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RESEARCH ARTICLE

Morphological evaluation of liposomal iron carriers

Michela Battistelli | Sara Salucci | Elisabetta Falcieri

Department of Biomolecular Sciences (DiSB), Urbino University Carlo Bo, Urbino, Italy

Correspondence
Michela Battistelli, Campus Scientifico, Via Ca' Le Suore 2, 61029 Urbino (PU), Italy.
Email: michela.battistelli@uniurb.it

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Riccardi B.; De Paoli T.

Abstract

Iron is one of the most important elements for human, because it plays an essential role in many metabolic processes. However, it is also recognized to be dangerous for its detrimental effect inside human cells, where, in the absence of homeostatic balance, it can induce free radicals formation. Moreover, an excessive accumulation of iron in tissues can produce iron overload, a condition incompatible with life. The use of liposomes as carriers can represent an interesting iron therapy to improve iron bioavailability and reduce its negative effects, in particular during pregnancy. In this study, a morphological analysis has been performed on commercial liposome vesicles at various drying times, both in saline solution and in distilled water. Furthermore, to highlight their possible interaction or internalization in cells, liposomes have been administered to human hemopoietic U937 cells. Ultrastructural analyses confirm that vesicle morphology and size are comparable with classical liposomal structures. Products are stable during specimen preparation and drying. Additionally, they have a good ability to penetrate into cells, interacting with cytoplasmic organelles, without inducing, at least apparently, any ultrastructural damage.

KEYWORDS

electron microscopy, iron, liposomes, nanotechnology, U937 cells, uptake

1 | INTRODUCTION

Iron deficiency is one of the most frequent health problems in the world, affecting approximately one third of the entire population. A high request for nutrients, especially iron, occurs during gestation to cover the losses of basal iron, the increase in maternal red blood cells and to supply the growing fetus and the placenta (Haider & Bhutta, 2017). This request may not be satisfied with a regular diet due to low iron bioavailability.

The imbalance between supply and demand is the most frequent cause of Anemia, particularly in developing countries. The integration with iron is considered a global economic and effective strategy for prevention and control of Anemia during pregnancy, with consequent reduction of maternal–infant morbidity and mortality (Kamau, Mirie, & Kimani, 2018).

Nanotechnology is a new approach that has obtained, in recent years, an excellent development. It has been used for many industrial applications to enhance the bioavailability of certain micronutrients in the body (Vivek et al., 2010).

In particular, in the biomedical area, nanotechnology is intensively studied to improve the results of detecting and treating the most common pathologies (Tamjidi, Shahedi, Varshosaz, & Nasirpour, 2014). This approach is also used to develop a new type of iron supplementation, with high absorption efficiency, and minimal toxicity.

Liposomes (Tamjidi, Shahedi, Varshosaz, & Nasirpour, 2013) are vesicular structures with double lipid membrane, which are proved to be particularly interesting in the biomedical field as a delivery system of pharmaceutically active substances (Keshari et al., 2014; Mikhaylov et al., 2011; Stuchliålk & Žaålk, 2001). They reveal a remarkable affinity and adaptability as carriers for biological systems and they can be easily produced and managed. The use of liposomes as carriers is significant for pharmacologically active substances that have a low therapeutic index (as some anticancer, antibiotics, etc), because they allow to reduce the concentration of the drugs and improve the bioavailability, with significant reduction of side effects (Cullen, 2001). Iron, widely used for dietary supplement production, is essential for human organism and in many metabolic processes. Nevertheless, it has numerous disadvantages related to its high reactivity with other organic molecules and to their strong tendency to produce free radicals, extremely harmful to living matter.

