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Photodynamic diagnosis of parathyroid glands with nano-stealth aminolevulinic acid liposomes

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ABSTRACT

Background The use of ALA to identify the parathyroid glands had been investigated both experimentally and clinically with promising results but the side effects from the systemic use of this photosensitizer reduce its widespread in clinical use. The aim of this study is to test the formulation of ALA in nano-stealth liposomes for better photodiagnosis of parathyroid glands intraoperatively with less ALA dose.

Materials and Methods: Preparation of ALA nanovesicles and in vitro characterization for the drug encapsulation percentage, vesicle size and Zeta potential then the study of nanovesicles stability and in vitro drug release profile was done. The study compared nano-stealth liposomes and nano-liposomes with the free ALA solution, intraperitoneal administration of these different ALA formulations in rats and observing the ability to identify parathyroids intraoperatively and evaluation of fluorescence differences between these groups.

Results and conclusion: Stealth liposomes were insignificantly higher in drug encapsulation%, in vitro drug release and zeta potential compared to conventional liposomes. Additionally, they needed less time for the start of the photosensitization and recorded the highest signal after spectrometry compared to the other two preparations. These data provide a new evidence of the potentiality of ALA-stealth Liposomes for identification of PTGs intraoperatively and could lead to propose a non-invasive procedure with reduced postoperative side effects.

1. Introduction

Anatomy of the parathyroid glands and their variability in number and location make them very difficult to detect and identify. However, the identification of parathyroid glands is challenging yet very important as it is one of the main reasons for postoperative hypoparathyroidism.

It is observed that during thyroid surgery it is difficult to differentiate parathyroid glands from other tissues adjacent to the thyroid gland. Subsequently, accidental removal of parathyroid glands or interruption of their vascularization ends frequently with postoperative hypocalcaemia or hypoparathyroidism.Hypoparathyroidism has a reported incidence at 0–43% [1,2]. Within 2–5 days after total or subtotal thyroidectomy, a decrease of serum calcium level, a condition known as hypocalcaemia, is reported to occur from 1.6% to more than 50% of operations. Hypocalcemia is the most common cause of malpractice litigation after endocrine surgery [3].

The principle of fluorescence diagnosis is based on the specific accumulation of administered agent, PS, in targeted cells. After I.V., oral or topical application, the PS predominantly concentrates in the site and remains inactive until exposed to the light of a specific wavelength. When light is delivered to the specific site, it causes fluorescence of the photosensitizer [4]. Fluorescence diagnosis with ALA-induced porphyrins offers the opportunity to study various tissue-types based on the differences in the ability to accumulate the endogenous photosensitizer protoporphyrin IX after the administration of ALA. Because PpIX has characteristic fluorescence properties, its preferential accumulation in certain tissue-types can be used as a diagnostic tool [5].

Intraoperative Fluorescence diagnosis using the photosensitizer ALA has been described to identify parathyroid glands in an experimental setting during bilateral neck exploration in rats [6].

Nonetheless, ALA solution is light-sensitive and unstable as ALA is a polar molecule and it is presented in physiological pH mainly as a charged zwitterion, which accounts for its low lipid solubility and reduced bioavailability. Besides, its hydrophilic character hinders efficient penetration through the cell membranes [9]. The chemical instability of ALA in aqueous solution is an irreversible process. The ability of the solution to stimulating porphyrin production in cells is

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Table 1
Lipids composition and characteristics of the liposomal formulations prepared.

Formula	DPPC: Cholesterol: PEG- PE (mg)	Entrapment Efficiency	Size (nm)		Zeta potential (mv)	
			Freshly prepared	Post 48 weeks	Freshly prepared	Post 48 weeks
ALA NL*	50: 25: –	60.7% ± 0.81	100 ± 10	240 ± 20	−57	−33
ALA NSL**	50: 25: 5	70.1% ± 0.7	100 ± 20	210 ± 12	−60	−42

Size and morphology of liposomes Liposomal formulations (10 μL) were diluted 10 times with PBS and were examined for morphology using Optical microscope type CKX41, Olympus, U.K attached to colour CCTV Camera Panasonic WV-CP 240/G Manufactured by Panasonic System solution Suzhou. Co., Ltd. Suzhou China. The number of vesicles/mm3 was measured for the size through the use of Micrometrics SE/CMOS Version 2.6, ACCU-SCOPE INC., using a hemocytometer (Feinoptik, Blakenburg, Germany).

* NL: NanoLiposomes.

** NSL: Nano Stealth Liposomes.

gradually lost upon breakdown [11,12].

Liposomes are vesicular systems can be formulated in nanosize of spherical structures, they are composed mainly of a phospholipids bilayer surrounding an aqueous core and are known to improve drug bioavailability, increase the solubility and/or stability of drugs in vivo, and reduce the undesired drug actions causing toxicity.

