IOCN 2022

MDPI

Proceeding Paper

Lipid-Based Nanosystems to Carry Manganese Derivatives for Diagnostic Purpose †

Maddalena Sguizzato [1], Walter Pula [1], Markus Drechsler [2], Lorenza Marvelli [1] and Rita Cortesi [1,*]

[1] Department of Chemical, Pharmaceutical and Agricultural Sciences (DoCPAS), University of Ferrara, I-44121-Ferrara, Italy; sgzmdl@unife.it (M.S.); walter.pula@edu.unife.it (W.P.); lorenza.marvelli@unife.it (L.M.)

[2] Bavarian Polymer Institute (BPI) Keylab "Electron and Optical Microscopy" University of Bayreuth, D-95440-Bayreuth, Germany; markus.drechsler@uni-bayreuth.de

* Correspondence: crt@unife.it

† Presented at the 3rd International Online-Conference on Nanomaterials, 25 April–10 May 2022; Available online: https://iocn2022.sciforum.net/.

Abstract: This study focuses on the design, production and characterization of lipid-based nanosystems to deliver manganese-based compounds for diagnostic purpose in multimodal imaging techniques. Anionic liposomes, obtained by direct hydration method and extrusion, were homogeneous, monodispersed and negatively charged. The loading of manganese-based compounds was almost quantitative, their magnetic properties retained and the in vitro cytotoxicity on human keratinocytes highlighted that liposomes loaded with hydrophilic manganese derivatives did not affect cell viability, while liposomes loaded with lipophilic manganese derivatives showed a dose-dependent antiproliferative effect. Further experiments need to be performed to elucidate both type and concentration of manganese derivative to be used.

Keywords: manganese; anionic liposomes; PET/MRI; lipid-based nanosystem

Citation: Sguizzato M, Pula W, Drechsler M, Marvelli L, Cortesi R. Lipid-Based Nanosystems to Carry Manganese Derivatives for Diagnostic Purpose. *Biol. Life Sci. Forum 2022, 2, x. https://doi.org/10.3390/xxxxx*

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

1. Introduction

Continuous research in pharmaceutical technology has led to the development of drug delivery systems capable of delivering different molecules within the body exploiting different areas of application. Among these systems, liposomes have proved to be excellent candidates able of modifying the site and the release time of the substances loaded within them [1]. This preliminary study focused on the description and characterization of these vesicular systems, as entities capable of carrying molecules with diagnostic activity.

The diagnostic application of liposomes was investigated by conveying complexes of manganese. This metal has both radioisotopes capable of emitting positrons, which can be used in Positron Emission Tomography (PET), and paramagnetic properties, in its Mn^{2+} and Mn^{3+} oxidation states, which allow it to be used as a contrast agent in Magnetic Resonance Imaging (MRI) [2]. PET / MRI, a multimodal imaging technique that combines the two aforementioned diagnostic analyzes, is useful in providing anatomical, morphological and at the same time metabolic characterizations and currently employs two different contrast media that are injected into the body simultaneously.

Manganese (Mn) exists in several oxidation states: +2 (the most stable), +3, +4, +6 and +7. The transition metal cations have unpaired electrons, which give paramagnetism. Specifically, Mn (II) is optimal for diagnostic use as it has a good number of unpaired electrons appropriate for both PET radiotracer and MRI paramagnetic substance. Therefore it has been taken into consideration for the present study. However, manganese in its free form is toxic, consequently the use of liposomes to encapsulate it has been

considered to reduce its toxicity. In this regard, negatively charged liposomes were produced by thin layer hydration method and extrusion [3]. Particularly, N-lauroylsarcosin sodium salt (NLS) and sodium lauroyl lactylate (SLL) have been used as anionic surfactants.

