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Liposomes containing nanoparticles: preparation and applications

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Abstract: The impetuous development of nanotechnology over the past two decades has enabled the production of a plethora of nanomaterials with outstanding optical, magnetic, electrical, catalytic and mechanical properties. The versatility of these materials attracted attention from the very beginning in the most disparate sectors of science and technology. The application of nanomaterials in the biological and biomedical fields soon benefited from the interaction with liposomes, which increased their biocompatibility and biostability. Liposomes indeed are versatile self-assembling supramolecular (nano)structures constituted of an aqueous core enclosed by a lipid bilayer, able to host hydrophobic and hydrophilic cargo, and with superior biocompatibility and great similarity with the biological membranes. The result is the construction of hybrid nanoscale architectures, in which nanoparticles (NPs) are allocated either in the aqueous core, in the palisade of the lipid bilayer or on the outer surface of the vesicles. In the first part of this review, the principal methods for the preparation of NP-loaded liposomes are carefully illustrated in a tutorial manner. In the second part, an overview of the great potentialities deriving from the conjugation of liposomes with NPs is presented. In each paragraph, the main characteristics of the most notable classes of NPs, the related issues, and the advantages arising from their association with liposomes are shown. Here, the most significant research works in literature for each kind of system are presented.

Keywords: hybrid liposome-nanoparticle; hybrid liposome-nanoparticle preparation; metallic NP loaded liposome, QD loaded liposome, silica NP loaded liposome, carbon NP loaded liposome.

Highlights:

- Current methods for incorporating NPs into liposomes are illustrated.
- The advantages of incorporating NPs into liposomes are discussed.
- A tutorial section for non-expert researchers in the field is included.
- An updated review of the main hybrid NP-liposomes and their applications is shown.
- The impacts in the field of innovative therapies, theranostics and sensing are shown.

Statistical summary of the article:

- total number of words (bibliography excluded): 10,578
- total number of words (including references): 16,657
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1. Introduction

In 1964 A. D. Bangham and R.W. Horne published for the first time electron microscopic images of phospholipid vesicles [1]. Since that moment, an impressive succession of research advancements and discoveries has made liposomes, similar in composition and structural organization to cell membranes, protagonists of numerous sectors of basic and applied research.

Liposomes are supramolecular aggregates formed by amphiphilic components, such as polar lipids, dispersed in an aqueous solution. These building blocks form bilayer structures organized in closed, spherical vesicles. Liposomes have an aqueous core and therefore possess core-shell structures that enable the encapsulation of both hydrophilic and hydrophobic molecules. Liposomes are relatively easy to prepare and, by modulating their composition, size, and degree of lamellarity, they can be adapted to several experimental and application needs. Furthermore, liposomes can be decorated with a great variety of molecules, acquiring specific properties that soon expanded the range of possible applications. By virtue of their peculiar characteristics, liposomes have been applied to the most diverse fields ranging from the administration of drugs and other bioactive molecules to the development of vaccines [2-5], from the study of protocells and the origin of life to the reconstitution of proteins membrane [6, 7]. Their use as micro / nanoreactors, sensing agents and pollutant removal systems is also reported [8, 9]. If the first generation liposomes suffered from important limitations (lack of stability, payload leakage etc), the extraordinary properties of these vesicles soon benefited from the integration with various natural and synthetic polymers to give rise to lipid-polymer hybrid structure, with improved performances in the most disparate contexts, mainly in the field of drug and diagnostic probe delivery [10]. In fact, liposomes are excellent carriers of drugs and molecular imaging probes for in vivo applications. Liposomes with a diameter of less than 200 nm are characterized by large blood circulation capacity and lead to increased accumulation of their payload at target tumor/inflamed tissues through passive targeting by enhanced permeation and retention (EPR) effect. In addition, active targeting can be achieved by modifying the vesicle surface with antibodies or other suitable targeting ligands [11].

Liposomes can also be loaded with nanoparticles (NPs) of various nature and size, thus extending the field of application of this class of materials with extraordinary characteristics and properties that have dominated the last decades of research [12]. The high surface area and quantum confinement effects give rise to new and unexpected physical and chemical properties in these materials. Due to their tunable properties (optical, magnetic, catalytic…), dependent on their size and shape as well as on the intrinsic properties of the constituent elements, NPs have found a plethora of applications in the fields of medicine, imaging, catalysis, energy-based research, sensing and environmental applications as well. Although there is no general consensus on the definition of nanomaterials, they are usually described as materials that have at least one dimension in the range of 1 to 100 nm. In 2011 the European Commission adopted indeed a definition of nanomaterial, describing them as *materials in an unbound*

state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm - 100 nm [13]. It follows that even the liposomes fall within this definition as long as they are prepared with certain dimensional characteristics and the hybrid structures made with various NPs are themselves nano-composite nanomaterials. The main applications of hybrid liposome-NP complexes have been proposed in the field of drug delivery, theranostic and sensing. The integration of nanomaterials into the structure of liposomes is motivated by the need to overcome some of the limitations of first-generation liposomes and / or add new desired properties to them. On the other hand, incorporating nanomaterials into the structure of liposomes is often a valid way to reduce their toxicity and in general increase their compatibility with biological milieus. What results is the emergence of new features and new application possibilities for these hybrid structures by virtue of a synergistic effect of the two constituent partners (Table 1).

Table 1. Mutual advantages which can derive from the integration of liposomes with NPs.

What nanoparticles can do for liposomes	Stabilize the liposomes with respect to fusion (avoid intra-vesicle fusion, unwanted) or on the contrary initiate fusion (favor vesicle-cell membrane fusion, desired) in response to a stimulus	**What liposomes can do for nanoparticles**	Making the nanoparticles soluble in water, preserving their peculiar "as synthesized" properties
	Avoid the premature release of the payload, modulate it, or initiate it in response to a stimulus (light, magnetic field, pH variation…)		Increase their biocompatibility
	Increase the carrying capacity of a drug, a biomolecule or another biologically active payload		Prevent the aggregation of nanoparticles in biological fluids and reduce recognition by the immune system
	Make the vesicles fluorescent, magnetic etc.		Offer an easy way to enter the cell by acting as a trojan horse

Although other nanostructured systems capable of incorporating NPs have been developed, liposomes remain highly attractive considering a series of undoubted advantages. Compared to polymer-NPs for example, liposomes have a good ability to carry hydrophilic and hydrophobic molecules and NPs at the same time. They have excellent biocompatibility and can effectively promote the diffusion of their payload across the plasma membrane due to their peculiar composition and structural organization. In addition, liposomes also have the advantages of easy modification and targeting potential, which could be implemented by modifying the surface with appropriate molecules to actively bind a target on specific cells or tissue. However, due to the limited bilayer thickness of liposomes, stable loading of hydrophobic NPs is limited to dimensions of approximately 4-5 nm. Compared to polymer-NPs, liposomes are generally less stable and have controlled release properties that are more difficult to

control. Nevertheless, the loading capacity and the encapsulation rate of polymer-NPs are lower, and due to the high molecular weight of polymers, polymer NPs can easily induce immune response [14]. Therefore, liposomes are often the first choice when carrying NPs in a biological environment.

The current review is focused on hybrid structures based on liposomes and synthetic NPs of a hard nature, such as metal NPs, semiconductor quantum dots, silica, carbon dots and carbon nanotubes. Compared to other previous works dealing with the same topic [15-17], our review aims to provide an updated and more general overview of the various liposome-NP complexes, taking into consideration materials and applications that have not been previously reviewed, such as uncommon metallic oxides, graphene, carbon nanotubes and carbon dots. Structures containing exclusively nanocrystalline drugs will not be considered, as this topic is covered by other specialized reviews [18]. Furthermore, only hybrids in which the structure of the liposome is strictly conserved (one or more closed lipid bilayers on an aqueous core) will be reviewed, and therefore will not be considered lipid bilayers supported on NPs. In the first part of the review there is a tutorial section where different methods of preparation of these hybrid structures will be discussed. This is a totally new and unprecedented section, which is proposed as a guide for the reader in choosing the most suitable method for the realization of the liposome-NP complexes of interest, depending on the characteristics of NPs to be embedded and the type of application envisaged. In the second part of the review different categories of NPs embedded in hybrid vesicles and their main characteristics and applications will be shown, focusing on the most recent publications without however neglecting the basic works for a simple and complete understanding by the reader.

2. Preparation Methods for Hybrid Vesicles

There are several methods for the preparation of liposomes and all of them can be adapted to incorporate NPs. Each method has its own peculiarities, leading to the formation of vesicles of different sizes, degree of lamellarity and vesicularity, polydispersion, etc. Figure 1 shows the structures and size range of the most commonly obtained vesicles to incorporate NPs and discussed in this review. Since the characteristics of the liposomes greatly influence their field of application and their performances, the method used for the preparation must be carefully evaluated. Furthermore, some preparation methods are more scalable than others, or are more suitable for the incorporation of hydrophilic rather than hydrophobic NPs.

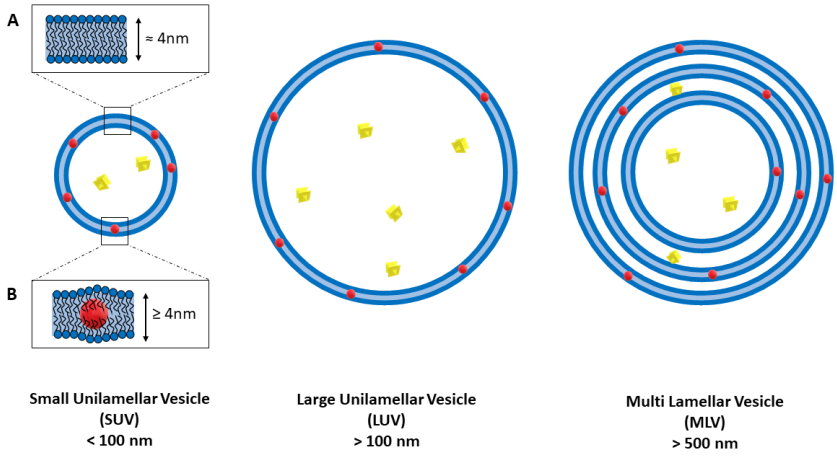

*Figure 1. Schematic illustration of the types of liposome-NP complexes discussed in this review and their size range. Hydrophilic NPs are represented in yellow, hydrophobic ones in red. (**A**) The thickness of a lipid bilayer of pure phosphatidylcholine is approximately 4 nm. (**B**) The lipid bilayer undergoes deformation to accommodate hydrophobic NPs of similar size to its native thickness. The dimensions of the various constituent elements are not to scale.*

