Nanoscale Advances

Analytical probing of membranotropic effects of antimicrobial copper nanoparticles on lipid vesicles as membrane models

Journal:	*Nanoscale Advances*
Manuscript ID	NA-ART-08-2023-000608.R1
Article Type:	Paper
Date Submitted by the Author:	12-Sep-2023
Complete List of Authors:	Izzi, Margherita; University of Bari, Chemistry Department Oliver , Miguel ; University of the Balearic Islands, Department of Chemistry Mateos, Helena; University of Bari, Chemistry Department Palazzo, Gerardo; University of Bari, Chemistry Department Cioffi, Nicola; University of Bari, Chemistry Department Miro, Manuel; University of the Balearic Islands, Department of Chemistry
Note: The following files were submitted by the author for peer review, but cannot be converted to PDF. You must view these files (e.g. movies) online.	
Manuscript nanoscale advances_R1_no highlighting.docx	

Nanoscale Advances

Guidelines for Referees

Thank you very much for agreeing to review this manuscript for *Nanoscale Advances*.

Nanoscale Advances is an international gold open access journal, publishing high quality, reproducible new work across the breadth of nanoscience and nanotechnology that makes an important advance to the existing literature.

Articles should aim to publish high quality work that is discussed appropriately in the context of the literature.

Nanoscale Advances' Impact Factor is **4.7** (2022 Journal Citation Reports®)

To view other metrics, visit the *Nanoscale Advances* website.

The following manuscript has been submitted for consideration as a

PAPER

For acceptance, papers should report important field-advancing work that has not been published previously. Full papers based on Communications are encouraged provided that they represent a substantial extension of the original material. There are no restrictions on the length of a paper.

When preparing your report please:

- Focus on the **originality**, **importance**, **impact** and **reproducibility** of the science.
- Refer to the **journal scope and expectations**.
- **State clearly** whether you think the article should be accepted or rejected and give detailed comments (with references) both to help the Editor to make a decision on the paper and the authors to improve it
- **Inform the Editor** if there is a conflict of interest, a significant part of the work you cannot review with confidence or if parts of the work have previously been published.
- **Provide your report rapidly** or inform the Editor if you are unable to do so.

Best regards,

Professor Chunli Bai
Editor-in-Chief, *Nanoscale Advances*

Professor Dirk Guldi
Editor-in-Chief, *Nanoscale Advances*

Dr Jeremy Allen
Executive Editor, *Nanoscale Advances*

Contact us

Please visit our reviewer hub for further details of our processes, policies and reviewer responsibilities as well as guidance on how to review, or click the links below.

What to do when you review

Reviewer responsibilities

Process & policies

Bari, September 12th, 2023

Dear Prof. Dufrêne,

We are pleased to submit the revised version of the manuscript entitled *"**Analytical probing of membranotropic effects of antimicrobial copper nanoparticles on lipid vesicles as membrane models**"* by Margherita Izzi, Miquel Oliver, Helena Mateos, Gerardo Palazzo, Nicola Cioffi * and Manuel Miró* (corresponding authors) for potential publication in *Nanoscale Advances.*

The manuscript has been revised according to the Reviewers' comments/requests. A response to the reviewers' comments in a point-by-point basis has been provided in a separate file.

Best Regards,

Nicola Cioffi & Manuel Miró

Corresponding Authors

Response to the reviewers' comments - Manuscript ID: NA-ART-08-2023-000608

We deeply thank the Reviewers for their constructive suggestions. In the following, all their comments/requests of revisions are itemized, and our replies (when needed) and the changes made to the manuscript are reported in a point-to-point basis.

Referee: 1

The manuscript entitled "Analytical probing of membranotropic effects of antimicrobial copper nanoparticles (CuNPs) on lipid vesicles as membrane models" describes the synthesis and characterization of small size CuNPs, i.e., 4 nm-sized, using glutathione as stabilizer, along with the investigation of its membranotropic effects on lipid vesicles by means of fluorescence studies involving laurdan and prodan, as well as dynamic light scattering measurements and calcein leakage assay. The topic is very interesting, and the investigation is timely, since CuNPs are widely being used as antimicrobials, while, as authors stated, few studies have been carried out on the investigation of the effect of CuNPs on the phospholipid membranes. The manuscript is very well written and clear, and the topic is novel. It could be published in Nanoscale advances journal after some issues are revised. The following suggestions are given for improving the manuscript:

1) Please define all the abbreviations the first time they are mentioned in the manuscript, e.g., DLS in the abstract and also in the main text (page 2).

CHANGE: Abbreviations were defined the first time they were mentioned in the text, and the manuscript was revised accordingly.

2) The second paragraph of the introduction could be divided into different paragraphs. Please revise this part.

CHANGE: The second paragraph of the Introduction was divided into three paragraphs, according to the reviewer's request.

3) For the theoretical estimation of the CuNPs concentration, is there in literature studies which support the hypothesis that the synthesis through such procedure is quantitative? If so, please include the reference and a comment on that. Authors could include also a comment justifying the selection of this method to calculate the concentration and the limitations encountered when using other techniques.

REPLY: We thank the Reviewer for this important consideration. The most common practice for the theoretical estimation of the metal nanoparticle concentration involves their optical properties and surface plasmon resonance (SPR) features. Specifically, considering the NP molar extinction coefficients, and the NP diameter, it is possible to correlate the SPR absorption of colloidal solutions with the NP concentration (10.1016/j.saa.2017.10.047, 10.1021/ac0702084). However, in the case of the CuNPs used in this study, we could not use the above-mentioned approach, because these particles do not show any useful SPR band. Therefore, an alternative calculation based on the average NP diameter, the metal density and assuming that all the Cu(II) precursor has been converted into elemental copper, in the form of CuNPs, was adopted. The main approximation resides in the latter aspect, e.g. in setting the NP synthesis yield at 100%. This means the highest accessible CuNP concentration, thus the worst available case.
To the best of our knowledge, all the published papers related to glutathione-capped metal nanoparticles (mainly gold, silver and copper NPs) do not report any information regarding their extinction coefficient nor the nanoparticle concentration estimation.
CHANGE: To justify the selection of our estimation method, we added the following sentence at pag. 2:
"*The common practice for the theoretical estimation of the metal nanoparticle concentration involves calculations based their optical properties, such as extinction coefficients relevant to surface plasmon resonance (SPR) features [10.1021/ac0702084]. Nevertheless, it could not be used in the present work because of the lack of suitable SPR signals. For this reason, a theoretical calculation based on the copper density and assuming that the Cu(II) precursor is quantitatively converted into elemental nanosized copper phases was carried out. The resulting estimation returns the highest available CuNPs concentration, which is the worst case scenario.*"

4) Has been the procedure used for the synthesis of large unilamellar vesicles previously described in literature? If so, please include the appropriate reference.

REPLY: We thank the reviewer for this comment. Liposomes were prepared by the well-known and widely used lipid film hydration method developed by Bangham in the 60's (10.1016/0009-3084(67)90030-8) to obtain MLVs, followed by repeated extrusion steps (10.1016/0005-2736(85)90521-8) to control the size and lamellarity in order to obtain the final LUVs of the desired size. The procedure used in this work has been optimised in our group with the aim of obtaining a very narrow polydispersity, good reproducibility and long-term stability.

CHANGE: To clarify the method used for liposome synthesis we added the following sentence: *Liposomes were prepared by lipid film hydration method (10.1016/0009-3084(67)90030-8) followed by extrusion (10.1016/0005-2736(85)90521-8) with some changes to improve the quality of the final product.*

5) Please revise the following sentence: "To determine the number of liposomes the average number of PC molecules in a liposome is first using equation 1 and considering that the liposomes are unilamellar."

CHANGE: The sentence was revised as follows: "*To determine the number of liposomes, the average number of PC molecules in a liposome is first calculated using equation 1 and considering that the liposomes are unilamellar.*"

6) Figure 1a, please include the units in the y-axis (a.u.). The same applies to Figure S1 left.

CHANGE: The units in the y-axis in Figure 1a and Figure S1 were added.

7) In figure 1f, the bands that are discussed within the text could be highlighted in the spectra. The other bands that appear in the spectra are not mentioned or ascribed in the text. Please, if possible, include some comments on them.

CHANGE: The following sentence was added to briefly describe the main bands attributed to GSH moieties: "*The IR spectra also showed several typical glutathione IR peaks, which are consistent with the glutathione spectrum in SpectraBase (John Wiley & Sons, Inc. SpectraBase, Compound ID=6507B5KJYT1). Among them, the ν(-COOH) stretching vibration at 1730 cm^{-1}, the asymmetric carboxylate vibration ν_{as}(COO-) at 1610 cm^{-1} and the COO- symmetric stretching vibration at 1410 cm^{-1} are the most evident.*"

8) In Table 1, please take into account the significant numbers and provide only the needed decimal values according to the standard deviation.

CHANGE: Table 1 was appropriately modified.

9) This study has been carried out with CuNPs of very small diameter. Why authors selected such small sized NPs? Some comments on the possible expected effect of the size of the CuNPs on the membranotropic effects would be interesting to be discussed. Even if the authors have some preliminary results with CuNPs of higher diameter they could be included.

REPLY: We thank the reviewer for bringing up this critical point about the NP size. Indeed, metal nanoparticles can cause NP internalisation within the membrane bilayer or NP absorption on the external membrane surface, according to the different NP size. However, the majority of the published papers about the membranotropic effects of metal nanoparticles on liposomes is related to gold nanoparticles with an average size > 10-20 nm and few papers focus on the investigation of the potential effects of the NP sizes, considering ultra-small metal NPs (size < 10 nm, e.g. 10.1038/s42004-020-00377-y). Since they are the most critical and potentially toxic NPs, we decided to start our investigation with ultra-small CuNPs. These particular particles offer the advances of being colourless and can be used as antimicrobial additives for paints and coatings that should retain the substrate pristine colour. On the other side, their size poses potential (nano)toxicological risks, thus they have been the first potential contaminant to study. At present, we don't have any preliminary data on other nanoparticle sizes. However, we agree with the reviewer that this could be

an interesting development and we have planned to continue the membranotropic studies considering other kinds of metals and sizes.

