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# A new approach for direct visualization of unlabeled lipid nanoparticles for intracellular pathway analysis

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## Background incl. aims

Lipid Nanoparticles (LNPs) are the most clinically advanced delivery vector for both small molecules (liposomes) and genetic material payloads, such as siRNA or mRNA (ionizable cationic LNPs).[1] Their recent development has unlocked a wide range of therapeutic approaches, overcoming the limitations of naked genetic payloads and of previous delivery strategies.[2] This is testified by the fact that the majority of clinically approved nano-systems are of lipidic nature.[3] However, much is still unclear about the release of genetic material in the cytoplasm upon LNPs cell internalization and trafficking in the endo-lysosomal compartment. This process, known as endosomal escape, is a crucial step of the delivery process but to date is inefficient and often considered the bottleneck for the clinical efficacy of LNPs.[4] Most of the methods for detection and quantification of endosomal escape are either indirect assays, usually based on detection of endosome damage indicators, or rely on fluorescent labelling of LNPs. The latter, however, has proven to significantly alter the surface properties of nanoparticles and thus their biodistribution and cell interaction properties.[5] While the direct imaging of LNPs in biological samples is impossible given the nature and elemental composition of LNPs, which are practically identical to the composition of cells. To overcome these limitations, a new hybrid Liposomal-Gold nano-system (namely Lipo-Gold) has been here developed for the direct analysis of cellular interaction and intracellular trafficking of LNPs.

## Methods

The Lipo-Gold have been synthetized by a modified thin lipid film hydration method: specifically, the lipid film composed by saturated phospholipid DPPC, cholesterol and PEGylated lipid DSPE-PEG (2000 Da), has been hydrated with an aqueous solution of biocompatible reducing agent, namely ascorbic acid (vitamin C). The vesicles encapsulating the reducing agent have been extruded to the desired dimension and a size-exclusion chromatography column has been employed for the quick removal of free ascorbic acid. Upon addition of metal precursor, $HAuCl_4$, AuNPs spontaneously and quickly formed in the LNPs aqueous compartment. The formation of hybrid Lipid-Gold nanoparticles has been optimized and characterized by quantification of encapsulated ascorbic acid, UV/Vis spectroscopy, dynamic light scattering (DLS), nanoparticles tracking analysis (NTA) and electron microscopy techniques. Then, MTS cytotoxicity assay, flow cytometry, confocal and hyperspectral darkfield microscopy, as well as TEM, have been used to evaluate the cellular biocompatibility and cell interaction properties of the nanoparticles and to verify whether the hybrid Lipo-Gold system could be used as a model for the study of LNPs.

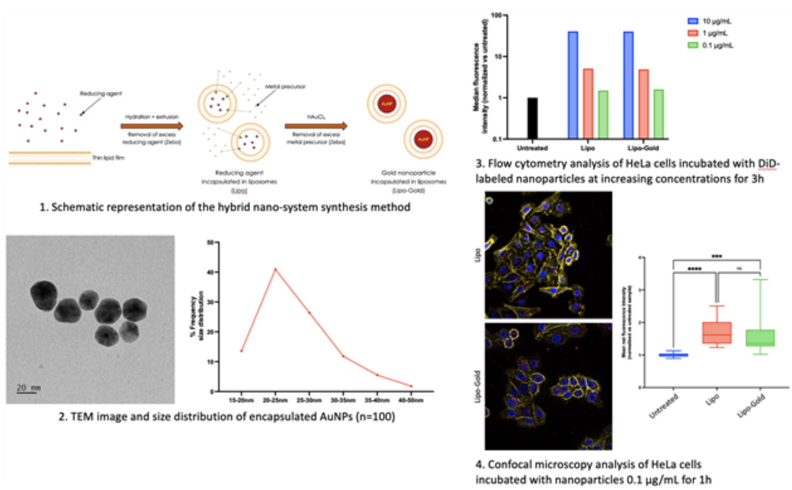
## Results

The optimization of the methods resulted in formation of very monodispersed ~130nm LNPs encapsulating 15-35nm AuNPs. The resulting nano-system has proven to be highly biocompatible through MTS cytotoxicity assay after 24 or 48 hours even at very high concentrations. Unlabeled hybrid Lipid-Gold nanoparticles have been employed for the analysis of cellular internalization by TEM and hyperspectral darkfield microscopy, while fluorescent labeling with DiD dye (Ex/Em: 647/663nm) have been used to compare the properties of the hybrid system in comparison with empty LNPs. Both flow cytometry and confocal microscopy clearly showed that the cellular internalization and intracellular localization is not altered upon AuNPs synthesis as compared to empty LNPs, both quantitatively and qualitatively. This proves that the hybrid Lipid-Gold nano-system is a potential good model for the direct analysis of LNPs interaction with cells, taking advantage of AuNPs optical properties.

Conclusion

This work resulted in the development of a hybrid Lipo-Gold nano-system that showed promising results towards its employment as a reporter system for the visualization of LNPs cellular internalization and intracellular trafficking. This could enable further and easier optimization of Lipid Nanoparticle platform, enhancing the efficacy of the endosomal escape and thus the overall clinical relevance. Furthermore, Dynamic TEM measurements are ongoing on this system, with the aim of getting real-time visualization of the intracellular path of nanoparticles in response to an external optical/thermal trigger. The method will be developed in standard biological samples prepared for electron microscopy analysis (i.e. dehydrated cells included with epoxy resin), thus with a low biological relevance. However, the method would be a proof of concept that could then be employed in state-of-the-art, and more biologically relevant, techniques such as Cryo-TEM for the time-resolved, high-resolution analysis of intracellular trafficking of LNPs in biological samples.

**Graphic:**




**Keywords:**

Lipid-Nanoparticles, Cellular internalization, Endosomal escape

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