NPs are usually incorporated into the liposomes during the assembly of the vesicles, a way often called passive loading. For the success of the incorporation, the dimensional affinity between the nanoparticle and the phase that will host it, should be evaluated. For example, the size mismatch between hydrophobic NPs and the thickness of the lipid bilayer of the liposomes can lead to a destabilization of the vesicle structure or to a lack of NPs incorporation. The thickness of a lipid bilayer of pure phosphatidylcholine (PC) is approximately 4 nm (Figure 1A) [19]. Ideally, NPs with a diameter below this threshold should be selected for a stable incorporation (Figure 1B), also considering the contribution of any capping agents on their surface. Still, the nature of the stabilizers on the surface of the NPs could have decisive effects for successful incorporation. For example, De Leo et al. found that the original capping of trioctylphosphine oxide (TOPO) on the surface of CdSe@ZnS quantum dots (QDs) was detrimental to the insertion of QDs into the lipid bilayer through the detergent depletion method. Conversely, alkyl thiols with different lengths of the carbon chain allowed for a successful QDs incorporation [20]. TOPO capping, on the other hand, proved to be compatible with insertion of CdSe@ZnS QDs into liposomes using the thin lipid film hydration protocol [21]. NPs can be also chemically conjugated to lipids and then incorporated into the liposome bilayer as a lipid building block. For this purpose it has been used for example 1,2-dipalmitoyl-sn-glycero-3-phosphothioethanol, a synthetic S–H-terminated phospholipid [20]. For the incorporation of hydrophilic NPs, the trapping yield depends on the volume of the aqueous core: a larger internal volume generally leads to higher encapsulation efficiency. In addition, to modulate the leakage of NPs, the properties of the bilayer should be optimized by appropriately choosing the lipid membrane composition, chain length of the phospholipid, superficial charge, etc.

NPs can be loaded even after liposome formation. In this way, rather than being incorporated into the vesicles, NPs decorate their external surface. One way to achieve this task is to exploit the electrostatic interactions between the nanoparticles and the lipid vesicle surface. Alternatively, NPs are functionalized with a molecular pendant equipped

with a hydrophobic anchor, capable of interleaving in the double layer, or with a functionality capable of interacting covalently with the hydrophilic end of a suitably functionalized phospholipid.

In any case, whichever method is chosen, it must be taken into account that size, type and concentration of embedded NPs affect the lipid packing, fluidity, and phase transition temperature of phospholipid bilayers [22, 23]. Therefore, a careful design and planning phase is necessary to obtain hybrid liposome-NP systems with the desired characteristics, avoiding unpleasant and frustrating experimental failures.

2.1. Thin-film hydration method

One of the most popular methods for the preparation of liposomes is the *thin-film hydration method*. It is an easy-to-perform method, requiring no special equipment and skills, and was the first to be adapted to the incorporation of both hydrophilic and hydrophobic NPs in liposomes [24-27]. As a first step, a solution of lipids in chloroform and/or methanol in a round bottom flask is dried to obtain a thin lipidic film adherent to the inner wall of the flask. This lipid film should be dried under vacuum for several hours to eliminate any trace of organic solvents, ideally until the weight of the dry film remains constant. Then, the film is hydrated with an aqueous solution or buffer under vigorous shaking to form liposomes by lipid swelling (Figure 2A). For lipids with T_m higher than room temperature, the thin lipid film and the buffer should be preheated above T_m before the hydration step.

Lipophilic NPs to be encapsulated in the liposome bilayer, can be dissolved in the lipid solution before the formation of the thin film. The lipid bilayer can distort to accommodate hydrophobic NPs with a diameter close to its thickness (Figure 1B), and this distortion reduces lipid ordering. This behaviour is similar to that reported for the accommodation of integral membrane proteins [20, 22]. Often, the NPs do not distribute uniformly in the bilayers: electron microscopy (EM) analyses return images of vesicles completely loaded with NPs, vesicles completely empty and others in which the NPs are grouped in limited portions of the bilayer. Thermodynamic evaluations of hydrophobic NPs insertion into the lipid bilayer have suggested that side by side association of NPs may reduce strained regions at the NP – lipid interface and void space around the NPs, thus minimizing the energy penalty to deform the bilayer due to NP insertion [28, 29].

Hydrophilic NPs to be trapped in the aqueous core of liposomes, can be dissolved in the buffer solution used for the hydration of the lipid thin film. Although the thin-film hydration method leads to low entrapment efficiencies, it is frequently used for loading with hydrophilic NPs due to its simplicity and reproducibility, despite its difficulty of scaling up.

With this method, multi-lamellar vesicles (MLVs) of different sizes are obtained. The size and degree of lamellarity of these vesicles can be reduced by subjecting these MLVs to subsequent treatments of resizing by sonication, extrusion, or freeze-thaw cycling. Sonication with a high-intensity ultrasonic horn leads to the formation of small unilamellar vesicles (SUVs). The extrusion method, that involves the forced passage of the MLVs through a thin polycarbonate membrane filter of desired pore size using an extruder, leads to the formation of SUVs or large unilamellar vesicles (LUVs). Freeze-thaw cycling is implemented not only to reduce the lamellarity of liposomes and form a less polydisperse system, but especially to increase the encapsulation efficiency within the aqueous lumen of the vesicles. The method consists in freezing the liposomes with liquid nitrogen and thawing at a temperature above

the phase transition temperature of the lipids for several cycles, causing fragmentation and reformation of the bilayer which thus encapsulates very efficiently the aqueous phase [30].

Although the loading of hydrophobic NPs can, within certain limits, be considered quantitative, an excess of hydrophilic NPs remains excluded from the liposome core and is found in the external solution. Surprisingly, many authors report no indications concerning the purification of these preparations, but a removal of the non-trapped material should always be accomplished by (ultra)-centrifugation, size-exclusion chromatography, or dialysis [31].

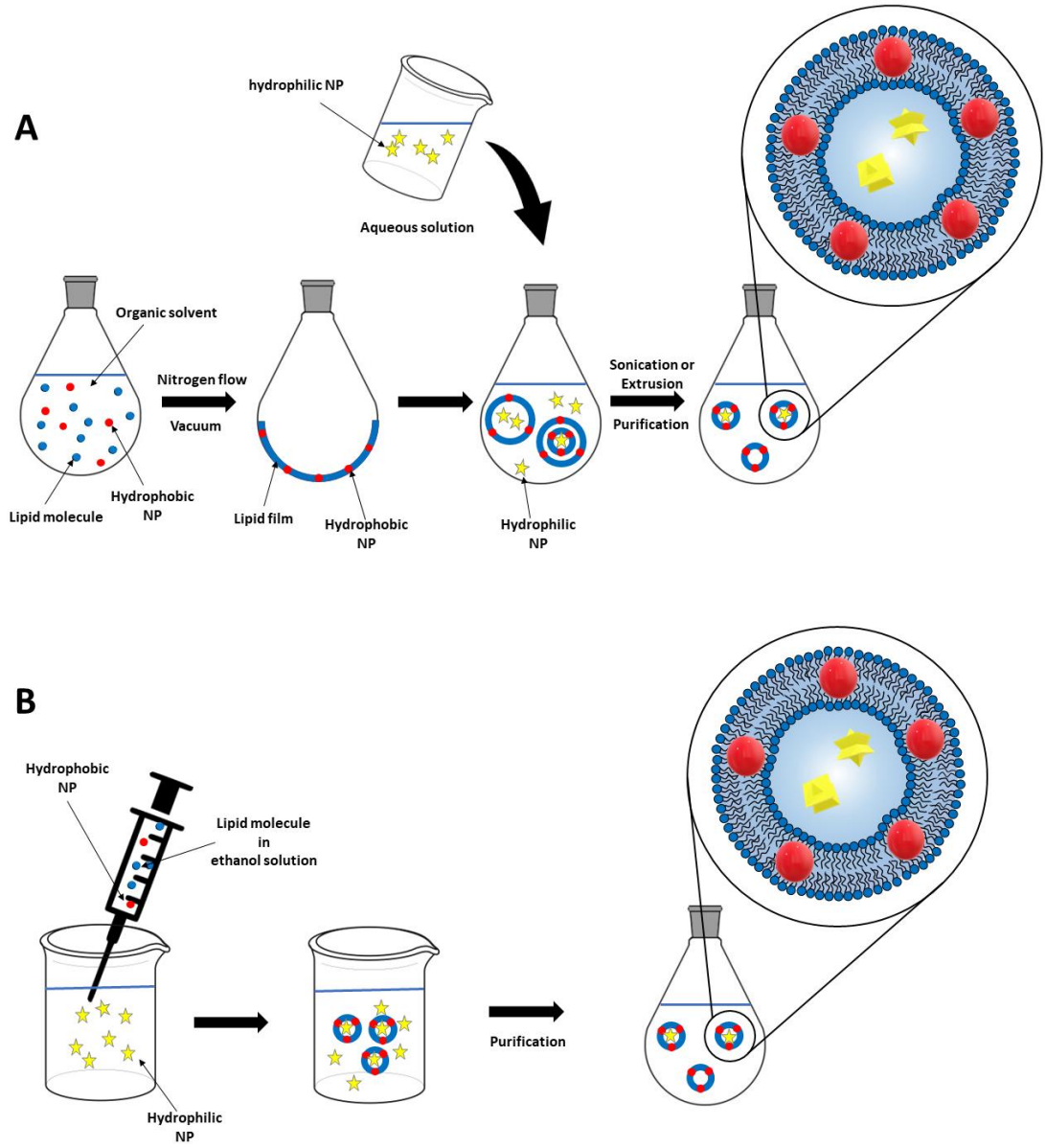

*Figure 2. (**A**) Schematic illustration of the thin-film hydration method for the preparation of liposomes (in blue) and the simultaneous encapsulation of hydrophobic (red spheres) and/or hydrophilic (yellow stars) NPs, followed by procedures of size reduction and purification. (**B**) Schematic illustration of the ethanol injection method for the preparation of NP-loaded liposomes and subsequent purification. Hydrophobic NPs (red spheres) are dispersed in ethanol with the lipid molecules (blue spheres) into the syringe, while hydrophilic NPs (yellow stars) are in water.*

2.2. Ethanol injection method

Ethanol injection method is the main alternative technique to the thin-film hydration method used to produce SUVs in a simple and rapid manner. The method belongs to the solvent injection techniques, in which a water-miscible organic solvent containing lipids is injected into a large amount of aqueous buffer. To prepare liposomes using this method, lipids are dissolved in ethanol and then are injected into a large volume of a water phase under vigorous stirring. After rapid dilution of lipid ethanol solution in water, the lipids first rearrange at the boundary phase between ethanol and water in the form of phospholipid bilayer fragments, and then these join together to form vesicles. Numerous parameters such as injection velocity, stirring rate, lipid concentration, and ethanol / water ratio, influence the process and determine the characteristics of the vesicles obtained and the encapsulation efficiency [32]. Hydrophobic NPs can be dissolved together with lipids in ethanol prior to injection in water, while hydrophilic NPs are dissolved in the aqueous receiving phase (Figure 2B).