Lipotech has a 25 year experience in the production of liposomes as nutrient carriers for food and pharmaceutical industry (Lysionek et al., 2002a, Lysionek et al., 2002b). One of the first liposome products was

Biofer (Iron sulfate and Vitamin C in liposomes, patented in 1994 as SFE 171). It is widely used in food industry, as additive for iron supplementation of milk and dairy products (Boccio et al., 1995, 1996, 1997a, 1997b, Boccio, Zubillaga, Caro, Lysionek, Calmanovici, et al., 1998a, Boccio, Zubillaga, Caro, Lysionek, Gotelli, et al., 1998b; Boccio, Zubillaga, Lysionek, & Caro, 1999; Boccio, Zubillaga, Lysionek, Caro, & Weill, 2000; Gotelli et al., 1996; Zubillaga et al., 1996). Biofer shows a long shelf life and an easy range of storage temperature, between +8 and +15°C.

These favorable conditions enhance the use of Biofer and differentiate it from its "native" product, called in this study "Reference Sample."

Thanks to a drying treatment in a hot air stream, at a controlled temperature, a better product has been observed with a shelf life of more than 2 years and preservable at room temperature. It can be used as a food additive in the form of powder, or in the form of capsules and/or tablets for production of supplements.

Another technological development was achieved by encapsulating liposome with five nutrients: Iron sulfate, Vitamin C, Folic Acid, Vitamin B6, and Vitamin B12 (Boccio et al., 2000; Lysionek et al., 1998, 2000; Lysionek, Zubillaga, Salgueiro, Caro, & Boccio, 2001; Lysionec, Zubillaga, Salgueiro, Caro, Weill, et al., 2001; Uicich et al., 1999). The new product has been approved and registered with the Lifervit brand in 2014. Encapsulation of five nutrients in a liposome represents a significant technological progress, with the advantage of improving the bioavailability of waterborne substances and favoring erythropoiesis. Therefore, in addition to iron, other elements such as folic acid, vitamin B12, and vitamin B6 have been included into liposome. Liposomal encapsulation of the five nutrients in carriers also improves their assimilation by far.

Lifervit liposomes were dried and they can be used for the production of supplements in the form of capsules and tablets. In this study, ultrastructural analyses have been carried out to verify if liposome dehydration process produces structural alterations of lipid membranes, which could compromise carriers integrity and function. In particular, to check integrity of Biofer vesicles, both in liquid form and in dried powder, an analysis at transmission electron microscopy (TEM) has been conducted to verify whether the typical morphology of the liposomes in liquid form is maintained throughout the drying process. A further investigation was conducted to ascertain the liposome interaction with U937 cell line, a well-known phagocytosis human model. In this process, the relationships with cell membrane and organelles could be documented in detail.

2 | MATERIALS AND METHODS

2.1 | Liposome production

Biofer and Lifervit liposome production was carried out through the formation of homogeneous suspension of phosphoglycerides in water, rapid cooling of the suspension at −12°C and slow thawing at room temperature, insertion of iron sulfate (II) in suspension in aqueous solution including the reducing agent Vitamin C and other microelements (folic acid, and Vitamin B6, and Vitamin B12) and repetition of the cycle of freezing and thawing and subsequent separation of the liposomes formed.

The procedure used for the production of the liposomes (Biofer or Lifervit) started with the formation of a homogeneous suspension of phosphoglycerides in water, rapid cooling to −12°C and slow thawing at room temperature. It was followed by the insertion of iron sulfate (II) in aqueous solution and by that of the reducing agents Vitamin C and other microelements (folic acid, and Vitamin B6 and Vitamin B12) by the repetition of the cycle of freezing and thawing and by subsequent separation liposomes were formed.

Liposome vesicles obtained are not uniform in diameter. The uniformity required for various applications, especially in medicine and cosmetics, is obtained with subsequent treatments, by means of ultrasound and by filtration through selective membranes with pores. Once obtained liposomes in solution are subjected to drying in the crush air stream at a controlled temperature, to form a fine powder.

2.2 | Sample preparation

Samples were obtained both in liquid/gel form "native" liposomes (reference sample); both in dried form, by taking samples at various time intervals during the drying process. The samples were collected every 15 min from the beginning of the drying process. All specimens have been observed and, among them 0, 45, 90, and 180 min were chosen.

