International Journal of Pharmaceutics 362 (2008) 189–192

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Note

Cold field emission gun-scanning electron microscopy: A new tool for morphological and ultrastructural analysis of liposomes

G. De Rosa [a,*], M. De Stefano [b], F. Ungaro [a], M.I. La Rotonda [a]

[a] Dipartimento di Chimica Farmaceutica e Tossicologica, Facoltà di Farmacia, Università degli studi di Napoli Federico II, Via Domenico Montesano 49, 80131 Napoli, Italy
[b] Dipartimento di Scienze Ambientali, Seconda Università di Napoli, Via Vivaldi 43 81100, Caserta, Italy

ARTICLE INFO

Article history:
Received 20 March 2008
Received in revised form 30 May 2008
Accepted 4 June 2008
Available online 7 June 2008

Keywords:
Liposomes
Microscopy
Surface morphology
PEGylated liposomes

ABSTRACT

Liposomes are lipid vesicles largely investigated in the past 30 years as pharmaceutical carriers. In the development of new liposome-based formulations, the study of liposome surface properties remains a crucial step. For this purpose, microscopy techniques can provide useful information, although each such technique suffers from some limitations. Here, we have used cold field emission gun-scanning electron microscopy (cFEG-SEM) to acquire detailed images of liposome surface. In particular, we observed PEGylated and non-PEGylated liposomes in different size ranges. In the case of nanosized liposomes (mean diameter about 200 nm), a morphological evaluation of the whole preparation was obtained. On the other hand, in the case of giant liposomes (mean diameter about 2 μm), it was possible to observe the different surface ultrastructures of the two formulations. In particular, a regular and only slightly wrinkled surface was observed in the case of non-PEGylated liposomes, while a very irregular surface ultrastructure was visible in the case of PEGylated liposomes. This study shows, for the first time, the potential of cFEG-SEM as a new and powerful tool to obtain information on liposome morphology and, at least in the case of giant liposomes, on ultrastructure of the liposome surface.

From their discovery in the early 1960s as model biomembranes (Bangham et al., 1965), liposomes have received great attention, especially as drug delivery systems, leading to several marketed liposome-based pharmaceutical products. Today, many efforts are focused on the modification of liposome surface in order to meet specific needs (Torchilin, 2005). The development of "long circulating liposomes", characterised by polar moieties on the vesicle surface, represents a milestone in drug delivery (Allen and Chonn, 1987; Gabizon and Papahadjopoulos, 1988). Liposome surface can also be modified in order to introduce chemical species able to interact with the target cells or tissues (Torchilin, 2005). A critical issue in the development of surface-modified liposomes is the characterization of the vesicle surface. For this purpose, measurement of the liposome zeta potential can be of help, although it is limited to the cases in which chemical modification leads to a change of the superficial charge. Despite this indirect analysis of the liposome surface, the visualization of its fine morphology remains appealing for many researchers. Vesicle lamellarity of chemically fixed or freeze-fractured liposomes has been estimated by transmission electron microscopy (TEM) that can provide a raw image of the liposome outline (Sommerville and Scheer, 1986; Hope et al., 1989). Atomic force microscopy (AFM) has been proposed as a non-invasive microscopic technique to observe liposome morphology in the hydrated state (Ruozi et al., 2005; Garg and Kokkoli, 2005). AFM can resolve morphological details of the liposome surface in the nanometer range, giving images that are electronic reconstruction of local properties, such as height, optical absorption, or magnetism, measured by a probe or "tip" placed very close to the sample. Unfortunately, this technique can be affected by several experimental constrains. The available end radii of tips confines atomic resolution to flat and periodic specimens or to a small portion with similar features in morphologically complex samples. In addition, soft structures tend to be distorted or destroyed by the tip–sample interaction. However, taking into account the relative advantages of TEM and AFM, their combination has been proposed to study liposome ultrastructure (Anabousi et al., 2005). Like AFM, environmental scanning electron microscopy (ESEM) has also been recently proposed (Mohammed et al., 2004) as an innovative non-invasive microscopic technique to observe liposome morphology in the hydrated state. However, this technique can provide clear 3D images of the liposome only at low magnifications, since the interference of the electronic beam with the hydrated atmosphere in the SEM chamber increases with the magnification, generating fog-like effects in the images. As a consequence, ultrastructural details of the liposome surface are hardly visualized using this technique.

* Corresponding author. Tel.: +39 081678666.
E-mail address: gderosa@unina.it (G. De Rosa).