Non-encapsulated NPs are removed by ultracentrifugation or size exclusion chromatography [33, 34]. Excess ethanol can also be removed from the liposome suspension either by rotary evaporation or by centrifugation through a silica gel column [35].

This method leads to the formation of SUVs with a better degree of monodispersity than the thin film hydration method. Therefore, resizing by extrusion or sonication is not necessary, with undoubted advantages in terms of speed of realization, cost-effectiveness and stability of lipids and encapsulated NPs. The method is also useful for the preparation of large quantities of liposomes on an industrial scale. However, both the lipids and the hydrophobic NPs that are desired for the realization of the hybrid vesicles may have a limited solubility in ethanol. Furthermore, the concentration of the obtained liposomes is generally low and the complete removal of traces of organic solvent can be difficult. This method is very frequently used for the incorporation of hydrophilic NPs in the aqueous lumen of liposomes, although the encapsulation efficiency is generally not high.

2.3 Reverse phase evaporation method

The reverse-phase evaporation technique is the most used among the liposome preparation methods that belongs to the category of emulsification methods, where an emulsion of water in organic phase (generally called water-in-oil emulsion) is formed. The lipid blend is dissolved in organic solvents, such as diethyl ether or isopropyl ether. A two-phase system is made after the addition of an aqueous solution, and a homogeneous dispersion is formed by sonication or vigorous shaking.

The water phase can be loaded with hydrophilic NPs while hydrophobic NPs are added to the organic phase together with the lipids for forming the liposome bilayer [24, 36, 37]. The organic solvent is at this point slowly evaporated under reduced pressure conditions. The emulsion is then converted into a viscous gel and subsequently into an aqueous suspension containing liposomes. The residual solvent can be removed by centrifugation, dialysis, or size exclusion chromatography [38]. Liposomes generated by the reverse-phase evaporation technique usually need to be reduced in size by a subsequent extrusion step (Figure 3A). This method is generally used to encapsulate hydrophilic NPs inside the aqueous lumen of vesicles since, compared to other preparation techniques, it allows to obtain higher NP entrapment efficiencies [39].

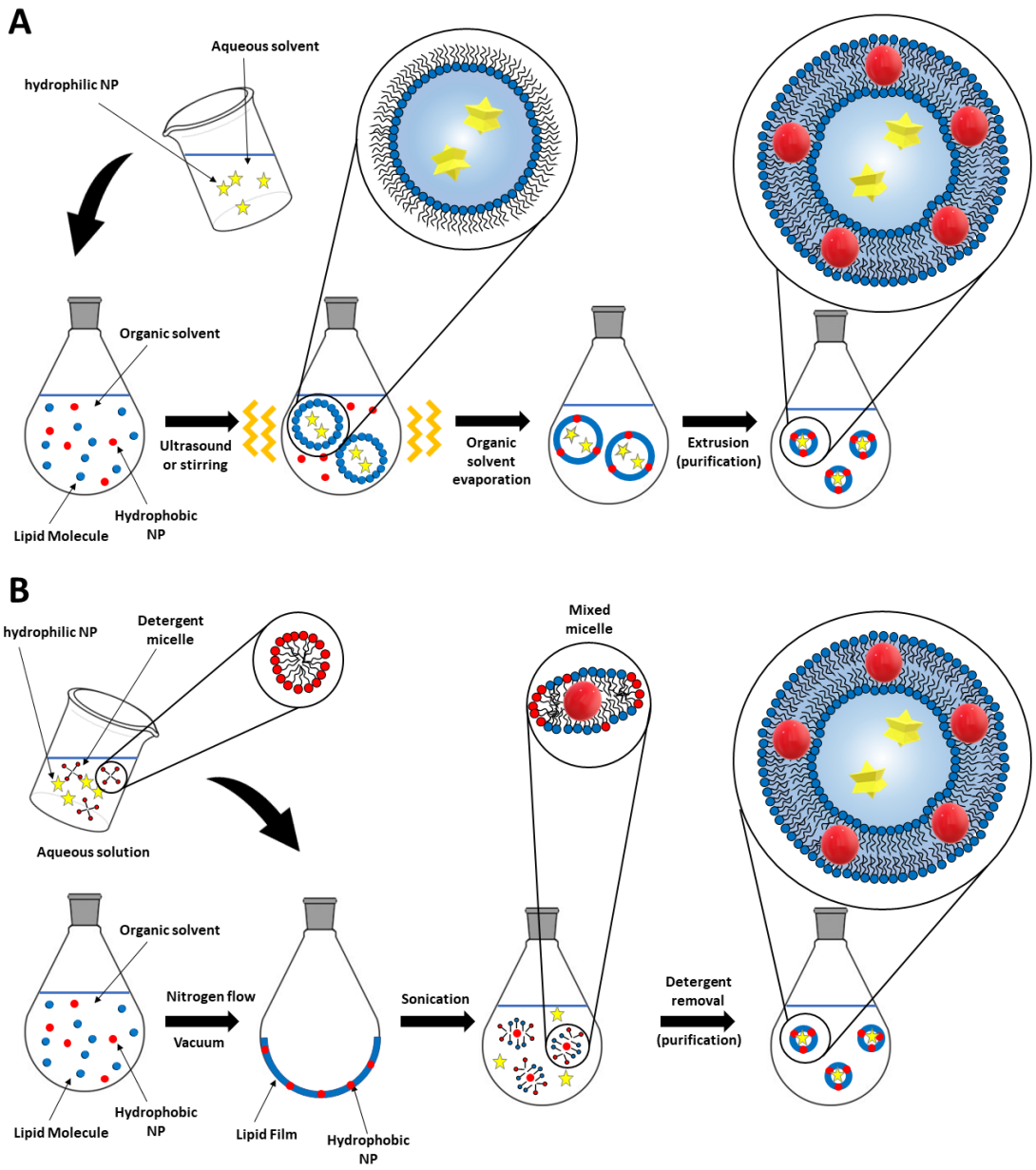

*Figure 3. (**A**) Schematic illustration of the phase reverse evaporation method to obtain liposomes (in blue) encapsulating hydrophobic (red spheres) or hydrophilic (yellow stars) NPs. (**B**) Schematic illustration of the detergent depletion method to obtain liposomes encapsulating hydrophobic (red spheres) or hydrophilic (yellow stars) NPs. First mixed micelles, composed of lipidic (blue) and detergent (red) molecules, are obtained and then they are converted into liposomes through the depletion of the detergent.*

2.4 Detergent depletion method

Detergent depletion method for liposome preparation is superior over other methods, when homogeneous populations of essentially SUVs of tailored size are needed. The size and size distribution of liposomes has indeed been demonstrated to strongly affect some important biological properties [4]. Furthermore, a spherical shape of the liposomes is better realized with this method rather than with mechanical procedures [40]. In addition, this

method is suitable for the reconstitution of integral membrane proteins in lipid vesicles [6, 41]. The first step of the preparation involves the formation of a dry lipidic film, as for the thin-film hydration method. Again, lipophilic NPs can be dissolved in the lipid solution before the formation of the thin film. At this point the NPs-lipid film is hydrated with a high critical micellar concentration (CMC) detergent solution. Hydrophilic NPs can be dissolved in this detergent solution. After vigorous vortexing or sonication, mixed micelles are formed. Mixed micelles may show variable appearance but essentially consist of a microheterogeneous suspension of lipid bilayer fragments in which the detergent molecules are mainly distributed at the hydrophobic edges (Figure 3B). NPs remain trapped within micelle lipid bilayer region [20]. Non aggregated monomeric molecules of detergent in the bulk phase are in rapid equilibrium with mixed micelles. By means of different techniques (size exclusion chromatography, dialysis, dilution…), detergent molecules can be easily removed from the solution. To maintain the equilibrium, detergent molecules move progressively from mixed micelles into the bulk solution, promoting the merging of bilayer fragments until closed vesicles are formed. The hydrophilic particles are partly trapped in the aqueous core of the vesicles although the entrapment yields are not high. A final purification step is therefore necessary to remove non-encapsulated material.

2.5 Surface decoration of preformed liposomes

Liposomes can be decorated with NPs on their outer surface after their preparation by exploiting both covalent and non-covalent interactions. Hydrophilic NPs should be suitably functionalized to interact in the desired way with the charged polar portion of the phospholipids or with hydrophilic functionalities suitably inserted on the vesicle surface during their preparation.

In order to achieve a covalent bond, a hydrophobic anchor is used to insert the desired functionality on the surface of the liposomes. Generally, a long chain fatty acid or modified phospholipid is used for this purpose. For example, variously derivatized phosphatidylethanolamine (PE) phospholipids are commercially available. At the same time, the hydrophilic particles are also enriched on the surface with suitable functionalities, thus being able to interact covalently with the liposomes (Figure 4A). In this way, Zhu and coworkers conjugated carboxyl-modified Au@Ag NPs with amino-ended, pegylated PE (1,2-distearoyl-sn-glycero-3-phosphoethanolamine- (DSPE)-PEG2000-NH2) on the surface of liposomes via amide bond formation [42]. Similarly DSPE, which bears a primary amine group, was used for liposome formulation in order to covalently bind vesicles to carboxylated multiwalled carbon nanotubes by amide bonds [43]. The reaction between thiol functions and maleimide groups can also be used to bind NPs to liposomes through a stable thioether bond formation. Maleimide-functionalized QDs were conjugated to fusogenic liposomes through binding to 1,2-dipalmitoyl-sn-glycero-3-phosphothioethanol (DHPTE), a thiolated phospholipid added at 10^{-3} mol% to the lipid formulation [44].

