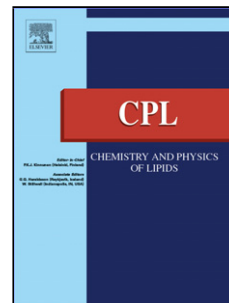


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Title Page

Lipopolymers and lipids from lung surfactants in association with N-acetyl-L-cysteine: characterization and cytotoxicity.

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Lipopolymers and lipids from lung surfactants in association with N-acetyl-L-cysteine: characterization and cytotoxicity.

Highlights

- Polymeric diacetylene liposomes constitute a possible NAC delivery system.
- Lipopolymers with lipids from lung surfactant presented high levels of polymerization.
- Formulations present small hollow fibers.
- Lipopolymers with lipids from lung surfactant are rigid and stable.
- Lipopolymers with lipids from lung surfactant are non-toxic and easy to produce

Abstract

In the present work, we obtained polymeric diacetylene liposomes that can associate N-Acetyl-L-Cysteine (NAC), a broad spectrum mucolytic. The reason for studying these formulations is that they could be applied in the future as NAC delivery systems, with a possible dose reduction but maintaining its effect.

Liposomes used herein are obtained by a photopolymerization reaction, thus gaining stability and rigidity. Lipids belonging to lung surfactant were added in different ratios to the formulations in order to maximize its possible interaction with the lung tissue. Because of lipopolymer stability, the oral or nasal route could be appropriated. This formulation could efficiently transport NAC to exert its mucolytic activity and help in diseases such as cystic fibrosis, which has abnormal mucus production. Also, this type of treatment could be useful in other types of diseases, interacting with the mucus layer and making the lung tissue more permeable to other therapies. Formulations so obtained presented high levels of polymerization. Also, they present small hollow fibers structures with a high number of polymeric units. These types of arrangements could present advantages in the field of drug delivery, giving the possibility of a controlled release. Lipopolymers with lipids from lung surfactant associated with NAC are promising complexes in order to treat not only respiratory illnesses. The stability of the formulation would allow its inoculation through other routes such as the oral one, helping the reposition of NAC as an antioxidant drug. Finally, these formulations are non-toxic and easy to produce

Keywords: lipopolymer; mucolytic; N-Acetyl-L-Cysteine; lung; respiratory illnesses

Introduction

Photopolymerizable lipids were obtained for the first time in the 80's and studied ever since (Johnston, Sanghera, Pons, & Chapman, 1980). Liposomes developed from photopolymerizable lipids are prepared by a light-triggerable reaction with UV light of 254 nm and are based in the photopolymerizable lipid DC8,9PC (1,2-bis (tricoso-10,12-diynoyl)-sn-glycero-3-phosphocholine). After UV irradiation, this phospholipid can form intermolecular crosslinking through the diacetylene group to produce a highly conjugated polymer within the hydrocarbon region of the bilayer (Yavlovich, Singh, Blumenthal, & Puri, 2011). Addition of other lipids to the formulation, especially those that present short-chain, increased polymerization efficiency. The ideal lipid to mix with DC8,9PC in order to induce the highest polymerization efficiency is 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) (Alonso-Romanowski et al., 2003; Chiramoni et al., 2010; Temprana et al., 2012, 2017).

Lipopolymer formulations were applied in several areas. We studied lipopolymers as drug delivery systems for small compounds and macromolecules as well (Chiaramoni et al., 2008; Chiaramoni et al., 2010; Julieta et al., 2013; Taira et al., n.d.; Temprana et al., 2017).

Nowadays, several liposomal formulations in the market have been clinically approved, and many others are in clinical trials. The benefits that liposome formulations bring are the reduction of toxicity and enhancement of the efficacy of conventional drugs, among others (Eng et al., 2001). Additionally, polymerizable lipid-based structures have been used in the controlled release of drugs that inhibit cell migration; these structures were microcylinders formulated based on a diacetylene lipid (Rudolph et al., 1992).

Respiratory illnesses are widespread and affect a large part of the world population. During airway infections, the body responds with an incremented mucus production in order to induce a rapid clearance (Tarrant et al., 2019).

Cystic fibrosis (CF) is a genetic disease. It is caused by a defect in the CFTR chloride channel that, as a result, ends in organ malfunction, particularly the lungs (Ewence & Jones, 2020).

Normal airways maintain a sterile environment by several defense systems, one of them includes mucus clearance via mucociliary. The loss of CFTR function in CF patients affects these defenses (Wine et al., 2018); as mucociliary clearance is hampered, the risk of infections and mucus accumulation increases (Bell et al., 2020).

With this in mind, it would be extremely useful to have a delivery system that transports drugs with mucolytic activity in order for them effectively reaching the airways where mucus production is abnormal.

Additionally, pulmonary fibrosis is a condition that occurs with the presence of scar tissue. As fibrosis increases, lung function is impaired. This condition can be due to various diseases. An example of them is silicosis, a pneumoconiosis caused by inhalation of silica particles. The inhalation of these particles causes inflammation and fibrosis and usually ends in irreversible lung disease and abnormal mucus production. This mucus could prevent that treatments effectively exerts its action. For example, there are reports of treatments with bone marrow-derived cells in an experimental model of silicosis. This treatment improves lung function but the benefit was not maintained in time (Lassance et al., 2009).