CHANGE: A sentence has been added at the end of the Conclusions section: *"Work is in progress to extend the study to antimicrobial nanoparticles of different size and composition."*

Referee: 2

The study demonstrates interactions of Cu nanoparticles with phosphatidylcholine (PC) based vesicles and model membrane disruption studies. Through characterization of Cu nanoparticles. It is good manuscript. However, it needs following points should be addressed with the corrections/modifications, before it may be considered for publication.

1) Do the authors try to examine the antibacterial effects of their Cu nanoparticles (CuNPs)? They should try to evaluate the antibacterial property of these CuNPs. 2) They can do the experiments for studying the antibacterial activity against a few Gram Positive bacteria (e.g. Staphylococcus aureus, Streptococcus pneumonia, Staphylococcus epidermidis, Bacillus subtilis, Streptococcus pyogenes) using the CuNPs. 3) They can do the experiments for studying the antibacterial activity against a few Gram Negative bacteria (e.g. Pseudomonas aeruginosa, Klebsiella pneumonia, Acinetobacter baumannii, E. coli) using the CuNPs.

REPLY: We thank the reviewer for the comments, but the biological experiments mentioned, and the list of bacteria cited would require a separate, dedicated study, not to count the fact that copper nanoparticles not only show antibacterial, but also antiviral and antifungal effects.

Copper nanomaterials are nowadays already accepted as broad-range antimicrobial agents. The intensity of the effect and the extent of the antimicrobial range of course depend on the CuNP properties, including size and capping agent. We already acknowledged the availability in the literature of numerous studies that demonstrate the efficacy, in general, of Cu-based nanomaterials against fungi, bacteria, and viruses (for instance, the review cited as ref. 1 offers a notable overview of the copper bioactivity in the current "copper age", doi: 10.1021/acsnano.0c10756).

Additionally, GSH-capped CuNPs have been investigated as bioselective reagents in cellular imaging (10.1016/j.microc.2020.105253)

Indeed, the present manuscript is not focused on the assessment of the antimicrobial properties of GSH-capped CuNPs, but the in-vitro investigation of their interaction with the human organism using liposomes as a model system. Such considerations were already present in the first paragraph of the Introduction.

CHANGE: Additional sentences were added, to stress the current spread of CuNP uses, including

(i) the several studies already published about their antimicrobial efficacy: "*In addition, in the last few years CuNPs have been successfully involved in various antiviral applications, exhibiting also a significant efficacy against the recent SARS-CoV-2 [10.1016/j.diagmicrobio.2020.115176, 10.1038/s41598-022-08766-0, 10.1007/s10534-021-00339-4].*"

(ii) the recent use of GSH-capped CuNPs as bioselective reagents for cellular imaging: *"Considering the current growth of CuNP uses as antimicrobials, and also their recent use as bioselective reagents for cellular imaging applications ,"*

4) Authors have done the experiments by taking only phosphatidylcholine, they can do these experiments by taking other phopoholipids like phosphatidylserine or even phosphatidylinositol or phosphatidylglycerol and check that all these experiments show similar results or some different results.

REPLY: We thank the reviewer for the comments. In fact, phosphatidylcholine is the primary lipid found in biological cell membranes, and liposomes synthesized with it are the most commonly used models for evaluating membranotropic effects in an artificial membrane model. To better mimic the biological lipid bilayer, we chose to use soybean-derived PC, a natural source, rather than pure PC. Additionally, liposomes made solely from PE or other phospholipids are not stable, as PC (a cylindrical shape molecule) must be present in at least 40-50% to obtain viable lipid vesicles. Therefore, we consider that liposomes made solely of natural PC provide us with a good approximation of the effect of these tiny CuNPs on the lipid membrane without the need for complex and expensive cell cultures. However, it could be interesting to attempt the use of liposomes composed of various phospholipid mixtures to evaluate potential composition-dependent changes. Nonetheless, this would necessitate an entirely new research endeavor, as it would require the repetition of all experiments for each liposome formulation.

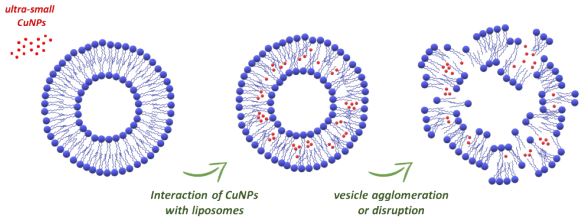
5) Some CuNPs are observed with the size bellow 3.0nm, it is interesting to note this. Do they show property like nanoclusters? Please check it.

REPLY: We thank the reviewer for this comment. Indeed, the whole population of CuNPs exhibited nanocluster-like behaviour, as proved by the absence of SPR bands in the visible range and by the fluorescence spectra. The discussion is already reported in the paragraph “Spectroscopic and morphological CuNPs@GSH characterization” (pag. 5).

6) Proper abbreviation of the journal’s name should be used like in Ref. 9. “ ……. International Journal of Molecular Sciences, 2021, 22, 6547.” should be “…….Int. J. Mol. Sci. , 2021, 22, 6547.”; in Ref 20. “ ……. Journal of Fluorescence, 1998, 8, 365–373.” should be ““ ……. J. Fluoresc. , 1998, 8, 365–373.”

CHANGE: we are sorry for the discrepancies. The Reference list was checked and modified, accordingly.

Fluorescence spectroscopic studies assess *in vitro* supramolecular interactions of ultra-small antimicrobial copper nanoparticles with phospholipids integrating biological membranes.



ARTICLE

Analytical probing of membranotropic effects of antimicrobial copper nanoparticles on lipid vesicles as membrane models

Received 00th August 20xx,
Accepted 00th August 20xx

DOI: 10.1039/x0xx00000x

Margherita Izzi,[†a] Miquel Oliver,[†b] Helena Mateos,[a] Gerardo Palazzo,[a] Nicola Cioffi *[a] and Manuel Miró*[b]

Copper nanoparticles (CuNPs) are worthwhile antimicrobial agents that are increasingly used in several real-life goods. However, concerns are arising about their potential toxicity and thus, appropriate legislations are being issued in various countries. *In vitro* exploration of the permeability and the distribution of nanoparticles in cell membranes should be explored as the first step towards the investigation of the toxicity mechanisms of metal nanoantimicrobials. In this work, phosphatidylcholine-based large unilamellar vesicles have been explored as mimic of cellular membranes to investigate the effect of ultra-small CuNPs on the physical-chemical features of phosholipid membranes. 4 nm-sized CuNPs were synthesized by a wet-chemical route that involves glutathione as stabilizer, with further characterization by UV–vis absorption spectroscopy, fluorescence spectroscopy, transmission electron microscopy , X-ray photoelectron spectroscopy (XPS), and Fourier transform infrared (FTIR) spectroscopy. Two fluorescent membrane probes bearing naphthalene moieties (laurdan and prodan) were used to monitor the bilayer structure and dynamics, as well as to demonstrate the strong membranotropic effects of CuNPs. The fluorescence spectroscopic studies were supported by dynamic light scattering (DLS) measurements and the calcein leakage assay. Additionally, the degree of perturbation of the phospholipid bilayer by CuNPs was compared against that of Cu^{2+} ions, the latter resulting in negligible effects. The findings suggested that CuNPs are able to damage the phospholipid membranes, leading to their agglomeration or disruption.

Introduction

Metals, such as copper and silver, have been utilized for thousands of years in antimicrobial applications such as water disinfection, food preservation, surgical bandages and sutures. In particular, with the nanomaterial growing interest and the raising emergence of antibiotic resistance, there has been reinvigorated interest in the use of metal nanomaterials as antimicrobial agents. Among them, the use of copper nanoantimicrobial agents is widely spread [1]. Copper is less expensive than other metals (such as gold and silver) and it is a trace element well tolerated by humans. Many aerobic organisms require copper for their metabolism as well as for the electron transport chain. However, if its concentration exceeds the biologically required, it becomes toxic and inhibits microbial growth.[1] Several applications of CuNP- antimicrobials are based on their use as nano-reservoirs, providing a controlled release of Cu^{2+} bioactive ions. Specifically, solid-state surface/films can embed biocidal NPs, allowing only the release of metal ions from their surface rather than the release of the entire nanoparticle. In this sense, CuNPs have been used as source of cupric ions in polymeric matrixes to develop bioactive coatings for food packaging applications [2]. The bioactivity of such coatings correlates with the Cu^{2+} release from the nanocomposite surface. In general, the effect is long-lasting, until the nanoparticle corrosion completely depletes the metal contained in the packaging outer layers.

In addition, in the last few years, CuNPs have been successfully involved in ~~different~~various antiviral applications, exhibiting also a significant efficacy against the recent SARS-CoV-2[3–5]

Considering the current growth of CuNP uses as antimicrobials, and also their recent use as bioselective reagents for cellular imaging applications [6], the investigation of the ecotoxicological effect of inorganic nanomaterials is arising, aiming to assess their influence on both human health and ecosystem processes. In addition, the legislation related to CuNP uses needs deeper toxicological evaluation [7]. To such aim, it is essential to understand how CuNPs and Cu^{2+} ions interact with cell membranes, which are the first barriers to be crossed to enter cells [8]. The investigation of toxicokinetics in the organism – including internal transport, metabolism, and excretion – is just the last step of human health risk assessments. Before that, elucidating the mechanisms at the molecular level *in vitro,* and inferring permeability and distribution of contaminants in cell membranes is a critical onset process that supports the prediction and assessment of toxicity pathways [9–11], in line with the EU REACH (Registration, Evaluation, Authorisation and restriction of Chemicals) regulations to promote *in-vitro* testing against ecotoxicity assays.

[a] *Chemistry Department, University of Bari Aldo Moro, Via Orabona, 4, 70126 Bari, Italy.*
[b] *FI-TRACE Group, Department of Chemistry, University of the Balearic Islands, Carretera de Valldemossa km 7.5, E-07122 Palma de Mallorca, Spain.*
† These authors contributed equally to this work.
Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

In this sense, liposomes have been consolidated as biomimetic artificial model of eukaryotic cell membranes. They are composed mainly of phospholipids, the same major component in biological membranes, and thus they are useful for the *in vitro* exploration of supramolecular interactions of xenobiotics with the lipid bilayer [12–14]. Therefore, their interaction with inorganic nanoparticles, as potentially toxic materials, should be also explored. Several studies have been published on the effect of gold nanoparticles on the membranes [15–17]. Lipid bilayers are highly flexible and can be deformed due to NP adhesion on its surface followed by NP engulfment that might end with the full NP uptake. The morphology, size and composition of the shell stabilizing the inorganic NP core has a great impact on its interaction with biological systems [18]. Indeed, the NP-membrane interactions can lead to different scenarios: NP internalisation within the membrane bilayer, full NP engulfment (similar to non-specific cellular endocytosis) or NP adsorption on the external membrane surface [8,16].