0378-5173/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2008.06.003

Table 1
Characteristics of nanosized and giant liposomes

Liposome formulations		Mean diameter (nm)		Zeta potential (mV)
Composition of lipid bilayer	Weight ratios between lipids	Extruded liposomes	Giant liposomes	
EPC/Chol/PG	10:4:0.1	215.4	2222.2	−45.1 ± 1.2
EPC/Chol/PG/DSPE-PEG2000	10:4:0.1:2.5	189.6	2248.7	−22.0 ± 1.1

In the present paper, we have investigated the potentiality of cold field emission gun-scanning electron microscopy (cFEG-SEM) to acquire ultrastructural images of the liposome surface. Furthermore, considering the high level of sharpness that can be reached in cFEG-SEM at high magnifications, we have also tested the resolution and sharpness of the images by comparing the surface morphology of PEGylated and not-PEGylated liposomes.

Cholesterol (Chol) was purchased from Sigma-Aldrich (Milan, Italy). Egg phosphatidyl choline (EPC) and phosphatidylglicerol (PG) were kindly provided by Lipoid GmbH (Cam, Switzerland). Analytical grade chloroform and methanol were supplied by Carlo Erba Reagenti (Milan, Italy). Glutaraldehyde and osmium tetroxide (OsO_4) were provided by TAAB Laboratories Equipment Ltd. (Berkshire, UK). Giant and nanosized liposomes, PEGylated and non-PEGylated, were prepared by a modified hand-shaking method (Bangham et al., 1965). The lipid mixture composed of EPC/Chol/PG (10:4:0.1 weight ratio) or EPC/Chol/PG/DSPE-PEG 2000 (10:4:0.1:2.5 weight ratio) was dissolved in chloroform/methanol (2:1 v/v); the solution was introduced into a 50-ml round-bottomed flask and the solvent was removed in a rotary evaporator (Laborota 4010 digital, Heidolph, Schwabach, Germany) until a lipid film formed on the wall of the flask. The lipid film was hydrated with 2 ml of 0.22 μm filtered distilled water and the resulting suspension was gently mixed in the presence of glass beads until the lipid layer was removed from the glass wall. The flask was then attached to the evaporator again, rotated at room temperature for about 30 min, and left at room temperature for 2 h to allow the formation of giant liposomes. To prepare nanosized liposomes, an aliquot of each formulation was extruded 10 times through polycarbonate filters with 400 and 200 nm pore sizes (Nucleopore Track Membrane 25 mm, Whatman, Brentford, UK). The size of the liposomes was measured by photon correlation spectroscopy (N5, Beckman Coulter, USA) and laser light scattering (LS 100Q, Beckman Coulter). The zeta potential of all formulations was measured in water by means of a Zetasizer Nano Z (Malvern, UK). For cFEG-SEM analysis, samples were prefixed in a mix of 4% formaldehyde and 1% glutaraldehyde in distilled water for 1 h. The quick fixation rate of 4% formaldehyde, coupled with the high fixation efficiency of glutaraldehyde, was necessary to partially harden liposome membrane, thus preserving vesicle integrity. Then, samples were rinsed in distilled water by ultracentrifugation (35,000 rpm) and post-fixed by adding 1% OsO_4 for 1 h to assure a full hardening of liposome membranes. After a further washing with distilled water, pellets were filtered on a polycarbonate filter (0.1 μm) (Nucleopore, Costar Corning, USA) in a Swinnex filtration apparatus (Millipore, USA). A second Nucleopore filter was placed over the first to form a sandwich in which liposomal pellets were trapped. Samples were then dehydrated in a graded alcohol series (10, 30 and 50% for 10 min, 70 and 80% for 30 min, 95% for 1 h and 100% overnight at 4 °C) and critically point dried. At the end of the treatment, the Swinnex was opened and both filters were placed on a stub cleaned with acetone to remove any grease. Double adhesive carbon disks (EMS, USA) were stuck onto the stub, and the filters containing the samples were placed over it. Finally, the stubs were sputter coated with a nanometric layer of platinum. The use of platinum as coating conductive metal, instead of the routinary gold–palladium, was mandatory to avoid over-coating of the morphological details of the liposome surface. Observations were carried out by a cold cathode Field Emission Gun Scanning Electron Microscope (FEG Jeol 6700F, Jeol Ltd., Japan). The pictures of the liposome surface at higher magnifications were taken at 2–5 kV. These low values of acceleration voltage improved the efficiency in the collection of secondary electrons from the surface of the samples. In addition, the low voltage of the acceleration avoided structural damages of the liposome surface that could be generated by the electron beam during the observation at high magnification. The reduction of the secondary electron collection from the samples generated by the low kV level of primary electron beam was overcome by the high efficiency of a cold inLens secondary electron detector. Low-magnification images of liposomes were obtained with a standard secondary electron detector.