2. Methods

2.1. Liposomes Preparation

Anionic liposomes were prepared by thin layer hydration and extrusion. Phosphatidylcholine (PC), cholesterol (CH) and the anionic surfactant (AS) in a 4:2:1 molar ratio were dissolved in dichloromethane:methanol (1:1 *v*/*v*) and the organic mixture was subjected to evaporation under vacuum (70 bar, 100 rpm for 40–45 min) using a Rotavapor R-200 (Buchi Italia, Cornaredo, Italy). The obtained film was then hydrated with PBS (pH 7.4) and subjected to ultrasounds for 5 min. Mn-based compounds (500 μM) were added to the organic phase (manganese acetylacetonate, lipophilic, MnL) or to water (manganese chloride, hydrophilic, MnH) during the hydration step. Liposomes with final PC content of 25 mg/ml, were then extruded (Extruder, Lipex Biomembranes, Vancouver, BC, Canada) through two stacked polycarbonate filters 0.2 μm pore size (Nucleopore Corp, Pleasanton, CA, USA) under 10–20 bars nitrogen pressure [4] and stored for further studies.

2.2. Liposomes Characterization

Liposomes were visualized by Cryo-Transmission Electron Microscopy (Cryo-TEM) after being vitrified and transferred to a cryo-holder (CT3500, Gatan Inc., Pleasanton, CA, USA) of a Zeiss EM922Omega instrument [5]. Sample temperature was maintained below −175 °C throughout the visualization. Specimens were examined with doses of about 1000–2000 e/nm^2 at 200 kV. Images digitally recorded by CCD camera (UltraScan 1000, Gatan Inc., Pleasanton, CA, USA) were processed by mean of GMS 1.4 software (Gatan Inc., Pleasanton, CA, USA).

Liposome size was measured by mean of Zetasizer Nano S90 (Malvern Instr., Malvern, UK) equipped with a 5 mW helium neon laser with a wavelength output of 633 nm on aqueous diluted liposome samples (1:20 by volume). Plasticware was cleaned with detergent washing and rinsed twice with milliQ water. Measurements were made at 25 °C at an angle of 90°, run time around 180 s. Data were interpreted by the "CONTIN" method [6].

The surface charge of the liposomes was measured by mean of zeta potential (ζ) (Zetasizer Ultra, Malvern Panalytical Ltd., Malvern, UK). The samples were diluted 1:20 by volume with deionized water and analyzed trice in triplicate at 25 °C.

2.3. Encapsulation Efficiency of Manganese

The encapsulation efficiency (EE) of manganese in liposomes was determined by mean of Atomic Absorption Spectrophotometry (AAS) on ultracentrifuged samples. Particularly 500 μL of liposomes, loaded on Microcon centrifugal filter unit YM-10 membrane (NMWCO 10 kDa, Sigma-Aldrich, St. Louis, MO, USA) were centrifuged at 8000 rpm for 20 min (Spectrafuge™ 24D Digital Microcentrifuge, Woodbridge, NJ, USA) and both the lipid and aqueous phases were analyzed with a AAS device (Analyst 800, Perkin-Elmer, Shelton, CT, USA), together with a sample of liposomes before centrifugation, used as control. The measure was performed at 279.5 nm. Each sample was prepared twice and subjected to analysis. The EE was determined using Equation (1)

$$EE = [Mn_L/(Mn_L + Mn_A)] \times 100 \quad (1)$$

where Mn_L is the manganese concentration in the lipid phase while Mn_A is the concentration of manganese in the aqueous phase, both measured by AAS.