We followed two different solutions for samples preparation:

1. Isotonic solution, identical to that used in the production of liposomes, to maintain the internal and external osmolarity solution and preserve liposome integrity:

Isotonic solution is obtained by mixing the following ingredients: 208 g, ascorbic acid: 6 g, sodium ascorbate: 6 g, dissolved in 500 g of distilled water;

2. Distilled water, to assess the liposomal vesicle stability in hypotonic solution.

We used these two solutions (isotonic solution and distilled water) to dissolve both liquid and powder liposomes. This procedure has been carried out only for Biofer, useful to verify liposome stability. The same liposomes have been employed in Lifervit preparation, even if this product contains inside different elements.

2.3 | Negative staining

Biofer liposomes were adsorbed to formvar–carbon coated 200 mesh grids (Agar Scientific Ltd) for 2 min, and briefly rinsed in filtered PBS. Sample on grids were immediately negatively stained with 2% (wt/vol) Na-phosphotungstate for 1 min. The observations were carried out by means of a transmission electron microscope at 80 Kv (Guescini et al., 2010).

2.4 | Cell culture

Human myelomonocytic cell line U937 was grown in RPMI 1640, supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 1% antibiotics, and was maintained at 37°C in humidified air with 5% CO_2 (Salucci et al., 2014). Cell behavior was monitored by means of Inverted Microscopy (Eclipse TE2000-S Nikon; objective

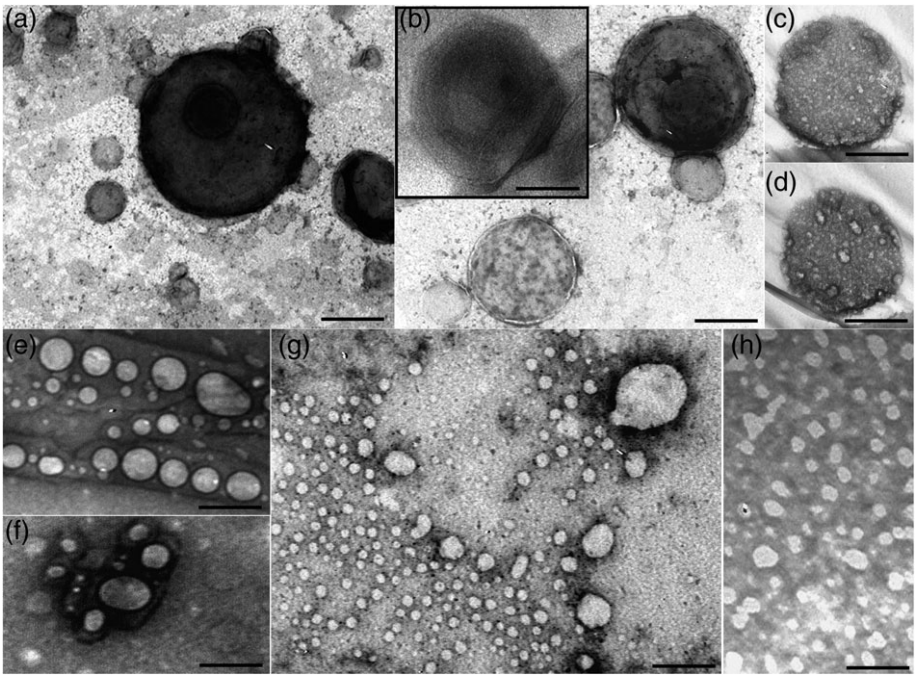

FIGURE 1 Morphological analysis of Biofer liposomes in saline solution at different drying times. In reference sample (a, b) and at time 0 (c, d) the vesicle size range are between 1.0 and 2 μm. After 45 min (e, f) or 90 min (g) of drying, vesicle size appears reduced to 200–300 nm and after 180 min (h) their size is further reduced to a 40–50 nm. a, b, inset b–h, Bar = 200 nm

10×). Different concentrations and administration times were evaluated (data not shown). Twenty-four hours of treatment and a concentration of 33 mg/ml, appear satisfactory for Biofer and Lifervit internalization. In addition, treated U937 cells were processed for TEM.