A treatment that efficiently transports drugs with mucolytic activity to where the bone marrow-derived cells must be implanted after instillation could cause the tissue to be more permeable to this type of therapy, thus increasing the probability of efficient grafting.

Drugs with mucolytic activity are commonly used. These drugs should lower the viscosity of mucoprotein solutions, reduce bronchial secretions, and increase mucociliary clearance (Cazzola, Calzetta, Page, Rogliani, & Matera, 2019).

Thiol-based drugs are considered as mucolytics because they can interact with mucoproteins, decreasing their viscosity by reducing disulfide bonds (Cazzola et al., 2019). N-Acetyl-L-cysteine (NAC) is one of these drugs, which means it interacts with mucus, disrupting its structure by breaking disulfide bonds of the mucin proteins (Aldini et al., 2018). This bond-breaking results in changes in rheological properties, decreasing mucus viscosity (Cazzola et al., 2019).

NAC is approved for human use as a mucolytic drug. However, it would be useful to have a delivery system that allows the inoculation of a lower dose, maintaining the same effect. Additionally, an efficient drug delivery system would allow NAC to exert its activity when an abnormal mucus production occurs, as in the case of cystic fibrosis (Callela, Valerio, Brodlie, Donini, & Siervo, 2018).

Additionally, drug repositioning has drawn significant attention in the field of drug development. It has advantages related to cost and time, in comparison with the development of new drugs (Yang & Zhao, 2019).

Thiol-based drugs, besides having a mucolytic effect, present other pharmacological activities such as antioxidant activity, anti-inflammatory action, and direct or indirect antibacterial activity (Cazzola et al., 2019). In the case of NAC, it was found that, besides its mucolytic effect, it presents properties like antioxidant action, making NAC a promising candidate for the treatment of other illnesses like cancer, parasite infections and even schizophrenia (Rizk, El-Sayed, AbouLaila, Yokoyama, & Igarashi, 2017; Sepehrmanesh, Heidary, Akasheh, Akbari, & Heidary, 2018).

Liposomes are well-accepted drug vehicles, often providing safety and controlled release carriers. These vehicles can encapsulate a wide range of drugs with a specific activity. Especially

for the respiratory route, they can be inoculated in respiratory airways through nebulizers and inhalers (Elhissi, 2017).

Lung surfactant is a mixture composed of lipids and proteins that are produced by pneumocytes presents in the alveolar epithelium. The function of this surfactant is the reduction of the surface tension of the alveolar air-water interface. Additionally, the surfactant is also present in the narrow bronchiolar and tracheal airways, although it has an alveolar origin. It has been suggested that the function of the surfactant in the upper airways is to maintained opened and unobstructed, allowing free airflow through narrow capillaries (Nag et al., 2007)

Regarding its lipid content, lung surfactant is mainly composed of phospholipids, particularly dipalmitoylphosphatidylcholine (DPPC), its main component. Other phospholipids that are present in lung surfactant are palmitoyl-myristoyl-phosphatidylcholine (PMPC) and palmitoyl-palmitoleoyl-phosphatidylcholine (POPC) (Bernhard, 2016).

One of our long-term goals is to maximize the arrival of the transported mucolytics to lung tissue.

This could be beneficial in diseases that have abnormal mucus production, such as cystic fibrosis or as a pre-treatment in chronic lung ailments such as some fibrosis, in which it may be necessary to make the lung tissue more permeable for the subsequent treatments increase their efficiency.

This research line aims to obtain a formulation that has high polymerization profile and a high probability of efficiently reaching the lung tissue, releasing its contents there, with a structure suitable for controlled release. Short-chain lipids that are present in lung surfactant were added to DC8,9; formulations so obtained were characterized by biophysical methods since this lipid addition might increase drug delivery to lung tissue.

The lipid choice was DPPC, DMPC, and PMPC. We avoided using POPC because the unsaturation would interfere with photopolymerization. In order for the polymerization to be effective, the acyl chains must remain close to each other, the presence of the double bond belonging to oleic acid will interfere with the correct lipid packing, thus reducing the polymerization efficiency (Puri et al., 2011).

DMPC was included in formulation because lipopolymer high stability was previously reported by our group (Alonso-Romanowski et al., 2003; Temprana et al., 2017). Besides, it was studied that lipopolymers produced by DC8,9PC and DMPC were stable in several solutions, making them suitable for drug delivery vehicles that have to pass through different barriers (Taira et al., n.d.).

In the first stage, we tested a wide variety of lipid mixtures and studied their polymerization efficiency in order to keep those mixtures that polymerized efficiently. Formulations so obtained were characterized by biophysical methods, focusing on its polymerization efficiency, particle size, and hydrophobicity profile. Its structure was assessed by transmission electron microscopy.

In this work, we study and characterize formulations that can associate NAC in order to apply them in the future as carriers which might increase NAC mucolytic activity, reducing its dose.

In order to test its safety, cell toxicity was studied in A549 cells. This cell line is derived from human lung carcinoma, so it is ideal for the analysis of the effect on cell viability and metabolic activity of the formulations developed in the present work.

Additionally, these formulations could be useful for repositioning NAC as an oral antioxidant treatment, since the lipopolymers have high stability and could be inoculated through the oral route.

