Experimental report

15/02/2021

Proposal: 9-10-1502 **Council:** 10/2016

Title: Squalene Nanomedecine: solvent andtemperature effect in controlling size distribution

Research area: Soft condensed matter

This proposal is a continuation of 9-10-1485

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Samples: C36H53F2N3O5
C36H55N3O5
Acetone, D2O

Instrument	Requested days	Allocated days	From	To
D33	3	3	22/02/2017	25/02/2017

Abstract:

Due to the quick adaptation of microorganisms to chemicals and the shrinking range of drugs available, medicine try to find a way to avoid drug resistance and treatment side effects. In 2016, Couvreur et al. discovered a new prodrug family via the squalenoylation of nucleoside analogues. The prodrug was nanoprecipitated via ouzo effect (solvent-antisolvent mixing) leading to nanoparticles with a liquid crystal structure. For squalene-gemcitabine and squalene-deoxycitidine, Saha et al. have shown the key role of the solvent in structure stability and nanoparticle size for naoprecipitation from ethanol. Recently, we found a different behavior when DMSO and acetone are utilized for the nanoprecipitation: Single nucleation and no aggregation was observed with increasing the organic solvent volume fraction. In addition, temperature appeared as very important in the control of polydispersity. With these news results, it becomes crucial to complete our data set with a study on a forth solvent and a more precise quantification of the temperature effect. This is the aim of this proposal (continuation of 9-10-1485) to finally explain the particularities of the squalenoyl NPs formation.

Aims of the experiment and results:

As large differences on the precise role of the parameters of preparation is found in the literature, systematic investigations are needed to optimize the protocol for each ternary system. This is particular important for nanomedicine, less studied than polymer for the nanoprecipitation methods. In this study, the squalenoylated deoxycytidine (SqDc) compound is the focus of our attention as a model system. Squalenoylation methods have been introduced by Couvreur et al in 2006 as a versatile strategy to formulate nanoparticles with high loading of active principle (Couvreur et al. 2006). Recent publications on the behaviour of the squalene derivatives under heating or solvent/anti-solvent ratio show that the preparation parameters affect the tendency to self-assemble and therefore the size of the particles (Saha et al. 2015),(Lepeltier et al. 2015). Here, we present a study on the nanoprecipitation of squalenoylated derivative. In the details, we investigate the effect of several parameters: i) the speed and regularity of the droplets addition, ii) the stirring rate, iii) the volume of the mixing solution, iv) the solvent removal methods, v) the impact of dilution, vi) the temperature effect and the impact of stabilizer addition on the nanoformation. Small angle neutron scattering (SANS) is our principal method for this purpose. It allows an in-situ quantitative characterization of the size, the size distribution, the density number of the particle, their shape and the internal structure. The systems are described both in their intermediate state, i.e. before the removal of the organic solvent, and at the final state (after removal). Thus, we are able to give a detailed description of our protocol and what we think are the optimal conditions for the nanoprecipitation via solvent dispersion method of squalene deoxycytidine

I) D33 first results: Influence of the stirring rate and the scalling on the nanoparticles size distribution

Examples of SANS results obtained with ethanol are presented in fig 1 and 2.

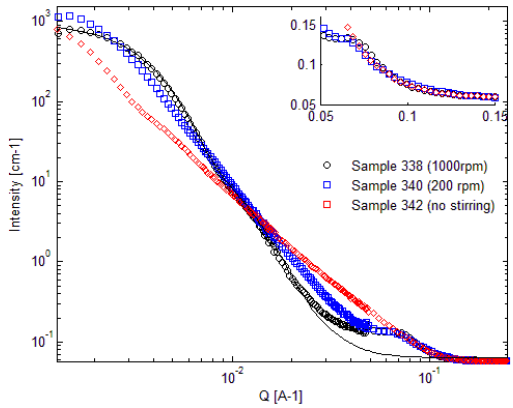


Fig 1: SANS pattern of SqDc nanoparticles in deuterated ethanol/D2O at intermediate state for 1.1 mg/mL of SqDc, nanoprecipitation is obtained with syringe pump, with 1000 rpm, 200 rpm or no stirring for the D2O phase during addition. As insert a zoom on the Bragg peaks

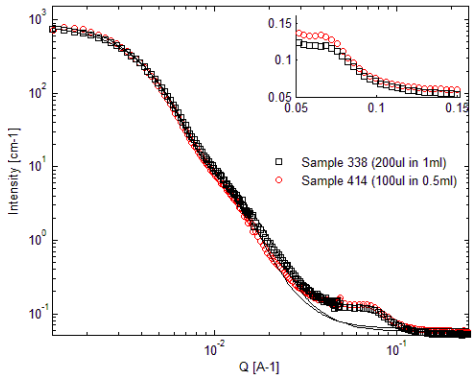


Fig 2: SANS pattern of SqDc nanoparticles in deuterated ethanol/D2O at intermediate state nanoprecipitated at equivalent ratio but for 2 different total volumes. As insert a zoom on the Bragg peaks

