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Photo-Enhanced Delivery of Genetic Material Using Curcumin Loaded Composite Nanocarriers

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Keywords

Photodynamic therapy; Photochemical internalisation; Gene therapy; Liposomes; Polyethylenimine; Lipopolyplexes

Introduction

Since several ages, light has been used as a therapeutic agent for the treatment of psoriasis, vitiligo, rickets, and skin cancer [1]. Photo Dynamic Therapy (PDT) is the therapeutic utilisation of light with or without the use of an adjuvant. An otherwise inter photosensitiser delivered to the target tissue is activated using light at a specific wavelength. Upon photo-activation, in presence of molecular oxygen, reactive oxygen species are generated which selectively destroy the tumour tissue [2,3]. PDT has already been approved by the Food and Drug Administration (FDA) and European Medicines Agency (EMA) for the treatment of certain pre-malignant and malignant diseases [4,5]. Curcumin, a naturally occurring multifaceted drug, obtained from the rhizomes of *Curcuma longa* has been used extensively for its antibacterial and antitumor properties and more recently for photodynamic therapy [6]. A subcategory of photodynamic therapy, Photo Chemical Internalisation (PCI) is the use of light for the breakdown of endo/lysosomal membranes to facilitate release of their respective contents. This process is driven by the presence of photosensitisers in these membranes which upon photo-activation initiate photochemical reactions causing rupture of the vesicles leading to the release of the endocytosed contents [7,8]. PCI could be used for both triggered release and enhanced release of therapeutically active substances and biomolecules [9,10].

Recent advances in LED technology have enabled development of flexible, portable and cost-effective light sources for photodynamic therapy. A prototype LED powered irradiating device, capable of irradiating at different wavelengths was used in this study. To solve the solubility problems of curcumin, we have used liposomes for encapsulating the curcumin. Liposomal encapsulation increases the bioavailability, biocompatibility and bio efficacy of the therapeutic substances ranging from drugs to biomolecules such as DNA, siRNA and other oligonucleotides [11-13]. Poly Ethylen Imine (PEI) on the other hand is a proven polymer for gene delivery and is often considered as a gold standard for transfection [14-16]. A combination of PEI/nucleic acid polyplexes and liposomes called Lipopolyplexes (LPP) has been proven to increase the efficiency of gene delivery and decrease the toxicity associated with polyethylenimine [17-19]. In this study, we present the use of a novel combination of curcumin loaded liposomes and PEI/DNA polyplexes for photochemical internalisation. Keeping in mind the toxicological aspect of PEI, whose toxicity is related to the chemical structure and molecular weight, a linear and highly deacylated variant of PEI, lPEI with a molecular weight of 22kDa was used in this study [20,21]. The cationic polyplexes were encapsulated inside anionic liposomes formulated using 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), Dipalmitoylphosphatidylcholine (DPPC), cholesterol and curcumin. Since the transfection efficiency is highly dependent on the surface charge, size, flexibility and stability of the liposomes, a helper lipid DOPE was incorporated in the liposomal formulation which influences the above parameters [22-24].

Ovarian carcinoma is one of the most common cancer types, ranking fifth in the number of deaths caused (WHO estimate). With a risk rate 1 in 75 women getting ovarian cancer and a mortality rate of 1 in 100, conjugative tumour therapeutics involving gene therapy and photodynamic therapy offer a promising adjunct to the prevailing therapies. We demonstrate the use of Curcumin Loaded Lipopolyplexes (cLPP) for photochemical internalisation in SK-OV-3 human adenocarcinoma cells (Figure 1). Cytotoxicity of the complexes has been

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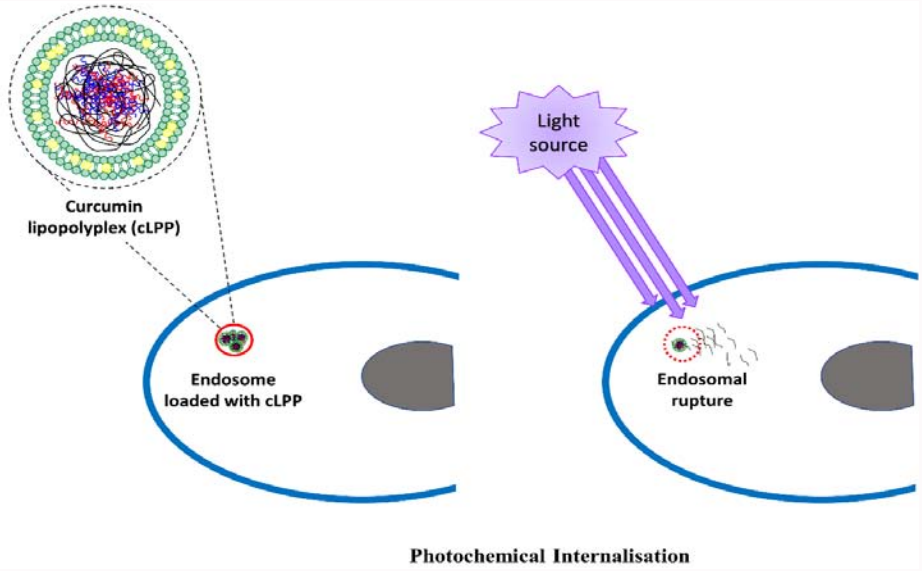