Charged NPs easily adsorb to lipid polar head groups of opposite charge or to the zwitterionic head groups of outer leaflet lipids through charge-dipole interaction in a non-specific way [45] (Figure 4B). The charge on the surface of the vesicles can be modulated by adding to the lipid blend suitable charged lipids or other charged lipophilic molecules, while the charge on the NPs is imparted by the capping and stabilizing agents on their surface. This route has been used extensively to decorate liposomes on the outer surface with hydrophilic NPs [12]. Aizik and coworkers used electrostatic interaction between the negatively charged QDs and the positively charged vesicles to obtain

liposomes coated with QDs for imaging applications. Hydrophilic and negatively charged CdSe@CdZnS QDs were obtained by replacement of the pristine TOPO hydrophobic coating with glutathione, using the ligand exchange method. Carboxylic acid residues on glutathione are deprotonated in physiological conditions, and impart negative charge to the QDs. The liposomes were obtained by the thin-film hydration method by adding to the lipid blend 1,2-dioleoyl-3-trimethylammonium propane (DOTAP), a well known cationic lipid [46].

Other types of non-covalent interactions can be used to bind NPs and liposomes, such as the interaction between a protein and a ligand or between two strands of DNA (Figure 4C). The first case is well represented by the interaction of biotin and avidin or streptavidin, which rapidly form a stable complex in a wide range of experimental conditions (pH, temperature, organic solvents ...). A supramolecular nano-hybrid based on carbon nanotubes and liposomes was obtained by using avidin – biotin interactions and self-assembly techniques. Carbon nanotubes and liposomes were decorated with avidin and biotin respectively, thanks to two pegylated phospholipids suitably modified on the polar head. Thus, the supramolecular complex was obtained spontaneously by combining the two building blocks [47]. The interaction between DNA strands was exploited by Neeshma and collaborators. They conjugated a strand of DNA to liposomes by reacting maleimidophenyl functionality introduced into the vesicles with a modified lipid (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[4-(p-Maleimidophenyl)butyramide] (MPB-PE)) and with thiol-modified DNA (DNA1). In parallel, AuNPs were functionalized with a different thiol-modified DNA (DNA2). By mixing the so modified liposomes and NPs in the presence of a DNA linker, they obtained the formation of assembled AuNP-liposome complexes, in a programmable and reversible way [48].

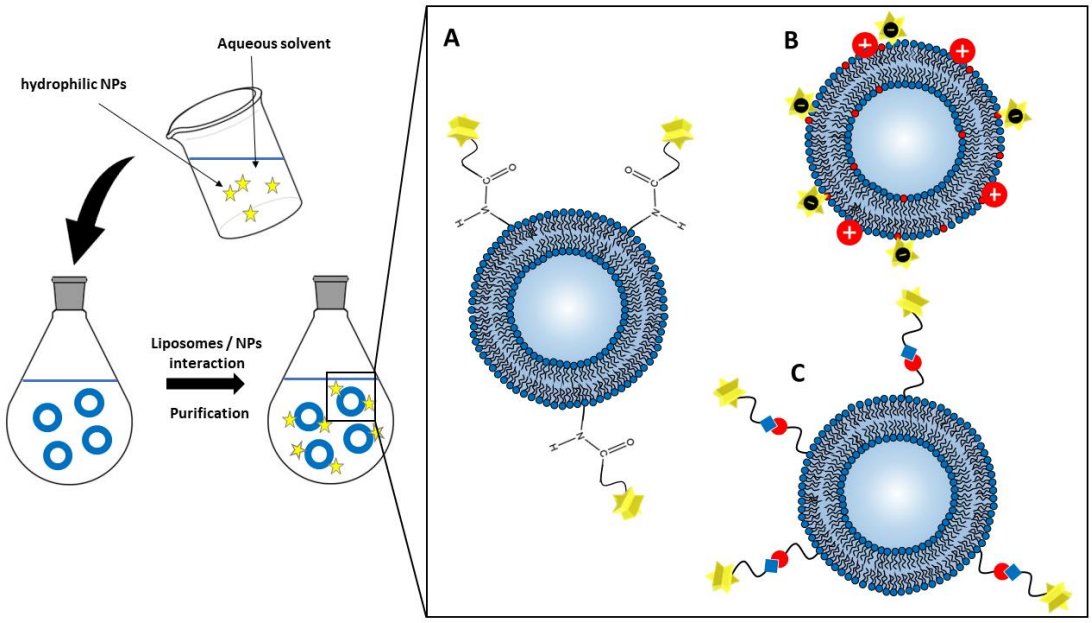

Figure 4. Decoration of preformed liposomes with hydrophilic NPs (yellow stars), through (A) covalent bonds, (B) electrostatic interactions, and (C) non-covalent interactions between two different ligands, respectively bound on the surface of liposomes and NPs.

3. Liposomes with metallic and metallic oxides nanoparticles

Metallic nanoparticles gained particular interest in the last decades thanks to the unique optical, electronic, chemical, and catalytic properties, which arise from the great surface-volume ratio obtained in the nano-regime. Metallic NPs have been exploited for drug delivery applications, chemotherapy, and antithrombotic therapies also due to their capability to interact with microbes and viruses [49]. The most used compounds are gold, silver, cerium oxide, zinc oxide, and titanium oxide. Furthermore, extensive scientific research focused on the subclass of the magnetic NPs, with particular attention on the hybrid systems prepared using iron oxides [50].

3.2 Gold

Gold nanoparticles (AuNPs) exhibit unique physical chemical properties, high stability, high reactivity, photothermal and plasmonic properties. In addition, AuNPs are characterized by a chemical inertness that assures good biocompatibility for *in vitro* and *in vivo* applications [51]. All these properties make AuNPs interesting in several fields, such as drug delivery, radiation therapy, diagnostics, electronics, biosensing, and much more.

Researchers have encapsulated AuNPs into different platforms in order to allow an easy biodistribution in the organism, increase their circulation lifetime and enhance their accumulation in the site of interest [52]. In detail, AuNPs have been conjugated with liposomes in several ways, by loading NPs into the phospholipid bilayer or in the aqueous core. Furthermore AuNPs were bound to the vesicle surface through chemical or physical adsorption [53].

There is a growing interest in the research of stimuli-triggered carriers able to release the drugs in the site of interest. Among the most exploited, there are pH-, heat-, photo- and metabolite-responsive systems. The coupling of AuNPs with liposomes offers a variety of opportunities to realize carriers with these desired properties. For example, AuNPs have been loaded into liposomes to obtain a pH-triggered system [54, 55]. Notably, Pornpattananangkul et al., prepared pH-stimuli responsive liposomes by attaching carboxyl-modified AuNPs to the surface of cationic (Egg-PC/DOTAP)-liposomes to obtain drug delivery systems for dermal applications [54]. It is noted that the human skin is characterized by acidic pH, especially when affected by lesions and acne. Therefore, they prepared hybrid systems in which, at neutral pH, the carboxyl group is deprotonated (Au-COO^-) and it strictly binds to the cationic bilayer, stabilizing the liposomes and preventing their reciprocal fusion and the fusion with undesired cells, such as bacterial cells. At pH < 5, the carboxyl group undergoes protonation, and the NPs detach from the liposomes, allowing them to interact and fuse with the skin cell membranes.

AuNPs loaded liposomes have been widely used also to obtain light-stimuli responsive drug carries in order to improve the release characteristics of thermo-sensitive liposomes into cells or to obtain a targeted delivery [56-60]. By virtue of the Surface Plasmon Resonance (SPR), which arises in the nano-regime, AuNPs absorb light and convert the acquired energy into heat that induces a phase transition within the membrane, with a consequent increase of the fluidity and the permeability. This kind of application is useful for the delivery of drugs to superficial tissues (skin, eyes, etc..) and to combine the synergistic effect of chemo- and photothermal therapy for the treatment of cancer [61-63]. Paasonen et al. for the first time tested this kind of approach by loading the AuNPs into liposomes in three ways: hydrophobic hexanethiol-AuNPs (Au-C_6SH) have been loaded into the lipid bilayer, negatively charged hydrophilic mercaptosuccinic acid-AuNPs (Au-MSA) have been encapsulated in the core of liposomes, and lipid 1,2-dipalmitoyl-sn-glycero-3-phosphorylethanolamine (DPPE)-Nanogold® have been bound to the inner and the outer

surface of the 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) / 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) bilayer. They saw that the light-triggered calcein release rate with Au-C_6SH and DPPE-Nanogold® was more than a hundred times greater than with plain liposomes [57]. Lajunen et al., tried to couple both light- and pH-triggers by modifying a DPPC/DSPC/LysoPC thermosensitive liposome with the pH-sensitive component diolein/cholesteryl hemisuccinate (CHEMS) and loading Au nanorods, in order to obtain a selective drug release into the cytosol [56]. In addition, bacterial toxin-stimulated drug release has been achieved by binding chitosan-AuNPs to the surface of liposomes. This binding prevents the fusion of liposomes in physiological conditions, but in presence of bacteria, their toxins insert into the liposomal membrane and open pores through which drugs are released [64]. Finally, DOX-loaded liposomes susceptible to pH and temperature were prepared by Garcia et al. for the treatment of breast and ovarian cancer. In detail, they prepared liposomes with nucleolipids having a negative charge on the polar head. The presence of this particular kind of lipids improves the cellular uptake and biodistribution of the liposomes and allows to anchor positive charged AuNPs on their surface, thus enabling to trigger the release of the DOX in correspondence of the cancer cells [65].

Considering the great use of AuNPs embedded liposomes, some studies have been conducted to understand the influence of the presence of AuNPs on the properties of a phospholipid membrane and to find the best formulation for the desired purpose. Park et al. highlighted how the presence of stearylamine-coated AuNPs into the lipid bilayer causes an increase of the fluidity over the transition temperature and a decrease of fluorescence anisotropy values, demonstrating as this stratagem can be used to obtain thermosensitive liposomes [66]. Moreover, Živanović et al. studied the interaction of citrate-AuNPs with PC and PC/sphingomyelin (SM) liposomes through Surface-Enhanced Raman Spectroscopy (SERS) and Cryo-EM, finding that there is a strong influence of the quantity of citrate bound to the AuNPs on the membrane properties: high citrate concentrations lead to a destruction of the lipidic membrane, while low concentrations favour AuNPs interaction with the liposomal surface [67].

Liposome-AuNPs hybrids were not designed only for biomedical applications, in which liposomes act as delivery carriers but, for instance, they have been also used for the fabrication of electrochemical sensors. In this field, Chen et al., designed a three-dimensional system composed of AuNPs, ferrocene and liposomes cluster to obtain an electrochemical biosensor able to detect liposaccharides in food matrices (Figure 5) [68].