Materials and Methods

Materials

Phospholipids were purchased in Avanti Polar Lipids (Alabama, USA) and were used without further purification.

NAC was from Sigma (St. Louis, USA) and all reagents used in cell culture were from Invitrogen (California, USA).

A549 cells were given by the cell bank of IMBICE (Argentina).

Liposome preparation.

Liposomes as multilamellar vesicles (MLVs) were obtained by the dehydration-rehydration method, as previously described (Bandeira et al., 2016; Bangham, 1972; Julieta et al., 2013; Marsanasco, Márquez, Wagner, del V. Alonso, & Chiaramoni, 2011)

Briefly, 10 mg of total lipids were dissolved in 0.2 mL of methanol. Then, the solvent was evaporated, and the film was resuspended in 1 mL of Phosphate Saline Buffer, pH 7.4 (PBS). Whenever required, N-Acetyl-L-Cysteine (NAC) was added when rehydration at two different final concentrations: 1 and 10 mg/mL

In order to obtain small unilamellar vesicles (SUVs), MLVs were sonicated as previously described (Chiaramoni et al., 2010).

Polymerization process.

Polymerization was accomplished by UV irradiation, as reported by our group (Alonso-Romanowski et al., 2003; Julieta et al., 2013; Taira et al., n.d.; Temprana et al., 2012). Briefly, a Stratalinker 1800 (Stratagene, La Jolla, CA) was used in order to cross-link and polymerize the SUVs. Polymerization was performed by UV cycles. Each cycle energy was 360,000 mJ/cm² and with a duration of 1650 seconds. There was a total of 15 cycles at a wavelength of 254nm. The temperature was kept at 4°C in between cycles.

Polymerization efficiency

Diacetylenic polymers used in this work had two shoulders in the visible spectra: one at 480 nm and the other at 520 nm. These two shoulders correspond to polymers formed by different numbers of effectively conjugated units (Chiaramoni et al., 2007). By following the presence of these peaks, we can measure the polymerization efficiency of the proposed formulations. Visible spectra were recorded between 400 and 600 nm on a Shimadzu 160-A double beam spectrophotometer (Shimadzu, Kyoto, Japan). Samples were measured before polymerization and after the last cycle.

In order to evaluate the effects of surfactant lipids in the polymerization process, several mixtures were tested. Those formulations that had the best polymerization efficiency were selected for further modifications, in order to combine the two lipids that are present in lung surfactant: DPPC and PMPC. Formulations thus obtained are detailed in table 1.

Membrane packing and surface hydrophobicity.

It is interesting to measure the packing level of the surface region since it is closely related to water entrance and liposome stability. Also, membrane packing is modified with polymerization: the more polymerization, the more packing of the membrane.

Surface membrane packing was measured by using merocyanine 540 (MC540). This probe is sensitive to its polar environment. MC540 is located in the membrane phospholipids with its polar sulphonated group toward the more polar outer surface of the head group region, and the rest is in the hydrocarbon chain region (Lelkes & Miller, 1980).

Liposome formulations (before and after polymerization) were used to measure surface packing. The probe/lipid ratio was 180/1 (Disalvo, Arroyo, & Bernik, 2003). Lipid concentration was kept at 1mg/mL. A scan of each sample between 400 and 600 nm was obtained with a UV–VIS spectrophotometer (Shimadzu). Measurements were performed at room temperature. Samples without polymerization were measured, then the probe was added and allowed to reach equilibrium. Spectra were measured, and baseline correction was done as previously described (Disalvo et al., 2003). Hydrophobicity factor (HF) was calculated as A_{570}/A_{500} ; this parameter relates to the concentration of probe in the non-polar environment concerning the aqueous phase (Chiaramoni et al., 2008). The higher the HF, the less packed the surface is. Measurements were performed in formulations with and without NAC. Three independent measurements were performed of each sample.

Transmission electron microscopy (TEM)

Liposomes with and without polymerization process were subjected to TEM with negative staining, as previously described (Alonso-Romanowski et al., 2003). A TEM EM109T was used, images were taken with an attached digital camera (Zeiss, Germany).

Particle size measurements.

Particle size was assessed by Dynamic Light Scattering (DLS). Briefly, samples were diluted in PBS in order to accomplish Brownian motion; the determination was performed in a Zetasizer Nano S (Malvern Instruments, UK).

Samples were measured before and after UV irradiation, but results of lipopolymers were withdrawn because polymerized formulations tend to form fibers and arrangements that are not spherical, as observed by TEM. Five independent measurements were performed of each sample.

Cell viability

For the study of the influence of the polymeric liposomes on cell culture, and to analyze whether there were cytotoxic effects or changes on metabolic activity, the A549 cell line was used. This cell line corresponds to a human carcinoma and belongs to lung tissue, so it is ideal for the analysis of the effects of these formulations, having in mind that they are thought for the application in respiratory illnesses as mucolytics vehicles. Briefly, A549 cells were seeded in a 96-well plate (7×10^3 cells/well). Passages 13-15 were used. Cells were grown in RPMI medium, supplemented with 10% v/v of bovine fetal serum (BFS), and a combination of antibiotics and antimycotics. Cells were allowed to grow at 37 °C in a 5% CO₂ atmosphere. Upon cell confluence, samples were seeded in the maintenance medium, without BFS. They were incubated for 24 hours; afterward, the medium was washed, and metabolic assay (MTT) was performed. MTT is a rapid colorimetric assay, based on the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide); living cells with active mitochondria are required to generate a strong signal and the amount of colored product generated per cell would depend on the level of cell metabolism. (Mosmann, 1983).

