOBEAUX

Local contacts: Anne MARTEL

Samples: D2O
bovine albumine
Leu-enkepanlin-squalene
Porc hemoglobine
foetal bovine serum

Instrument	Requested days	Allocated days	From	To
D22	2	2	21/09/2021	23/09/2021

Abstract:

To face the global health challenges of new pain killer, an efficient pharmacologically efficient nanomedecine based on nanoparticles (NPs) suspension was developed. The NPs are formed by nanoprecipitation of a bioconjugate (LENK-SQ) obtained by a versatile linkage between squalene (SQ) and the otherwise metabolized Leu-enkephalin (LENK) neuropeptide. Three type of linkage (amide, dioxycarbonyl or diglycolic spacer) have been explored to ensure the formation of NPs with similar composition but different surface charges. For these three bioconjugates, no evident relation have been found between their in vivo analgesic profile after intravenous injection and their chemical stability and release profil of the LENK in mouse serum. To help the understanding of these NPs bio-activity, the aim of this proposal is to investigate the protein corona formation around these organic nanoparticles with different surface charges using model system for the proteins (BSA and hemoglobin). Beyond this particular system, we hope with this study to give a better description of corona protein around deformable organic Nps.

Experimental Report / Experiment 9-13-1028 / D22

Fate of Enkephalin-based nanomedicines and stability in protein solutions:

Investigation of the protein adsorption as a function of the surface of the NPS.

Frédéric GOBEAUX, Fabienne TESTARD, Serge PIN,
Jean-Philippe RENAULT, Sinda LEPETRE, Anne MARTEL

21st-23rd septembre 2021

https://doi.ill.fr/10.5291/ILL-DATA.9-13-1028

To face the global health challenges of new painkillers, a pharmacologically efficient squalene-based nanomedicine carrying endogenous neuropeptide was developed.[1] In this study, it was shown that the rapidly metabolized Leu-enkephalin (LENK) neuropeptide may become pharmacologically efficient owing to a simple conjugation with the lipid squalene (SQ) using 3 different chemical linkers. The resulting LENK-SQ bioconjugates were able to self-assemble into nanoparticles by nanoprecipitation method. The different chemical linkers were designed in order to modulate the release of LENK from the nanoparticles. This new SQ-based nanoformulation prevented rapid plasma degradation of LENK and conferred on the released neuropeptide a notable antihyperalgesic effect that lasted longer than after treatment with morphine in a rat model of inflammation (Hargreaves test). The use of brain-permeant and -impermeant opioid receptor antagonists indicated that LENK-SQ NPs act through peripherally located opioid receptors. These results were confirmed by biodistribution studies in inflammation-bearing rats, using in vivo fluorescence imaging, thus highlighting the accumulation of LENK-SQ NPs toward the peripheral inflamed tissue. This study represents a novel nanomedicine approach, allowing the specific delivery of LENK neuropeptide into inflamed tissues for pain control. We intend now to study how the supramolecular organization of these LENK-SQ nanomedicines as well as the composition of a possible surrounding protein corona after their intravenous administration, may affect their pharmacokinetics. Thus, the aim of these experiments was to investigate the protein corona formation around these LENK-SQ nanoparticles using the two main blood proteins (BSA and hemoglobin) as model systems in order to propose a mechanism accounting for their pharmacodynamics and bioactivity. Because of chemical synthesis issues, we could not test the role of the 3 linkers as planned in the proposal. Finally, only LENK-SQ nanoparticles with amide bond (SLA NPs) and the Met-enkephaline-squalene also with amide bond (SMA NPs) were analysed. As we will show, the permutation of a single amino acid in the peptide sequence with another amino acid has considerable outcome on the structure and behaviour of the NPs.

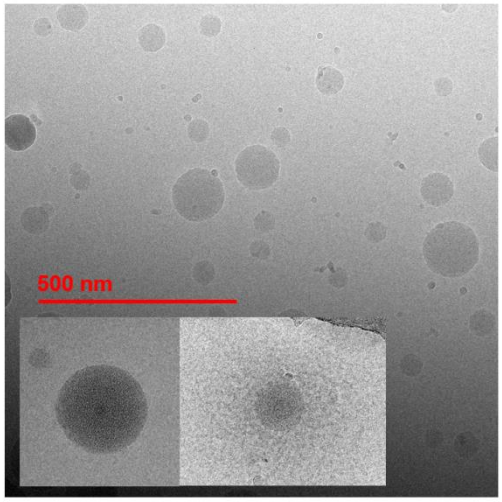

Figure 1 : SQ-ENK NPs observed by cryoTEM. Inset: close-up in the absence and in the presence of BSA.

