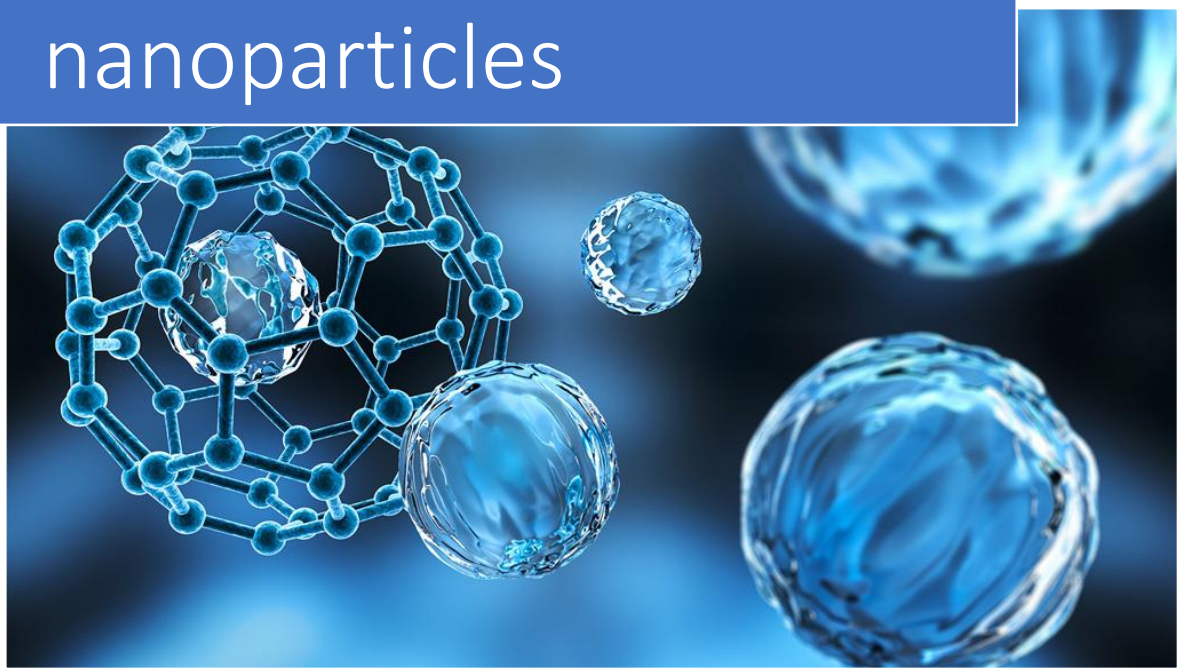
UNIVERSITAT JAUME I

Synthesis of Ultra Magnetic Liposomes coupling with gold nanoparticles



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Chemistry Degree Research Project

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Abbreviations

LUM Ultra Magnetic Liposome

PEG Polyethylene Glycol

DLS Dynamic Light Scattering

TEM Transmission Electron Microscopy

HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

DPPC 1,2-dipalmitoyl-sn-glycero-3-phosphocholine

DSPC 1,2-distearoyl-sn-glycero-3-phosphocholine

DSPE-PEG (2000) Amine 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N- [amino (polyethylene glycol) -2000] (ammonium salt)

MNPs Maguemite Nanoparticles

EDC 1-ethyl-3- (3-dimethylaminopropyl) carbodiimide

APTS (3-Aminopropyl) triethoxysilane)

Acknowledgements

This project has been possible thanks to the opportunity that the 'Pierre et Marie Curie' University (Paris) has given me. I'm highly gratefully to Christine Menager, associate professor at UPMC University and to the other members of Laboratory Phénix whose colaboration has been crucial to ensure the smooth running of the project. Thanks also to Beatriz Julian, associate professor at 'Jaume I University', who has supervised my work and who has been of great help during this project.

During these weeks of research in the laboratory, I've learned more about a field that I consider very important which offers many possibilities for the future such as nanotechnology. I think the implantation of this field in medicine as a new research path is fascinating.

On the other hand, this project has allowed me to become even more familiar with the characterization techniques used daily in synthesis, in particular, the electron microscopy of transmission, of which I hadn't had so much knowledge at this time.

SUMMARY

1. Introduction

Nanoparticles are widely used as carrier materials for biomedical applications such as functional and morphological imaging diagnosis and therapeutic drug delivery[1]. Based on their performance in these biomedical applications, nanoparticles have attracted great interest as a platform for combining diagnostics and therapy. Research efforts are currently underway to design effective theranostic nanoparticles that enable imaging diagnosis and therapy simultaneously. Liposomes, which have been used clinically for drug delivery systems, are a principal platform for theranostic applications.[2]

Specifically gold nanoparticles (AuNPs) are emerging as promising agents for cancer therapy and are being investigated as drug carriers, photothermal agents, contrast agents and radiosensitizers. [3] The use of NPs as radiosensitizers is also related to nanoparticle enhanced X-ray therapy or NEXT.[4] They are very inert and highly biocompatible; they induce the radiation effect over a large tumor area; they are well absorbed into the systemic circulation; they have low systemic clearance, which allows enough time for photosensitizing material to be absorbed into the tumor tissue, and lower clearance rates also result in an enhanced permeability and retention (EPR) effect; and they can be designed in different sizes or shapes (such as cubes, spheres, cones or rods), to optimize their delivery to get the best results.[5]

Another type of nanoparticles with a clear prospect of being approved in the clinic are magnetic nanoparticles (made up of metals such as iron). These nanoparticles are used in hyperthermia protocols, in which an alternating magnetic field is used to heat the nanoparticles. Thus, they reach temperatures of about 45 ° C locally inducing damage to the tumor cells in the treated area.

[1] Sagar R. Mudshinge, Amol B. Deore, Sachin Patil, Chetan M. Bhalgat, *Saudi Pharmaceutical Journal* **2011**,19, 129-141

[2] Keitaro Sou, *Recent Patents on Nanomedicine* **2014**,4,95-101

[3] Jain, S, D G Hirst, and J M O'Sullivan. "Gold Nanoparticles as Novel Agents for Cancer Therapy." *The British Journal of Radiology* **2012,** 101–113

[4] Praetorius NP, Mandal TK. Engineered nanoparticles in cancer therapy. *Recent Pat Drug Deliv Formul*. **2007**;1:37–51.

[5] Hainfeld JF, Slatkin DN, Smilowitz HM. The use of gold nanoparticles to enhance radiotherapy in mice. *Phys Med Biol*. **2004**;49:N309.

On the other hand, liposomes, the vesicles of phospholipid bilayer, can encapsulate both hydrophilic and lipophilic drugs and protect them from degradation. Liposomes have been extensively studied and continue to create intense interest in research since their discovery in the mid-1960s. Since then, liposomes have been considered to be the most successful nanocarriers for drug deliver and have made their way to the market. Currently, a number of liposomal formulations are on the marker for cancer treatment and many more are in pipe line.[6]

Concretely magnetic liposomes offer opportunities as theranostic systems. The prerequisite for efficient imaging, tissue targeting or hyperthermia is high magnetic load of these vesicles. Here we describe the preparation of Ultra Magnetic Liposomes (UMLs), which may encapsulate iron oxide nanoparticles in a volume fraction of up to 30%. This remarkable magnetic charge provides UMLs with high magnetic mobilities, MRI relaxivities, and heating capacities for magnetic hyperthermia. Moreover, these UMLs are rapidly and efficiently internalized by cultured tumor cells and, when they are administered to mice, they can be vectorized to tumors by an external magnet.[7]

The coupling of the ultra-magnetic liposomes with the gold nanoparticles permits us to combine the properties of both particles in radiotherapy to reduce non-specific side effects and enable higher dose delivery to target tissues.

[6] Pandey, Himanshu et al. "Liposome and Their Applications in Cancer Therapy." **2016**

[7] Béalle, G.,Di Corato, R., Kolosnjaj-Tabi,J., and Dupuis, V., Clément, Olivier and Gazeau, F., Wilhelm,c., Ménager, C. "Ultra Magnetic Liposomes for MR Imaging, Targeting, and Hyperthermia" *Langmuir*,**2012**, *28* (32), pp 11834–11842

1.1 Liposomes

Liposomes, sphere-shaped vesicles consisting of one or more phospholipid bilayers, were first described in the mid-60s[8]. Liposomes are small artificial vesicles of spherical shape that can be created from cholesterol and natural non-toxic phospholipids. Due to their size and hydrophobic and hydrophilic character (besides biocompatibility), liposomes are promising systems for drug delivery. It has been displayed that phospholipids impulsively form closed structures when they are hydrated in aqueous solutions. Such vesicles which have one or more phospholipid bilayer membranes can transport aqueous or lipid drugs, depending on the nature of those drugs.