Here, the study is focused on ultra-small copper nanoparticles (CuNPs). To the best of our knowledge, few studies have been carried out on the investigation of the effect of CuNPs on the phospholipid membranes, focusing mainly on the cytotoxicity studies of Cu- or CuO-NPs on cell lines [19–21]. No papers have yet been published on the investigation of the membranotropic effects of CuNPs, especially with respect to ultra-small CuNPs. Tiny NP size allows for obtaining colourless colloidal solutions [22], which is sought for the development of transparent bioactive nanocomposites. Such CuNP feature makes them the best candidates for the preparation of antimicrobial coatings, useful to cover common-touched surfaces, textiles or other industrial goods. However, potential health risks related to the CuNP small size should be considered [23]. For this study, 4 nm-sized CuNPs were synthesized by means of a wet-chemical route that involves glutathione (GSH) as a stabilizer. The resulting CuNPs were characterized by UV–vis absorption spectroscopy, fluorescence spectroscopy, transmission electron microscopy (TEM), X-ray photoelectron spectroscopy (XPS), and Fourier transform infrared (FTIR) spectroscopy. Phosphatidylcholine (PC) is the most abundant phospholipid in eukaryotic membrane cells. Therefore, *in situ* synthesized large unilamellar vesicles (LUVs)/liposomes from natural sources of PC containing both saturated and unsaturated chains have been herein selected as model of biological membranes [10,11]. The incorporation of environment-sensitive fluorescent membrane dyes was used to monitor structure, dynamics and interactions of CuNPs with biomembranes. In particular, two fluorescent membrane probes bearing naphthalene moieties (viz., 6-propionyl-2-dimethylaminonaphthalene (prodan) and 6-dodecanoyl-2-dimethylaminonaphthalene (laurdan)) have been chosen to ascertain low-resolution changes in bilayer organization by generalized polarization (GP), that serves to indicate alterations in lipid ordering, packing and hydration [11,24,25]. The chemical-physical studies were supported by dynamic light scattering (DLS) measurements and calcein leakage assay. Additionally, the comparison of the membranotropic effects of CuNPs versus Cu^{2+} ions was investigated in detail, so as to decouple the role of the whole particle from that associated to ions released from its surface.

Materials and methods

Materials. Ascorbic acid (AA) and PBS (Phosphate Buffered Saline) tablets were purchased from Sigma-Aldrich/Merck KGaA. Copper(II) chloride dihydrate ($CuCl_2{\cdot}2H_2O$, reagent grade ACS) was received from Scharlau. L-glutathione (GSH, , reduced, 98+%) was obtained from AlfaAesar. Natural soybean L-α-phosphatidylcholine, LIPOID S100, was purchased from LIPOID GmbH (Ludwigshafen, Germany) with a concentration of L-α-phosphatidylcholine not less than 94% and a lipid tail distribution of linoleic acid (C18:2, (9Z,12Z)-octadeca-9,12-dienoic acid) as the main fatty acid, followed by palmitic acid (C16:0, n-hexadecanoic acid) and oleic acid (C18:1, cis-9-octadecenoic acid) with percentages of ca. 63%, 15% and 11%, respectively. The fluorescent membrane probes 6-dodecanoyl-N,N-dimethyl-2-naphthylamine (laurdan) and N,N-dimethyl-6-propionyl-2-naphthylamine (prodan) were also obtained from Sigma-Aldrich/Merck KGaA.

Synthesis of ultra-small CuNPs@GSH. CuNPs were prepared following the wet-chemical synthesis proposed by Huang *et al.* [22], using $CuCl_2$ and ascorbic acid as precursor and reducing agent, respectively. The GSH was used as stabilizer. Typically, a solution of 0.15 mM $CuCl_2$ in 0.2 mM GSH was prepared. Then, 112.5 µL of freshly-prepared 100 mM AA was added to 5 mL of the $CuCl_2$ - GSH solution and the reaction was kept under stirring for 4 h at 65°C. Afterwards, the colloid was allowed to cool to room temperature, and stored at 4°C for further use.

Theoretical estimation of CuNPs concentration. ~~In order to have an estimation of the number of CuNPs per millilitre, a theoretical calculation based on the copper density was carried out.~~ The common practice for the theoretical estimation of the metal nanoparticle concentration involves calculations based on their optical properties, such as extinction coefficients relevant to surface plasmon resonance (SPR) features [26]. Nevertheless, it could not be used in the present work because of the lack of suitable SPR signals. For this reason, a theoretical calculation based on the copper density and assuming that the Cu(II) precursor ~~i~~was quantitatively converted into elemental nanosized copper phases was carried out. The resulting estimation returns the highest available CuNPs concentration, which in turn leads to the worst e case scenario. In particular, assuming that all the amount of Cu^{2+} from the metal precursor used in the synthesis (0.15 mM) was quantitatively turned into elemental Cu, the mass of copper in 1 mL of colloid was calculated (9.8 µg). Then, using the CuNP average diameter estimated from TEM images (3.7 nm) to determine the average volume of a single spherical CuNP, the mass of one CuNP was calculated from the copper density (8940 kg/m^3). Hence, the total mass to single NP mass ratio gives an estimation of the

number of CuNPs, in our case, $4 \cdot 10^{13}$ particles/mL of colloid solution.

Synthesis of large unilamellar vesicles (LUV). Liposomes were prepared by lipid film hydration method [27] followed by extrusion [28] with some changes to improve the quality of the final product. To this end, Aa solution of 50 mM of Soy PC was prepared in a mixture of chloroform:methanol at a ratio 3:1. Then 200 µL of this solution was added to a 25mL round-bottom flask covered by aluminium foil to protect lipids from photooxidation. For laurdan-containing liposomes, 100 µL of 1 mM Laurdan solution prepared in the same organic solvent mixture was added. Then, the solution of PC alone or with laurdan was diluted up to 3 mL with the same organic mixture. The increase of the volume was intended to improve the formation of a thin lipid film by increasing the surface area of deposition after evaporation. To this end, the solution of PC was dried using a rotavapor at 30ºC and reduced pressure (290 mbar) for 2 hours to obtain a well-dispersed homogeneous lipid film on the walls of the flask. Subsequently, the flask was kept under vacuum for 2 more hours to ensure the complete removal of any organic solvent traces that might have remained tightly attached to the lipid film. The hydration of the dry lipid film was achieved by adding 1 mL of filtered aqueous phosphate buffered saline (PBS) solution to obtain a final concentration of 10 mM of PC and 100 µM of fluorescent probe (ratio 100:1), and then vortexing for 1 minute every 5 minutes over a period of 1 hour to aid in the swelling and complete resuspension of the PC layers. The obtained milky suspension of multilamellar vesicles (MLVs) was refrigerated overnight to stabilize the emulsion. Finally, the size, lamellarity and homogeneity of MLV suspension was controlled by extrusion using a mini extruder (Avanti Lipids) at room temperature through a 100 nm polycarbonate membrane. The procedure was repeated 29 times, to enable obtaining large unilamellar vesicles (LUVs).

Theoretical estimation of liposome concentration. To determine the number of liposomes, the average number of PC molecules in a liposome is first calculated using equation 1 and considering that the liposomes are unilamellar.

$$N_{lipid} = \frac{4\pi\left(\frac{d}{2}\right)^2 + 4\pi\left(\frac{d}{2} - h\right)^2}{a} \quad \text{Eq. 1}$$

Where "d" is the diameter of the LUV, in our case 100 nm, "h" is the average thickness of the lipid bilayer (c.a. 5 nm) and "a" is the lipid head group area that for PC corresponds to 0.71 nm^2. The number of liposomes/mL (Eq. 2) is obtained by dividing the initial concentration of lipids (M_{lipid}) multiplied by the Avogrado's number over the number of lipids that were previously calculated, giving a final concentration of 10^{12} liposome/mL.

$$N_{liposomes/mL} = \frac{M_{lipid} \times N_A}{N_{lipid} \times 1000} \quad \text{Eq. 2}$$

Spectroscopic and morphological CuNPs@GSH characterization. UV-Vis spectra were acquired with a double beam spectrophotometer (Shimadzu UV-1601) in the 250–800 nm wavelength range. Excitation and emission fluorescence spectra were acquired using a Varian Cary Eclipse fluorescence spectrometer (Agilent technologies). Quartz cuvettes (optical path 1 cm, Optech, München, Germany) were used. Transmission electron microscopy (TEM) was performed with a FEI Tecnai 12 microscope (Eindhoven, Netherlands), equipped with a LaB_6 filament operating at 120 kV. Size distribution histograms were obtained after TEM images processing by ImageJ software [29], manually highlighting individual NPs on each micrograph. Histograms were produced on three replicates, counting more than 500 nanoparticles. X-ray photoelectron spectroscopy measurements were performed on CuNP colloids deposited on silicon substrates using a PHI Versaprobe II (Chanhassen, MN, USA) spectrometer equipped with monochromatized Al-Kα radiation (1486.6 eV). Binding energy (BE) scale was corrected on C1s component at 284.8 eV. $Cu2p_{3/2}$ region was fitted using MultiPak® version 9.9.0.8 that selected, selecting an asymmetric function for the Cu(0)/Cu(I) component and a Gaussian-Lorentz function (with a Gauss% = 90%) for the satellite peaks. Infrared spectra were recorded by a Perkin Elmer Spectrum-Two. Dried samples were analysed in transmission with attenuated total reflection (ATR) mode, using an ATR accessory with a diamond crystal at a fixed 45° incidence angle. Each spectrum was averaged over 32 scans in the range 400–4000 cm^{-1}, at a spectral resolution of 2 cm^{-1}.