Lipid concentration was 10 mg/mL, NAC concentration was 1 and 10 mg/mL. These concentrations were thought based on the following:

Lipopolymer concentrations between 2 and 10 mg / mL have been tested in previous works, proving that they were not highly toxic (Chiarmoni et al., 2010; Julieta et al., 2013).

NAC was added in the same relationship as the previous polymerization efficiency, DLS, HF, and TEM tests were done.

Also, cells were treated with NAC and liposomes alone. Cells with maintenance medium were also used as control.

Results and discussion

When exogenous compounds access the respiratory system, first they find a layer of mucus which usually prevents its progression to the lung tissue. In addition to these barriers, there is also a steric impediment as a consequence of the lung geometry since there is a progressive wall thickness that facilitates gas exchange. However, the gradual reduction of pulmonary ducts

prevents the delivery of drugs to the deeper parts of the respiratory system (Hidalgo, Cruz & Perez Gil, 2017).

From this point of view, a mucolytic treatment that permeabilizes lung tissue and makes it sensitive to certain treatments that require the arrival of active ingredients in alveolar tissue could be useful, leaving only then the geometry of the tissue as a barrier.

The airway secretions from tracheal aspirates contain a significant amount of surfactant with a phospholipid composition similar to the alveolar but with decreased protein concentration (Hohlfeld, 2002). However, the function of the surfactant in this part of the respiratory tract is still been discussed. Despite its function, its presence might be used as a target for drug delivery systems that transport mucolytics and help mucus clearance in the upper airways.

In our research group, we developed lipopolymeric formulations with the addition of lipids from the surfactant to be used in the near future as systems for delivering NAC to lung tissue.

In this work, we perform the biophysical characterization of the formulations and evaluate their effect on a lung cell line.

Polymerization efficiency.

The phospholipid polymers were synthesized several years ago (Johnston et al., 1980). From that work until today, several characterizations were performed, and applications were developed. The photopolymerizable 1,2-bis (tricoso-10,12-diynoil)-sn-glycero-3-phosphocholine (DC8,9PC) can form intermolecular crosslinking through the diacetylene group to produce a highly conjugated polymer within the hydrocarbon region of the bilayer. Due to this backbone, the obtained polymer is colored (Johnston et al., 1980). The addition of saturated short-chain lipids such as 1,2- dimyristoyl-sn-glycero-3-phosphocholine was found to increase polymerization efficiency. (Alonso-Romanowski et al., 2003; Temprana et al., 2010).

We studied the influence of adding lipids found in lung surfactant such us PMPC and DPPC. Formulations studied are described in Table 1. These were formulations with 50 mol % of DC8,9PC and 25-38 mol % of DMPC. They were called 1A, 2A, and 2B. After 15 cycles of polymerization (each cycle was irradiation with UV light of 254 nm during 90 seconds), the sample was withdrawn and diluted to a 1mg/ml lipid concentration.

Lipopolymers formed herein present two shoulders in the visible spectrum: one at 480 nm and another at 520 (data not shown), these absorbances are in agreement with previous work, regarding DC8,9PC (Chiaramoni et al., 2010; Julieta et al., 2013; Temprana et al., 2017). These absorbances reflect the number of units effectively conjugated, as previously described (Chiaramoni, Speroni, Taira, & Alonso, 2007) In systems incorporating DC8,9PC and moderately short-chain lipids such as DMPC and DPPC, small rigid polymeric areas interrupted by nonpolymeric DC8,9PC are obtained (Temprana, Duarte, Taira, Lamy, & Del Valle Alonso, 2010).

Absorbances at 480 and 520 nm of these formulations with and without NAC were studied in order to evaluate the influence of the modified amino acid in the polymerization process. The results are shown in figure 1.

Concerning short lipopolymers (figure 2A), the presence of NAC actively interferes with the polymerization process in formulations with less DMPC (2A and 2B).

Regarding longer lipopolymers, NAC increased the presence of these species, in the case of formulation 2B. Formulation 2B is the one that has more amount of PMPC, the only asymmetric lipid present in the formulations. The presence of this lipid could facilitate interdigitation between acyl chains, incrementing lipid packing, thus improving the formation of more extended polymeric units.

NAC reduces disulfide bonds in mucoproteins, thus exerting its mucolytic activity. Additionally, some evidence suggests that acetylcysteine may exert an anti-apoptotic effect due to its antioxidant activity (Aldini et al., 2018). Photopolymerization has a particular dependence, in order to be successful, acyl chains had to be close between each other. Additionally, new double bonds are formed so that the presence of an antioxidant component could also influence covalent formation. NAC could interfere with the rearrangement of electrons during the formation of lipopolymer (Puri et al., 2011).

In the case of formulation 1A, there is a higher amount of DMPC. Our group previously reported that an equimolar ratio between DC8,9PC and DMPC was ideal for polymerization efficiency (Alonso-Romanowski et al. 2003). As seen in figure 2, NAC does not affect polymerization efficiency. So, it could be that the higher amount of DMPC could counteract the antioxidant

effect of NAC by rapidly forming the polymer units. When DMPC concentration is lower, as, in the case of formulations 2A and 2B, the antioxidant effect of NAC could be significant, avoiding the formation of polymer units.