The strategy followed by this project is to prepare Ultra Magnetic Liposomes (UMLs), suitable for systemic delivery characterized by an outstanding loading potential for magnetic nanoparticles. A high amount of MNPs in liposomes is critical for efficient MRI detection, magnetic targeting and heating of the sites of interest while minimizing the injected dose.[7]

Liposomes are vesicles constituted by a concentric phospholipid bilayer (Figure 1), which involves an aqueous central compartment of 50–100 nm.[9]

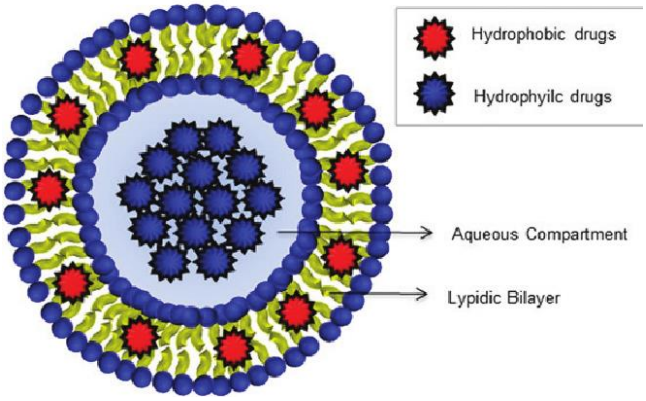


***Figure 1*. Liposome drug delivery system**

[8] Akbarzadeh, Abolfazl et al. "Liposome: Classification, Preparation, and Applications." *Nanoscale Research Letters* 8.1 (**2013**): 102.

[9] Cukierman E, Khan DR. The benefits and challenges associated with the use of drug delivery systems in cancer therapy. *Biochem Pharmacol*. **2010** Sep 1;80(5):762–70.

Due to its larger size, when compared to most particles, liposomes can carry larger amounts of drugs.

The organisation of this system is based on the presence of water, since the orientation of the bilayer may be determined by the nature of the polar groups and carbon/alkyl chains.

The amphipathic nature of liposomes allows transportation of hydrophobic compounds interacting with the carbon/alkyl chains of their phospholipids and also of hydrophilic molecules in the interior aqueous cavity. The maintenance of the drug inside the liposome is dependent on the concentration, chemical nature, electric charge of the phospholipid, ionic strength of the media and the size of the drug.[10]

[10] Israelachvili JN. Intermolecular and Surface Forces. 3th ed. Amsterdam: Academic Press; **2011**

2. Objectives

The general objective of the work is divided in four specific objectives:

1. The synthesis of magnetic liposomes made by the reverse-phase evaporation technique.

2. The synthesis of gold nanoparticles

3. The coupling between the magnetic liposomes and gold nanoparticles

4. Characterization of magneto-plasmonic liposomes by fluorimetry, dynamic light scattering, optical spectrophotometry and transmission electron microscopy.

3. Experimental Section

3.1 Synthesis of LUMs

The synthesis of magnetic liposomes is made by the reverse-phase evaporation technique.[11] This technique consists of the formation of reverse micelles after sonication of a mixture of an organic phase and an aqueous phase. This aqueous phase consists of an aqueous suspension of iron oxides nanoparticles prepared to be encapsulated and an organic phase composed of phospholipids solubilized in chloroform and diethyl ether. Then, an external coating of PEG molecules will be performed in order to help the stabilization of the liposomes. Furthermore, an amine function (PEG-NH2) will be anchored, which will facilitate coupling with gold because both have a high affinity (*Figure 2*).

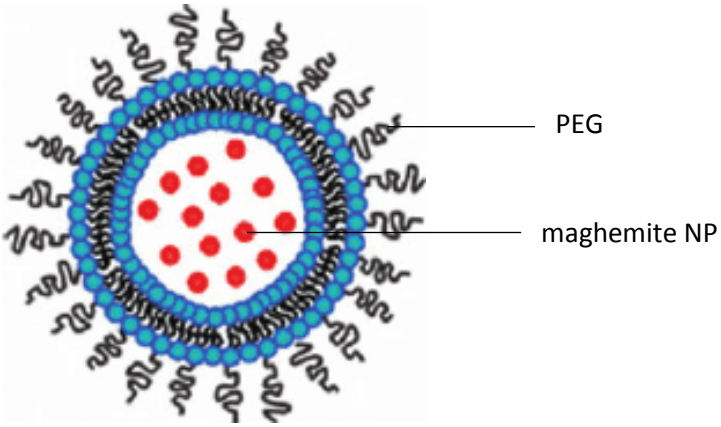


Figure 2. Magnetic Liposome with a coating of PEG molecules. Red dots represent maghemite nanoparticles.

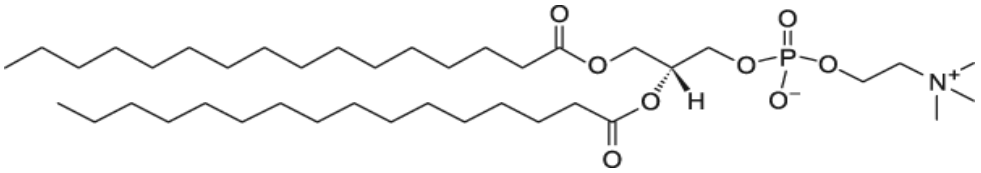
The nanoparticles of maghemite (γ-Fe_2O_3) which were provided by the host laboratory were synthesized according to the Massart method[12] by the aqueous mixture of ferrous and ferric solution in the form of hydrated chloride salts in alkaline medium. These nanoparticles had a size of 7 nm and they were dispersed in a buffer solution of HEPES, sodium chloride and water. They are concentrated in iron oxide to 1 mol / L.

[11] Cortesi R, Esposito E, Gambarin S, Telloli P, Menegatti E, Nastruzzi C.
"Preparation of liposomes by reverse-phase evaporation using alternative orgànic solvents" .*PubMed* **1999** 16(2):251-6.

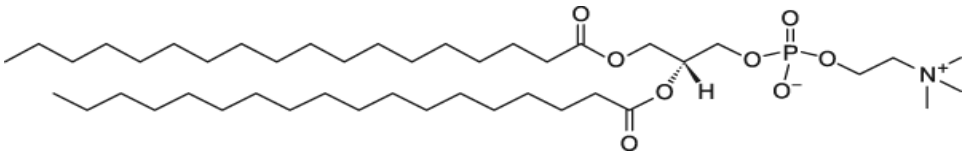
[12] Ang Bee Chin, Iskandar Idris Yaacob. *Journal of Materials Processing Technology* **2007,** 191(1), 235 - 237

The three phospholipids used to prepare liposomes are:

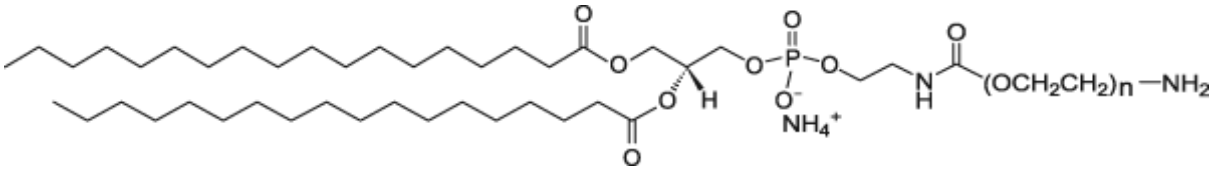
DPPC



DSPC



DSPE-PEG(2000) Amine, which will be abbreviated from now as PEG-NH2



The PEG-NH_2 is a widely used phospholipids-polymer conjugate in drug delivery applications[13].

The use of these phospholipids allows the formulation of liposomes which will be used as nanocarriers for achieving prolonged blood circulation time, improved stability and enhanced encapsulation efficiency.

[13] Che J, Okeke CI, Hu ZB, Xu J. *Curr Pharm Des*. **2015**;21(12):1598-605

The reverse-phase evaporation technique[14] is used. A schema is represented below (*Figure 3*).