Identification of CuNP permeation across lipid by fluorescence measurements. The potential alteration of phospholipid packing and ordering at distinct depths of the membrane of LUVs in the presence of bioavailable contaminants was investigated by fluorescent membrane probes (laurdan and prodan) at 37 °C. A stock solution of liposomes was prepared and diluted to 100 µM PC in PBS and stored in a brown glass vial. As mentioned before, Laurdan probe was added to the phospholipid solution during the lipid film formation, to ensure appropriate molecule stabilization across the bilayer. On the contrary, a metered volume of prodan in 3:1 chloroform/methanol was added to the liposomal suspension after their preparation and kept in darkness at room temperature for 15 min, to allow its partition into the membrane. In both cases, a final probe concentration of 1 µM was used. Finally, a proper volume of copper colloid was added, to have a final concentration of 10^{11} CuNPs/mL. Steady-state fluorescence assays were performed using a Varian Cary Eclipse fluorescence spectrometer (Agilent technologies). The samples were incubated at 37 °C in the Peltier multicell holder for 5 min prior to start the measurements. Fluorescence emission spectra were recorded from 400 to 600 nm by setting the excitation wavelength to 360 nm, and the photomultiplier detector voltage to 540 V with slit widths of 10 nm. The potential modification of lipid packing and ordering, thus hydration of membrane upon interaction with CuNPs into the bilayer was measured by the Generalized Polarization (GP) equation:

$$GP = \frac{I_B - I_R}{I_B + I_R} \quad \text{Eq. 3}$$

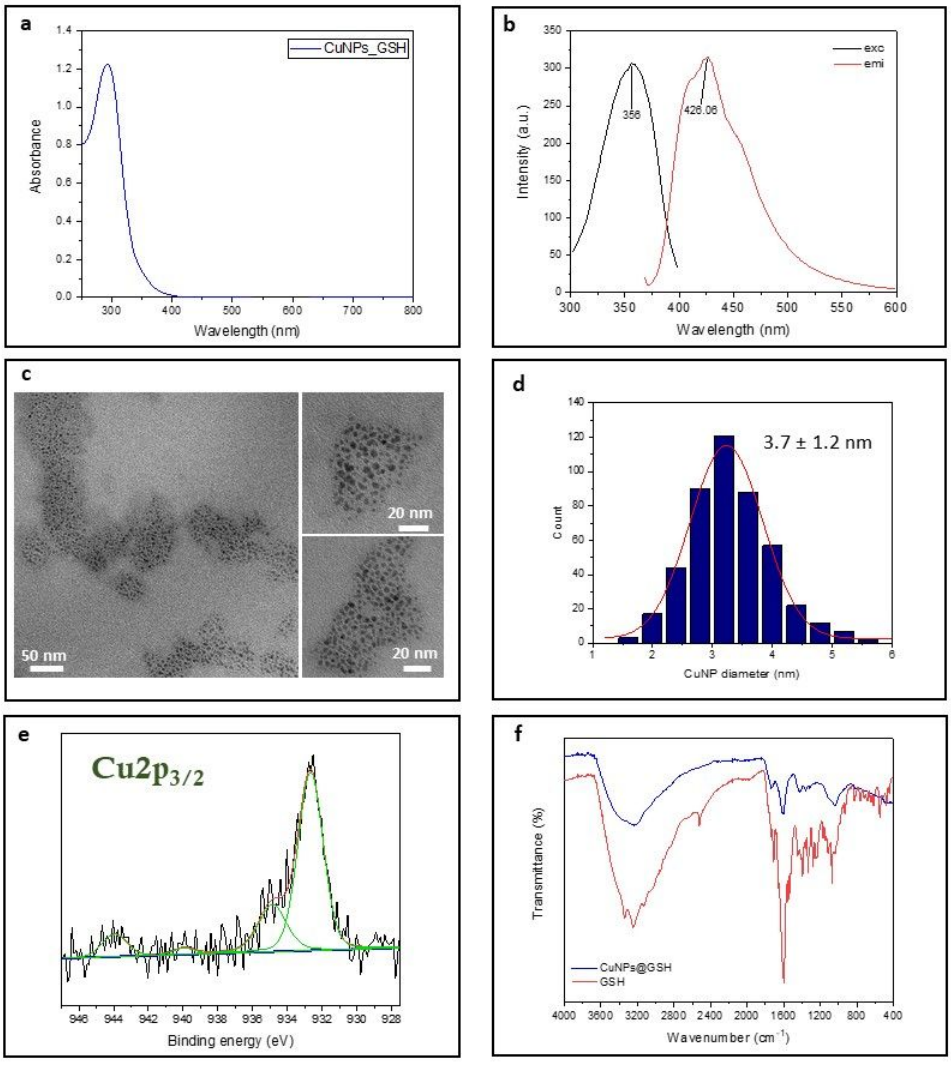
where I_B and I_R stand for the fluorescence emission intensities at 440 nm and 490 nm, respectively, that correspond to the maximum emission of the membrane probes in nonpolar media

and polar environment, respectively. Theoretically, GP values range from -1 to +1. Negative values refer to a liquid-crystalline phase, while positive values are obtained with the gel phase, which is characterized by a more ordered and dehydrated membrane. The change in the GP values were monitored for 24 hours by recording an emission spectrum each hour. Biological membranes are usually characterized by having negative GP values, due to the presence of highly unsaturated acyl chains that allow the permeation of metabolites and other molecules for their proper functioning. The same instrumentation was used to measure the fluorescence emission of calcein from vesicles. The excitation and the emission wavelengths were 495 nm and 516 nm, respectively. Excitation and emission slits were changed, depending on the concentration of total calcein in the solution analyzed. The measured calcein fluorescence was used to quantify the calcein leakage from the vesicles. However, the strong quenching by the CuNP does not allow the measurement of customary leakage kinetics. To overcome this aspect, the *ad-hoc* strategy described in the Result and Discussion section has been used.

Results and discussion

Spectroscopic and morphological CuNPs@GSH characterization. Glutathione is a natural tripeptide widely used to prepare ultra-fine metal nanoparticles [22,30,31]. Herein, it is utilized as a stabilizing agent for the synthesis of small CuNPs. The as-prepared copper colloid is almost colorless in visible light. In fact, the UV–Vis absorption spectrum shows a strong absorption in the UV region, ascribed to the molecule-like properties of tiny CuNPs. The typical plasmonic peak of CuNPs, falling at around 490 nm, is indeed absent for these fine particles (Fig. 1a). The fluorescent CuNPs showed maximum excitation and emission peaks at 356 nm and 426 nm, respectively, that are in agreement with the findings of Huang

Commented [MI1]: This figure will be replaced with a new version in the final manuscript, according to the suggestions of the Referee 1



and coworkers [22] (Fig. 1b). No UV absorption and fluorescence emission was observed for the reactants at room temperature (Fig. S1). This demonstrated that the formation of CuNPs only occurred when the synthesis was carried out at the activation temperature of 65°C. Although the CuNP excitation wavelength is very closed to the laurdan and prodan's excitation wavelength, we tested that the emission fluorescence intensity of CuNPs was neglectable at the concentrations used for GP studies. The size of CuNPs was assessed by TEM analysis (Fig. 1c-d). According to the size analysis of more than 500 individual particles, the mean diameter of CuNPs was 3.7 ± 1.2 nm. Fig. 1e shows the Cu2p$_{3/2}$ XP high-resolution spectrum relevant to the CuNPs@GSH sample. Two photoelectron components were detected: the component centered at 932.7 ± 0.2 eV, attributed to Cu species in a lower oxidation state, Cu^0 and Cu^{1+}; and the second one, at 934.8 ± 0.2 eV, attributed to Cu^{2+} [32]. The presence of cupric species was confirmed by the shake-up features, fitted by two peaks falling at 941.2 ± 0.2 eV and 943.7±0.2 eV. These results suggest that CuNPs undergo a partial surface oxidation, reasonably due to the unavoidable exposure of colloidal solution to air, both during the synthesis and the sample preparation for the XPS characterization. In addition, the ultra-small size scale of NPs implies an extremely high surface/volume ratio, thus increasing the availability of Cu atoms on the NP surface for potential oxidation. Fig. 1f reports the ATR-FTIR spectra of CuNPs@GSH and pure GSH. As expected, the –SH stretching band (2519 cm^{-1}) of GSH disappears from the surface of CuNPs, indicating the formation of the covalent bonds (Cu-S-R) between GSH and CuNPs [22]. The IR spectra also showed several typical glutathione IR peaks, which are consistent with the glutathione spectrum in SpectraBase [33]. Among them, the ν(-COOH) stretching vibration at 1730 cm^{-1}, the asymmetric carboxylate vibration ν_{as}(COO-) at 1610 cm^{-1}, the COO- symmetric stretching vibration at 1410 cm^{-1}, and ν(C-N) around 1050–1000 cm^{-1} are the most significant [34].

Fluorescence Generalized Polarization Spectroscopy. Steady-state fluorescence emission spectra and Stokes shifts of the fluorophore-bearing laurdan and prodan probes are good indicators of structural and dynamic alterations of liposome lipid bilayers. The studies were carried out in 24 hours, to examine the GP trend over time as function of the CuNP interaction with the liposomes. The emission spectra showed a decrease of the peak maximum throughout time for both probes, as shown in Fig. S2. In particular, the emission spectra of laurdan showed a remarkable blue shift of the emission peak up to 470 nm (Figure S2a), with the subsequent significant variation of the GP (calculated according to Eq. 1), which increased towards positive values from -0.39 to 0 (see Fig. 2a). Moreover, it reached a plateau level around 20 hours. On the other side, the GP values relative to prodan fluorescent emission, characterized by a short acyl chain composed of a propyl group, showed a smaller variation over time, from -0.50 to -0.40. This observation can be attributed to the ability of prodan to interact with both the external water molecules and the polar head group of the phospholipids as corroborated by the more negative GP values compared to those of laurdan [10]. Consequently, the higher polarity of the medium around prodan is much less affected by the presence of CuNPs. Interestingly, the increment of GP-prodan values did not keep constant throughout time. As shown in Fig. 2b, it reached a maximum GP value around 6 hours, and then decreased again towards more negative GP values. Additionally, the mobility of Prodan probe is identified by the higher variability of GP values after an incubation time of 10 h.