2.4. Cell Viability Test

The colorimetric assay based on the use of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT [7] was conducted to evaluate cell viability. Cultured Human Keratinocyte (HaCaT) were grown in Dulbecco's modified Eagle's medium High Glucose (Lonza, Milan, Italy), supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine. Cells were incubated at 37 °C for 24 h in 95% air/5% CO_2 until 80% confluence. Different dilutions of each liposome formulation (namely 1:50, 1:100, 1:200, 1:500, corresponding to liposome concentrations of 0.5, 0.25, 0.125, 0.05 mg/mL, respectively) were dispersed in cell culture medium. Seeded cells were exposed to the selected formulations for 24 h, and, after complete removal of the treatment, 110 μL of MTT (0.5 mg/mL) was added and incubated for 4 h. The conversion of MTT solution into a violet colored formazan was obtained after the addition, incubation (15 min) and shaking of 100 μL of DMSO. The solution absorbance, proportional to the number of living cells, was measured using a spectrophotometer at 590 nm and converted into percentage of viability.

Statistical analysis was performed by the analysis of variance (ANOVA). The level of significance was taken at p-values < 0.05.

3. Results and Discussion

3.1. Production and Characterization of Liposomes

This preliminary study was focused on the development of anionic liposomal systems able to carry the manganese ion (i.e., Mn^{2+} or Mn^{3+}), with the aim to control its toxicity and release into biological fluids [8,9].

A preformulatory study was previously conducted allowing the selection of two standard compositions, containing as anionic surfactants, NLS and SLL [10]. Afterwards Mn-loaded L-SLL and L-NLS were produced testing as model compounds either a hydrophilic (MnH) or a lipophilic Mn-based compound (MnL).

The extruded liposomes composed of PC, CH and SLL or NLS were analyzed in terms of size and charge by evaluating their average diameter, polydispersion index and ζ potential. The obtained results are summarized in Table 1. It can be underlined that the presence of Mn-compound (either MnH or MnL) within the lipid bilayer has no effect on the size of the anionic vesicles, whose average diameter never exceeds 200 nm. Moreover, the ζ potential values showed the presence of a net negative surface charge for all the produced liposomal dispersions, greater than 30 mV in absolute value, improving the stability of the dispersion by avoiding the agglomeration of the liposomal vesicles [11].

Table 1. Size and ζ potential values of extruded anionic liposomes.

Liposome Dispersion	Mean Size (nm ± s.d.)	PdI ± s.d.	ζ potential (mV)
plain SLL	183.20 ± 1.01	0.08 ± 0.01	−57.12 ± 0.64
SLL-MnH	192.51 ± 4.13	0.13 ± 0.02	−51.72 ± 1.37
SLL-MnL	193.33 ± 1.54	0.12 ± 0.03	−54.61 ± 0.90
plain NLS	171.22 ± 0.98	0.09 ± 0.01	−57.77 ± 1.26
NLS-MnH	172.91 ± 3.81	0.12 ± 0.02	−48.02 ± 0.70
NLS-MnL	176.82 ± 4.91	0.09 ± 0.02	−59.75 ± 0.75

From the macroscopic point of view, after extrusion the liposomal dispersions appear translucent. In depth visualization, the morphology of all the prepared liposome dispersions was investigated by cryo-TEM and the obtained images reported in Figure 1.

As expected, the extrusion process allowed the obtaining of homogeneous sizes, as confirmed by low polydispersity index (PdI) of size measurements (Table 1), mainly

characterized by unilamellar liposomes [12]. Indeed, PdI values lower than 0.3 are evidence of a monomodal distribution of vesicles [13]. Moreover, it has to be underlined that liposomal preparations with NLS and SLL in their composition were dimensionally stable over time [10].