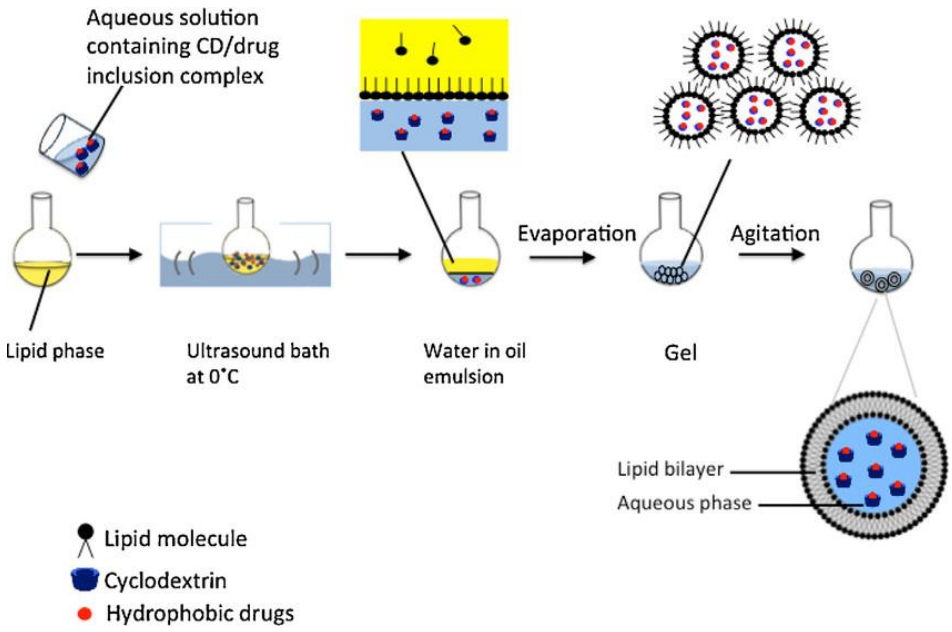


Figure 3. Schematic diagram of phase-reverse evaporation method.

LUMs were synthetized using two amounts of PEG-NH_2: 40 µL and 60 µL. This permits us to compare the size and potential zeta of two types of liposomes. The amounts of 90 µL of DPPC, 10 µL of DSPC and 40/60 µL of PEG-NH_2 were taken using a micropipette and all three were inserted into a glass tube. They were dissolved by 3 mL of diethyl ether and 1 mL of chloroform were collected by syringe and added to the mixture. The function of chloroform is to solubilize lipids. Then 1 mL of MNPs dispersed in a buffer 0.108 M NaCl, 0.02M citrate and 0.01M HEPES, (pH=7,4) was introduced on the tube.

Two phases were obtained (*Figure 4*). An organic phase which contains the solubilized phospholipids in the solvent and an aqueous phase containing the nanoparticles of iron oxides in the buffer were clearly separated.

Figure 4. Tube containing the liposome preparation

[14] Skoza, F. C.; Papahadjopoulos, D. Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. Biochemistry 1978, 75 (9), 4194–4198.

The tube was put in the ultrasound bath for 20 minutes. During the sonication, the action of homogenizer produced a water-in-oil emulsion between the two phases. Phospholipids were organized in the form of reverse micelles surrounding the nanoparticles of maghemite.

After 20 minutes, the solution was immediately transferred to a 50 mL round-bottom flask with the aim of preserving the emulsion and then it was placed on a rotary evaporator at a temperature of 28 ° C. The ether was evaporated at a pressure of 335 mbar and then chloroform at 119 mbar.

Evaporation of the solvent allows reverse micelles to approach each other; they will aggregate and form a gel. When all the solvent has been evaporated, the gel phase becomes liquid and the liposomes are formed. This passage is crucial and difficult to observe, so it's necessary to be very careful.

The solution containing the liposomes was filtered through a 400 nm filter in a plastic vial. 10 ml of buffer solution were added into the flask to recover a maximum amount of liposomes and the whole was filtered over 400 nm in the same vial. At the end, 10 ml of liposome solution was obtained.

Purification of liposomes from non-encapsulated maghemite MNPs was performed by magnetic sorting using a strong magnet purchased from Calamit (*Figure 5*). Along the walls, there was a black and dense liquid. The supernatant was brown clear and the liposomes had adhered to the walls of the syringe *(Figure 6)*.The repetition of three times of this operation allows to separate liposomes from the supernatant and recovered.

Figure 5. Decantation of liposomes

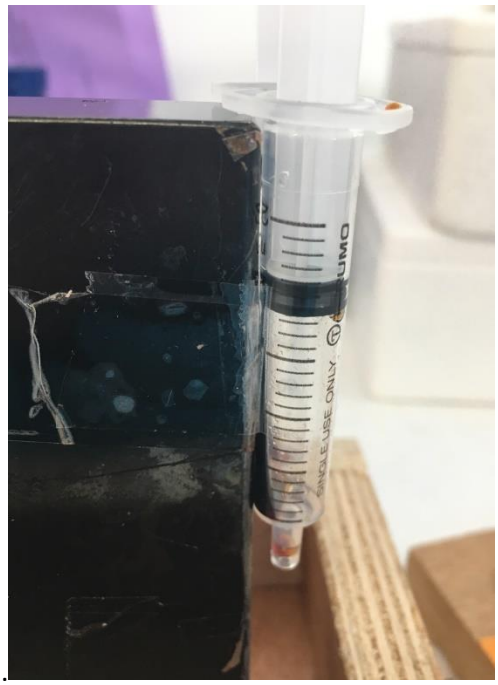


Figure 6. Recovery of liposomes

3.2 Synthesis of gold nanoparticles

Gold nanoparticles of ~ 20 nm diameter were synthesised according to Frens et al[15] method. This synthesis consists in chemical reduction of $AuCl_4^-$ at pH 6.3-6.5 by dissolved trisodium citrate Na_3Ctr at 100°C. 100 mL of 1mM $HAuCl_4$ containing 200 µl of 1M NaOH was prepared in a 250 mL flask equipped with a condenser. The solution was brought to boil while being stirred and then 38.8 mM Na_3Ctr was rapidly added. In this synthesis Na_3Ctr simultaneously acts as (i) reducing agent (driving the reduction of Au^{III} to Au^0) (ii) capping agent (electrostatically stabilizing the AuNP colloidal solution), and (iii) pH mediator (modifying the reactivity of Au species involved in the reaction).[16] After waiting 15 min of reaction, the reflux system was shut down and deionized water was added to the AuNp seed suspension and make up to 100 mL.

Growth reaction was performed in a 100 mL Erlenmeyer flask. An aliquot of seed suspension (Nseed= 6.54 $x10^{12}$ particles per ml) and 227 µL of 44.7 mM $HAuCl_4 \cdot 3H_2O$ were added to the flask with water. Subsequently, an aliquot of 176 µL of 38.8 mM $Na_3Ctr \cdot 2H_2O$ was added to the flask under constant stirring. *(Figure 7).*

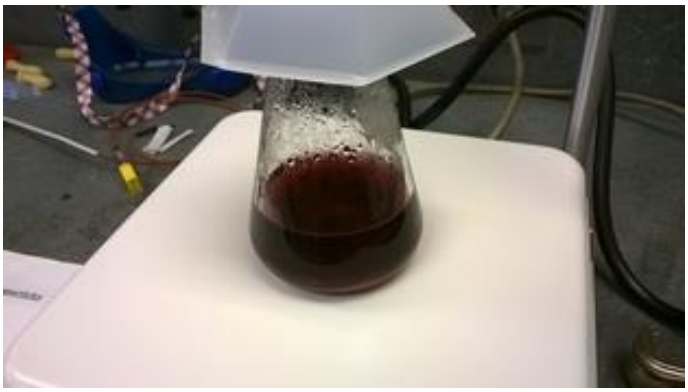


Figure 7 . Gold nanoparticles in solution

[15] Xia H., Xiahou Y., Zhang, P.,Ding, W. and Wang, D. *Langmuir* **2016** 32 (23), 5870-5880

[16] Leng, W., Pati,P. and Vikesland,P.J. 'Room temperature seed mediated growth of gold nanoparticles: mechanistic investigations and life cycle assesment' *Environ*. Sci.: Nano, **2015**, 2, 440

3.3 Synthesis of magneto-plasmonic liposomes (Coupling)

Magneto-plasmonic liposomes were synthesized by performing a coupling between the magnetic liposomes and gold nanoparticles (*Figure 8).*

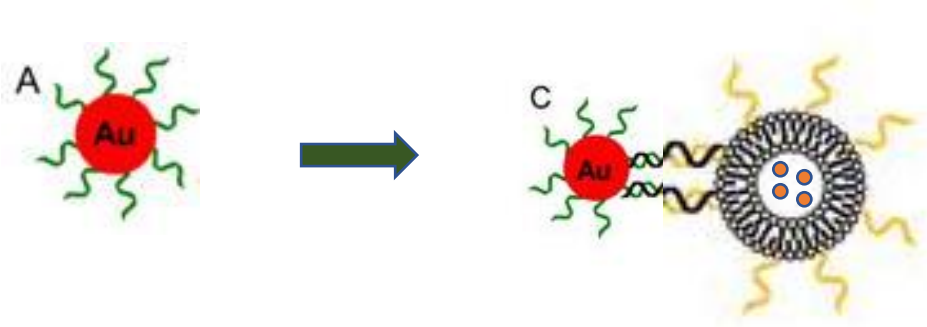


Figure 8. Coupling between the magnetic liposomes and gold nanoparticles

Gold nanoparticles being citrated, they have COO^- ions on the surface. It will be able to realize a peptide coupling with the magnetic liposomes which contains NH_2 functions on their surface. To achieve this coupling, it is necessary to use a coupling agent. It was used EDC (Figure 9). The carbodiimide is a crosslinker that activates carboxyl groups for spontaneous reaction with primary amines, enabling peptide immobilization and hapten-carrier protein conjugation.