In the case of laurdan, the increment (hypsochromic shift) of GP values is indicative of the packed structure of the lipid bilayer in the presence of CuNPs, thereby suggesting enhanced lipid packing and ordering at the glycerol level with concomitant membrane dehydration [10]. Our hypothesis is that CuNPs interact firstly with the polar head group of the external leaflet of the membrane, and then penetrate through the outermost part of the liposome membrane during the first incubation hours (prodan-responsive zone). During this period, CuNPs accumulate on the membrane/water interface. With longer assay times, CuNPs are able to reach the wider bilayer zone and interact with the phospholipid acyl chains (laurdan-responsive

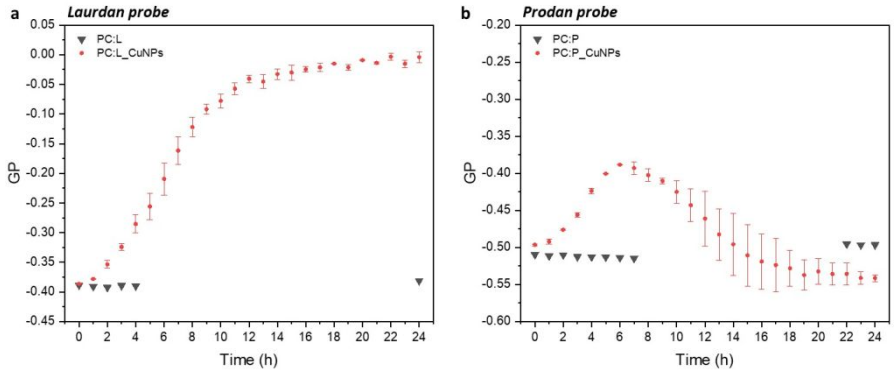


Figure 2. Time-dependent GP variation for laurdan (a) and prodan (b) in the presence of 10^{11} CuNPs/mL.

zone) with potential accumulation in the bilayer zone or penetration into the inner liposome part (*vide infra* Fig. 5). A similar trend was reported by Luchini and colleagues [17] on lipid-functionalized AuNPs, although they observed a more significant GP variation in a time-frame of about one hour. Such discrepancies are probably due to the different metal, as well as to the different liposome/NP concentration ([18-lysophosphocholine]AuNPs= 250 μM). Indeed, to the best of our knowledge, no papers have been published about the study of membranotropic effects of CuNPs to date. However, it was demonstrated by means of density functional theory and molecular dynamics simulations that Cu^{2+} ions can cause the decrease of phospholipid bilayer fluidity, due to the phospholipid coupling by a Cu-Cu bond [35]. It has been reported that upon Cu^{2+} binding to the negatively charged phosphate group of the polar head of the phospholipid, Cu^{2+} is reduced to Cu^{+}. As a consequence of the anomalous attraction between Cu^{+} cations, the binding between two close lipids is trigged, resulting in the formation of PL-Cu-Cu-PL structure. Thus, in order to investigate the membranotropic effects of the Cu^{2+} ions, which could be potentially released from the surface of the CuNPs, steady-state fluorescence studies involving the fluorophore probes were also carried out on Cu^{2+} rather than CuNPs. To this aim, Cu^{2+} ions were added to the liposome solution using $CuCl_2$ at the same concentration of the CuNPs synthesis. Fig.3 a and b report the GP temporal profiles of laurdan and prodan, respectively, as a function of the Cu^{2+} interaction with the liposome. It is evident that the effect of the Cu^{2+} ions is less significant than that of CuNPs, especially for the outer part of the lipidic membrane (prodan-responsive probe). In fact, the GP increase for the laurdan was just moderate, compared to that obtained for the CuNPs, and the GP increment for the prodan was almost negligible. These findings indicate that the main reason of the change of the membranotropic properties of the membrane is occasioned by CuNPs; however, the Cu^{2+} ions potentially released from the CuNP surface may still play an additional or synergistic role on deeper lipidic layer alterations. The potential effect caused by the stabilizing agent GSH was also evaluated, showing no significant changes in the Laurdan GP values over the course of 24 h (data not shown).

Fluorescence leakage assay. The effect of CuNPs on the membrane permeability can be investigated by measuring the fluorescence of liposomes entrapping a high concentration of calcein. Calcein is an aqueous soluble fluorophore that self-quenches at high concentrations, so in the customary leakage assay, one probes the kinetics of fluorescence emission associated to the vesicle breakdown and subsequent calcein release and dilution and detection in the outer medium[14]. Such an approach cannot be regrettably used in our case because both the CuNPs or the Cu^{2+} ions are very efficient quenchers of calcein emission. Indeed, the addition of CuNPs or Cu^{2+} to a diluted solution of highly fluorescent calcein (without any vesicle) induces in a few minutes the quenching of more than 90% of the signal. Accordingly, the fluorescence emission of LUV containing calcein challenged by CuNPs does not change in time because even in the presence of slow leakage events the emission of calcein molecules in the matrix solution is efficiently quenched by the CuNPs. For this reason, we modified the leakage assay as follows: various samples having the same concentration of LUV and CuNPs have been prepared simultaneously and let to incubate at different times. After a given incubation time, the vesicles were disrupted by adding the detergent Triton X100 (TX), which induced the prompt release of all the calcein molecules entrapped in the LUVs, and the fluorescence emission was collected immediately. Operating in such a way, the emission of calcein that permeated during the incubation time (before TX addition) is lost because of the CuNP quenching and the fluorescence emission recorded is due only to the remaining calcein that is released from the liposomes after the TX addition. The fraction of calcein that remained in the intact vesicles during the incubation time can be evaluated by Eq. 4:

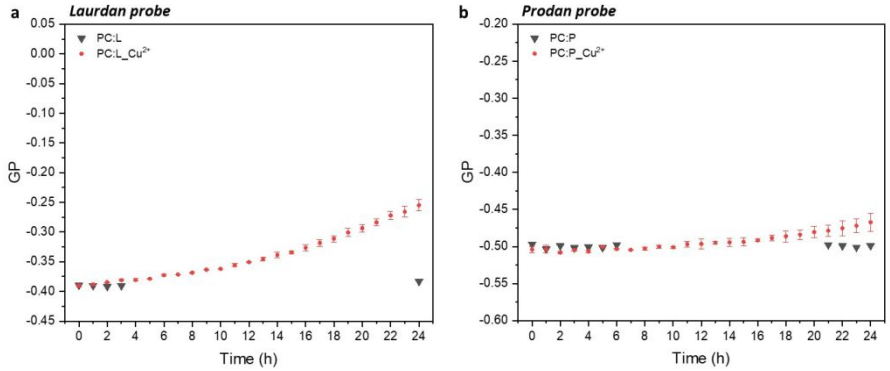


Figure 3. Temporal profiles of the GP value for laurdan (a) and prodan (b) without and with Cu^{2+} ions at the same concentration than that of the synthesis of CuNPs, diluted 100 times as in the case of CuNPs (0.0015 mM).

$$Residual\ Calcein = \frac{F_{TX}(t) - F_0(t)}{F_{TX}(0) - F_0(t)}$$ Eq. 4

Where $F_{TX}(t)$ and F_0 (t) are the fluorescence intensity values measured immediately after and before, respectively, the addition of TX to a solution of LUV incubated with CuNPs for a time *t*. $F_{TX}(0)$ is the fluorescence emission taken by adding simultaneously TX and CuNPs. The dependence of the residual calcein in intact liposomes on the incubation time with the CuNPs is shown in Figure 4. The fraction of calcein entrapped in intact liposomes decreased linearly with the time they were in contact with the CuNPs. After 24 hours almost all the calcein (80%) was quenched by the CuNPs. This indicates that in the presence of CuNPs the phospholipid membrane becomes gradually more permeable and the calcein is released in a controlled manner.

Liposome hydrodynamic diameter over time. Our findings in this work indicate that CuNPs might trigger changes of liposome size over time. The hydrodynamic diameter was then monitored for 24 hours by DLS measurements. As shown by the trend in Fig. S4, the liposome size significantly increased approximately 11 hours after mixing with CuNPs, suggesting agglomeration of vesicles or their disruption and transformation into wormlike micelle (which are expected to have a larger hydrodynamic size). Table 1 compiles the temporal size profiles of the liposomes before and after mixing with CuNPs (10^{11} CuNPs/mL) or Cu^{2+} (0.0015 mM). Interestingly, the vesicle size does not undergo any change over time when Cu^{2+} ions are added to the liposome solution. These results definitively are consistent with the fluorescence studies, providing evidence that ultra-small CuNPs are able to alter the physical-chemical features of the phospholipid membranes and jeopardise the stability of liposomes.

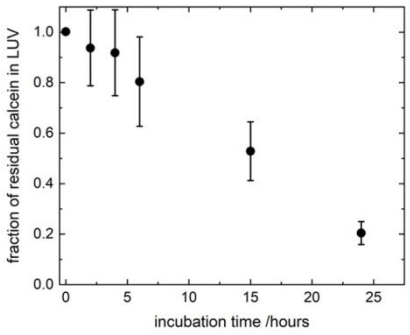


Figure 4. Fraction of calcein molecules remaining in liposomes after mixing with CuNP at different incubation times, as obtained by liposome solubilization with TritonX100.

Table 1. Hydrodynamic diameter of liposomes over time measured by DLS after interaction with CuNPs (10^{11} CuNPs/mL) or Cu^{2+} (0.15 mM).