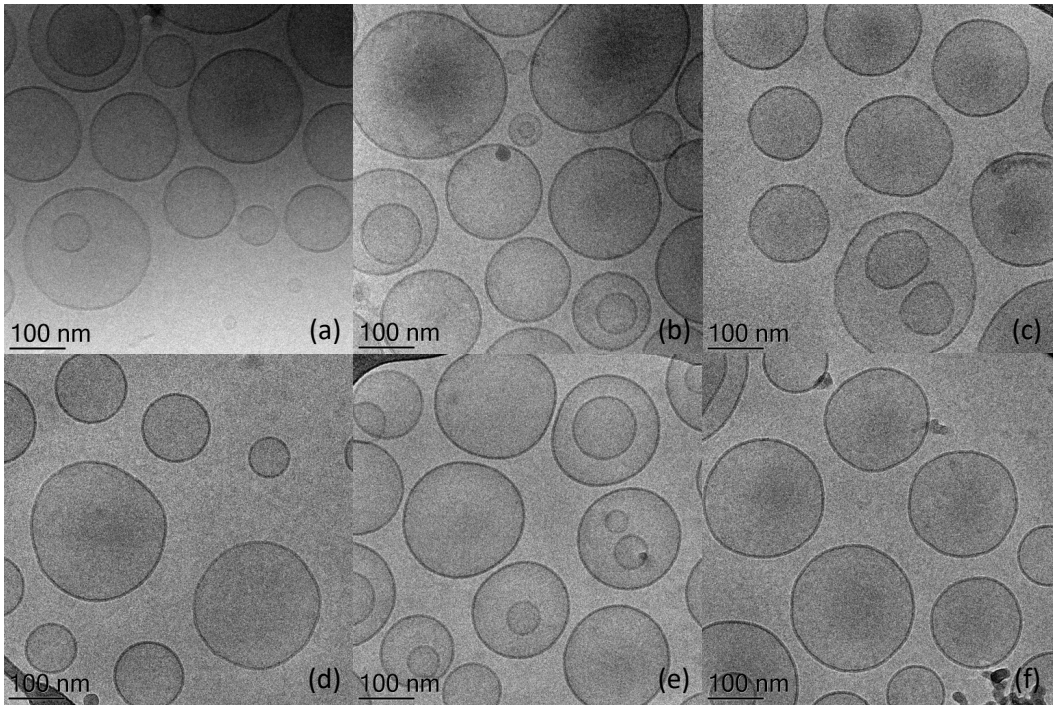

Figure 1. Cryo-TEM images of unloaded and Mn-loaded NLS and SLL liposomes. Plain SLL (**a**), SLL-MnH (**b**), SLL-MnL (**c**), plain NLS (**d**), NLS-MnH (**e**) and NLS-MnL (**f**).

3.1.1. Mn Encapsulation Efficiency

The encapsulation efficiency of MnH and MnL in liposomes with NLS or SLL was evaluated by AAS analyses. The obtained results indicate that both Mn compounds were retained by the lipid portion of liposomes. Particularly the actual concentration of manganese compound with hydrophilic characteristics is retained almost completely within liposomes (namely 99.83% in NLS liposomes and 99.72% in SLL vesicles), whilst lipophilic manganese compound is loaded around the 90% (e.g. 91.66% in NLS liposomes and 87.88% in SLL). Possibly, MnH could establish electrostatic interactions between the cationic charge of Mn^{2+} ion and the negative charged phospholipid surface. Considering that the negative charge of the nanovesicular carriers is located either in the inner and/or outer surface the electrostatic binding occurs mainly close to the phospholipid bilayer thus allowing the retaining of the MnH.

Therefore, anionic liposomes are able to carry manganese ions, possibly using also an electrostatic binding with the surfactant negative charges on the surface of lipid bilayer.

3.2. In Vitro Effect on HaCaT Cultured Cells

The anionic liposomal formulations were tested *in vitro* on the human keratinocyte cell line (HaCaT) by MTT assay to get quick information on their effect on cell viability, proliferation and cytotoxicity. However, as the MTT test is a sensitive and reliable indicator of cellular metabolic activity, in the future it will likely also be used for further experiments focusing on the negative effects on cell growth. HaCaT cells were employed as a model of human non-tumor cell line.

Unloaded and manganese-loaded anionic liposomes were tested at different concentrations after appropriate dilutions, and the effect was compared to those of untreated

cells used as control. The results were expressed as the percentage of cell viability with respect to control cells (100% viable) and are summarized in Figure 2.