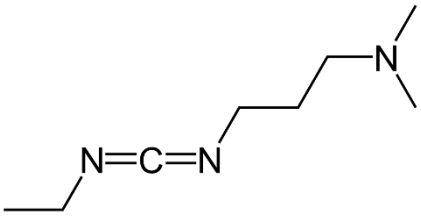


Figure 9. Molecule of EDC

Coupling is carried out with liposomes synthesized with 60 µL of PEG-NH2 and also with 40 µL of PEG-NH2.

The procedure was: 20 µL of magnetic liposomes were taken to the micro-pipette and inserted into a glass vial. 1 ml of buffer solution and 1 mL of gold nanoparticles were added together with 10 mg of EDC weighed to the precision balance. The flask was set to stir for 2 hours on the orbital shaker.

After two hours, the flask was recovered (*Figure 10)*. Purification of liposomes was performed by magnetic sorting using a strong magnet purchased from Calamit (*Figure11).* This step enables the separation of the magneto-plasmonic liposomes gold nanoparticles have not been coupled.

The next day, the liposomes coupled with gold nanoparticles were recovered. It was observed in the syringe, a good decantation. Along the walls, there was a dark brown liquid in small quantities. The supernatant was light orange or brown.

Figure 10. Flask containing liposomes after coupling with gold nanoparticles.

Figure 11. Magnetic sorting of new liposomes synthetized.

4. Characterisation of synthetized compounds

4.1 Characterization of magnetic liposomes

The principles of characterization techniques are summarized in the annex.

For the characterization of magnetic liposomes a dosage of amine functions by fluorimetry was realised. The size of the liposomes was determined by dynamic light scattering (DLS) and zeta potential is measured.

4.1.1 Dosage of amine functions by fluorimetry

The amount of amine functions on the surface of liposomes was quantified. The method used was a fluorimetry. Unfortunately, an incorrect calibration curve made the results of this fluorimetric measurement not usable. Thus, the amine functions couldn't be quantified. Despite of this, the sample preparation procedure is described now.

The purpose of this method is to combine our liposomes to a fluorescent molecule and then measuring the fluorescence of the compound thus synthesized. As a fluorescent molecule is used Fluram (Figure 12) which will allow us to produce a peptide coupling with the amine functions.

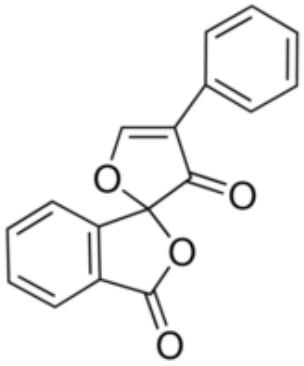


Figure 12. fluorescent molecule: 4 - Phenylspiro- [furan-2 (3H), 1-phthalan] -3,3'-dione (Fluram)

For this technique, it is necessary to make a calibration curve. It's used for this purpose a standard APTS (Figure 13), which is a molecule containing an amine function. To avoid that they are too concentrated, the standards are diluted twice. The solvent used is ethanol.

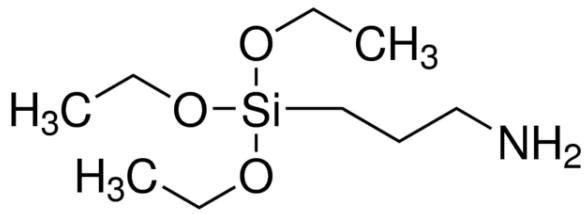


Figure 13. Molecule of APTS

Procedure

APTS solution: APTS was removed from the vial, taking care not to introduce air. For this, a needle surmounted by a balloon flask filled with nitrogen was introduced into the septum. The APTS was drawn into a syringe and introduced into an Eppendorf. 0, 56 g of PTSA were taken in a beaker and supplemented with 10 ml of ethanol, until 7,91g.

Standards APTS: 6 standards were prepared containing respectively 0 µL, 20 µL , 40 µL, 80 µL, 140 µL and 200 µL solution of APTS and supplemented with 10 ml of ethanol, until 7,91g.

Fluram solution: 5.6 mg of Fluram were removed under a hood, and introduced into a beaker filled with 100 ml of ethanol, until 79.1 g.

Three samples of magnetic liposomes were provided. Each one contains 15 µL, 30 µL and 40 µL of PEG-NH2.

Preparation of fluorescent samples: 20 µL of each standard of PTSA were removed, introduced into a glass bottle and supplemented with 5 ml of Fluram solution until 3,96g. Similarly, 40 mL of each sample magnetic liposomes were removed, introduced into a glass bottle and supplemented with 5 ml of Fluram solution until 3,96g. The fluorescent sample was stirred vigorously and placed in the dark. It is necessary to take into account the time difference between the preparation of the first fluorescent sample and the last because there will be a slight delay. As soon as the first sample is placed in the dark, it takes 2 hours before measuring the fluorescence.

4.1.2 Dynamic Light Scattering (DLS)

The size of the magnetic liposomes was measured before and after separation step. This technique is very sensitive to dust, so the tanks are kept in the sealed packaging. The instrument used was the Zetasizer Nano ZS from Malvern. Some settings were changed, including equilibration time which was set to 60 s and the temperature at 25 ° C. The chosen solvent was water, and in the material parameter, 'ferrofluid' was chosen.

One drop of liposome solution was taken and diluted in the buffer solution which was prepared before. The measuring vessel was filled to 1/3 with reference to the legend on the device and the measurement was performed. Intensity curves were obtained according to size.

From the curves obtained there is a particle size of about 18 nm that doesn't change before and after sorting. This size corresponds probably to the maghemite particles. Before sorting (*Figure 14)*, the liposomes have a size of approximately 255 nm which is a coherent result. After sorting *(Figure 15*), the measure has probably been distorted by other environmental factors such as dust. It is also possible that both liposomes have merged and we get twice the size before the sorting step.

Before sorting:

Two different sizes are obtained: 18 nm and 255 nm.

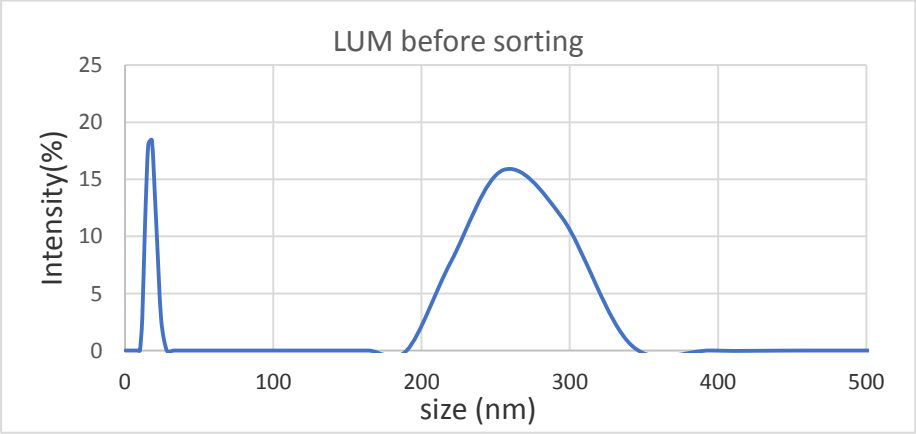


Figure 14. Curve representing the intensity as a function of the size before sorting

After sorting:

Two different sizes are obtained: 18.2 nm and 531 nm.