2.5 | Transmission electron microscopy

Samples were washed and immediately fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer for 1 hr, post fixed with 1% OsO_4 in the same buffer for 2 hr, alcohol dehydrated, and embedded in araldite, as previously reported (Battistelli et al., 2014). Thin sections were collected on 400 mesh nickel grids, stained with uranyl acetate and lead citrate, and analyzed with an electron microscope Philips CM10 at 80 kV.

3 | RESULTS AND DISCUSSION

The first morphological analysis was performed on liposome samples at various drying times both in saline solution (Figure 1) and in distilled water (Figure 2). Samples have been processed for negative staining, a technique that allows to highlight the cell membranes, by coloring the outside, if the membrane is intact, and leaving the lighter interior.

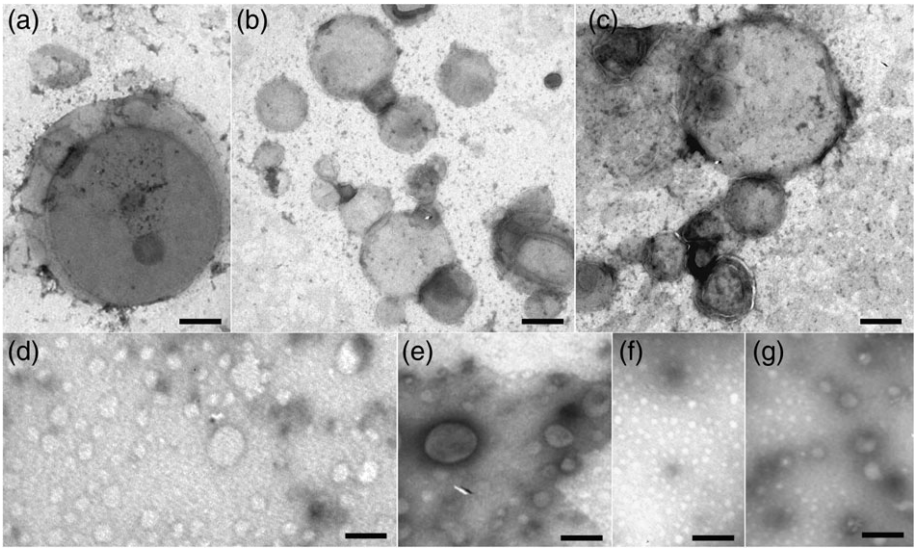

FIGURE 2 Morphological analysis of Biofer liposomes in distilled water at different drying times. a, b = reference sample; c = 0 min; d = 45 min; e = 90 min, f = 180 min. A comparable size reduction can be observed in this condition. a–g, Bar = 250 nm

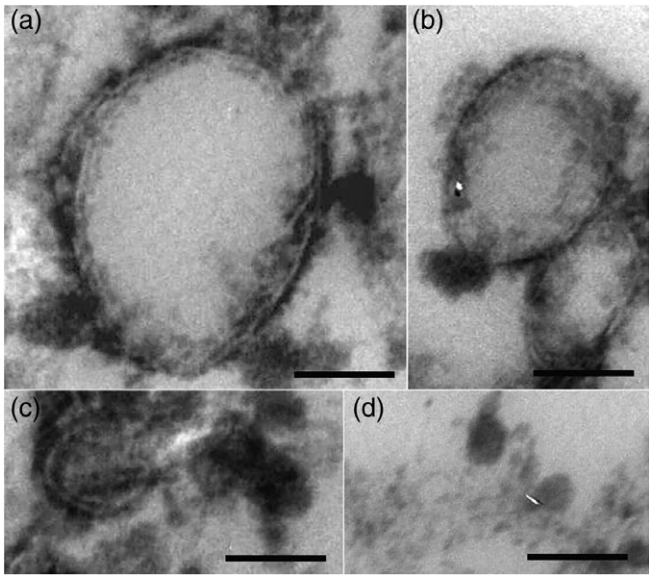

FIGURE 3 TEM of Biofer vesicles at time 0 (a), and after 45 min (b), 90 min (c), and 180 min (d) of drying time. a–d, Bar = 250 nm

These observations permitted to characterize liposomes and demonstrated their stability during the drying process, comparing them to the reference sample, the original product.