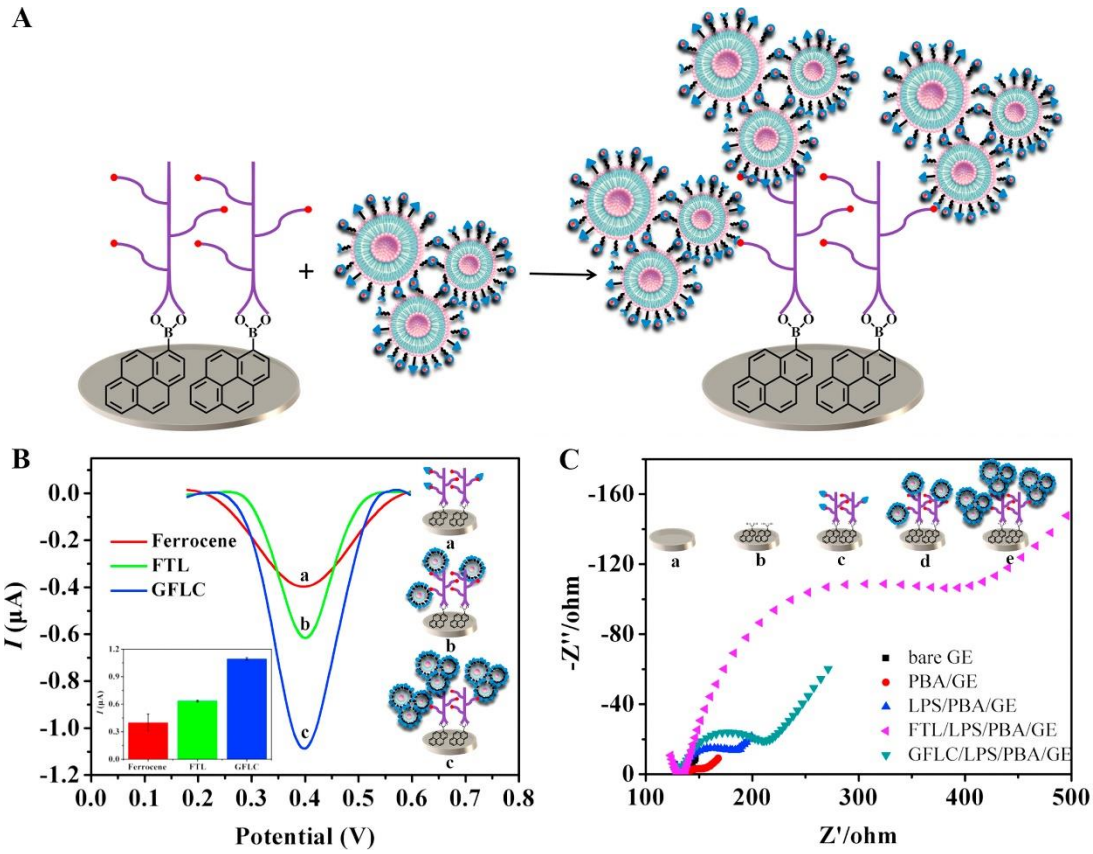

*Figure 5. (**A**) Illustration of the electrochemical biosensor composed from a AuNPs/ferrocene/liposome cluster. (**B**) Differential pulse voltammograms and (Inset) peak current for electrochemical biosensor composed by ferrocene, ferrocene tagged liposome, and AuNPs/ferrocene/liposome cluster, respectively. (**C**) Complex plane plot for the electrochemical impedance measurements of the graphite electrode at different modification stages and illustration of the different types of electrodes. [68]*

Instead, Aili et al. performed a colorimetric bioassay for the detection of the phospholipase activity by loading the liposomes with a polypeptide that associates with a different polypeptide immobilized onto AuNPs. The phospholipase induces the release of the polypeptide from the liposomes, which aggregates with the polypeptide bonded on the AuNPs leading to a colour change [69].

Finally, in literature, liposomes have been also used as nanoreactors for the synthesis of AuNPs [70, 71], for example by encapsulating chloroauric acid and using a reducing agent. This kind of compartmentalized synthesis allows obtaining homogeneous NPs in shape and size.

3.3 Silver

Silver nanoparticles (AgNPs) are characterized by high chemical stability, thermal end electrical conductivity, catalytic activity, but above all by antibacterial properties [51]. In fact, it is commonly noted the use of silver as a disinfectant agent. AgNPs in physiological conditions suffer from problems of low stability, aggregation, and oxidation [72], causing cytotoxic effects for the organism. Hence, they have been usually encapsulated into biocompatible systems, such as liposomes, niosomes [73] and silica NPs.

Researchers have long exploited the AgNPs-liposomes to obtain antimicrobial and antioxidant systems, going through the preparation of coatings, films, and drug delivery carriers [74-78]. Among these, Wu et al. designed Lignin-AgNPs encapsulated liposomes containing laurel essential oil and mixed them with chitosan to obtain an antimicrobial and antioxidant coating for polyethylene films for the pork packaging [79]. AgNPs have been loaded into liposomes also to improve their use as active agents against cancer and arthritis targets, as macrophages. It has been seen that this encapsulation suppresses the reactive oxygen species (ROS) production and reduces the glutathione (GSH) level, generating a redox imbalance which leads to a DNA damage and to the death of the macrophages [80]. Moreover, as explained before, metallic NPs are usually exploited to obtain stimuli-responsive drug release. For this purpose, Al-Ahmady et al. compared the efficiency of gold, silver, and iron oxide NPs in obtaining Doxorubicin (DOX)-lysolipid-containing thermosensitive liposomes (LTSL) for the treatment of cancer. They found that there are no significant differences among the three types of NPs in terms of physicochemical properties and drug release profiles and that all three systems induced lower rates of DOX release compared to simple LTSL liposomes [81]. Recently, Skora et al proposed to encapsulate AgNPs into the aqueous core of the liposomes to take advantage of the cytotoxic effect of AgNPs against cancer cells, while preserving healthy cells. These hybrid systems were tested on human keratinocytes, used as skin model, and it was found that the encapsulation process mitigates the toxic effect of the NPs. Then, similar systems labelled with the epidermal grow factor, were designed and investigated for the targeted treatment of human lung carcinoma and human tongue squamous carcinoma [82, 83].

Shmakov et al. used lipid vesicles as a template to obtain AgNPs embedded hollow polymer nanocapsules, developing a method which allows simultaneously the reduction of the Ag^+ in the aqueous core and the polymerization of the monomer embedded into the lipid bilayer [84].

As for gold, also the influence of AgNPs on the membranes properties is object of several studies. Bothun and coworkers prepared Ag-decanethiol NPs and studied their interaction with DPPC bilayers, finding that the dispersion is stable if in liquid-crystalline phase, while a phase separation occurs if the bilayer is in the gel state. Moreover, by increasing the NPs concentration, the melting temperature decreases, and the gel phase disappears, demonstrating the capability of the lipid bilayer to distort in order to accommodate the NPs. Finally, the AgNPs SPR wavelength resulted to be independent on the bilayer phase, but the absorbance changes [22]. Another study compared the interaction of citrate-AgNPs and phytomolecules-AgNPs with zwitterionic (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC)) and anionic (DMPC:1,2-Dimyristoyl-sn-glycero-3-phosphorylglycerol (DMPG)) monolayers and with *E. coli* membranes and it has been found that the interaction is stronger for the phytomolecules-AgNPs [85]. Finally, Wehbe et al. used curcumin as a probe to evaluate the influence of the AgNPs on DMPC liposomal bilayer, finding that low AgNPs concentrations cause a decrease of the curcumin partition into the liposomes and that in presence of the NPs, curcumin tends to collocate near the stern layer. Notably, the presence of AgNPs broads the phase transition temperature of the membrane, which spans from 20 °C to 35 °C [86].

3.4 Zinc oxide

Zinc oxide (ZnO) NPs possess interesting antibacterial properties thanks to their capability to act as photocatalyst agents. Moreover, they are recognized as GRAS (Generally Recognized As Safe) compounds by the FDA (Food and Drug Administration) [87]. These properties have been particularly exploited for food packaging applications [88]. Furthermore, the optical properties arising in the nano-regime allow the generation of ROS under UV and X-rays

exposure, hence this behaviour has been exploited in photodynamic cancer therapy (PDT) [89]. In literature, there are not a lot of works in which this kind of NPs have been used with liposomes. In one interesting application, the PDT of ZnO NPs action has been coupled with chemotherapy by preparing ZnO NPs-liposomes encapsulating Daunorubicin able to induce the release of the drug under acidic conditions [90].

3.5 Cerium oxide

Cerium oxide nanoparticles, also known as "nanoceria", show excellent catalytic, redox and antioxidant properties, due to the presence of defects onto the surface of the material caused by the switch of cerium between two different oxidation states, Ce^{3+} e Ce^{4+} [91, 92]. Their high reactivity has been exploited both in environmental remediation and biomedical fields [12].

As for ZnO NPs, also nanoceria's capability to modulate the production of ROS in tumor environment has been used for cancer therapy applications [93], however they suffer from problems of aggregation, low half-life *in vivo* fluids, and their surface properties are sensitive to adsorption phenomena, which can occur *in vivo*. Grillone et al. loaded nanoceria into liposomes to improve their colloidal stability and use them for therapeutic purposes. Besides, they found that the encapsulation preserves their antioxidant activity and generates systems well tolerated by cells [94]. Before them, Liu et al. studied the interaction between nanoceria and lipid membranes through a fluorescence quenching assay performed using phosphocholine-liposomes. They observed that nanoceria adsorb onto the surface of the liposomes through electrostatic interactions and that the system is stable at acidic pH, thanks to the presence of a positive charge on the NPs, while at neutral pH large aggregates form [95].

3.6 Titanium dioxide

Titanium dioxide (TiO_2) NPs have been widely used for environmental remediation, electronic devices, cosmetics, sun creams, and biomedicine [96, 97]. Among their most useful properties, there is the capability to act as a photocatalyst under UV irradiation [98]. Their applications in the biomedical field concerns targeted drug delivery, cancer therapy, construction of scaffolds, implants, etc [96]. Heidari Khoee et al. exploited the porous surface of TiO_2 nanotubes by loading them with 5-fluorouracil, an anticancer drug, and capping them with liposomes in order to obtain an extended-release of the drug and so, reduce the side effect of chemotherapy [99].