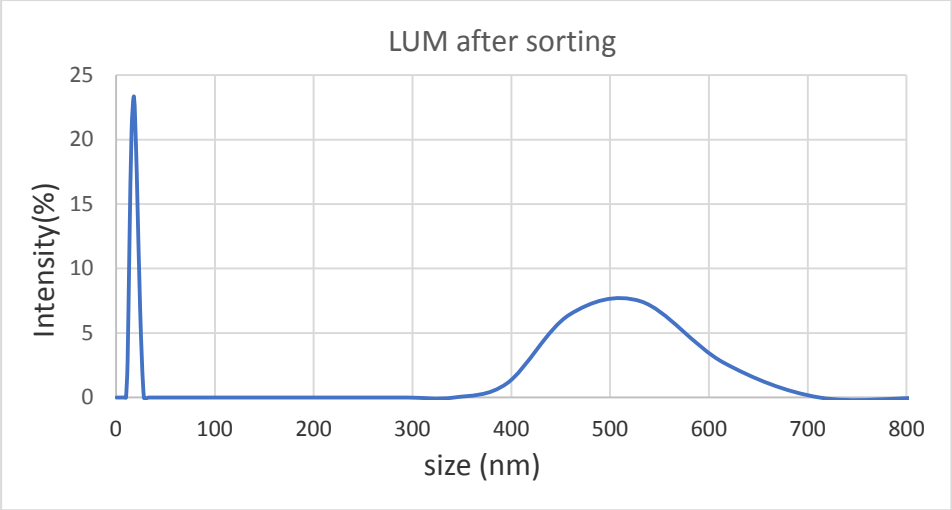


Figure 15. Curve representing the intensity as a function of the size after sorting

4.1.3 Determining the zeta potential

The zeta potential, which gives an indication of the surface charge of the vesicles or micelles, was also measured using the Zeta Nanosizer ZS. A special cuvette with electrodes was used for this measurement. (*Figure 16*). The folded capillary cell was completed in full and corked and the zeta potential of the liposomes after the sorting step was measured.

A zeta potential of 23.1± 2 mV is obtained with pH 7.4. This value is coherent because the measured zeta potential corresponds to the amine functions, so it must be positive. On the other side, the value represents a medium- stability solution. It should be noted that a good stability is comprised for values between 40-50 mV.

Figure 16. Folder capillary cell for measurement of zeta potential

4.2 Characterization of gold nanoparticles

Gold nanoparticles are characterized by measuring the size by DLS and the zeta potential. UV-Visible spectroscopy is also used.

4.2.1 Dynamic Light Scattering (DLS)

The sample was diluted in water. *Figure 17* shows the particle size distribution of AuNPs. Two size values are obtained of 21 nm and 91 nm. The presence of two distributions may be due to aggregation of AuNPs. The first peak corresponds to smallest gold nanoparticles while second pick is attributed to an aggregation of gold nanoparticles.

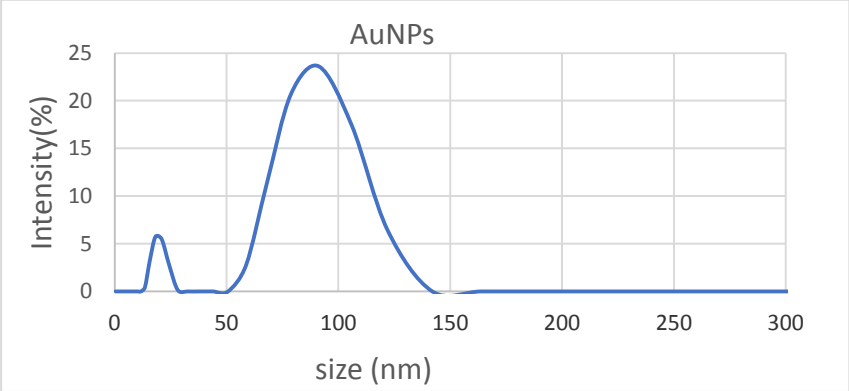


Figure 17. Curve DLS gold nanoparticles

4.2.2 Zeta potential

After measuring zeta potential it was obtained an average charge of -33.2 ± 2 mV. This value is consistent. Indeed, the gold nanoparticles that were citrated, they have COO- ions that provides them a negative charge. It was suggested that the emulsions with zeta potential values of −11 to −20 mV were close to the threshold of agglomeration, while the emulsions with zeta potential values of −41 to −50 mV had good stability.

The significance of zeta potential is that its value can be related to the short- and long-term stability of emulsions. Emulsions with high zeta potential (negative or positive) are electrically stabilized while emulsions with low zeta potentials tend to coagulate or flocculate, possibly leading to poor physical stability. In general, when the zeta potential of an emulsion is high, the repulsive forces exceed the attractive forces, resulting in a relatively stable system.

4.2.3 UV-visible.

Gold nanoparticles are characterised to possess surface plasmons. When they are excited by light at a given frequency, the free electrons of the nanoparticle oscillate collectively. This is the surface plasmon resonance. They can therefore be characterized by UV-visible spectroscopy.

The UV-visible spectroscopy gives an indication of the size of the gold nanoparticles. Indeed, as the size of the nanoparticles increases, as the absorbance band will move towards the higher wavelengths.

The measurement was made using a fibre optic spectrophotometer. The following spectrum was obtained (*Figure 18*).

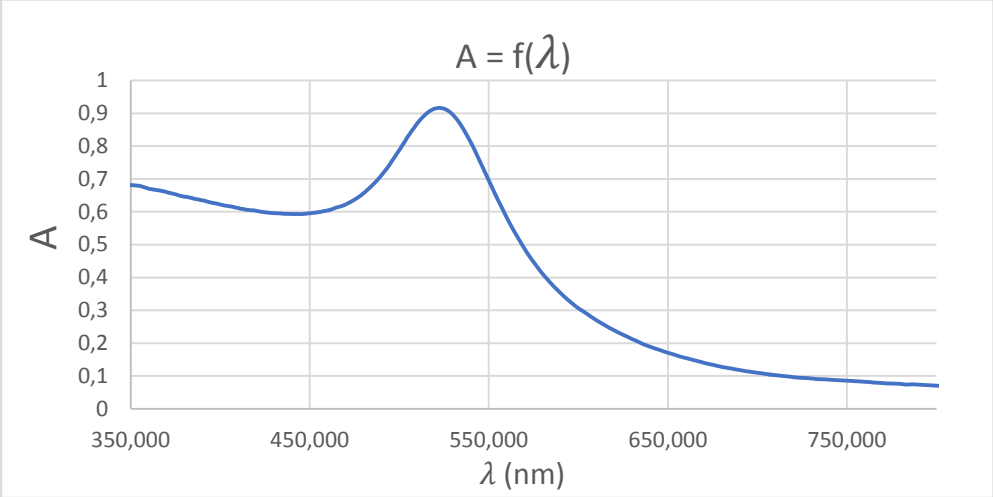


Figure 18. Curve UV-visible of gold nanoparticles

For the gold nanoparticles, an absorbance band was observed at 518±12 nm. The absorbed light is green. So the observed light is the complementary colour of green that is pink (*Figure 19*). This is consistent because the colour of the gold nanoparticle solution synthetized was pink.

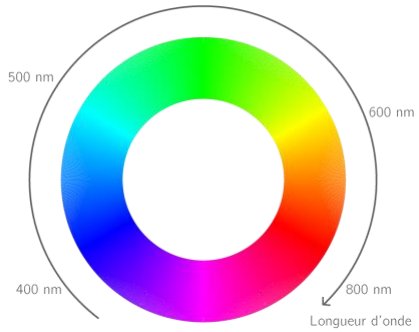


Figure 19. Diagram that represents the colour as a function of wavelength

4.3 Characterization of magneto-plasmonic liposomes

We characterized by UV-visible the magneto-plasmonic liposomes synthesized with 60 µL and 40 µL of PEG-NH2 after sorting. Then, the size of liposomes was also measured.

4.3.1 Dynamic Light Scattering

The samples were diluted in the buffer solution.

On one side, with 60 ml of PEG-NH2 a size of 190 nm was obtained (*Figure 20*). A size of 21 nm is also distinguishable from the liposomes which correspond at maghemite particles that have probably been released from the liposome.

On the other hand, with 40 µL of PEG-NH2, it was obtained a bit small size of 106 nm *(Figure 21).* As in the previous graph, the presence of particles of maghemite is clearly observable in this case.

In view of the results achieved, coupling with 60 µL of PEG-NH_2 produced particles with a size about twice as large as with the coupling with 40 µL of PEG-NH_2.