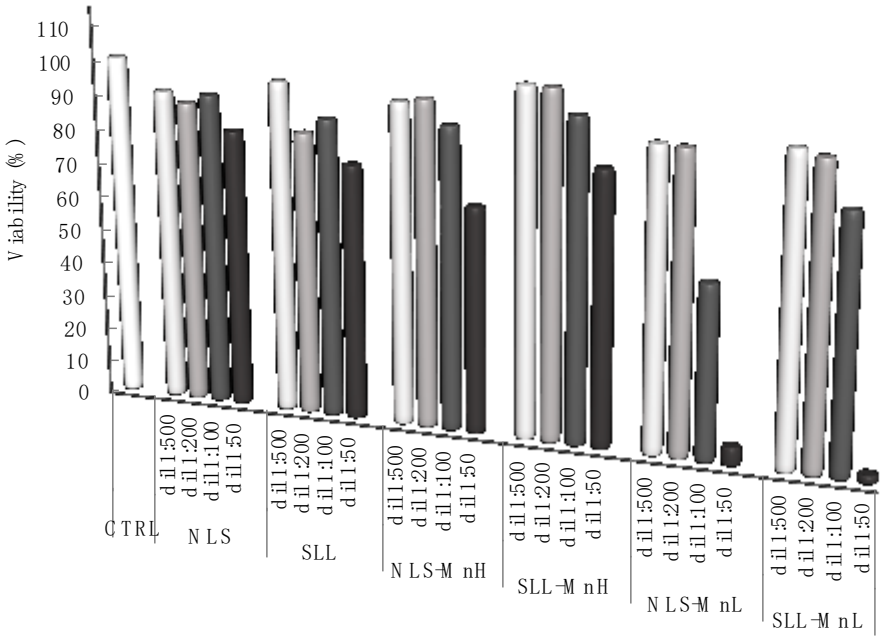

Figure 2. MTT test results of in vitro effect on HaCaT cell proliferation of unloaded and loaded anionic NLS and SLL liposomes. Data are the mean of three independent experiments conducted in triplicate. P-values always <0.01 vs. CTRL.

It was found that all the formulations indicated a dose-dependent antiproliferative effect on HaCaT cells. Precisely, the higher the liposome concentration, the lower the viability. However, both unloaded anionic formulations did not affect cell proliferation for all concentrations tested. Additionally, anionic liposomes loaded with MnH did not affect cell viability compared to unloaded formulations. On the other hand, anionic liposomes loaded with MnL showed a higher antiproliferative effect. It is probable that the lipophilic nature of MnL contributes to increase the lipophilic grade of the system affecting the interaction with cells. Indeed, at higher concentrations, the viability drastically decreases, reaching 10%.

Taking into consideration the results, further experiments, possibly using other cell lines, such as L929 fibroblasts and/or RAW 264.7 or J774 macrophages, need to be performed in order to elucidate the optimal concentration of Mn compound could be used.

4. Conclusions

Currently, the present study is still in progress with the aim of determining the magnetic properties of the obtained liposomal system, a relevant factor for the potential application in diagnostic imaging.

However, this work underlined the importance of pharmaceutical technology in the design of anionic liposomes carrying Mn using a simple preparation method. In particular, morphological and dimensional analyses, together with the loading capacity and in vitro activity, gave the chance to select SLL liposomes as the composition to be used for future investigations.

Author Contributions: Conceptualization, R.C., L.M.; methodology, R.C.; preparation of formulations, M.S., W.P; liposomal analyses, W.P., M.S., M.D.; in vitro experiments and discussion M.S.; original draft preparation, R.C.; writing and editing, R.C., M.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by University of Ferrara (FAR2019 and FAR2020).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: We acknowledge Antonella Pagnoni of Ferrara University for AAS analyses.