In Figures 1 and 2, it is possible to observe the reduction in size of the vesicles during the drying process. In reference sample (Figures 1a,b and 2a,b) and at time 0 (Figures 1c and 2c) the size of the vesicles ranges from 1 to 2 μm. In addition, well preserved double membranes can be observed in Reference sample (Figures 1a,b and 2a,b) and at time 0 (Figures 1c,d and 2c). In both condition, frequently, it is possible to observe several membranes associated to the single vesicles, probably suggesting their fusion or disaggregation in smaller particles (Figures 1a–d and 2a–c).

After 45 min (Figures 1e,f and 2d) or 90 min (Figures 1g and 2e) of drying, vesicle size reduced to 200–300 nm and after 180 min (Figures 1h and 2f,g) of drying liposomes with a 40–50 nm size can be observed. To better evaluate liposome membrane integrity the samples have been processed for TEM analysis (Figure 3a–d) that evidenced the presence of preserved liposomes with double membrane. The large size liposomes showed numerous membranes within the same vesicle.

After liposome morphological analysis, a further investigation was conducted to evaluate the liposome interaction with U937 cell line, a well know phagocytotic human model. Biofer (Figure 4) and Lifervit (Figure 5) can easily penetrate inside of cells. To exclude possible artefacts due to the staining/contrast of thin sections the analysis was carried out on unstained and stained sections.

Biofer appears as a electron-dense agglomeration both in unstained (Figure 4a,b, inset) and stained sections (Figure 4e, inset). We can clearly identify the single vesicles near plasmatic membranes or inside cytoplasm. Moreover, within the cytoplasm, we can often observe the Biofer liposomes enclosed within vacuolar structures (Figure 4c–h, arrows).

Therefore, Biofer diffusely localizes outside the cell and near cell membranes, where it forms electron-dense clusters of different sizes (Figure 4a–c,e–h). In the inset in Figure 4a,b,e, it is possible to appreciate liposome aggregates near the plasmatic membrane at high magnification. Moreover, Biofer can be internalized in the cellular cytoplasm through endocytosis (Figure 4c–h). Sometimes, inside the cell, Biofer liposomes seem to release, the transported material into the cytoplasm (Figure 4b arrowhead). To evaluate the possible diffusion from the vacuole to the cytoplasm it may be necessary to prolong the culture time. Further study are in progress to evaluate internalization process.

Lifervit formes electron-dense agglomerates of greater size if compared to Biofer. This compound shows membrane and vesicles well preserved and for that easily distinguishable at TEM, at low magnification too (Fig. 5a,b,h).

Large endocytic vesicles incorporated Lifervit (Figure 5d,f and inset, g) then released it into the cytoplasm (Figure 5c,e,f). In this case, as appeared in Figure 5, a high amount of product penetrated inside