First, we characterized the NPs suspension in D_2O before and after a 8x dilution in D_2O (Figure 2). Both NPs suspensions can be modelled by a lognormal distribution of spheres. The absence of feature in the wide angles (> 0.05 $Å^{-1}$) indicate the absence of a strong inner organization. While the SLA NPs are barely affected by the dilution (albeit maybe a small swelling), the SMA NPs size distribution is clearly shifted towards smaller sizes. This indicates a dependence of the particles size distribution with concentration and ionic strength. The fit parameters are reported in the Table 1 and are broadly consistent with cryo-TEM observations.

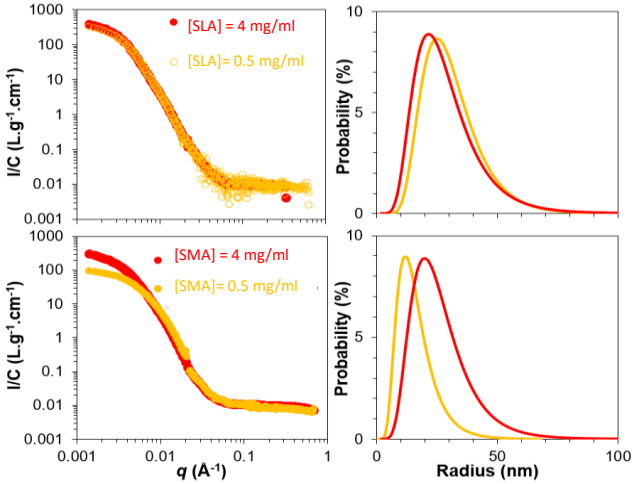

Figure 2 : (left): SANS patterns of SLA (top) and SMA (bottom) suspensions at two concentrations (4 mg/ml and 0.5 mg/ml). On the right side, the corresponding radius distributions obtained from the curve-fitting with a lognormal distribution of spheres.

Table 1: Fitting parameters from curve-fitting of SANS patterns with a lognormal distribution of spheres (Figure 2)

	Radius (nm)	PDI
SLA 4 mg/ml	26±0.4	0.44
SLA 0.5 mg/ml	28.8±0.04	0.38
SMA 4 mg/ml	23.9±0.1	0.49
SMA 0.5 mg/ml	14.5±0.5	0.46

We then characterized the protein solutions and their mixtures with the NPs, keeping the NPs concentration constant. The addition of proteins to the SMA suspension result in their destabilization: flocculation can be observed visually. The resulting SANS patterns (see Figure 3) shows that the proteins remain in solution and do not precipitate with the NPs. The data analysis are still under progress.

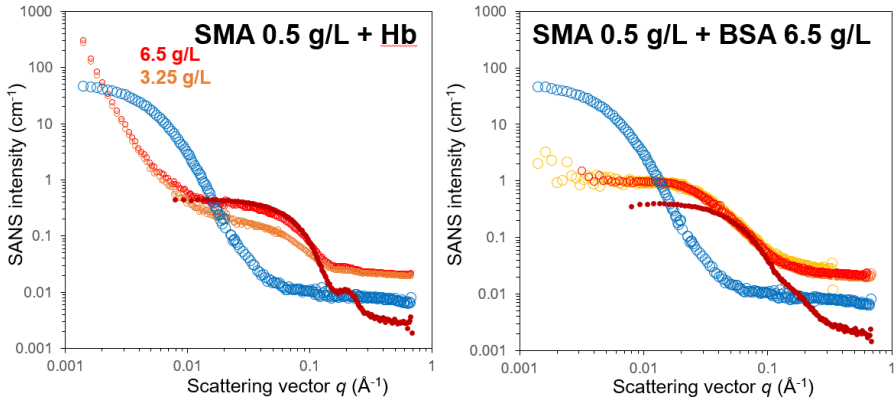

Figure 3 : SANS patterns of SMA in the presence of haemoglobin (left) and BSA (right). Blue circles: SMA suspension in D_2O; Left: open orange and red circles: mixtures with Hb (3.25 and 6.5 g/L respectively); Right: open yellow and red circles: mixtures with BSA with and with buffer. Closed dark red circles: 6.5 g/L protein suspension (left: Hb, right: BSA).