Conflicts of Interest: The authors declare no conflict of interest

References

1. Ta, T.; Porter, T.M. Thermosensitive Liposomes for Localized Delivery and Triggered Release of Chemotherapy. *J. Controlled Release* **2013**, *169*, 112–125, doi:10.1016/j.jconrel.2013.03.036.
2. Brandt, M.; Cardinale, J.; Rausch, I.; Mindt, T.L. Manganese in PET Imaging: Opportunities and Challenges. *J. Label Compd. Radiopharm.* **2019**, *62*, 541–551, doi:10.1002/jlcr.3754.
3. Aiello, M.; Cavaliere, C.; Marchitelli, R.; d'Albore, A.; De Vita, E.; Salvatore, M. Hybrid PET/MRI Methodology. *Int. Rev. Neurobiol.* **2018**, *141*, 97–128, doi:10.1016/bs.irn.2018.07.026.
4. Cortesi, R.; Romagnoli, R.; Drechsler, M.; Menegatti, E.; Zaid, A.N.; Ravani, L.; Esposito, E. Liposomes- and Ethosomes-Associated Distamycins: A Comparative Study. *J. Liposome Res.* **2010**, *20*, 277–285, doi:10.3109/08982100903443057.
5. Esposito, E.; Mariani, P.; Ravani, L.; Contado, C.; Volta, M.; Bido, S.; Drechsler, M.; Mazzoni, S.; Menegatti, E.; Morari, M.; et al. Nanoparticulate Lipid Dispersions for Bromocriptine Delivery: Characterization and in Vivo Study. *Eur. J. Pharm. Biopharm.* **2012**, *80*, 306–314, doi:10.1016/j.ejpb.2011.10.015.
6. Pecora, R. Dynamic Light Scattering Measurement of Nanometer Particles in Liquids. *J. Nanopart. Res.* **2000**, *2*, 123–131, doi:10.1023/A:1010067107182.
7. Singh Hallan, S.; Sguizzato, M.; Pavoni, G.; Baldisserotto, A.; Drechsler, M.; Mariani, P.; Esposito, E.; Cortesi, R. Ellagic Acid Containing Nanostructured Lipid Carriers for Topical Application: A Preliminary Study. *Molecules* **2020**, *25*, 1449, doi:10.3390/molecules25061449.
8. El-Hammadi, M.M.; Arias, J.L. An Update on Liposomes in Drug Delivery: A Patent Review (2014-2018). *Expert Opin. Ther. Patents* **2019**, *29*, 891–907, doi:10.1080/13543776.2019.1679767.
9. Shah, M.R.; Imran, M.; Ullah, S. Liposomes. In *Lipid-Based Nanocarriers for Drug Delivery and Diagnosis*; Elsevier, Amsterdam, Netherlands, 2017; pp. 63–110 ISBN 978-0-323-52729-3.
10. Sguizzato, M.; Pula, W.; Bordin, A.; Pagnoni, A.; Drechsler, M.; Marvelli, L.; Cortesi, R. Manganese in Diagnostics: A Preformulatory Study. *Pharmaceutics* **2022**, *14*, 108, doi:10.3390/pharmaceutics14010108.
11. Has, C.; Pan, S. Vesicle Formation Mechanisms: An Overview. *J. Liposome Res.* **2021**, *31*, 90–111, doi:10.1080/08982104.2020.1730401.
12. Kulkarni, S.B.; Betageri, G.V.; Singh, M. Factors Affecting Microencapsulation of Drugs in Liposomes. *J. Microencapsulation* **1995**, *12*, 229–246, doi:10.3109/02652049509010292.
13. Danaei, M.; Dehghankhold, M.; Ataei, S.; Hasanzadeh Davarani, F.; Javanmard, R.; Dokhani, A.; Khorasani, S.; Mozafari, M. Impact of Particle Size and Polydispersity Index on the Clinical Applications of Lipidic Nanocarrier Systems. *Pharmaceutics* **2018**, *10*, 57, doi:10.3390/pharmaceutics10020057.