Time (h)	Liposome (nm)	CuNPs-liposome (nm)	Cu^{2+}-liposome (nm)
0	110~~.3~~ ± 4~~.1~~	105.3 ± 1.1	103.5 ± 1.1
2	104.5 ± 1.1	108.3 ± 2.0	107.5 ± 2.1
4	106.1 ± 0.5	10~~7.68~~ ± 3~~.0~~	107.4 ± 1.2
20	110~~.5~~ ± 3~~.3~~	~~702~~ 700 ± 160	107.2 ± 0.8
24	116.7 ± 0.1	550 ± ~~345~~350	111.0 ± 0.8

Conclusions

In this work, the *in vitro* supramolecular interactions of 4 nm-CuNPs with phospholipids integrating biological membranes were studied. For this purpose, PC-liposomal nanoparticles tailored with two distinct polarity-sensitive membrane probes, prodan and laurdan, were harnessed for discrimination of membranotropic effects at the polar head group and glycerol level/acyl chains of PC, respectively. The use of the two fluorescence probes is suitable to ascertain low-resolution changes in bilayer organization by GP measurements that indicate alterations in lipid ordering and membrane hydration due to the presence of CuNPs. In particular, the increase of laurdan GP towards more positive values suggested the enhancement of lipid packing and the order at the glycerol level, with concomitant membrane dehydration. In addition, the investigation over time of calcein leakage and liposome hydrodynamic diameter confirm that ultra-small CuNPs are able to damage the phospholipid membranes, with the subsequent vesicle agglomeration or disruption and transformation into wormlike micelle (Fig. 5). Interestingly, the comparison of CuNPs against Cu^{2+} demonstrated that the potential deleterious effects are mainly attributed to the presence of whole nanoparticles themselves, rather than to Cu^{2+} released from their surface. This is an interesting finding for the antimicrobial application of CuNPs, corroborating once more the advantages of their use as nano-reservoirs of bioactive Cu^{2+} ions, entrapped in solid state films/coatings, because in this way the direct release of potentially toxic CuNPs is avoided. Fluorescence spectroscopy proved to be a suitable tool for the investigation of membranotropic effects of nanoantimicrobial particles on the phospholipid membranes, paving the way for further insights about the potential toxicity of nanoantimicrobials. Work is in progress to extend the study to antimicrobial nanoparticles of different size and composition.

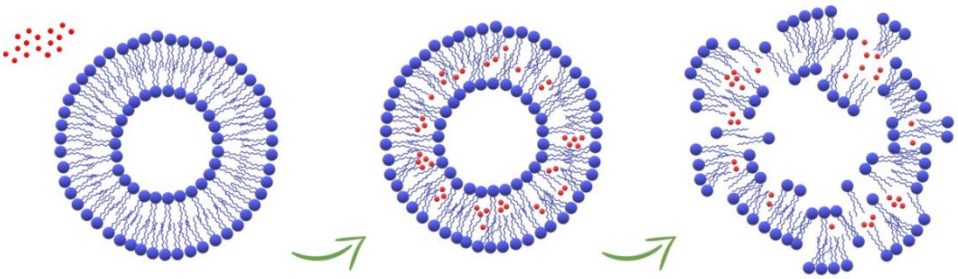


Figure 56. Schematic representation of the damage of the liposomes caused by the accumulation of CuNPs in the phospholipid membranes.

Author Contributions

Conceptualization: M.M., N.C., M.I., M.O.; Methodology: M.O, M.I., M.M., G.P., N.C.; Investigation: M.I., M.O., H.M.; Writing - Original Draft: M.I.; Writing - Review & Editing: M.O., H.M., G.P., N.C., M.M.; Supervision: M.M., N.C.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

This work was partially carried out during the PhD secondment of M.I. at the University of the Balearic Islands. Prof. Rosaria Anna Picca is kindly acknowledged for her contribution in XPS analyses. Dr. Maria Chiara Sportelli is kindly acknowledged for suggestions on the CuNPs synthesis. Partial financial support is acknowledged from HORIZON EUROPE SEEDS ~~-SCOOP~~ – Codice identificativo progetto S152 (N.C.) and from the MUR PNRR Extended Partnership initiative on Emerging Infectious Diseases -project no. PE00000007, INF-ACT (G.P.). H.M. was funded by POR PUGLIA FESR-FSE 2014/2020 Research for Innovation (REFIN)-Codice Pratica: 7BDC8679. M.M. acknowledges financial support from the Spanish State Research Agency (Agencia Estatal de Investigación, AEI/10.13039/501100011033), and the Spanish Ministry of Science and Innovation (Ministerio de Ciencia e Innovación, MCIN) through project PID2020-117686RB-C33 (AEI/MCIN). M. O. is grateful to the Spanish Ministry of Universities and European Union (NextGenerationEU) for granting a "Margarita Salas" post-doctoral position in the framework of the Recovery, Transformation and Resilience Plan.

References

1 M. L. Ermini and V. Voliani, *ACS Nano*, 2021, **15**, 6008–6029.
2 M. C. Sportelli, M. Izzi, A. Volpe, V. Lacivita, M. Clemente, C. Di Franco, A. Conte, M. A. Del Nobile, A. Ancona and N. Cioffi, *Food Packaging and Shelf Life*, 2019, **22**, 100422.
3 A. Purniawan, M. I. Lusida, R. W. Pujiyanto, A. M. Nastri, A. A. Permanasari, A. A. H. Harsono, N. H. Oktavia, S. T. Wicaksono, J. R. Dewantari, R. R. Prasetya, K. Rahardjo, M. Nishimura, Y. Mori and K. Shimizu, *Scientific Reports*, 2022, **12**, 4835.
4 A. A. Cortes and J. M. Zuñiga, *Diagnostic Microbiology and Infectious Disease*, 2020, **98**, 115176.
5 V. Govind, S. Bharadwaj, M. R. Sai Ganesh, J. Vishnu, K. V. Shankar, B. Shankar and R. Rajesh, *Biometals*, 2021, **34**, 1217–1235.
6 R. Rajamanikandan, B. Aazaad, S. Lakshmipathi and M. Ilanchelian, *Microchemical Journal*, 2020, **158**, 105253.
7 Directorate-General for Health and Food Safety, SCCS - Final Opinion on Copper (nano) and Colloidal Copper (nano), https://health.ec.europa.eu/latest-updates/sccs-final-opinion-copper-nano-and-colloidal-copper-nano-2021-03-05_en, (accessed July 25, 2023).
8 K. L. Chen and G. D. Bothun, *Environmental Science & Technology~~Environ. Sci. Technol.~~*, 2014, **48**, 873–880.
9 J.-J. Ortega-Calvo, J. Harmsen, J. R. Parsons, K. T. Semple, M. D. Aitken, C. Ajao, C. Eadsforth, M. Galay-Burgos, R. Naidu, R. Oliver, W. J. G. M. Peijnenburg, J. Römbke, G. Streck and B. Versonnen, *Environmental Science & Technology~~Environ. Sci. Technol.~~*, 2015, **49**, 10255–10264.
10 M. Oliver, A. Bauzá, A. Frontera and M. Miró, *Environmental Science & Technology~~Environ. Sci. Technol.~~*, 2016, **50**, 7135–7143.
11 M. Oliver, M. Adrover, A. Frontera, J. Ortega-Castro and M. Miró, *Science of The Total Environment*, 2020, **738**, 140096.
12 S. Andrade, M. J. Ramalho, J. A. Loureiro and M. C. Pereira, *Journal of Molecular Liquids*, 2021, **334**, 116141.
13 L. Šturm and N. Poklar Ulrih, *International Journal of Molecular Sciences*, 2021, **22**, 6547.
14 H. Mateos, A. Mallardi, F. Blasi and G. Palazzo, *Colloids and Surfaces B: Biointerfaces*, 2022, **220**, 112885.
15 A. Bhat, L. W. Edwards, X. Fu, D. L. Badman, S. Huo, A. J. Jin and Q. Lu, *Appl. Phys. Lett.*, 2016, **109**, 263106.
16 C. Contini, J. W. Hindley, T. J. Macdonald, J. D. Barritt, O. Ces and N. Quirke, Communications Chemistry ~~*Commun Chem*~~, 2020, **3**, 1–12.
17 A. Luchini, G. D'Errico, S. Leone, Z. Vaezi, A. Bortolotti, L. Stella, G. Vitiello and L. Paduano, *Colloids and Surfaces B: Biointerfaces*, 2018, **168**, 2–9.
18 C. Contini, M. Schneemilch, S. Gaisford and N. Quirke, *Journal of Experimental Nanoscience*, 2018, **13**, 62–81.
19 H. L. Karlsson, P. Cronholm, Y. Hedberg, M. Tornberg, L. De

Battice, S. Svedhem and I. O. Wallinder, *Toxicology*, 2013, **313**, 59–69.
20 J. Hedberg, H. L. Karlsson, Y. Hedberg, E. Blomberg and I. Odnevall Wallinder, *Colloids and Surfaces B: Biointerfaces*, 2016, **141**, 291–300.
21 N. Li, H. J. Sui and D. M. Gao, *Advanced Materials Research*, 2012, **356–360**, 2274–2277.
22 H. Huang, H. Li, J.-J. Feng, H. Feng, A.-J. Wang and Z. Qian, *Sensors and Actuators B: Chemical*, 2017, **241**, 292–297.
23 I. Pacheco and C. Buzea, in *Metal Nanoparticles*, John Wiley & Sons, Ltd, 2018, pp. 237–293.
24 T. Parasassi, E. K. Krasnowska, L. Bagatolli and E. Gratton, *Journal of Fluorescence*, 1998, **8**, 365–373.
25 G. Gunther, L. Malacrida, D. M. Jameson, E. Gratton and S. A. Sánchez, *Accounts of Chemical Research~~Acc. Chem. Res.~~*, 2021, **54**, 976–987.
26 W. Haiss, N. T. K. Thanh, J. Aveyard and D. G. Fernig, *Analytical Chemistry~~Anal. Chem.~~*, 2007, **79**, 4215–4221.
27 A. D. Bangham, J. De Gier and G. D. Greville, *Chemistry and Physics of Lipids*, 1967, **1**, 225–246.
28 M. J. Hope, M. B. Bally, G. Webb and P. R. Cullis, *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 1985, **812**, 55–65.
29 ImageJ, https://imagej.nih.gov/ij/, (accessed May 31, 2019).
30 X. Yuan, Y. Tay, X. Dou, Z. Luo, D. T. Leong and J. Xie, *Analytical Chemistry~~Anal. Chem.~~*, 2013, **85**, 1913–1919.
31 G. Zhang, Y. Li, J. Xu, C. Zhang, S. Shuang, C. Dong and M. M. F. Choi, *Sensors and Actuators B: Chemical*, 2013, **183**, 583–588.
32 N. Cioffi, L. Torsi, N. Ditaranto, G. Tantillo, L. Ghibelli, L. Sabbatini, T. Bleve-Zacheo, M. D'Alessio, P. G. Zambonin and E. Traversa, *Chemistry of Materials~~Chem. Mater.~~*, 2005, **17**, 5255–5262.
33 Glutathione (reduced) - SpectraBase, https://spectrabase.com/compound/6507B5KJYT1, (accessed September 9, 2023).
34 J. P. Monrás, V. Díaz, D. Bravo, R. A. Montes, T. G. Chasteen, I. O. Osorio-Román, C. C. Vásquez and J. M. Pérez-Donoso, *PLOS ONE*, 2012, **7**, e48657.
35 X. Jiang, J. Zhang, B. Zhou, P. Li, X. Hu, Z. Zhu, Y. Tan, C. Chang, J. Lü and B. Song, *Scientific Reports,~~Sci Rep,~~* 2018, **8**, 14093.