As said before, TiO_2 NPs are used for several applications, so they can be easily found in the environment. As a consequence, several methods for their quantification in aqueous wastes and for evaluation of their effect on human health have been developed. Some of them exploit the use of liposomes. Zhao et al., proposed a protocol in which TiO_2 and ZnO NPs are first covered with the polydopamine to prevent their aggregation, and then encapsulated into liposomes, in order to obtain better performances in terms of peak area in a quantitative gel filtration chromatography [100]. Moreover, to evaluate the effect of the presence of the commercial metal oxide NPs on human health, it has been studied how liposomes can stabilize metal oxide NPs, preventing their fusion and sedimentation. Specifically, DPPC/phosphatidylglycerol (PG) are typical lungs surfactant, so DPPC/PG-liposomes have been prepared in order to simulate the lipid corona which forms when NPs present in the air were inhaled [101].

3.7 Magnetic nanoparticles

Magnetic nanoparticles (MNPs) based on ferric or ferrous oxides have been widely used in the biomedical field thanks to their nontoxicity, biocompatibility, injectability, strong accumulation in tissues or organs, superparamagnetic properties, and capability to be carefully transported to specific sites using an electromagnet [102, 103]. In iron oxides, iron can have different oxidation states, and magnetite (Fe_3O_4) is the most used in biomedical applications, but it is susceptible to oxidation processes [51]. The main applications of superparamagnetic iron oxide nanoparticles (SPIONs) fall within the scope of chemotherapy, hyperthermia, and magnetic resonance imaging [49, 104]. Cobalt and Nickel can be further exploited to enhance the magnetic properties of MNPs, but they have less stability to the oxidation and they are considered more toxic for the organism [103]. However, alternatively to the magnetite, other bimetallic MNPs are gaining interest for their application in nanomedicine, such as Fe-Co, Fe-Ni, Fe-Pt, Zn-Fe [105-109].

Generally, to increase the colloidal stability of the MNPs and avoid aggregation processes, their surface is coated with citrate, sodium bis(2-ethylhexyl)sulfosuccinate (AOT), peptides, polymers, or other ligands [25, 110, 111]. The presence of these coatings has also been exploited to give a hydrophilic or hydrophobic character to the NPs. Besides, to reduce their toxicity and immunogenicity, facilitate their transport in aqueous media, increase their bioavailability and obtain stealth systems, researchers have encapsulated MNPs into vehicles, such as liposomes, chitosan, protein, silica NPs [112, 113]. MNP-embedded liposomes, also known as magnetoliposomes [114, 115], were prepared mainly with the thin-film hydration method, the reverse phase evaporation method, and the ethanol injection method described previously, and were exploited for numerous applications, integrating the multiple properties of the MNPs with the capability of liposomes to deliver them in an easy and safe mode.

Liposomes with iron oxide MNPs have been used as contrast agents for magnetic resonance imaging [116], cancer therapy and controlled drug release [116, 117], as iron supplies for the treatment of iron deficiency anaemia [118] and bioassays [24].

Moreover, it is well known that MNPs accumulate in the tumour region and here can act as hyperthermia-inducing agents by application of a high-frequency magnetic field to raise the local temperature around the cells and cause cell death [26, 119, 120]. Exploiting the same principle, MNPs can be used to deliver drugs in the site of interest. However, problems for thermosensitive drug-release systems can be encountered when the delivery must take place in tissues susceptible to temperature, such as the brain. To overcome this limit, thermosensitive liposomes with iron oxide NPs capable to induce the drug release under a non-invasive alternating magnetic field (AMF) exposure, have been synthesized [121]. This goal has been achieved because the released heat induces the break of the liposomal membrane instead of the tissue warming-up. Hyperthermia process was further exploited in synergy with antitumoral agents for the treatment of cancer by the development of folate magnetic liposomes with iron oxide NPs and DOX [31]. In addition, Lu et al. designed thermal and magnetic dual-responsive thermosensitive liposomes which co-encapsulate in the aqueous core Camptosar, an anticancer drug, citric acid-coated Fe_3O_4 NPs and conjugate the monoclonal antibody Cetuximab on the surface, to allow the specific recognition of the epidermal growth factor receptor, overexpressed in different brain tumors. This system can trigger the release of the encapsulated drug at cancer cells when exposed to a high-frequency AMF [27]. Moreover, Di Corato et al., developed nanovehicles based on dually loaded hybrid liposomes: the aqueous core has been loaded with iron oxide NPs and the lipidic bilayer with a photosensitizer payload, in order to couple PDT to hyperthermia [37]. Iron oxide nanoflowers loaded into

thermosensitive liposomes were also proposed for the treatment of lung adenocarcinoma. Since the magnetic properties are strictly dependent on the size of the NPs, nanoflowers of about 15 and 35 nm were compared, and it was observed that nanoflowers of 35 nm are more effective as hyperthermia-inducing agents [122]. Beyond the use in hyperthermia therapy, magnetite NPs were used as photothermal agents and were encapsulated into liposomes loaded with the anticarcinogenic Bufalin to obtain a system able to be guided from a magnetic field and to act both as a photothermal and chemotherapeutic agent for the treatment of lymph metastatic breast cancer. Results showed an effective capacity of these systems to inhibit and lessen the growth of the tumor compared to free Bufalin (Figure 6) [123].

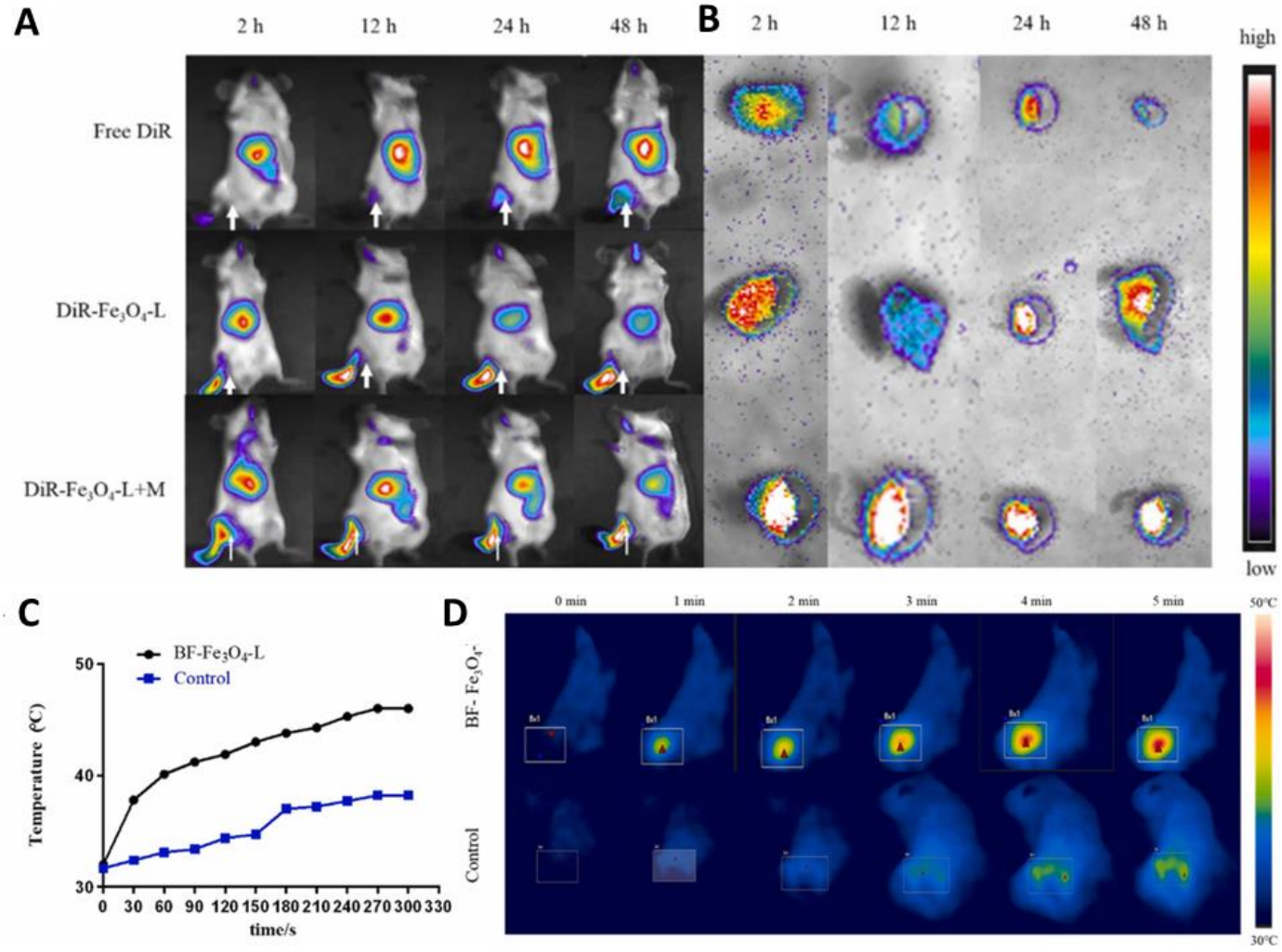

*Figure 6. (**A**) In vivo fluorescence images obtained using a mouse model of lymph metastatic breast cancer following i.v injection of free near-infrared fluorescent cyanine dye (DiR) and DiR-Fe_3O_4-Liposomes (**B**) In vitro fluorescence images of excised sentinel lymph nodes (SLNs). (**C**) Temperature change in the area of SLN obtained using a mouse model of lymph metastatic breast cancer, following i.v injection of Bufalin-Fe_3O_4-Liposomes. (**D**) Thermographic images of mouse model of lymph metastatic breast cancer following i.v injection of Bufalin-Fe_3O_4-Liposomes* [123].

Magnetic resonance imaging (MRI) is based on the principle that after the excitation with a radio-frequency, a system can return to the equilibrium through two types of relaxation with a proper time constant: the spin-lattice relaxation time (T_1) and the spin-spin relaxation time (T_2), and that the relaxation time depends on the local

environment and the mobility of the molecules. SPIONs are able to enhance the local magnetic fields experienced by water molecules, increasing the rate of relaxation and so they can be used as T_2 contrast agents, while gadolinium derivatives can enhance the T_1 relaxation rates [124]. In this field, the ability of SPION-liposome hybrids to act as a T_2 MRI probe and the possibility to properly functionalize the surface of liposomes with antitumoral ligands have been exploited for the *in vivo* guidance and monitoring of the drug delivery into tumoral sites in a non-invasive way [125-127]. Notably, Han et al. suggest that the use of hydrophobic magnetite-liposomes reduces the side effects typical of hydrophilic MNPs, especially their leakage from the core of the liposomes and their dispersion into human tissues [128].