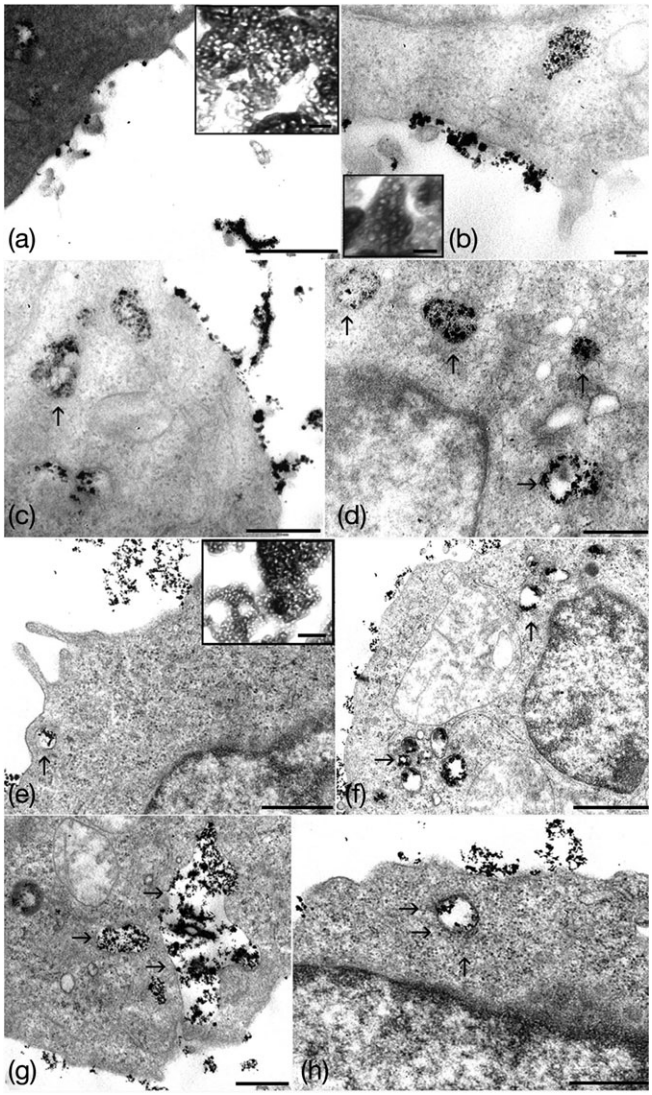

FIGURE 4 TEM U937 cells incubated with Biofer liposome. Biofer vesicles appear as a electrondense agglomerations both in unstained (a–d, inset a, b) and stained samples (e–h, inset e). We can observe liposomes near the cellular membrane (a, b, c, e, f, g, h) and, mostly, inside the cell in small vesicles (c, d, f, h). a, f, Bar = 1 μm; b, c, d, e, g, h, Bar = 500 nm; inset a, b, e, Bar = 500 nm