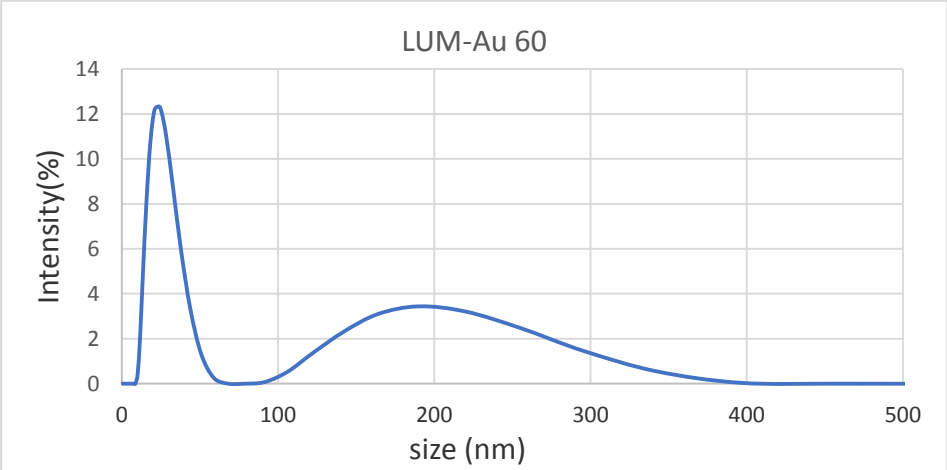


Figure 20. Curve DLS LUM-Au (60 µL PEG-NH2)

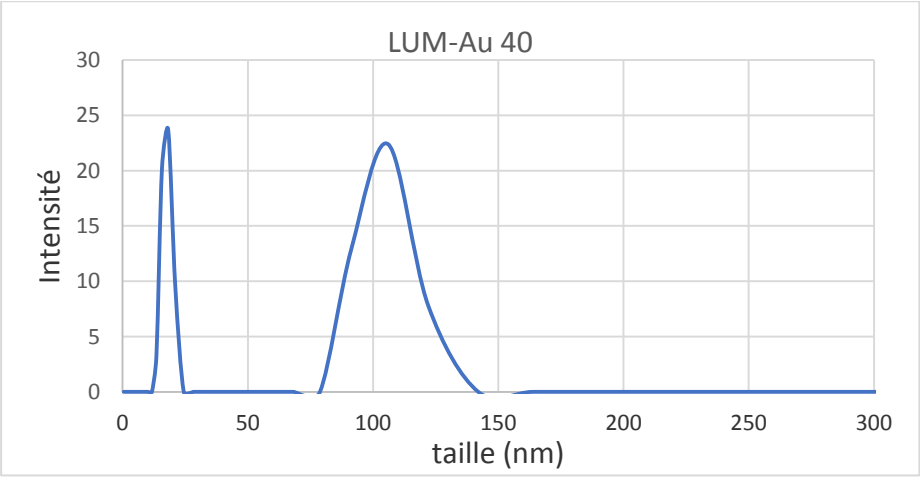


Figure 21. Curve DLS LUM-Au (40 µL PEG-NH2)

4.3.2 Zeta potential

After measuring zeta potential of both samples of liposomes, a value of 28.3±5 mV was obtained for liposomes avec 40 µL and a value of 15.2±3 mV was obtained for liposomes synthetized with 60 µL of PEG-NH_2. These results indicate that the first one have a quite good stability compared with the second one.

For particles that are small enough, a high zeta potential gives it stability, that is, the solution or dispersion will resist aggregation. When the potential is low, there is attraction between the particles, the repulsion is overcome and flocs are formed instead of dispersion. Therefore, high potential zeta colloids are electrically stabilized, while colloids with low zeta potentials tend to coagulate or flocculate.

This can explained the result obtained after measuring the size of liposomes. The size of liposomes with 60 µL is approximately twice the liposomes with 40 µL. Taking into account that the stability of the first one is not very high, it's possibly that flocs were formed during the synthesis, which would explain the very high size of them.

4.3.3 UV-visible

The samples were diluted in the buffer solution. The following curves were obtained.

The UV-Vis spectrum was obtained for the two complexes LUM-Au with 40µL of PEG-NH_2 (*Figure 22)* and 60 µL of PEG-NH_2 (*Figure 23)*.

The gold surface plasmon resonance band is present but it is moved to lower wavelengths (about 480 nm). This is probably due to the aggregation of gold nanoparticles in the salt content in the buffer solution.

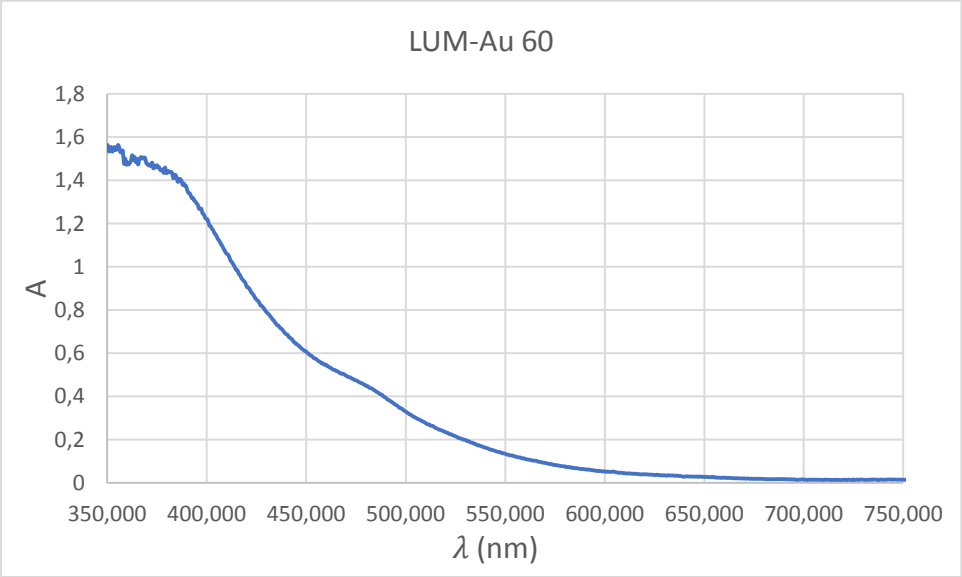


Figure 22. Curve UV-Vis LUM-In (60 µL PEG-NH2)

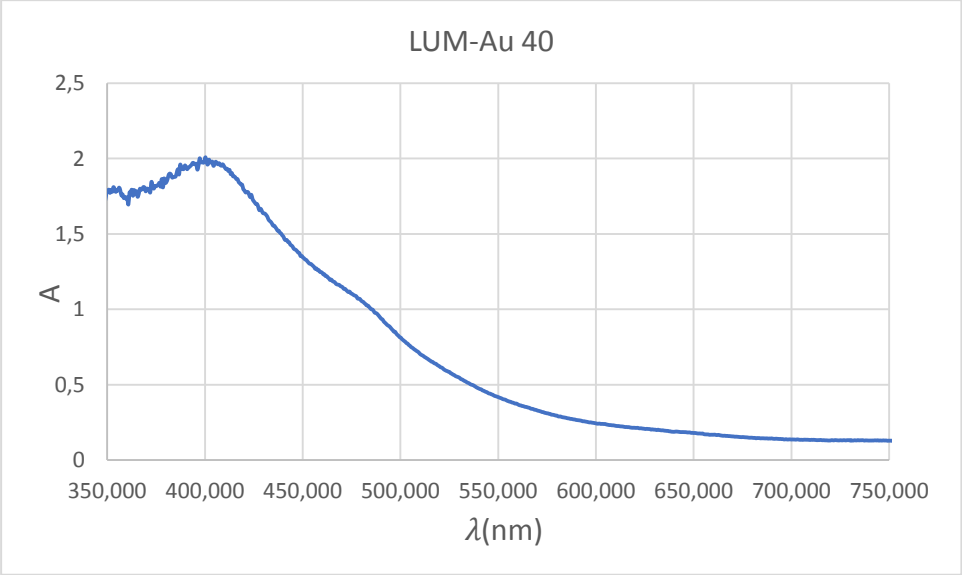


Figure 23. Curve UV-Vis LUM-In (40 µL PEG-NH2)

4.3.4 Transmission Electron Microscopy (TEM)

Liposomes magneto-plasmonics synthesized with 40 µL of PEG-NH_2 were observed using Transmission Electron Microscopy

For sample preparation it was preceded as follows: magnetic liposomes coupled with gold were recovered and diluted in 1 ml of buffer solution. Then 20 µL of the liposome solution were diluted again in the buffer solution until obtaining a light colour (*Figure 24).* One drop of the diluted solution was twice deposited on the microscope grid covered with a dried inert carbon layer. After the drop had dried, microscopy grid was placed in a Petri dish (*Figure 25).*

Figure 24 Sample preparation

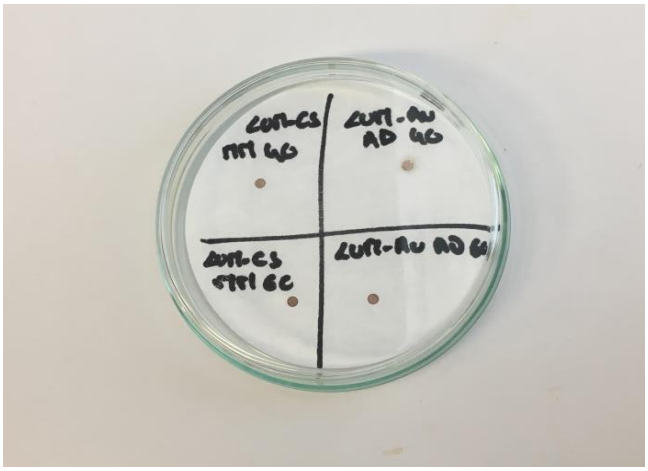


Figure 25. Preparation of samples microscopy grids in a Petri dish

The principle of this technique is detailed in the annexe.