The influence of stirring variation and thus, the mixing time scale is illustrated in Figure 1. At low Q, the scattering curve of the sample 340 (200rpm) is above the scattering curve of sample 338 (1000 rpm). The two I vs Q curves cross each other in the small Q region. No oscillation is visible in the intermediate Q and the slope of Q-3 is lower than the characteristic Q-4 from sphere. The scattering curve of sample 342 (without mixing) does not have a Guinier plateau in the investigated Q-range and the slope (Q-2.3) is not characteristic of spherical object. In addition, to the loss of the spherical shape, no internal structure is visible. From these observations, we can conclude that the stirring rate has a strong influence on the final size distribution.

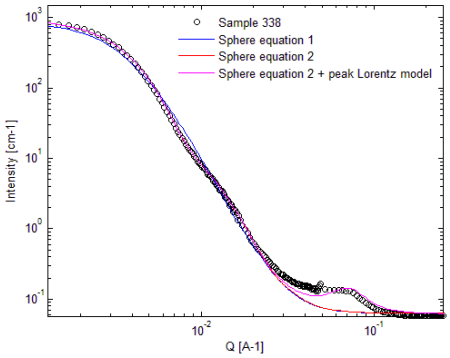
Squalene deoxycytidine particles are prepared by automated addition under stirring (1000rpm). Sample 414 has the same ratio of organic and aqueous phase with respect to sample 338 at half of its volume. As the stirring rate is the same for both samples, it is expected that the efficiency of mixing of sample 414 is equal to or better than sample 338.

The SANS scattering patterns are presented in Figure 2. The scattering curves are almost superimposed. The fit of the data reveals particles distributions of 45 and 14nm for the sample 338 (200□l in 1mL), while sample 414 (100□l in 500□l) has particles of 42 nm and 12 nm. For the two samples, the volume fraction of particles are similar and within the error bar (ØLarge,swell 338 = 1.48 10-3 and ØLarge,swell414 = 1.25 10-3; ØSmall,swell

338 = 5.25 10-4 and ØSmall,swell414 = 5.5 10-4). Similar size distributions are obtained with decreasing the scaling volume preparation.

Treatment for SANS patterns:

The D33 data for Squalenoylated nanoparticles were fitted with SASview software. The intensity of Squalenoylated Deoxycitidine curves were fitted with two populations of polydisperse sphere:



Fixed parameters for the fit	
$I_{q\to 0}$	1.03 10^3
Scale pop 1	0.00044448
Radius pop 1 (nm)	44.4
Polydispersity pop 1	0.25
Scale pop 2	0.00013337
Radius pop 2 (n^)	
Polydispersity pop 2	0.25
$SLD_{solvent}$ (Ă$^{-1}$)	6.33 10^{-6}
SLD_{SqDc}(Ă$^{-1}$)	1.24 10^{-6}

Fig 3: SANS pattern of the SqDc nanoparticles in deuterated ethanol/D2O for 1.1 mg/mL of SqDc (intermediate state, n° 338). The continuous lines are obtained from the fit with either one lognormal spheres distribution (ie one population)(blue) or two lognormal spheres distribution (ie two populations)(red) models. The fitting parameters are given in table beside.

A typical pattern of SqDc NPs dispersed in D2O is shown in Figure 3. The nanoparticles are prepared following the automated protocol with the addition of SqDc/deuterated ethanol solution to D2O at a stirring rate of 1000rpm (sample 338). The SqDc concentration is 1.06 mg/mL. At low Q, the Guinier regime is visible as the curve almost reaches the plateau, strong evidence that no large aggregates are present in solution. In the intermediate Q range, between 5 10-3 and 2 10-2 Å-1, the curve follow a Q-4 behaviour, typical for spherical object with a thin interface. In addition, around 8 10-3Å-1 a small oscillation is visible. At large angle, there is a large correlation peak as the signature of the internal structure of the nanoparticle. Two different models were considered for the fitting. First, a single population of polydisperse sphere is considered giving particles of 40 nm with a high polydispersity Index of 0.5. The fit underestimate the scattering at low Q and completely smoothes the oscillation in the intermediate Q range. The analysis is strongly improved considering the coexistence of two populations with radii of 45 and 14 nm and a σ equal to 0.25 for both. We observe that no difference in the fitting parameters is obtained when the intensity is considered for the full Q range or on a restricted one: limited to Q = 0.05 Å-1 at the end of the Porod region (see Table 2), without the Bragg peaks.

Experimental results:

From experimental results obtained for the different cases we want to propose the key to control the size distribution for squalenoyl nanodrugs.