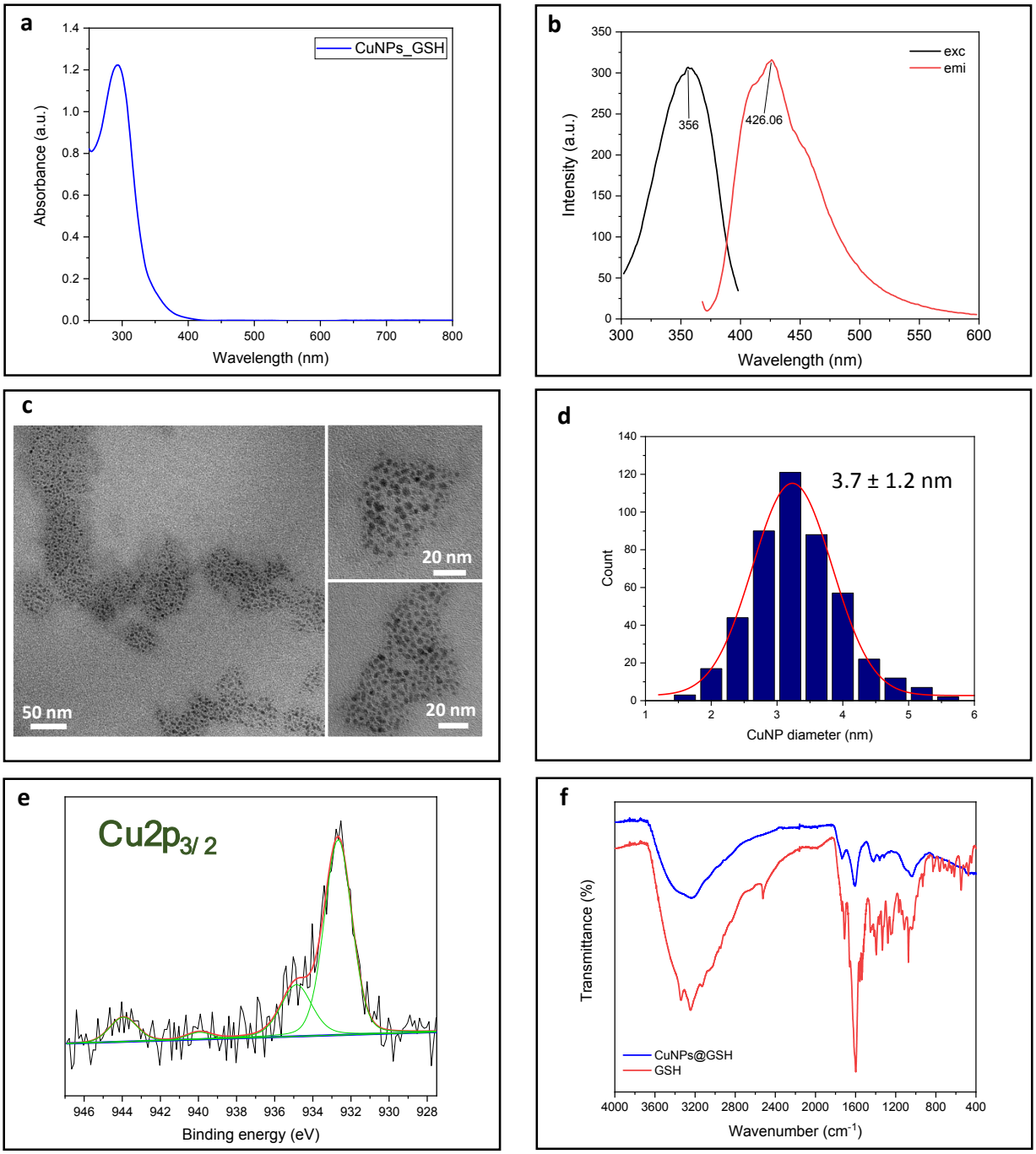


Figure 1. Spectroscopic and morphological characterization of CuNPs: (a) UV-Vis spectrum; (b) Fluorescence spectra: (c) TEM images; (d) size histogram; (e) $Cu2p_{3/2}$ XPS spectrum; (f) ATR-FTIR spectra.

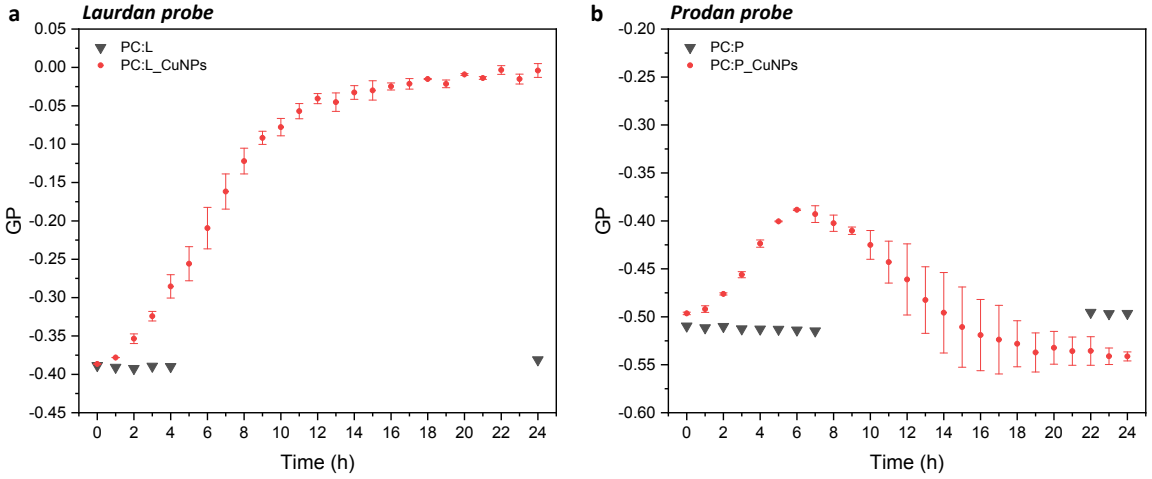


Figure 2. Time-dependent GP variation for laurdan (a) and prodan (b) in the presence of 10^{11} CuNPs/mL.

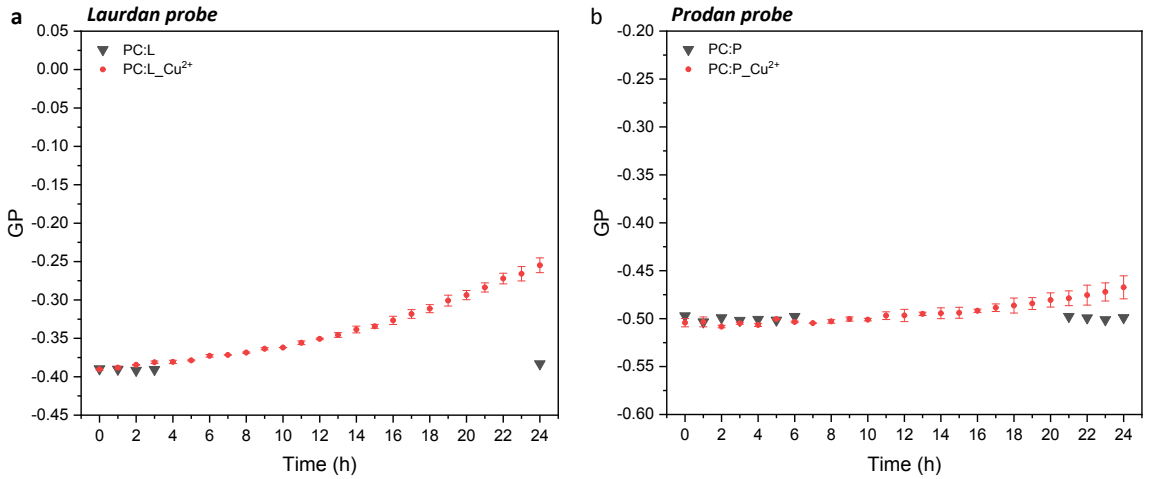


Figure 3. Temporal profiles of the GP value for laurdan (a) and prodan (b) without and with Cu^{2+} ions at the same concentration than that of the synthesis of CuNPs, diluted 100 times as in the case of CuNPs (0.0015 mM).

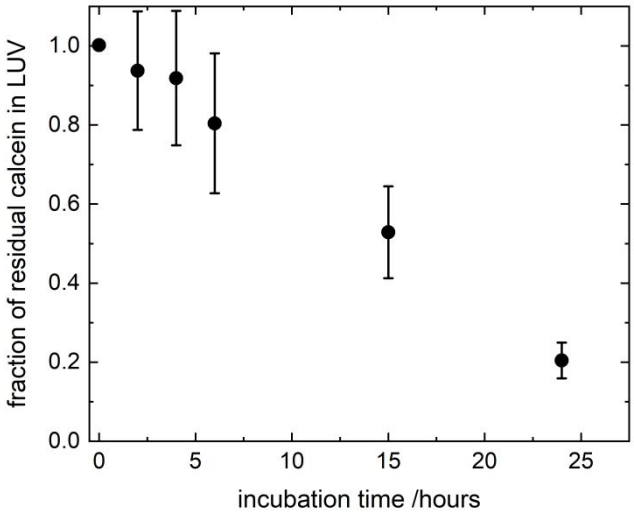


Figure 4. Fraction of calcein molecules remaining in liposomes after mixing with CuNP at different incubation times, as obtained by liposome solubilization with TritonX100.