In Table 1, liposome characteristics are reported. For both nanosized and giant liposomes, the mean size was not significantly different between PEGylated and non-PEGylated formulations. The increase in zeta potential of the liposomes, from about −45 to −22 mV, provided the first evidence of surface modification when using DSPE-PEG in the lipid mix (Table 1). Fig. 1a–d shows cFEG-SEM images of nanosized non-PEGylated and PEGylated liposomes. In the case of the formulation prepared without DSPE-PEG, liposomes appear as irregularly shaped particles with a rough surface (Fig. 1a). Acquisitions performed at very high magnification of the same sample (about 100,000×) confirmed this morphology (Fig. 1b). On the other hand, liposomes containing DSPE-PEG were more spherical and presented a smooth surface (Fig. 1c–d). This difference could be ascribed to the presence of DSPE, a phospholipid with fully saturated fatty acid chains, which should improve membrane rigidity, thus leading to more regularly shaped vesicles.

Since in the case of nanosized liposomes, it was not possible to investigate in detail the ultrastructure of the external surface, or to evaluate the influence of PEGylated lipids on its appearance, we observed giant PEGylated and non-PEGylated liposomes by cFEG-SEM (Fig. 1e–i). Liposomes appeared as spherical particles with a surface morphology that was dependent on the presence or absence of PEG. Indeed, liposomes prepared without DSPE-PEG were characterised by a regular and only slightly wrinkled surface (Fig. 1e and f). In the case of PEGylated liposomes, the surface was very irregular and the particle outline seemed to be “downy”. This shell should be due to PEG being bounded to DSPE and exposed, because its hydrophilicity, to the polar environment of the external medium (Fig. 1g–i).

In conclusion, cFEG-SEM provided the first 3D images of liposomes and of their surface ultrastructure. Furthermore, in the case of giant vesicles, the liposome surface morphology was found to differ greatly depending on the presence or absence of PEG on the lipid membrane. Although in the case of nanometric particles, surface details are still not clearly visible, the use of cFEG-SEM opens new horizons in liposome characterization, providing a unique tool to observe the liposome surface. Clear information on the ultrastructural details of liposome surface could be very useful for multiple purposes. Firstly, this new technique can allow to evidence morphological changes of liposome occurring by varying lipid composition or when species of different nature, i.e. hydrophilic polymers, ligands, monoclonal antibodies, etc., are introduced on the liposome surface. Moreover, morphological changes occurring during

