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OPA quantification of amino groups at the surface of Lipidic NanoCapsules (LNCs) for ligand coupling improvement

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ABSTRACT

Lipidic NanoCapsules (LNCs) were prepared via an emulsion phase inversion method. Nanoparticles with hydrodynamic diameter of 25, 50 and 100 nm were easily obtained. Their surfaces are covered with short PEG chains (PEG 660) which are not bearing any chemical reactivities. Thus, in order to overcome this handicap towards post-functionalization possibilities, post-insertion of DSPE-PEG2000 amino (DSPA) can be employed. In order to characterize the insertion step, we have developed a chemical assay for the quantification of amino group inside the PEG shell of LNCs. Subsequently, the post-insertion yield was found to be comprised between 60 and 90% whatever the hydrodynamic diameter of the LNCs is. By means of simple calculations, the density of amino group is estimated to be closed to 0.2 and 1.2 molecules/nm^2. The formulation of LNCs and their controlled functionalization represent an interesting system for the development of bionanoconjugates in a short and effective process.

1. Introduction

The nanoparticle (NPs) family is composed by a broad range of nanoobjects as liposomes, vesicular carriers (nanocapsules, polymerosomes, niosomes), catanionic vesicles, polymer nanoparticles (nanospheres, solid lipid nanoparticles), micelles, dendrimers and hybrid organic/inorganic nanoparticles (magnetic nanoparticles, gold nanoparticles, quantum dots). All of these NPs have evoluted with time from first generation NPs, unable of targetability, to actual multifunctional nanoparticles (MFNPs).

Such nanoparticles are especially made to be used as nanomedecine (Riehemann et al., 2009) drug delivery vehicles, in particular for the treatment of diseases which are hardly cured such as cancers (Jabr-Milane et al., 2008; Davis et al., 2008) or infective pathologies as HIV. Indeed, NPs have been made to target cancer cells (McCarthy and Weissleder, 2008; Ganta et al., 2008; Cho et al., 2008) and to deliver drugs inside (Breunig et al., 2008) and/or in the close environment of the target such as tumors (Wagner, 2007; Nie et al., 2007). Anticancer drugs such as taxans or organometallic compounds have been used in both vitro/vivo experiments and were first investigated in order to answer to the urgent need for pharmaceutical forms with less counter side effects. In a second time, biomacromolecules (Sinha et al., 2006; Farokhzad et al., 2006) such as plasmids (Crystal, 1995; Meyer and Wagner, 2006; Donkuru et al., 2010), siRNA/shRNA (Elbashir et al., 2001; David et al., 2010) and proteins (Des Rieux et al., 2006) has been also investigated with promising results but also facing new challenges as these molecules are fragile both *in vitro* or *in vivo*. Furthermore, DNA based drugs need to reach the nucleus of the targeted cells, which means additional barriers to cross.

These obstacles reinforce the need of an engineered (Yih and Al-Fandi, 2006; Van Vlerken and Amiji, 2006) surface with responsive and biodegradable (Feng, 2004; Panyam and Labhasetwar, 2003) chemical bonds. All of these strategies (Allen and Cullis, 2004) imply the presence of ligands such as peptides (RGD, TAT), sugars (galactose) or proteins (growth factors, antibodies). Thus, RGD (Ruoslahti and Pierschbacher, 1987; Pasqualini et al., 1997) or TAT (Fawell et al., 1994; Vivès et al., 1997; Richard et al., 2003) possess great potentials towards targetability or enhancement of cell uptake; sugars as galactose (Morille et al., 2009) or mannose (Irache et al., 2008) has been used with success to target a whole organ (liver) or tumors, respectively. Regarding antibodies, they have been demonstrated to enhance targetability, leading to a new class of NPs, immunoNPs (Huwyler et al., 1996; Torchilin, 2006).

The introduction of these ligands involves a coupling between these ligands and NPs presenting reactive chemical groups such as primary amine, carboxylic acids, alkenes, azide/alkyne. In fact, numerous strategies (Perrier et al., 2010a,b) are available such as the well known carbodiimides chemistry (amines/carboxylic acids to form amide bonds) but also more recent developments like metathesis between alkene groups or reactions derived from the click chemistry school as copper catalyzed azide-alkyne cycloaddition (CuAAC) or thiol-ene chemistry. Hence, it is very important to quantify the amount of reactive entities with the best sensibility

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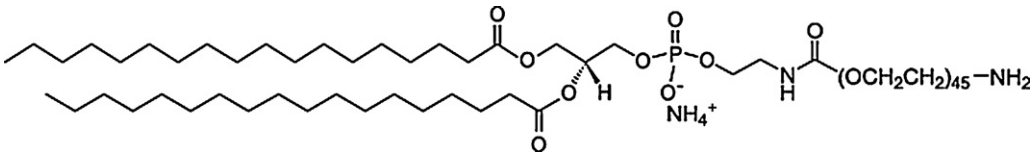

Fig. 1. Molecular structure of DSPA.

whatever the NPs are. It is as much important to set up methods suitable for the quantification of the ligands itself. This last point is emphasized by the fact that ligands are often present in the nanomolar range or less.

Our main objective is to develop a method for the quantification of reactive groups once purification step have been carried out. This information is highly relevant for the assessment of the yield of either formulation or post-formulation processes. It is also the central point in the establishment of a relationship between the density of reactive groups and/or ligands and expected biological activities.