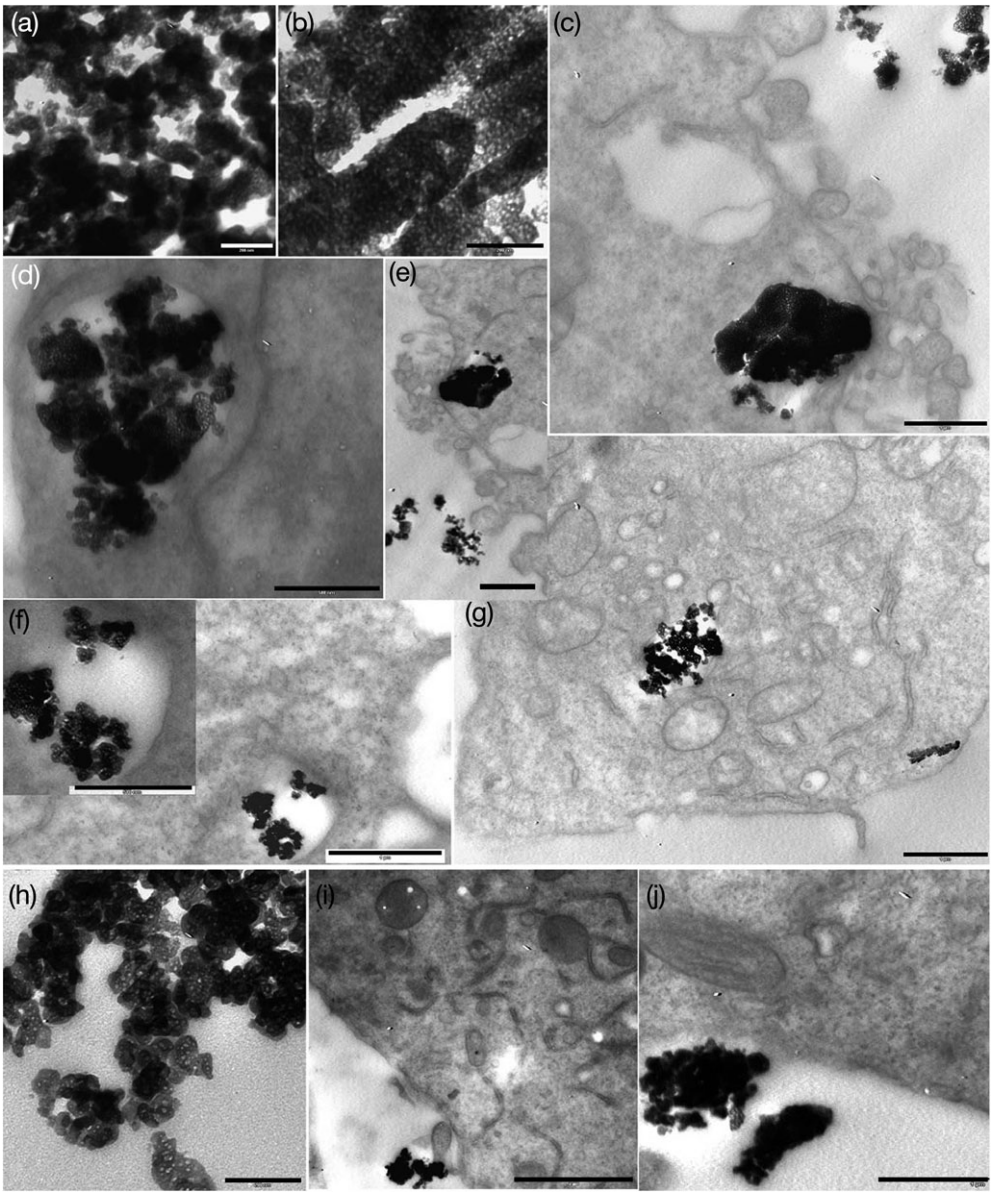

FIGURE 5 TEM of U937 cells incubated with Lifervit liposome: Unstained (a–g) and stained (h–l) sample are observable. Lifervite liposomes appear as electron dense agglomerates (a, b, h), near cellular membrane (e, i, l) or inside large vacuoles (c, d, e, f, inset f, g). a, b, Bar = 200 nm; c, e, f, g, l, Bar = 1 μm; i, Bar = 2 μm; h, d, inset f, Bar = 500 nm

the cells and appears as free aggregates in the cytoplasm or internalized in vacuoles. After both carrier administrations, cellular ultrastructural morphology appears well preserved, suggesting the absence of toxicity effects. A good preservation of nuclear membrane and diffuse nuclear chromatin can be observed; moreover, a high amount of free ribosome, that demonstrated a good metabolic cellular activity, appeared.

4 | CONCLUSION

Considered together, our results demonstrate that vesicle morphology and size are compatible with liposomes according to Tamjidi et al. (2014). Liposomes show a typical spherical shape and a size comprises between 100 nm and 1 μm. Both the samples, in liquid form (reference sample), and those dried, maintain the spherical shape, suggesting that the two procedures applied do not alter their morphology.

Biofer and Lifervit are easily internalized by U937 cells. At the same inoculation amount, Lifervit penetrates with different modality if compared to Biofer. In fact as appeared after ultrastructural observations, it forms electrondense aggregates of greater diameter that appear inside large vacuoles. Biofer and Lifervit do not induce cellular damage, and for that, they can be considered potential candidates for the iron vehicolation in agreement with the current literature. Further studies are in progress to evaluate their interaction with intestinal cell models.

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CONFLICT OF INTEREST

The authors declared no potential conflicts of interest with respect to the research, authorship, and publication of this article.

ETHICAL APPROVAL

This article does not require ethical approval.

ORCID

Michela Battistelli http://orcid.org/0000-0003-4028-0652
Sara Salucci http://orcid.org/0000-0002-3507-4451

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