Membrane packing and surface hydrophobicity.

Results corresponding to HF of each formulation are shown in figure 2.

In all formulations, the HF of polymerized mixtures was lower than non-polymerized ones. This result was expected, since polymerization induces intercatenary bonds that bring acyl chains closer between each other, thus decreasing hydrophobic defects in the surface (Chiaramoni et al., 2008; Chiaramoni et al., 2010).

There are no significant differences in HF with the addition of NAC. This result indicates that interaction with this modified amino acid is not at the surface level because it does not alter hydrophobic defects. Therefore, differences in polymerization efficiency had to be mainly due to the antioxidant effect of NAC, as previously mentioned.

Before the polymerization process, formulations are in the fluid phase, as obtained values of HF are in the range of 16-18. It is essential to mention that before UV irradiation, samples were cooled on an ice bath since ordered gel phase is needed in order for the polymerization reaction to be efficient (Alonso-Romanowski et al., 2003).

Transmission Electron Microscopy (TEM)

Images of formulations with 1 mg/mL of NAC, polymerized, and non-polymerized are shown in figure 3 while TEM of formulations with 10 mg/mL NAC are shown in figure 4

Regarding non-polymerized samples, size and lamellarity coincide with DLS determinations, the diameter is in the range of 100 nm, as observed in the upper panel (Figures 4 and 5 A, B, and C). As seen in the lower panel, UV irradiation-induced the presence of fibers. These fibers presented a smaller size in the case of formulation 2A (Figure 3 and 4E). Isolated vesicles were also observed.

Regarding NAC concentration, a higher amount of this modified amino acid-induced a mild disaggregation, as more separated fibers are observed in figure 4

Additionally, tubular structures appear to be hollow, particularly in the case of formulation 2B, since a lighter center is observed in the core of the fiber (Figure 3F). These hollow fibers are due to the presence of diacetylene lipids and also to the photopolymerization method, as found in the literature. Tubular structures were reported in other works like Schnur and co-workers (Schnur et al., 1987) that observed the presence of these kinds of structures obtained from diacetylene lipids. They reported the formation of hollow cylindrical microstructures with diameters of 500 nm and length of around 20 μ m. Michael Markowitz and Alok Singh worked in 1996 with a modified diacetylene phospholipid. In water, they observed the formation of flexible fibers with diameters in the range of 10 to 100 nm (Markowitz & Singh, 1996). The authors also reported that the formation of tubules should be useful in the development of technologies related to controlled release because these structures could be disassembled slowly, giving; as a result, the possibility of content leakage in several stages.

Tubular structures are the result of helical wrapping of the lipid bilayer in water, as obtained for diacetylene lipids (Markowitz, Schnur, & Singh, 1992). Additionally, these fiber structures grow via liposome intermediates in water, as previously reported (Markowitz & Singh, 1996).

Although in the latest reports found in the literature, the applications of lipopolymers do not include drug delivery, we strongly believe that it is an interesting option to explore since, due to their structure, they could have the necessary stability to provide a controlled release.

Particle size

Particle size distribution was studied in samples without the polymerization process. Intensity measurements give information about particle size distribution. DLS determination was not performed in polymerized samples because with the lipids used in this work, tubules are formed, as observed by TEM. Particle size cannot be determined in non-spherical arrangements, like fibers formed herein.

Results regarding DLS are shown in figure 5.

Particles obtained in this work are circa 100 nm in diameter; this is expected for small unilamellar vesicles (SUVs) produced by sonication (Hammond, Reboiras, Lyle, & Jones, 1984). NAC had a mild effect on particle size; however, it significantly reduces the size in formulation 2A when concentration is 10 mg/mL, in comparison with samples with 1 mg/mL. This mild effect could be related to a rearrangement of NAC, modifying the surface charge. If the modulus of surface charge is high, the particles repel each other, and aggregation events are prevented. A greater amount of NAC bound to the surface would increase the surface charge. However, this could not be observed through the methodologies used in this work, so further studies are necessary to analyze this.

The fiber structures obtained in water grown via liposome intermediate, as previously reported (Markowitz & Singh, 1996). The fibers obtained for formulation 2A appear to be shorter than the other two formulations (Figures 3 and 4, lower panel).

This mild lower particle size obtained for formulation 2A with 10 mg/mL of NAC could be the reason that induces the formation of smaller fibers.

Formulations developed in this work present the addition of lipids that belong to lung surfactant since one of our goals was to explore the possible application of lipopolymers as delivery systems to lung tissue. For the inoculation to be safe in the airways, they should be small so they cannot induce pulmonary embolism. Having this in mind, all formulations could be safe since TEM images show fibers that did not exceed 200nm in length. It is noteworthy that formulation 2A presents TEM images with the smallest fibers (Figures 3 and 4, bottom panel). However, further studies are needed to assure the safety and effectiveness of the formulations.

Fibers formed from liposomes were recently used as drug delivery systems, demonstrating that these kinds of structures are helpful in promoting drug penetration in cell culture (Chen, Duan, Pan, Yang, & Pan, 2019).