The assay we have developed relies on a fluorescence quantification after a straightforward labeling step. The feasibility of fluorescence labeling of primary amino groups at the surface of NPs has been made for organic/inorganic NPs. However, these systems are different from LNCs used in this study. Indeed, LNCs are made of triglycerides and pegylated surfactants with a low amount of phospholipids unlike liposomes which are composed mainly of phospholipids. In this article, we present the setting up of an assay for the quantification of primary amino groups inside the shell of LNCs. The interface of LNCs has been modified by the post-insertion process (Perrier et al., 2010a,b) carried out with pegylated amphiphilic phospholipids, DSPE-PEG. This assay allowed us to determine the number of amino groups onto the LNCs surface after all the steps of the process, leading to the establishment of the post-insertion yields. All the more post-insertion has been widely used on liposomes or LNCs without the ability to characterize the amount of inserted molecules during the process. This assay is based on the o-phtalaldehyde (OPA) which is highly reactive towards amino groups and subsequently leads to fluorescent indole derivatives following Fig. 2. In spite of OPA is commonly used to label molecules after HPLC column with fluorescence based detection, it has not been used in the quantification of the structural components of NPs, in particular at the interface between NPs and external medium. However, this methodology has been used to assay gentamycin encapsulation yields inside liposomes (Gubernator et al., 2006).

2. Experimental

2.1. Materials

Labrafac® WL 1349 (Gattefossé S.A., Saint-Priest, France) is a mixture of capric and caprylic acid triglycerides. NaCl was purchased from Prolabo (Fontenay-sous-Bois, France) and water was obtained from a Milli-Q-plus® system (Millipore, Paris, France). Lipoïd® S75-3 (Lipoïd GmbH, Ludwigshafen, Germany) is a soybean lecithin made of 69% of phosphatidylcholine, 10% phosphatidylethanolamine and other phospholipids, and Solutol® HS 15 (BASF, Ludwigshafen, Germany) is a mixture of free polyethylene glycol 660 (PEG) and polyethylene glycol 660 hydroxystearate, corresponding to 13 units of PEG. The dialysis membrane was purchased from Spectrapore and has a molecular weight cut-off point equal to 100,000 Da.

1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[amino(polyethylene-glycol$_{2000}$)] (DSPA) (see Fig. 1), corresponding to 45 units of PEG, were supplied by Avanti® Polar Lipids Inc. (Alabaster, USA). o-Phtalaldehyde (OPA) was purchased from ThermoFischer and used as received.

2.2. Preparation of LNCs

This formulation method has already been well documented (Heurtault et al., 2002a, b) and can be briefly presented as follows: all components (Solutol HS-15, Lipoïd S75-3, sodium chloride, Labrafac CC and water) are mixed under magnetic stirring at an agitation speed of 200 rpm at room temperature leading to an O/W emulsion. After progressive heating at a 4 °C/min rate, a short interval of transparency at temperatures close to 70 °C can be observed, and the inverted phase (water droplets in oil) is obtained at 85 °C. At least three temperature cycles alternating from 60 to 85 °C at the same rate are applied near the phase-inversion zone. Thereafter, the mixture undergoes a fast cooling–dilution process: it is diluted 1:3.5 with 12.5 ml of cold water at 4 °C and stirred for 30 min., leading to the formation an LNC suspension of the desired size. LNCs are liquid core nanocapsules made of medium-chain triglycerides (Labrafac CC) surrounded by a surfactant shell assembled in a mixed monolayer of Solutol HS-15 and Lipoïd S75-3. The obtained size distribution depends on the relative amount of surfactants and oily phase i.e. triglycerides as described in Table 1.

2.3. Preparation of post-inserted LNCs

Post-inserted LNCs were prepared using the post-insertion technology previously developed on liposomes and adapted for the LNCs system by our group. The process is the incubation of DSPE-PEG micelles with LNCs and a molecular transfer of DSPE-PEG molecules from micelles to LNCs occurred. We have demonstrated that the preparation of post-inserted LNCs with DSPE-PEG bearing PEG moieties from 750 to 5000 g/mol. In each case, stable LNCs with narrow size distribution and specific electrokinetic properties are obtained (Perrier et al., 2010a,b).

In this article, the post-insertion is used for the preparation of LNCs with chemical reactive surface, ideally designed for the grafting of ligands. To a suspension of LNCs (1.75 ml) was added 1.30 ml of DSPA micelles in MilliQ water with a variable DSPA concentration. This concentration is expressed in % of the amount of surfactants employed in the formulation step. The post-insertion was performed during 105 min at 60 °C under continuous magnetic stirring. The reaction was stopped in an ice bath for 1 min. LNCs suspension were either used for the OPA quantification or were dialyzed against MilliQ water (4 l MilliQ water for 3 ml of LNCs

Table 1
Amount of excipients for the preparation of Lipidic NanoCapsules.

Excipients	20 nm LNCs	50 nm LNCs	100 nm LNCs
SolutolHS15®(g)	1.930	0.846	0.484
Lip old 75-3® (g)	0.075	0.075	0.075
NaCl (g)	0.089	0.089	0.089
Labrafac® (g)	0.846	1.028	1.209
Water (g)	2.055	2.962	3.143
Water 4 °C (g)	12.5	12.5	12.5
Total volume (ml)	17.5	17.5	17.5
Concentration (g/l)	168	115	104