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Short communication

DLS and AF4-MALS/DLS as powerful techniques for *in vitro* evaluation of nanoliposomes stability in biorelevant medium

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Introduction

The process of protein corona formation could affect the stability of the nano-systems as well as their *in vivo* behavior. Thus, of crucial importance is to be able to easily monitor the particle's average diameter, which is considered an effective tool for observing the agglomeration process and for predicting their stability in presence of complex biomolecules (Klein et al., 2020). Therefore, the aim of this study was to compare the DLS as robust method and AF4-MALS/DLS as high size resolution method for particle size characterization of nanoliposomes in a complex biorelevant medium.

Materials and methods

Materials

Soybean lecithin (SL) was purchased from Vitalia, Macedonia, LIPOID PE 18:0/18:0-PEG 2000 (PEG) and hydrogenated soy phosphatidylcholine (LC3) from Lipoid, Germany. Cholesterol (CH) and EndoGRO-MV Complete Culture Media Kit SCME004 were obtained from Sigma Aldrich (St. Louis, USA). All the other chemicals were commercially available and used as received.

Preparation of pegylated nanoliposomes

Pegylated nanoliposomes (NLPEG) were prepared by the modified lipid film hydration technique described in detail in Mihailova et al. (2022), where SL, PC3, CH and PEG were used in mass ratio 17.3:1:1:2, respectively.

NLPEG characterization using DLS and AF4-MALS/DLS

The alterations in the mean particle size (nm) of NLPEG (1 mg/mL) incubated with serum free and 5% serum supplemented cell culture medium were evaluated using Dynamic Light Scattering in batch mode (Zetasizer Nano-ZS, Malvern Instruments Ltd., UK) as well as AF4-MALS/DLS analysis (Asymmetrical Flow Field Flow Fractionation (AF4) coupled to Multiangle light scattering detector (MALS) and Dynamic light scattering (DLS).

Proteomic profiling of the adsorbed proteins onto nanoliposome surface using electrophoresis

The proteomic profiling and the changes in the adsorbed proteins on the particle's surface were performed using automated high-resolution electrophoresis (2100 Bioanalyzer using the High Sensitivity Protein 250 kit assays) after protein isolation from complex biological systems, denaturation and appropriate preparation for analysis after 1 and 4 hours incubation time.

Results and discussion

The z-average diameter (nm) was monitored for 4 hours (in DLS batch mode) incubation of NLPEG in a

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supplemented cell culture medium, which is believed to be the average time of dynamic protein corona formation (Fig. 1). The obtained results showed that there isn't a statistically significant change in the particle size of the NLPEG formulation when incubated in serum supplemented cell culture medium ($p=0.6804$; $p>0.05$, ANOVA).

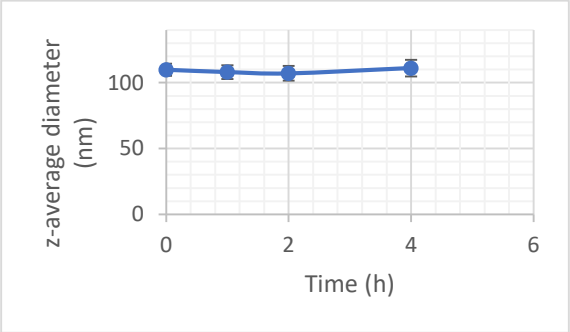

Fig. 1. Mean particle size (z-average) of NLPEG incubated in a supplemented cell culture medium.

After incubation of the prepared formulation with serum free (M) and serum supplemented (S) cell culture medium, there wasn't noticed any alterations in the size of the nanoliposomal vesicles neither with MALS nor with flow-DLS (Fig. 2). Moreover, the obtained results showed comparable particle size values with both techniques.

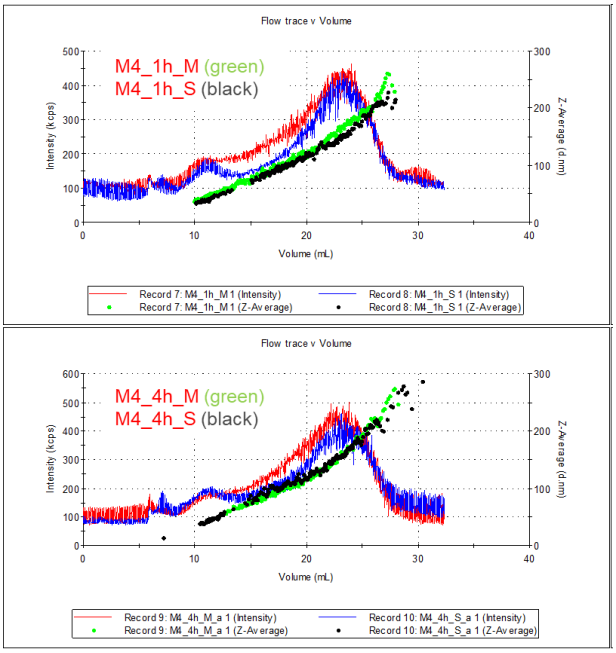

Fig. 2. Overlaid fractograms after 1 and 4 hours incubation time of NLPEG fractionated with AF4 (blue and red lines: MALS 90° scattering signal; black and green lines: DLS z-average).

In order to confirm the formation of the protein corona, high resolution electrophoresis was performed. The results clearly show that protein adsorption occurs already after 1 hour of incubation of NLPEG in serum supplemented cell culture medium, which is represented by a strong band at about 60 kDa, which most likely originates from the presence of albumin as the most abundant protein in the serum (Fig. 3).

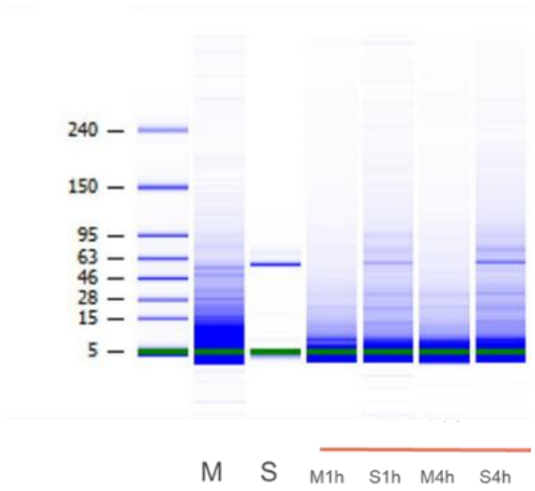

Fig. 3. Electrophoresis of NLPEG after incubation in serum free and serum supplemented cell culture medium.

Conclusion

Based on the obtained results from the investigation of the stability of the liposomal formulation, we can conclude that no change in the particle size of NLPEG can be observed in the investigated time intervals and in the presence of serum proteins (neither by MALS nor by flow-DLS), even though the process of protein corona formation was confirmed already in the first hour through the high-resolution electrophoresis. Moreover, we can confirm that DLS could be specific enough to achieve accurate particle size determinations even in a biorelevant medium, as more complex environment than the physiological buffers.

References

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