Cell viability: MTT method

In figure 6, samples incubated with formulation 1A (A), 2A(B), and 2B(C) are shown. Cells treated with liposomes were taken as the control in Dunnet's test in order to analyze the effect of the vehicle on cell viability.

Regarding the effect of NAC, a concentration of 1 mg/mL had a mild effect on cell viability and metabolic activity. On the other hand, the concentration of 10 mg/mL reduces metabolic activity to circa 50%.

Regarding the influence of lipid vehicles on cell viability, formulations studied in this work had no appreciable toxic effect (Figure 6). Metabolic influence of similar formulations in other cell lines was reported, giving, as a result, mild toxic effects (Chiarmoni et al., 2010, 2007).

A mild cell viability reduction was observed in formulation 1A when NAC 1mg/mL was present (Figure 6A). This effect could be due to a possible interaction of polymers with cell culture. As observed by TEM (figure 3), fibers of formulations 1A with NAC are slightly longer than the rest of the formulations. These fibers could form a network that would interfere with the normal exchange of nutrients. This was previously reported by our group when studying the effect of polymers that associate DNA in cell cultures (Chiarmoni et al., 2010).

Cell viability decreased with NAC transported by all formulations, except for 2B. In previous work, lipopolymers were used as L-tryptophan transporters. The associated aminoacid induced more profound effects in CACO2 cells, in comparison with the free drug, meaning that the entering to the cell was improved by the presence of the lipid transporter (Julieta et al., 2013). This obtained result for 2B may suggest that that formulation 2B did not transport NAC efficiently into the lung cells, but further studies are needed.

Conclusions

Formulations obtained herein were associated with N-Acetyl-L-Cysteine, a broad spectrum mucolytic. Also, lung surfactant lipids DPPC and PMPC were included in the formulations. The presence of NAC in formulations with a lower amount of DMPC interferes with the polymerization efficiency, probably because of its antioxidant activity. The antioxidant activity could prevent the polymerization reaction from occurring efficiently and the intercatenary double bonds from forming. With a higher concentration of DMPC, as in the case of formulation 1A, this does not happen since this lipid improves the proximity of the DC8,9PC molecules and, consequently, the negative effect of the antioxidant activity would be reduced. Nevertheless, all samples presented a high polymerization profile.

Regarding cell viability, formulations 1A and 2A possibly result in a modification of the activity of mitochondrial enzymes in A549 cells, decreasing it. Thus, these formulations present a mild toxic effect on cell viability.

The structures that result from UV irradiation are tubular and small, with a high number of polymeric units. Notably, the formulation that presented the smallest fibers was the one with the smaller amount of PMPC. This formulation also has the lowest amount of DMPC, resulting in polymers with small units effectively conjugated in the presence of NAC.

Small, mucus-penetrating lipopolymer arrangements are meant to enhance pulmonary delivery. Additionally, and because of lipopolymer stability, these complexes could be inoculated via the oral route, giving the possibility of repositioning NAC as an antioxidant treatment as well. Nevertheless, further studies are needed regarding cellular uptake to assure the efficiency of the lipopolymer formulations.

Declaration

The authors want to express that there are no conflict of interest in the work presented herein

Dr. Nadia S. Chiaramoni, PhD

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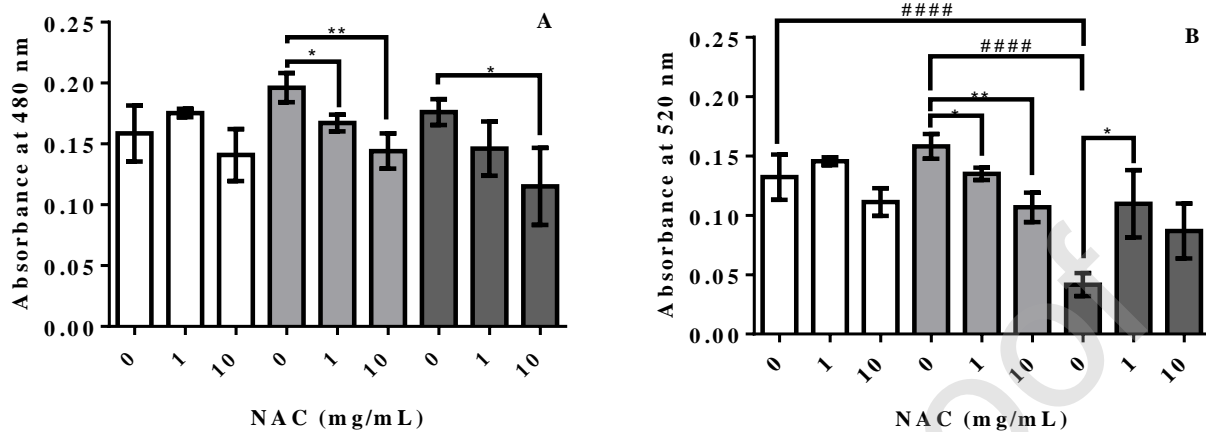


Figure 1: Analysis of the polymerization efficiency

The graph shows absorbance at 480 nm (A) and 520 nm (B) of lipopolymers. White bars belong to formulation 1A, light gray to 2A, and dark gray to 2B. Data are shown as mean \pm SD of three independent measurements. One-way ANOVA was performed.