4. Liposomes with semiconductor quantum dots

QDs are small (<10 nm) fluorescent semiconductor nanocrystals composed of groups II – VI, III – V or IV elements. The most common ones are based on a combination of zinc, cadmium, selenide, sulfide, and several additional components (e.g. stabilizing surface coatings) and dopants [129]. QDs exhibit superior optical and electronic properties with respect to traditional fluorescent organic dyes in terms of size- and composition-tuneable fluorescence properties, brightness, high quantum yield (QY), resistance to quenching and photo-stability [130]. All these properties have allowed their application in very different sectors such as luminescence, electronics, catalysis, and optoelectronics. Furthermore, QDs have emerged as a versatile tool for the investigation of biological systems at the molecular scale, with multiple applications in the study of complex processes in cells and biological tissues [129]. However, some factors limit their applications, especially in the biological field. In fact, QDs with the best performances in terms of fluorescence and QY are usually synthesized in a non-polar environment and are insoluble in water. Furthermore, in a biological milieu, they can release toxic ions (the core of these structures contains heavy metals) which can cause toxicity problems for in vitro and, above all, in vivo applications. The capping agents used to limit crystal growth and increase the stability of QDs also raise concerns about their safety [129]. Several strategies have been put in place to circumvent these obstacles, such as the exchange of ligands on the crystalline surface or the encapsulation of QDs in various biocompatible shells, such as silica, natural and synthetic polymers, micelles, and liposomes [20, 131-133].

The incorporation of QDs into liposomes has proved to be a good strategy to make these NPs compatible with the biological environment, reducing their cellular toxicity. Furthermore, the high QY of the QDs makes low concentrations of fluorophore sufficient for cell labelling experiments, thus lowering concerns on safety of the hybrid vesicles. The liposomal carrier has been shown to be able to rapidly deliver QDs into the cells. Delivery experiments towards HeLa cells were evaluated by fluorescence investigation and confocal microscopy, and showed that hydrophobic, red emitting and non-fusogenic QD-Liposomes are internalized by the cells through the endocytic pathway, then enter the early endosomes and reach the lysosomes in 1 h [129]. To better preserve the peculiar optical properties of the QDs it is necessary to: i) achieve a good interdigitation between the capping molecules and the lipid molecules of the bilayer (for hydrophobic QDs); ii) choose an encapsulation method that leads to the formation of small vesicles, in order to minimize the scattering; iii) appropriately determine the final concentration of QDs in the vesicle to avoid self-absorption and, therefore, self-quenching phenomena. For example, with the detergent depletion method it was possible to obtain the entrapment of hydrophobic, 1-dodecanethiol capped CdSe@ZnS QDs in liposomes of about 50 nm, while preserving their emission properties. The same method allowed

to encapsulate integral membrane proteins simultaneously and in a non-denaturing way, obtaining hybrid liposomes [20]. It is known that liposomes can passively influence the biodistribution of NPs, through the EPR effect, a unidirectional extravasation from the blood towards the inflamed tissue or tumor. For this purpose, QDs were mainly trapped in neutral charged, PEGylated vesicles, with "stealth" properties. However, it is possible to obtain an active delivery by modifying the lipid composition and the surface charge of the liposomes, as well as decorating them with suitable targeting agents. For example, Aizik and collaborators trapped hydrophilic and anionic QDs in cationic vesicles, making them able to interact with monocytes / macrophages. In this way they used these cells as trojan horses to achieve monocyte-mediated delivery [134]. Liposomes were also used to combine QDs and SPIONs in the same system to obtain multiple imaging agents for targeted delivery to glioma, guided under exogenous magnetic field. The dual glioma imaging, including MRI negative contrast imaging and fluorescence imaging, was successfully confirmed in vitro and in vivo. This system is therefore potentially useful for accurately localizing the tumour during imaging-guided cancer surgery, avoiding non-specific distribution of QDs, and preventing homogeneous leakage of individual SPIONs below 10 nm into healthy tissues [135].

In a recent work, black phosphorus (BP) QDs were entrapped into the aqueous core of targeted and temperature-sensitive liposomes by thin-film hydration method. In this case both the inherent photothermal and chemotherapeutic properties of BP were exploited for in vivo treatment of resistant tumours. Near-infrared (NIR) light irradiation induces a direct BP photothermal effect, which results in hyperthermia ablation of tumour. Simultaneously the rise in temperature triggers the liposome phase transition, with the consequent release of the BP QDs (Figure 7), obtaining a synergistic chemotherapeutic effect [136]. Interestingly, Karabuga and coworkers developed a multifunctional radiosensitizer by encapsulating Chlorine-e6-modified QDs in folic acid-modified PEGylated liposomes in an attempt to reduce the X-ray doses currently used clinically for cancer therapy [137].

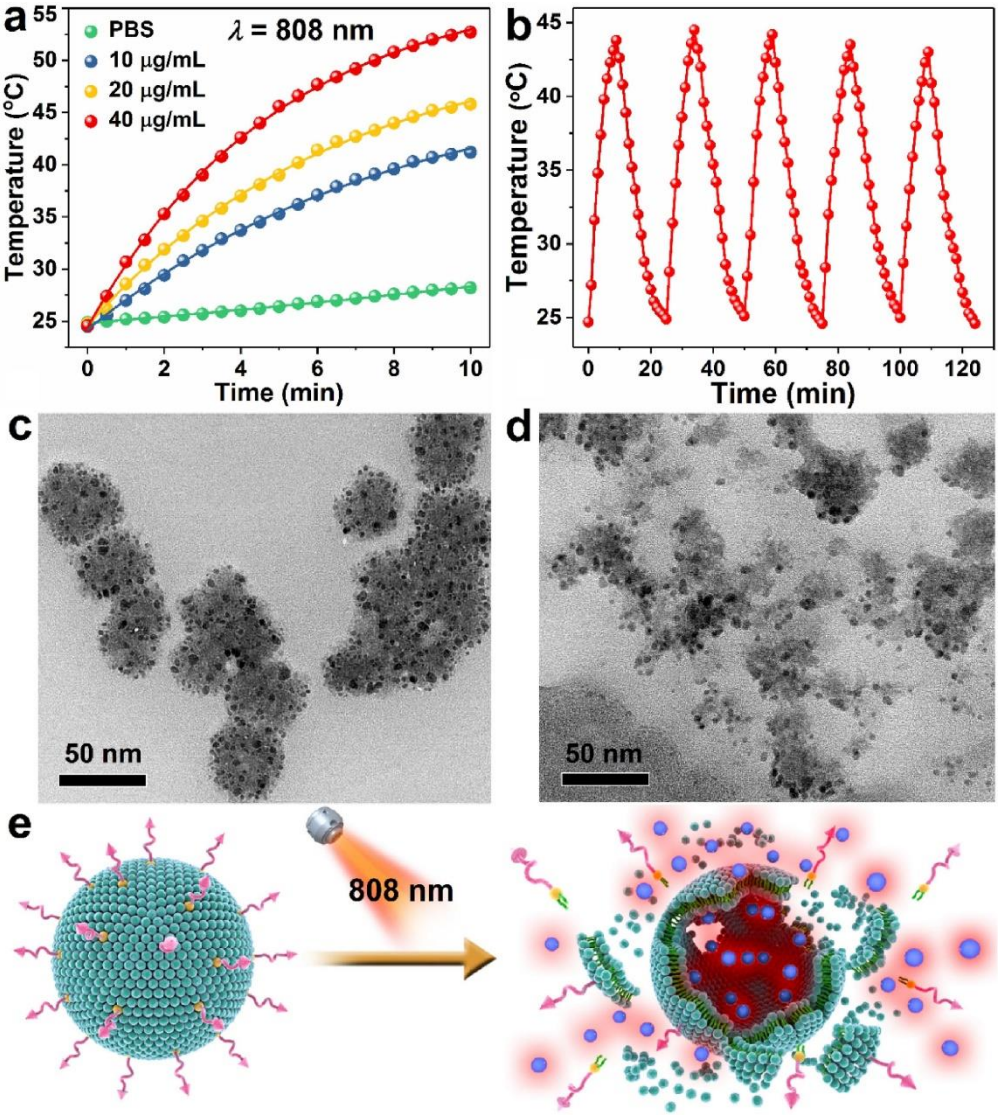

*Figure 7. (**A**) Photothermal effect of BP QD-Liposomes induced by NIR irradiation, by changing the BP concentration; (**B**) Temperature variation during five laser on/off cycles; (**C-D**) TEM micrographs of BPQDs-Liposomes after 5 and 10 minutes of NIR irradiation, respectively; (**E**) Simplified sketch illustrating the release of BP QDs from liposomes under NIR irradiation [136].*

5. Liposomes with silica nanoparticles

Silica (SiO_2) nanoparticles (SiNPs) are among the most used inorganic non-metallic nanomaterials for biomedical and environmental applications. They, in fact, possess outstanding optical, electrical, and mechanical properties, are biocompatible, and easy to functionalize [138, 139]. Moreover, by modulating the synthesis parameters, SiNPs with different shapes, sizes varying from 10 to 500 nm, and with porosity between 2 and 50 nm can be obtained. When used as drug carriers, the size and the shape greatly influence the interaction of the SiNPs with the biological media, while the porosity determines the loading capacity and the release behaviour [138]. Depending on their structure, SiNPs are generally classified into conventional non-porous SiNPs, mesoporous SiNPs (MSNs), hollow mesoporous SiNPs and core-shell silica NPs [138]. In particular, MSNs possess high surface area, tunable and uniform size and

volume of pores, which generate elevated loading capacity; therefore, they have been the most exploited silica derivatives for drug delivery purposes [140, 141].

Although SiNPs are usually recognized as safe for the organism, they retain inner cytotoxicity deriving from their surface chemistry, shape and pore size ([142]). Besides, they suffer from premature drug leakage during the administration; so, to enhance their biocompatibility and reduce the leakage of the drugs outer of the sites of interest, MSNs were loaded into liposomes [143-145]. For instance, Sun and coworkers prepared systems composed of DOX-Gd-MSNs loaded into thermosensitive indocyanine green (ICG)-liposomes able to achieve simultaneously imaging and cancer therapy [143]. These hybrid structures constituted of SiNPs loaded into liposomes were obtained mainly in two ways: by hydrating a thin lipid film with an aqueous suspension of SiNPs [143] or preparing separately liposomes and SiNPs, mixing them and subjecting everything to ultrasounds [144, 146, 147]. On the other hand, SiNPs around 20 nm in diameter were made to adsorb onto the surface of 200 nm insulin-loaded liposomes through electrostatic interactions to protect the vesicles from degradation into the gastrointestinal tract [148]. An application which involves the combined use of liposomes, SiNPs and polymer NPs of poly(dimethyldiallylammonium chloride) (PDDA) was developed by preparation of multi-layer carriers for the delivery of curcumin by using a layer-by-layer assembly method. Curcumin was used as a natural anticancer agent. Othman and co-workers compared curcumin-loaded liposomes coated with a single shell of PDDA, and curcumin-loaded liposomes coated with a triple shell of PDDA-silica-PDDA or with a PDDA-silica-curcumin-PDDA shell. They found that the presence of multiple layers improves the encapsulation efficiency of the curcumin by limiting its unwanted release and slows its diffusion [149].