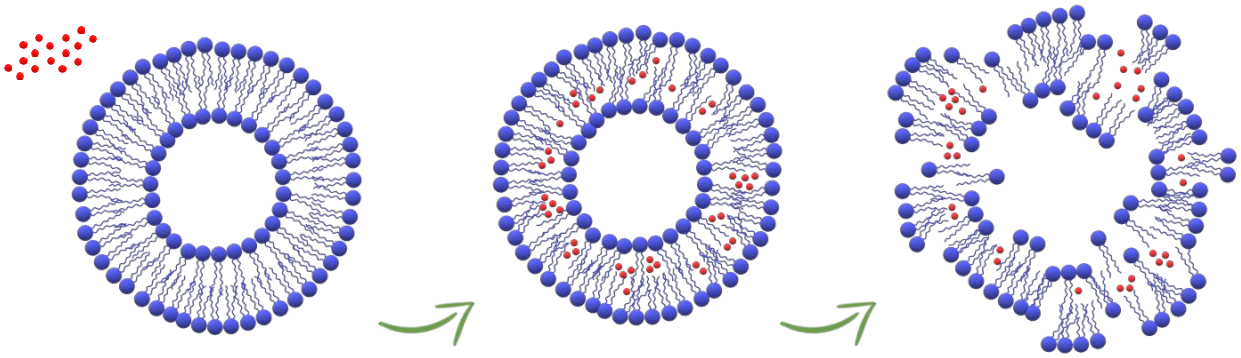


Figure 5. Schematic representation of the damage of the liposomes caused by the accumulation of CuNPs in the phospholipid membranes.

SUPPORTING INFORMATION

Analytical probing of membranotropic effects of antimicrobial copper nanoparticles on lipid vesicles as membrane models

Margherita Izzi,[†a] Miquel Oliver,[†b] Helena Mateos,[a] Gerardo Palazzo,[a] Nicola Cioffi *[a] and Manuel Miró*[b]

[a.] Chemistry Department, University of Bari Aldo Moro, Via Orabona, 4, 70126 Bari, Italy.
[b.] FI-TRACE Group, Department of Chemistry, University of the Balearic Islands, Carretera de Valldemossa km 7.5, E-07122 Palma de Mallorca, Spain.

[†] These authors contributed equally to this work.

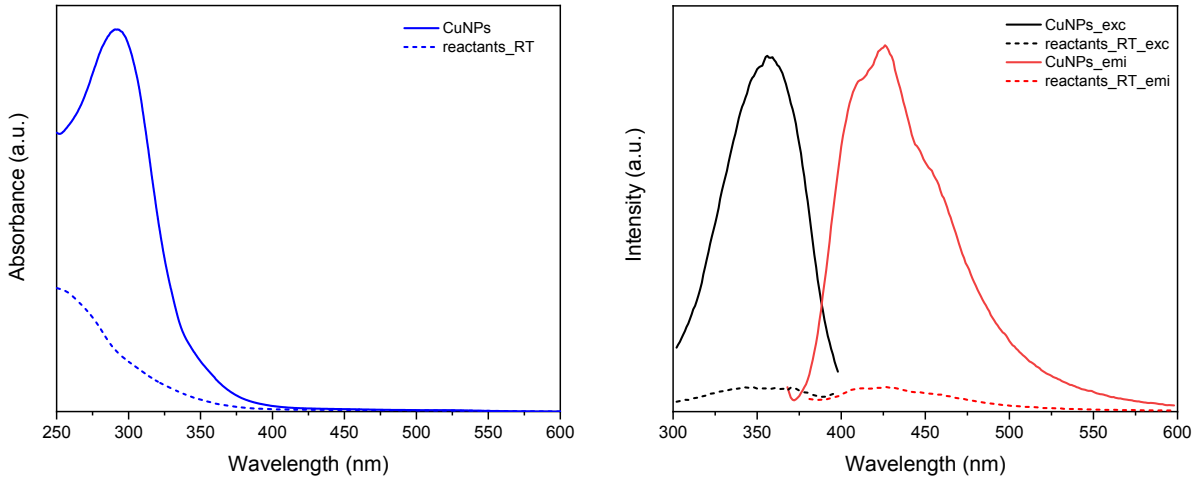


Figure S1. UV-Vis (left) and fluorescence spectra (right) of the reactants at room temperature, compared with the CuNPs synthesized at 65°C.

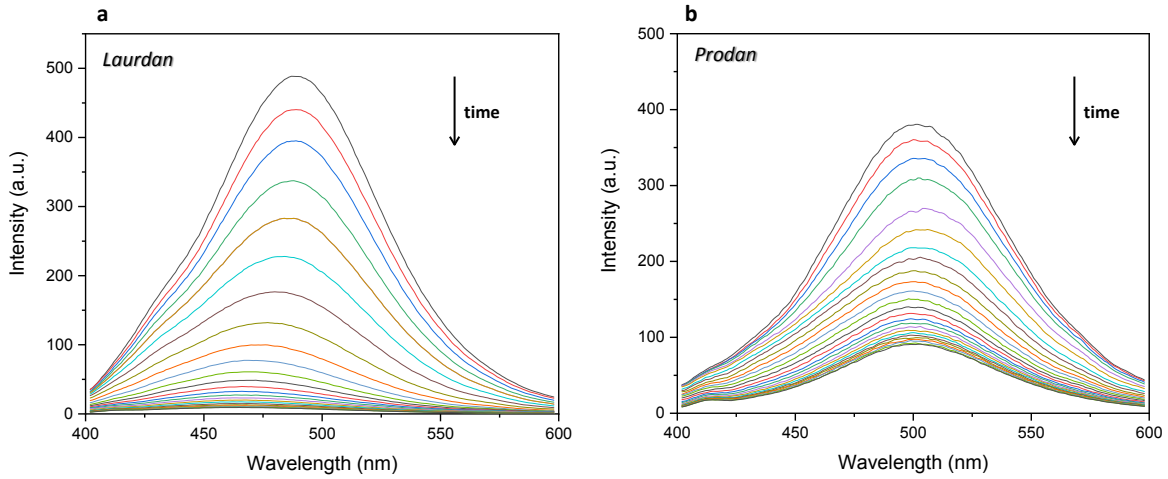


Figure S2. Emission spectra of laurdan (a) and prodan (b) over time in a suspension containing 100 µM PC and 10^{11} CuNPs/mL. The spectra were acquired under the following conditions: temperature 37°C; excitation 360 nm; emission 400–600 nm; data interval 1 nm; voltage to 540 V; slit widths of 10 nm.

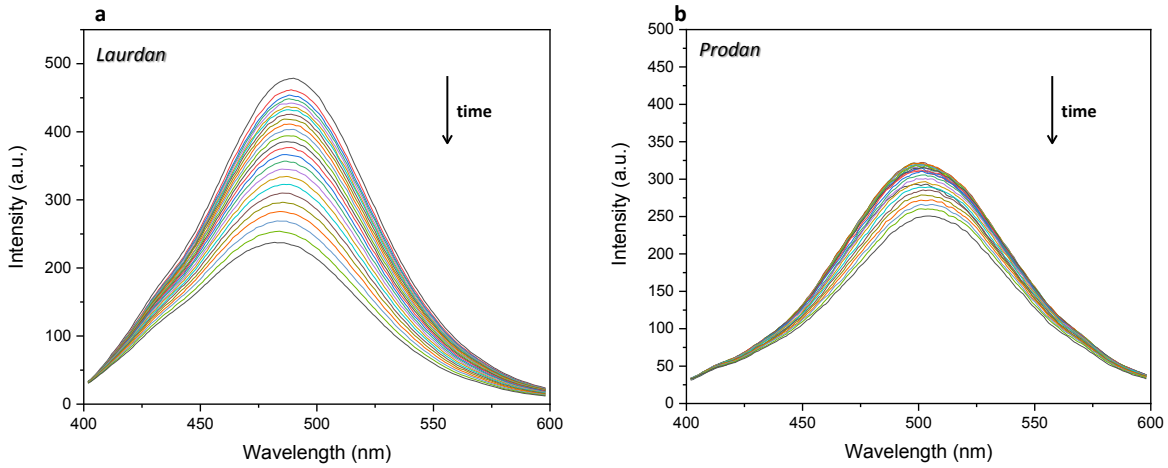


Figure S3. Emission spectra of laurdan and prodan over time in a suspension containing 100 µM PC and Cu^{2+} ions at the same concentration than that of the synthesis of CuNPs (0,15mM, ultimately resulting in 0.0015 mM, after the usual 1:100 dilution, operated prior the emission measurements). The spectra were acquired under the following conditions: temperature 37°C; excitation 360 nm; emission 400–600 nm; data interval 1 nm; voltage to 540 V; slit widths of 10 nm.

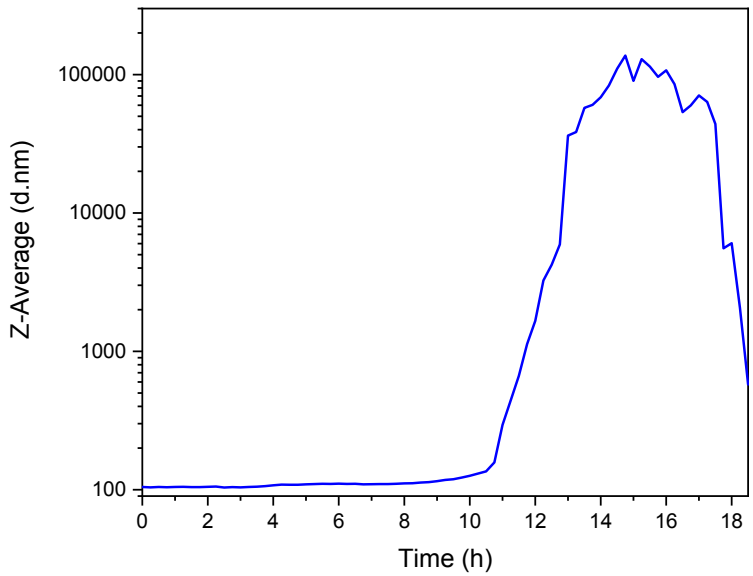


Figure S4. Hydrodynamic diameter of liposome over time as measured by DLS.