* significant differences after Dunnet's test ($p < 0.05$), samples without NAC were taken as the control group.

** significant differences after Dunnet's test ($p < 0.01$), samples without NAC were taken as the control group.

**** significant differences after Dunnet's test ($p < 0.0001$), samples without NAC were taken as the control group.

significant differences after Tukey's test ($p < 0.0001$)

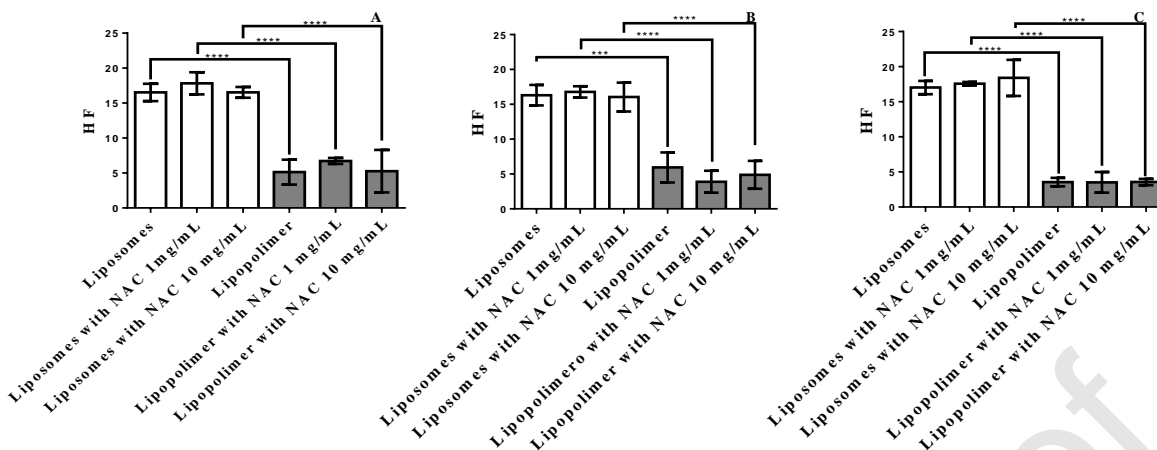


Figure 2: HF of formulations with and without NAC

The figure shows the ratio between absorbance at 570 nm vs. absorbance at 500 nm (HF) of formulation 1A (A), 2A (B), and 2B (C). White bars belong to non-polymerized samples and gray bars are values corresponding to polymerized samples. Data are shown as mean \pm SD of three independent measurements. One-way ANOVA was performed.

*** significant differences after performing Tukey's test ($p < 0.001$).

**** significant differences after performing Tukey's test ($p < 0.0001$).

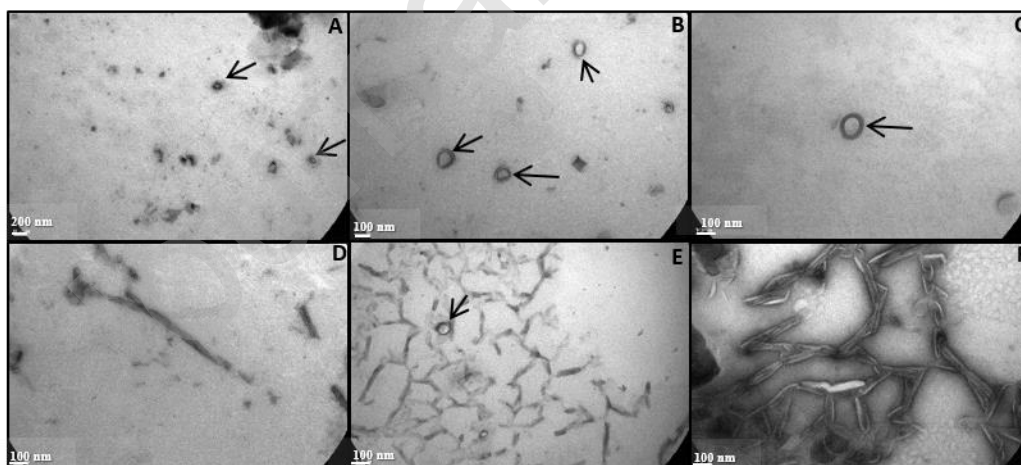


Figure 3: TEM images of negatively stained samples with 1 mg/mL NAC.

In upper panel images of non-polymerized samples are shown: (A) 1A; (B) 2A and (C) 2B.

In lower panel images of polymerized samples are shown: (D) 1A; (E) 2A and (F) 2B. Arrows indicate the presence of lipid vesicles.