By contrast, SLA/proteins mixtures remain colloïdally stable upon addition of proteins. However, we observe a decrease of the scattering intensity assigned to the NPs proportionally to the protein concentration (Figure 4). This was attributed in a previous study[2] to the disassembly of the NPs through the formation of a protein-bioconjugate complex. However, here the effect appears to be stronger than with other squalene-based NPs such as SQ-adenosine or SQ-siRNA.[2,3] The effect of Hb was investigated here for the first time and overall it seems very similar to that of BSA, despite our anticipation. However, complementary characterization techniques (cryoTEM (**Figure 1**), WAXS, DLS) suggest different mechanisms for each protein; this discrepancy still need to be explained.

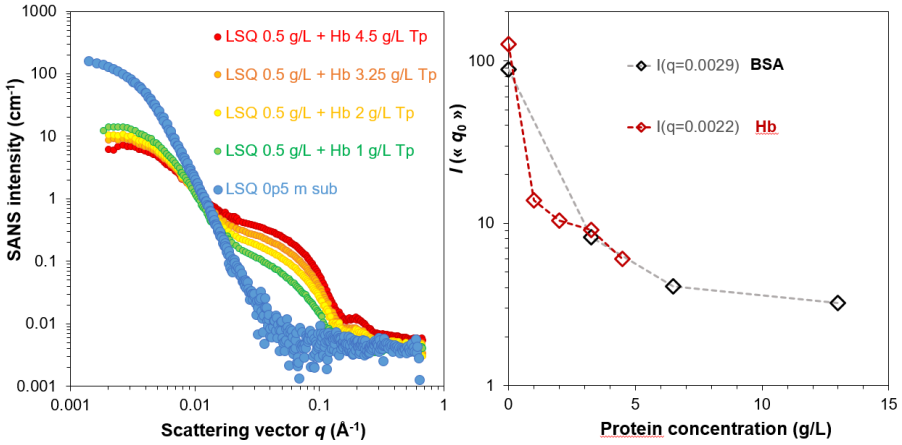

Figure 4 : (Left) SANS patterns of the Hb-SLA mixtures in 10 mM phosphate buffer/D_2O. All the mixtures have initially the same amount of NPs and the Hb content is increasing from blue (0 g/L) to red (4.5 g/L). SLA is noted LSQ in the figure. (Right) Intensity in low q range in function of protein concentration for two different proteins: BSA (black diamond) and Hb (red diamond).

REFERENCES

(1) Feng, J.; Lepetre-Mouelhi, S.; Gautier, A.; Mura, S.; Cailleau, C.; Coudore, F.; Hamon, M.; Couvreur, P. A New Painkiller Nanomedicine to Bypass the Blood-Brain Barrier and the Use of Morphine. *Science Advances* 2019, *5* (2), eaau5148. https://doi.org/10.1126/sciadv.aau5148.

(2) Gobeaux, F.; Bizeau, J.; Samson, F.; Marichal, L.; Grillo, I.; Wien, F.; Yesylevsky, S. O.; Ramseyer, C.; Rouquette, M.; Lepêtre-Mouelhi, S.; Desmaële, D.; Couvreur, P.; Guenoun, P.; Renault, J.-P.; Testard, F. Albumin-Driven Disassembly of Lipidic Nanoparticles: The Specific Case of the Squalene-Adenosine Nanodrug. *Nanoscale* 2020, *12* (4), 2793–2809. https://doi.org/10.1039/C9NR06485K.

(3) Caillaud, M.; Gobeaux, F.; Hémadi, M.; Boutary, S.; Guenoun, P.; Desmaële, D.; Couvreur, P.; Wien, F.; Testard, F.; Massaad-Massade, L. Supramolecular Organization and Biological Interaction of Squalenoyl SiRNA Nanoparticles. *International Journal of Pharmaceutics* 2021, 121117. https://doi.org/10.1016/j.ijpharm.2021.121117.