6. Liposomes with carbon-based nanoparticles

6.1 Graphene

Graphene is constituted by carbon hybridized sp^2 hexagonally arranged. It has a strong and flexible structure, with considerable electronic properties, broadband adsorption, and the capability to act as a photothermal agent under NIR irradiation. It is commonly considered biocompatible, though the nature of the source from which it is obtained and the procedure of synthesis can strongly influence its properties [150]. Moreover, graphene is difficult to disperse in water, with consequent limits for its applications. For this reason, Zappacosta et al., exploited liposomes to induce the exfoliation of graphite and obtain a stable aqueous dispersion of graphene sheets, stabilized by the phospholipid chains [151]. Anyway, to increment its solubility in water, graphene is usually oxidized into graphene oxide (GO), which shows numerous oxygenated groups on its surface. In such a manner, GO can be also easily functionalized in a covalent and non-covalent way, but its electrical and mechanical performances are poorer compared to graphene. An intermediate material between graphene and GO is the reduced graphene oxide (rGO), which still retains some oxygenated groups of the GO [152]. In addition, graphene quantum dots (GQDs) were synthetized. They are particles constituted from graphene, with a size less than 100 nm and with interesting fluorescence properties [153]. Concerning the use of nanostructured graphene (and derivatives) in conjugation with liposomes, Hashemi et al. exploited GO to obtain a stimuli-responsive system for the release of DOX from liposomes for the treatment of breast cancer [154]. Specifically, they prepared a multilayer system composed of alternated GO sheets and GO-poly-L-lysine sheets deposited on DOX-liposomes. The GO absorbs the NIR radiation and converts it into heat, while the poly-L-lysine improves the cellular uptake. Instead, Tajvar et al. loaded DOX and graphene nanosheets into

liposomes to obtain a multifunctional carrier for tumor tracking, photothermal and photodynamic therapy [155]. Notably, Prasad and collaborators co-loaded AuNPs, GQDs and DOX into liposomes, decorated with folic acid on the surface for an accurate diagnosis and ablation of 4T1 breast cancer, further tested on mouse models [156]. Recently, 4 nm GQDs were loaded into the aqueous core of the liposomes, through a modified reverse-phase evaporation method and the formation of the GQD-loaded liposomes was found dependent on the applied pressure during the evaporation of the organic phase [157]. Furthermore, the ICG was integrated into the system to obtain a NIR-triggered release of the GQDs, which showed good performances *in vitro*. Taking advantage of their superior luminescence properties, Awad et al., loaded hydrophilic GQDs into liposomes for bioimaging applications, obtaining in this way an increase in their biocompatibility and a controlled release in space and time under low-frequency ultrasound by exploiting the sonoporation mechanism and the similarity of the liposomes with the biological membranes [158].

6.2 Carbon nanotubes

Carbon nanotubes (CNTs) were discovered in 1991 in Japan by Iijima [159] and constitute another allotrope of Carbon. They are composed of enrolled graphite sheets and can be multi-walled (MWCNTs) or single-walled (SWCNTs). SWCNTs have a diameter from 0.4 to 2 nm, while MWCNTs from 2 to 100 nm. Both have micrometric lengths. Therefore, CNTs possess an aspect ratio of about 1:1000, which confers them the capability to easily penetrate the cell membranes and opens the door for their application in biomedicine. In addition, CNTs have excellent conductivity, thanks to their aromatic structure, great chemical and thermal stability, and their elevated surface area allows an easy functionalization in a covalent and non-covalent way [160, 161]. They also act as photothermal agents since they show a great absorption of the NIR radiation with consequent conversion in heat [162]. In general, they are considered not significantly cytotoxic for the organism. However, numerous debates are in course about this, because of their similarity to asbestos fibers [163]: longer structures seem to be more cytotoxic, and residual metals used in the synthesis process can induce some side effects when used for biomedical applications [160]. Another problem correlated to the use of CNTs for drug delivery purposes is their low encapsulation efficiency, which leads to the necessity of a massive dose to obtain significant therapeutic effects and therefore to a possible increment of the cytotoxicity [160]. Therefore, some researchers proposed to conjugate CNTs with liposomes, in order to join the directionality of the transport induced by the CNTs and their ability to enter into the cells with the great encapsulation efficiencies of the liposomes [43, 164]. This conjugation was realized exploiting ligands bonded on the surface of the CNTs and liposomes, such as biotin and avidin [47, 165], through electrostatic interactions [166], or in a covalent way [43]. For instance, Miyako et al. first prepared CNTs-liposomes to obtain a directional transport and a photo-triggered release of the cargo in order to induce an enzymatic reaction in a specific area of interest [165] and then tried to exploit a similar system to obtain a biomimetic parasite and symbiotic system able to allow a remote spatial-temporally control over the neuronal processes in *C. elegans* worm [47]. Moreover, Madani et al. proposed an external stimuli-responsive drug delivery system by conjugating anionic DNA-CNTs with cationic drug-loaded liposomes and introducing this system into a hydrogel to get an implantable NIR-responsive device [166].

6. Carbon Dots

Carbon dots (CDs) are spherical carbon NPs with a size less than 10 nm, a marked sp^2 character, and a passivated surface. CDs can be obtained from natural or synthetic carbon sources. Since their chance discovery in 2004 [167], they acquired increasing attention as they show combined properties of QDs and carbon-based materials. CDs, in fact, possess outstanding optical properties, with high absorption in the UV spectrum, high photostability, elevated fluorescence intensity and QY, and a λ_{em} generally dependent on the λ_{ex}. However, compared to classical QDs, they are less cytotoxic, cheaper, more eco-friendly and possess high conductivity [168, 169]. In addition, their surface can be easily functionalized with carboxyl, amino, and hydroxyl groups which confer them a good solubility in water [170]. For these reasons, they were proposed for safer applications in agriculture and nanomedicine [169]. Hydrophobic CDs are rarer because their synthesis is more difficult to realize, and they suffer from practical limitations being incompatible with aqueous environments. To overcome these problems, Fan et al., proposed a one-step procedure for the synthesis of blue and green-emitting hydrophobic CDs, which have been further loaded into liposomes and acrylate films [171].

In general, CDs were encapsulated into liposomes principally for bioimaging purposes [172, 173] and the loading frequently led to an increment of the cell viability as well as to increased stability and enhanced optical properties of the CDs. For instance, Guan et al. embedded hydrophobic CDs into D-mannose surface-modified liposomes to obtain targeted imaging of HepG2 cells [174]. Notably, Xue et al. proposed smart nanocarriers by loading DOX-CDs into the liposomal core and ICG into the lipid bilayer. ICG can enhance the fluorescence of the carrier and simultaneously act as a photothermal agent [175]. Similarly, Ren et al. prepared a multifunctional nanocarrier, loading cinobufagin, a hydrophobic cancer therapeutic agent, into the liposome bilayer and hydrophilic red-emitting CDs into the aqueous core [176]. This encapsulation led to an enhancement of the CDs photoluminescence of 5-folds, a 10 nm blue shift, a QY increment of 6-fold and a reduction of the lifetime. Moreover, the photoluminescence was found to be stable for at least one month. This carrier shows integrated sustained-drug release of the cinobufagin into MCF-7 cells and HepG2 cells, bioimaging detection and specific targeting of the tumor site in mice.

Instead of exploiting the CDs fluorescence, Zhang et al. proposed to use CDs as MRI contrast agent to be used also for *in vivo* imaging in deep tissues [177]. They tried to take advantage of the inherent diamagnetic Chemical Exchange Saturation Transfer (diaCEST) properties of the CDs generated by the presence of exchangeable protons on their surface, without doping them with superparamagnetic metals. To enhance the diaCEST contrast, arginine was used as dopant during the synthesis of the CDs. Moreover, to obtain a significant cellular uptake of CDs, they were encapsulated into the aqueous core of liposomes and, in this way, they succeeded to label human glioma cells, confirming once again the great advantages which arise encapsulating NPs into liposomes when they have to be used for biomedical purposes.

CD-liposomes were also used for the detection of nucleic acids. Divya et al. proposed composite systems based on CD-AgNP-decorated liposomes bonded on a gold electrode for a label-free DNA detection [178], while Jiang et al. developed an analytical method for the detection of nucleic acids based on the chemiluminescence of the CoOOH-luminol, using CDs instead of antioxidant reactants [179]. Here, the encapsulation of the CDs into the liposomes avoids their complex-modification and preserves their free-radical scavenging properties. Finally, Chen et al. fabricated micropatterns of photo-switchable CDs-liposomes through a photolithographic approach [180]. In

particular, they obtained CD-liposomes keeping triolein into a furnace at 220 °C for 3 days and then extruding at 100 nm.

5. Conclusions

NPs are versatile materials with distinct chemical, optical, magnetic, electrical, catalytic, and mechanical properties. They are able to respond to the numerous needs of the world of pure and applied research, finding already application in numerous products on the market. The properties and applications of NPs can usefully be extended thanks to the integration with liposomes of various compositions and architecture, especially in the field of biomedical and biosensing. In a complementary way, traditional applications of liposomes can take advantage of these innovative building blocks.

In this review, a representative overview of the main methods for the integration of hydrophilic and hydrophobic NPs in liposomes was presented, providing indications for a correct planning of a successful experimental approach. In the second part of the manuscript, an extensive review of the main applications of hybrid NP-liposome systems was provided, taking into consideration the most significant and recent literature relating to the main types of nanomaterials.

The challenge in the future will be to improve the integration of NPs into lipid systems, thus stably integrating various properties within the same liposome. This will greatly influence the real applicability of hybrid systems especially in the field of nanomedicine. It will be possible to exploit the multi-compartmentalization of liposomes for the delivery of drugs of different nature and polarity, trigger their release thanks to the properties of the NPs integrated in the bilayer and at the same time expand the therapeutic possibilities of the system with the photodynamic and hyperthermic properties. The diagnostic aspect can also be simultaneously solved by these hybrid nano systems.

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