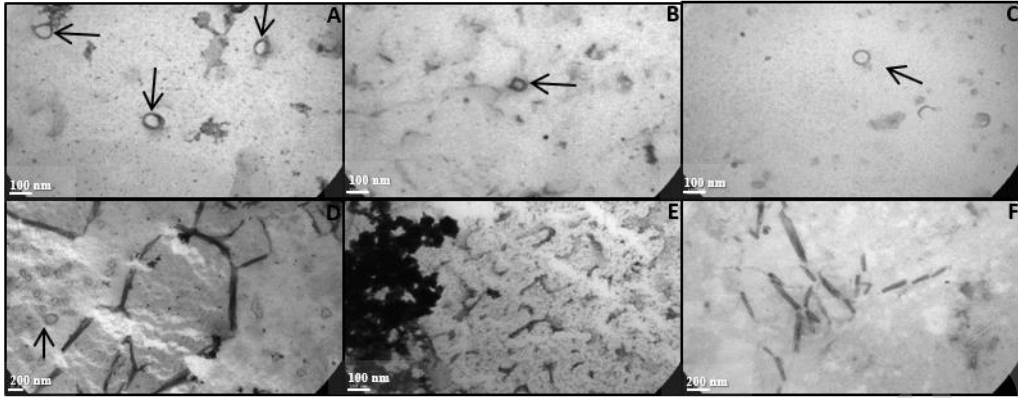


Figure 4: TEM images of negatively stained samples with 10 mg/mL NAC.

In upper panel images of non-polymerized samples are shown: (A) 1A; (B) 2A and (C) 2B.

In lower panel images of polymerized samples are shown: (D) 1A; (E) 2A and (F) 2B.

Arrows indicate the presence of lipid vesicles.

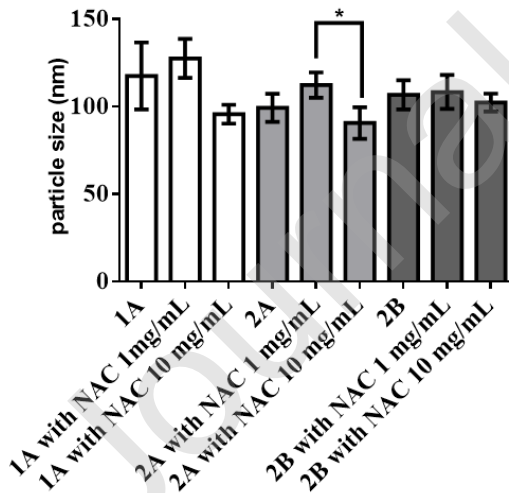


Figure 5: Particle size determination

Figure shows the particle size of each liposome formulation before the polymerization process.

White bars represent formulation 1A, light gray represents 2A, and dark gray are values corresponding to 2B. Data is shown as media \pm SD of five measurements. One-way ANOVA was performed.

* Significant differences respect to control after performing Tukey's test ($p < 0.05$).

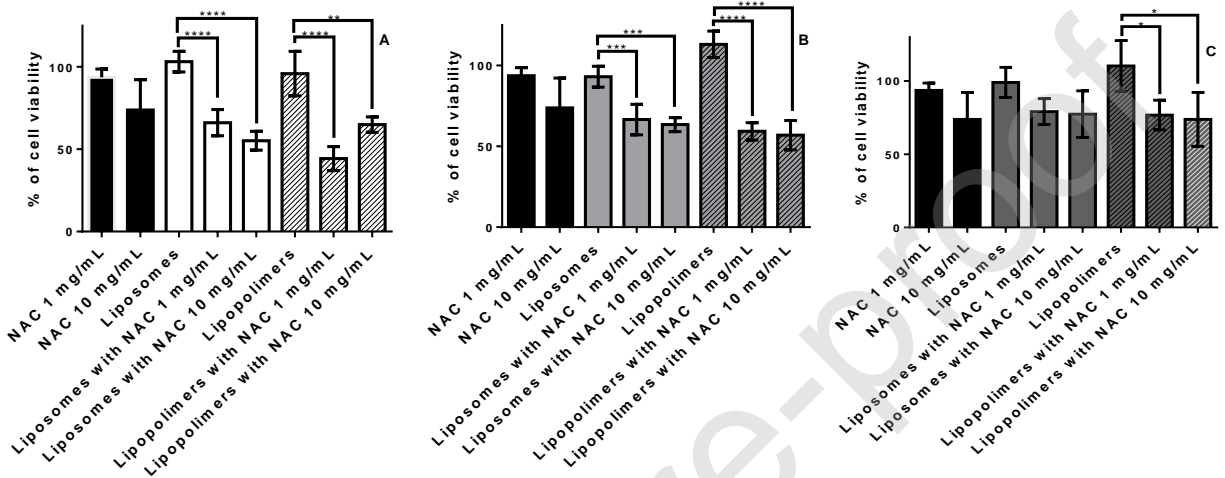


Figure 6

Data are presented as mean \pm SD of five determinations. Results corresponding to formulation 1A are shown in figure 6A (white bars), 2A are in figure 6B (light gray bars), and 2B are in 6C (dark gray bars). Dashed bars correspond to polymerized formulations. Black bars correspond to cells treated with NAC (1 and 10 mg/mL)

One-way ANOVA was performed.

*Significant differences after performing Dunnet's test taken correspond formulation without NAC as control ($p < 0.05$).

**Significant differences after performing Dunnet's test taken correspond formulation without NAC as control ($p < 0.01$).

***Significant differences after performing Dunnet's test taken correspond formulation without NAC as control ($p < 0.001$).

***Significant differences after performing Dunnet's test taken correspond formulation without NAC as control ($p < 0.0001$).

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Table 1: Lipid composition of the studied formulations.

Formulation	DC8,9PC (mol%)	DMPC (mol%)	DPPC (mol%)	PMPC (mol%)
1-A	50	38	6	6
2-A	50	25	20	5
2-B	50	25	15	10