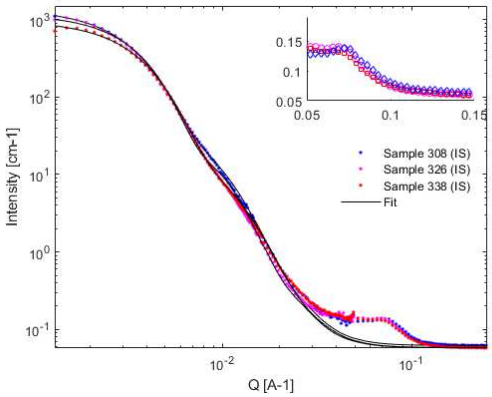


Fig 4 : SANS pattern of SqDc nanoparticles in deuterated ethanol/D2O at intermediate state. The samples are nanoprecipitated with the same conditions. The SANS patterns have been analysed after 1 day, 3 days or 1 week of ageing in controlled condition

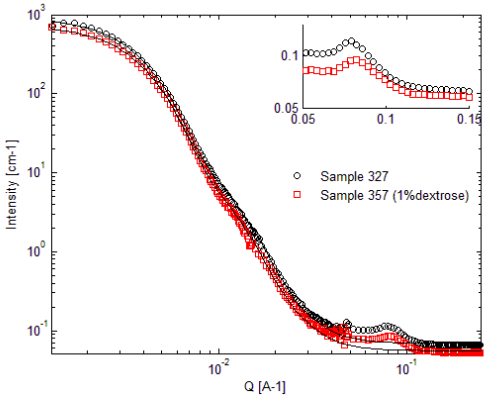


Fig 5: SANS pattern of SqDc nanoparticles in deuterated ethanol/D2O at final state with or without dextrose addition. As insert a zoom on the Bragg peaks

(RT=20°C, protected from light, no stirring, close vial and ATM pressure). Age of the sample 308: 1 week, sample 326: 3 days and sample 338 analysis in the day.

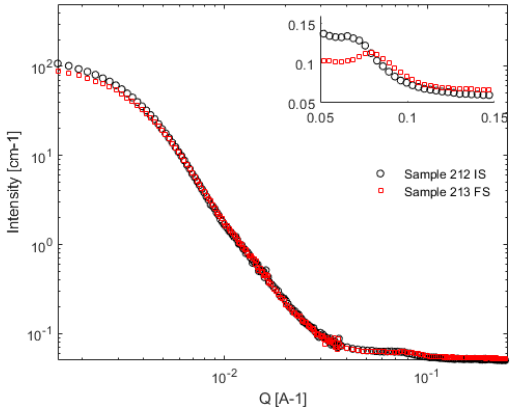


Fig 6: SANS pattern of SqDc nanoparticles in deuterated ethanol/D2O at intermediate and final state before and after removing the organic solvent by dialysis. As insert a zoom on the Bragg peaks

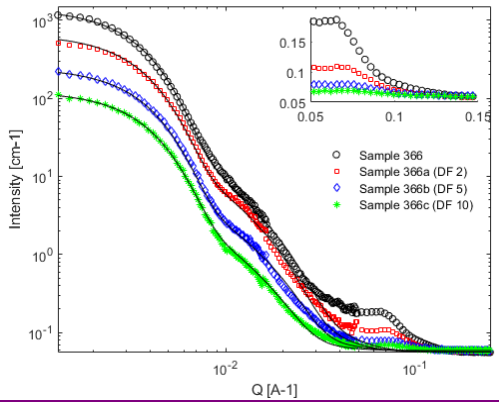


Fig 6: SANS pattern of SqDc nanoparticles prepared in deuterated ethanol/D2O at intermediate state. Effect of different dilutions factors (2, 5, 10) by D2O after nanoprecipitation. As insert a zoom on the Bragg peaks

Main conclusions and perspectives:

We show here that the solvent shifting method applied to the squalenoyl derivatives is sensitive to the nanoprecipitation parameter such as the concentration of monomer in the organic phase, the stirring efficiency and the solvent removal methods. They are the main parameters controlling the nanoparticles size and polydispersity. We proved that the used protocol produces reproducible nanoparticles that are stable. Our results do not corroborate previous publication with polymers. For difference between small and macromolecules, polymers may induce additional solvent effects due to conformational and structural constraints that may not be discriminated for the small molecule. The deoxycytidine squalene is about 400Da and 25Å length, it belongs to the small molecule category. This could explain the impact of parameters such as the initial concentration. Other effect like the mixing efficiency seems to be more related on the driving force (local event or nonlocal event) controlling the nucleation.

References:

1. Couvreur, P. et al. Squalenoyl Nanomedicines as Potential Therapeutics. Nano Lett. 6, 2544–2548 (2006).
2. Saha, D. et al. The role of solvent swelling in the self-assembly of squalene based nanomedicines. Soft Matter 11, 4173-4179 (2015).