However, the interaction of liposomes with plasma lipoproteins; which destabilize the vesicles, significantly reduces the circulation time of liposomes in the bloodstream and promotes the leakage of the PS before it can reach the target site [23]. Coating liposomes with Polyethylene glycol (PEG) reduce the binding of serum opsonins and uptake by the mononuclear phagocyte system (MPS). The consequence of avoiding opsonisation is the prolongation of the liposome and particle stability in the bloodstream from few seconds to several hours [26,27,25]. Liposomes with prolonged circulation times due to modification with glycolipids or PEGylated lipids are referred to as 'sterically stabilized' liposomes or 'Stealth liposomes' [24]. Therefore, PEGylated liposomes (Stealth® liposomes) exhibit longer circulating times with enhanced vascular permeability and increased accumulation [13].

In this study, we have found the effect of incorporating ALA in liposomes on the photodynamic diagnosis of parathyroid glands.

2. Materials and methods

2.1. Materials

Aminolevulinic Acid (approximately 98% pure) was attained from Sigma-Aldrich. 1, 2 dipalmitoyl-sn-phosphatidyl choline (DPPC), Cholesterol (CHOL) and HEPES buffer was purchased from Sigma Chemicals St. (Louis M.O.USA). Distearyl-phosphatidyl ethanolamine derivatized at the amino position of polyethylene glycol (PEG-PE) was obtained from Liposome Technology (LTI) USA. The purity of the lipids was 99% and the materials were used without further purification. Chloroform, used as a solvent, was obtained from BDH Chemical Ltd (ARISTAR GRADE). Other chemicals used in the study were of reagent grade.

2.2. Determination of ALA spectrophotometrically

The absorption spectrum of ALA solution in 10 mm HEPES buffer was measured and found to be a λ max = 218 nm with a non-linear relationship between different concentrations of ALA and their absorbance. Therefore, to study ALA spectrophotometrically a colourimetric assay based on the measurements of the UV absorption spectrum of the fluorescent derivative of ALA was carried out. This derivative was prepared on the basis of a modification of the Hantzsch reaction, in which amine compounds react with acetylacetone and formaldehyde [14]. Acetylacetone reagent (100 ml) was prepared by the mixing of 15 ml of acetylacetone and10 ml ethanol in deionized water (75 ml). One ml ALA sample was prepared for measurement by adding 3.5 ml of Acetylacetone reagent and 0.45 ml formaldehyde, heated for 15 min & cooled in ice then scanned in UV–vis spectrophotometer in range of 200 nm to 400 nm to determine λ max of the ALA derivative for further studies.

2.2.1. Standard calibration curve for ALA

In order to determine the exact drug content, a standard calibration curve for ALA derivative was made. Serial dilutions of ALA derivative in HEPES buffer of pH 7 were carried out to make concentrations of 0.25, 0.125, 0.625 mg/ml. The Absorbance using UV/VIS spectrophotometer was measured at previously determined λ max. The standard curve was prepared in triplicate (n = 3) covering the range of 250 − 625 μg/ml ALA. Precision and linearity were calculated from the coefficient of variation (C.V%) and linear regression of standard curve.

2.3. Preparation and characterization of liposomes

Liposomes were prepared according to the thin-film hydration method [16]. The lipids DPPC/Chol/PEG-PE in w/w ratios as described in Table 1 were dissolved in chloroform and ethanol mixture in ratio of (2:1 v/v) and then deposited from organic solvent in a thin film on the walls of the round bottom flask of the rotary evaporator under reduced pressure and nitrogen gas. The film was hydrated with 300 mg ALA dissolved in 10 mM HEPES buffer of a pH7 above the lipid transition temperature (50° C) for 30 min. In order to get small vesicles, the samples were sonicated by bath sonicator with frequency 35 kHz and temperature control system type US1 manufactured by Retsch GmbH & Co, Germany, for a period of 30 min. Non-encapsulated ALA was separated from liposomes by centrifugation. Vesicle suspensions were centrifuged at 10,000 rpm at 20 °C for 30 min.

The supernatant was separated and the liposomal pellets were resuspended in HEPES buffer. Liposomes samples were washed twice, the supernatant was measured for free unloaded drug using previously described spectrophotometric method and the prepared liposomes suspension was seized by extrusion using a stainless steel extrusion device at 50° C through polycarbonate filters (Nucleopore Corporation, Pleasanton, Calif, USA) to an initial pore size of 100 nm. The final liposomes were stored in sterile 10 ml vials, filled with nitrogen gas and stored at 4 °C for further studies.

2.3.1. Determination of encapsulation efficiency (EE)

The encapsulation efficacy can be obtained as the mass ratio between the amount of the drug incorporated in liposomes and the ratio was used in the liposome preparation. The separation of non-encapsulated and encapsulated ALA in the liposomes was obtained by centrifugation. One ml of the washed liposomes was dissolved in 1 ml ethanol and treated with acetylacetone reagent and formaldehyde as previously described. The absorption spectrum of the loaded drug was measured and estimated from the prepared standard calibration curve using dissolved lipids in methanol as a reference standard. The encapsulation capacity (EC) estimated through the following relationship:

$$EC = (\text{concentration of loaded drug/Initial concentration}) \times 100 \quad (1)$$