In the Figure 26 we can observe aggregated gold nanoparticles in a black intense which are more distinguishable. In the Figure 27 we can also see little particles less coloured which correspond to maghemite nanoparticles. There is also a significant salt layer from the buffer causing the aggregation of the nanoparticles of oxides of iron and gold which appear in Figure 28. The last image, Figure 29 shows magneto-plasmonic liposomes, clearly distinguishable by his spherical shape containing maghemite nanoparticles and gold nanoparticles. This liposomes have and smallest size of initial liposomes.

TEM gives the following micrographs (scale 20 nm):

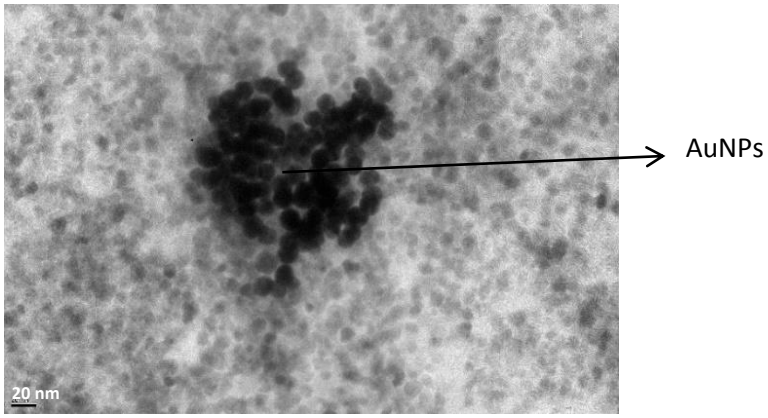


Figure 26. Aggregated gold nanoparticles

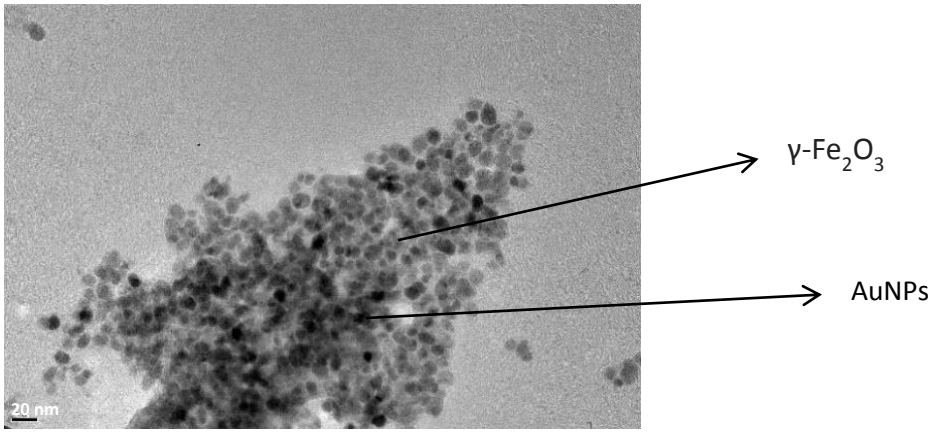


Figure 27. Gold and iron oxide

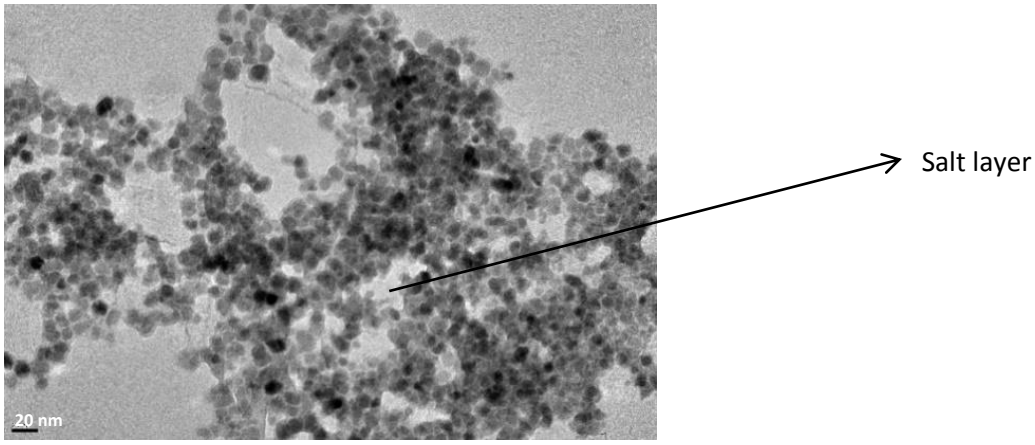


Figure 28. Gold and iron oxides with salt layer from the buffer.

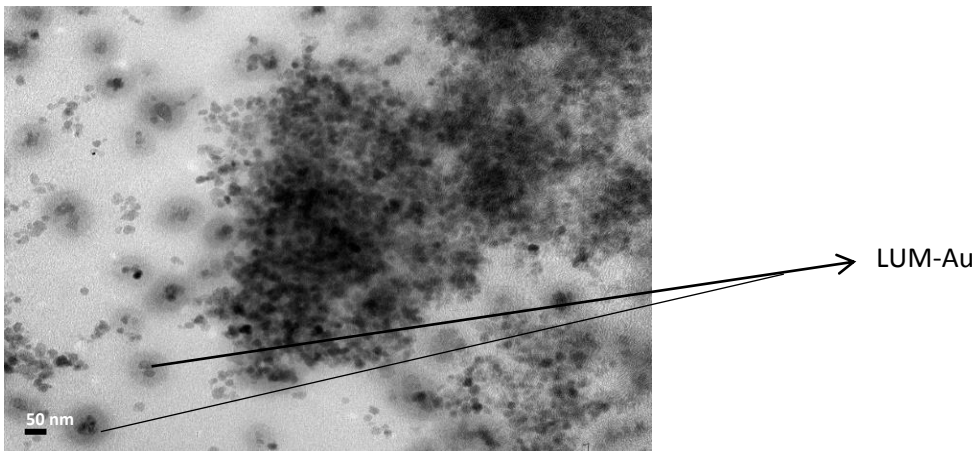


Figure 29. Liposomes magneto-plasmonics

5. Discussion and conclusion

In view of the results obtained it can be said that the synthesis has evolved in a satisfactory manner.

We used quite sensitive techniques such as DLS, which may explain some imprecise results.

The synthesis of gold nanoparticles was developed successfully obtaining a size of about 21 nm and a potential zeta consistent with their structure.

Furthermore, by the reason that liposomes are very fragile, the synthesis is complicated and should be performed with great care. This explained that liposomes coupling with 60 µL weren't observed in TEM. In addition, the low zeta potential of these liposomes shows the little stability of them.

Finally, a size of ~100 nm of magneto-plasmonic liposomes was obtained, with a value of zeta potential which shows a not very high stability. Despite of this, the spherical vesicles were observed with TEM, clearly distinguishable from the other particles in the sample. The reason why size of liposomes has decreased during the synthesis may probably due to a reorganization of the phospholipids, producing smaller liposomes containing both gold and maghemite nanoparticles. Further studies are necessary to understand the parameters influencing the stability of the liposomes in order to get efficient magneto-plasmonic liposomes.

After the synthesis, it would be necessary to see how the properties of these liposomes can be exploited in the medical field, particularly in theranostic applications, in favour of the relentless struggle against this terrible disease.

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7. Annex

7.1 Characterization Techniques

7.1.1 Fluorimetry

Fluorimetry is an assay method using the property of some molecules to be fluorescent. It consists of measuring the fluorescence which is proportional to the concentration.

A fluorescent molecule (either natural or obtained by formation of a derivative) has the property of absorbing light energy (excitation light) and quickly restores a portion of this absorbed light as fluorescent light (light of emission) at a higher wavelength. The molecule is excited by absorption of photons from the light source. The excitation results in a fluorescence radiation sent in all directions. The basic law of fluorimetry is that the intensity of the radiation emitted by fluorescence is proportional to the concentration of the molecule intercepting the light beam.

7.1.2 DLS

The dynamic light scattering (DLS) is an analytical technique spectroscopic non-destructive who allows to determine the size of the molecules. The analysis is based on the Brownian motion of the particles. It provides access to sizes of 1 to 500 nm. The sample to be analysed may be dispersed in various solvents. It is necessary to know the viscosity of the solvent and its refractive index. It works very middle diluted to avoid multicast and thus not analyse reissued makers.