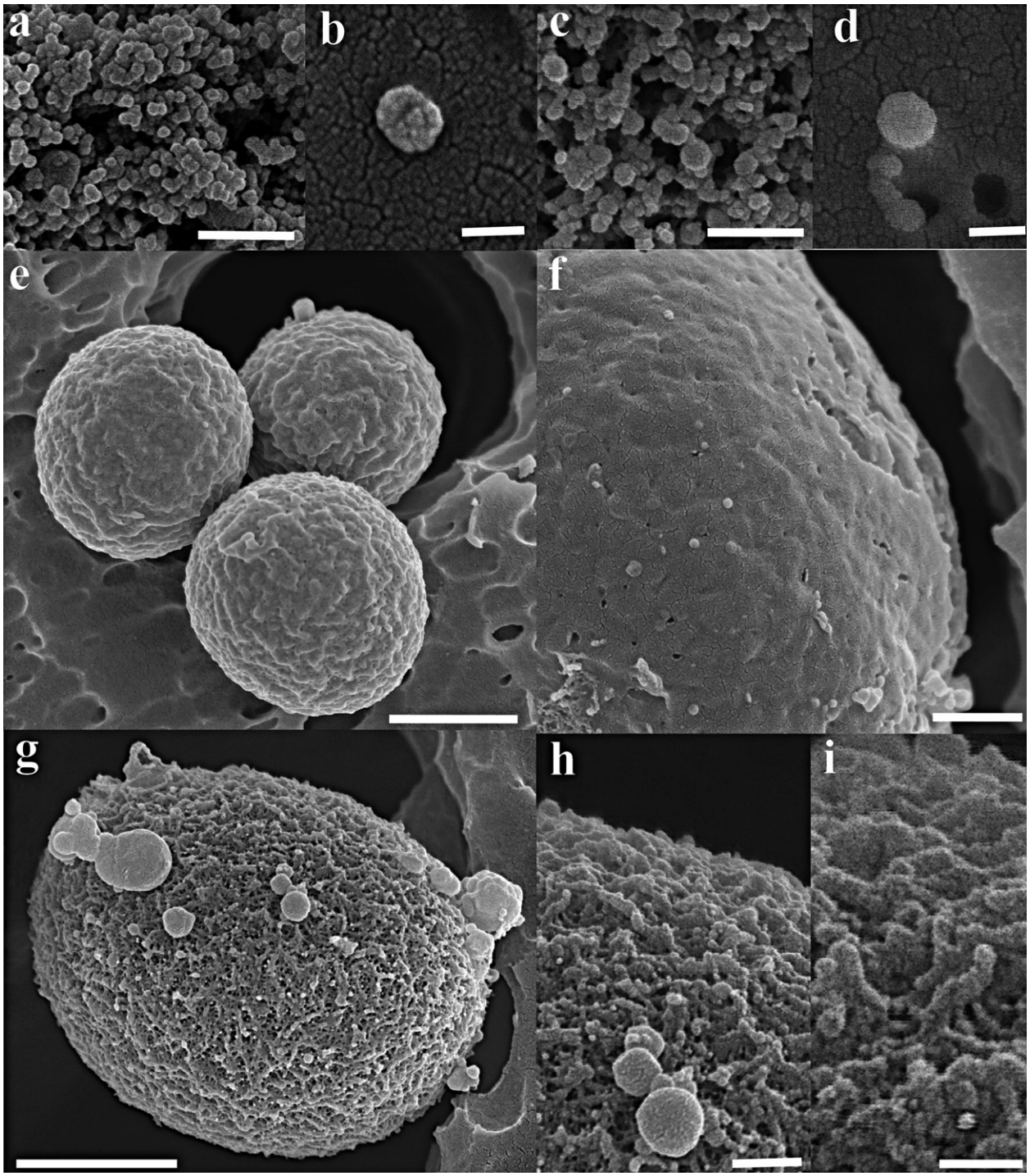

Fig. 1. cFEG-SEM images of liposomes. (a) Nanosized non-PEGylated liposomes at low magnification; scale bar = 0.5 μm. (b) Nanosized non-PEGylated liposomes at high magnification; scale bar = 100 nm. (c) Nanosized PEGylated liposomes at low magnification; scale bar = 0.5 μm. (d) Nanosized PEGylated liposomes at high magnification; scale bar = 100 nm. (e) Giant non-PEGylated liposomes; scale bar = 0.5 μm. (f) Detail of a giant non-PEGylated liposome surface; scale bar = 100 nm. (g) Giant PEGylated liposomes; scale bar = 1 μm. (h and i) Surface details of giant PEGylated liposomes at higher magnifications; scale bars: 200 and 100 nm, respectively.

storage/incubation in different conditions could be monitored by this technique, thus providing additional information on the physical and chemical stability of the liposome suspension. Finally, cFEG-SEM could be a new and powerful tool to study the formation of complexes between liposomes and macromolecules such as proteins and DNA.

Acknowledgements

The authors wish to thank Franco and Gennaro Iamunno for electron microscopy technical assistance. Thanks are also due to Rick Wehr who offered constructive comments and linguistic assistance for the final version of the manuscript.

References

Allen, T.M., Chonn, A., 1987. Large unilamellar liposomes with low uptake into the reticuloendothelial system. FEBS Lett. 223, 42–46.

Anabousi, S., Laue, M., Lehr, C.M., Bakowsky, U., Ehrhardt, C., 2005. Assessing transferrin modification of liposomes by atomic force microscopy and transmission electron microscopy. Eur. J. Pharm. Biopharm. 60, 295–303.

Bangham, A.D., Standish, M.M., Watkins, J.C., 1965. Diffusion of univalent ions across the lamellae of swollen phospholipids. J. Mol. Biol. 13, 238–252.

Gabizon, A., Papahadjopoulos, D., 1988. Liposome formulations with prolonged circulation time in blood and enhanced uptake by tumors. Proc. Natl. Acad. Sci. U.S.A. 85, 6949–6953.

Garg, A., Kokkoli, E., 2005. Characterizing particulate drug-delivery carriers with atomic force microscopy. IEEE Eng. Med. Biol. Mag. 24, 87–95.

Hope, M.J., Wong, K.F., Cullis, P.R., 1989. Freeze-fracture of lipids and model membrane systems. J. Electron Microsc. Tech. 13, 277–287.

Mohammed, A.R., Weston, N., Coombes, A.G., Fitzgerald, M., Perrie, Y., 2004. Liposome formulation of poorly water soluble drugs: optimisation of drug loading and ESEM analysis of stability. Int. J. Pharm. 285, 23–34.

Ruozi, B., Tosi, G., Forni, F., Fresta, M., Vandelli, M.A., 2005. Atomic force microscopy and photon correlation spectroscopy: two techniques for rapid characterization of liposomes. Eur. J. Pharm. Sci. 25, 81–89.

Sommerville, J., Scheer, U., 1986. Electron Microscopy in Molecular Biology: A Practical Approach. Oxford University Press, USA.

Torchilin, V.P., 2005. Recent advances with liposomes as pharmaceutical carriers. Nat. Rev. Drug Discov. 4, 145–160.