The laser beam illuminates the sample, the light is scattered in all directions at different intensities due to Brownian motion. The signal is collected on a detector placed at an angle theta with respect to the direction of the incident beam. (*Figure 30)*

The sensor analyses the intensity fluctuations over time. A small particle because of the higher intensity fluctuations than large particle as it moves faster. By mathematical processing of these data by a correlator, a self-correlation as well as the diffusion coefficient is obtained D.

Hydrodynamic diameter was determined using the Stokes-Einstein equation: $d_h = \frac{k_B * T}{3\pi * \eta * D}$

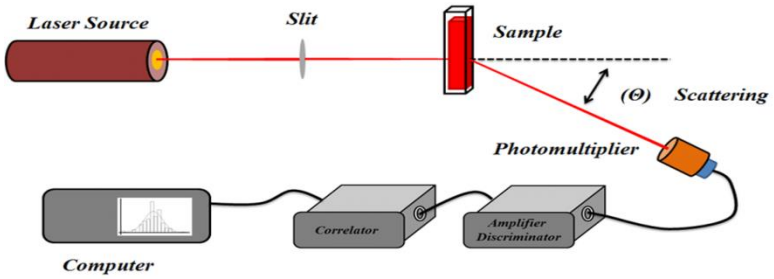

Figure 30. Diagram representing the equipment of the DLS technique.

7.1.3 Zeta potential

Zeta potential is the electric charge that a particle acquires through the cloud of ions that surround it when it is in suspension or in solution. Indeed, when she is moving in a liquid, she surrounds herself with ions organized in an "electric double layer": a portion of the ions clings to the particle thereby forming an ion layer adhering this dense layer. The other part of the ions forms an unbonded said diffuse layer.

The "shear plane" defines these two layers *(Figure 31).* It's the potential difference between the dispersion medium and the potential at the shear plane which defines the Zeta potential. This potential is a measure of the intensity of repulsion or electrostatic attraction between particles. Its measurement therefore provides an understanding of the causes of dispersion, aggregation or flocculation and a solution to improve the formulation of dispersions, emulsions or suspensions.

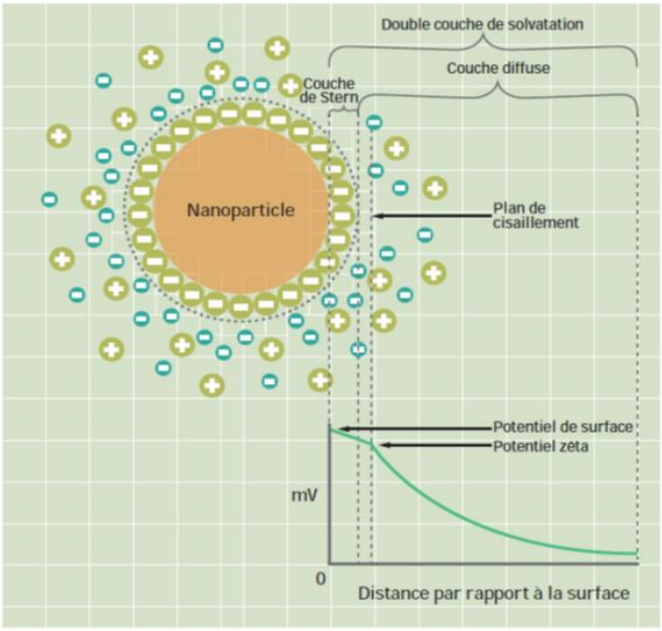

Figure 31. Schema of electric double layer of a particle.

Zeta potential of nanoparticles is relatively easy to measure. It provides extensive information on the properties and behaviour of nanomaterials in different environments: in vitro assays, soil, surface water ... The goal is to identify the properties that control the fate and effects of nanomaterials and eventually be able to predict the behaviour of a new nanomaterial based on the measured zeta potential.

Malvern Zetasizer Nano ZS equipment (*Figure 32)* can measure the size and zeta potential of nanoparticles.

Such equipment makes it possible to measure sizes of 0.3 nm to 10 µm (diameter).

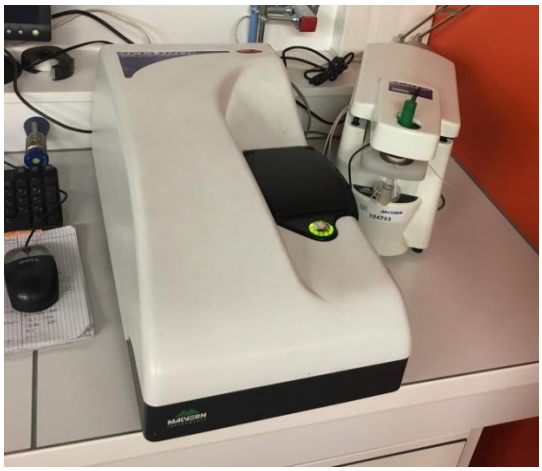

Figure 32. ZetaSizer Malvern Nano ZS used to measure the size and zeta potential

7.1.4 UV-Visible

The ultraviolet-visible spectroscopy is a spectroscopic technique involving photons whose wavelengths are in the ultraviolet range (200 nm - 400 nm), visible (400 nm - 750 nm) or near infrared (750 nm 1400 nm). Subjected to radiation in this range of wavelengths, molecules, ions or complexes may undergo one or more electronic transitions. The analysed substrates are mostly in solution but can also be in the gas phase and more rarely in the solid state.

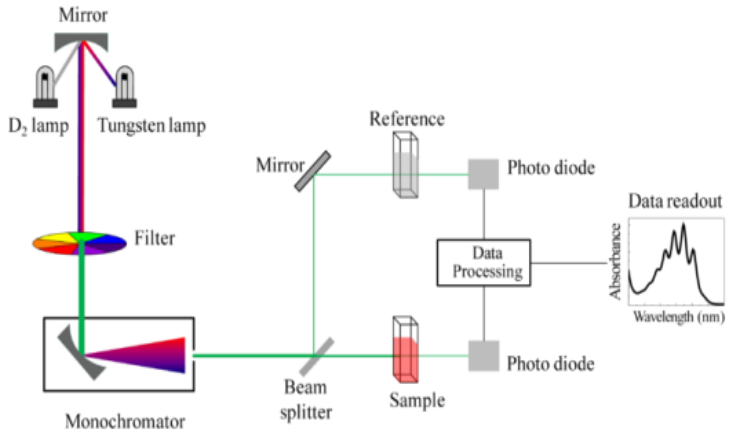

Figure 33. Schema of UV-Vis device

The electronic spectrum is the function which connects the light intensity absorbed by the sample analysed according to the wavelength. The spectrum is often presented as a function of absorbance depending on the wavelength. It can also be described as the molar extinction coefficient as a function of wavelength, then the spectrum is independent of the length solute concentration that absorbs.

The technical analysis is often used in a quantitative method for determining the concentration of a chemical entity in solution, using the Beer-Lambert law:

$$A_\lambda = -\log_{10} \frac{I}{I_0} = \varepsilon_\lambda \cdot \ell \cdot C.$$

wherein I / I_0 is the transmittance of the solution (no unit), A is the absorbance or optical density at a wavelength λ (unitless)ε_λ is the molar extinction coefficient (in L/mol·cm).

7.1.5 Transmission Electron Microscopy (TEM)

The principle of transmission electron microscopy is based on an electron beam extracted from a heating filament (or field effect) and accelerated by a high voltage (of the order of 50000-3000000 V) to level of the barrel, which is focused by magnetic fields of the order of tesla.

The beam of electrons can be treated either as particles or as waves. The physics of the formation of the image is similar to that of optical photonics. This is a first approximation, with the single tool like optical geometric that we can study the formation of the image by transmission electron microscopy. A schema of a transmission electron microscope is represented in *Figure 34.*

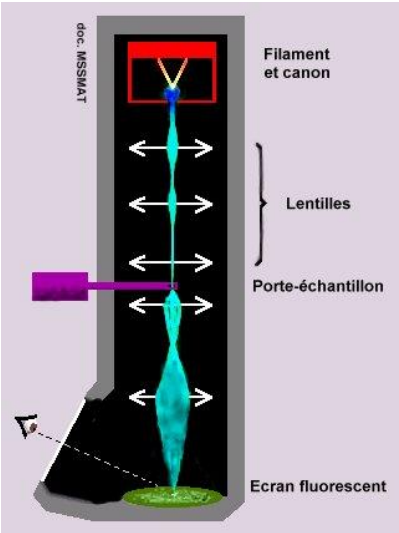

Figure 34. Scheme transmission electron microscope.