Figure 1: Scheme of photochemical internalisation showing endosomal rupture following photo-activation of curcumin lipopolyplexes leading to release of the endosomal contents into the cytosol. Inset showing an enlarged illustration of curcumin lipopolyplex with curcumin entrapped between the liposomal bilayer and a polyplex encapsulated within the liposome.

evaluated using MTT assay. Haemolysis assay using fresh blood and activated partial thromboplastin time test using plasma were used to demonstrate the biocompatibility of the curcumin loaded lipopolyplexes. Physicochemical and structural analysis was done using dynamic light scattering, laser Doppler velocimetry and Transmission Electron Microscopy (TEM) respectively.

Materials and Methods

Liposomes were prepared using thin film hydration method as described previously [25]. Briefly, chloroform, methanolic mixtures of lipids (DOPE, DPPC and cholesterol; molar ratios 70, 15, 15 respectively) were added to a round bottomed flask and evaporated using a rotary evaporator equipped with a vacuum pump (Laborota 4000, Heidolph Instruments, Schwabach, Germany) to obtain a thin lipid film. In case of curcumin containing liposomes, curcumin was dissolved in methanol was added to the lipid mixture at a ratio of 1:300 (curcumin: total lipid). The lipid film was rehydrated using 20 mM HEPES buffer (pH 7.4) and sonicated in a bath sonicator to facilitate liposome formation. The liposomes were extruded through 400 nm and 200 nm polycarbonate membranes to obtain small unilamellar liposomes. A fully deacylated 22kDa variant of linear PEI containing 11% more protonable nitrogens was used for polyplex formation with luciferase expressing pCMV-luc plasmid. Lipopolyplexes were prepared as previously described [26]. Briefly, both polyplexes and liposomes (mass ratio 1:0.5) were mixed vigorously and incubated for 1h at room temperature to facilitate lipopolyplexes formation. Dynamic Light Scattering (DLS) and laser Doppler velocimetry (using Zeta Sizer Nano ZS; Malvern Instruments Ltd., Malvern, UK) were used for determining the hydrodynamic diameter and zeta potential of the lipopolyplexes respectively. For transmission electron microscopy, the lipopolyplexes containing curcumin were diluted (1:10) using 10 mM HEPES buffer (pH 7.4) and mounted onto 300 mesh formvar coated copper grids. The samples were negatively stained using 2% uranyl acetate and examined under an accelerating voltage of 300 kV, with a current density of 55pA/cm^2 using a JEM 3010 UHR transmission electron microscope (JEOL Ltd, Tokyo, Japan). Images were acquired using a high-res slow scan CCD camera (Gatan Inc., Pleasanton, USA).

For transfection experiments, SK-OV-3 cells seeded in a 96-well plate (seeding density 10,000 cells/well) were incubated with lipopolyplexes (with and without curcumin) containing 0.2 µg pDNA/well mixed with IMDM culture medium containing 10% foetal bovine serum. Lipopolyplexes without curcumin and untreated cells were used as controls. 4h after incubation with lipopolyplexes, the cells were irradiated at a wavelength of 457 nm with a radiation fluence of 0.5 J/cm^2, 1 J/cm^2 and 3.2 J/cm^2 using a prototype LED irradiation device (Generation I LED irradiator; Lumundus GmbH, Eisenach, Germany) custom manufactured to fit microtiter plates. Post-irradiation, the cells were incubated at 37°C and 7.5% CO_2 under humid conditions for 48h. The cells were lysed using cell culture lysis buffer (Promega GmbH, Mannheim, Germany) and analysed using luciferase assay and Pierce protein assay to evaluate luciferase expression. Luciferase release was used as function transfection efficiency and was determined by measuring the luminescence using luminometer (BMG Labtech, Offenburg, Germany) followed by the addition of luciferase assay reagent mixture.

Similar steps were followed for the cytotoxicity assay with the exception that after 48h MTT dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added to cells and incubated for further 4h. Subsequently the medium was aspirated and the formazan crystals were dissolved using dimethyl sulphoxide. The absorbance was read at 570 nm in a plate reader. Haemolysis was performed using erythrocytes isolated from fresh blood obtained after prior consent. The erythrocytes were diluted 1:50 with isotonic NaCl (0.9%) and mixed 1:1 with lipopolyplexes containing 2µg pDNA. As controls, 1% Triton™ X-100, isotonic NaCl and blood were used. Haemolysis caused by Triton™ X-100 were considered as 100%. For activated partial thromboplastin time test, plasma separated from fresh blood was mixed with lipopolyplexes containing 2 µg pDNA and evaluated using an TEClot aPTT-S kit (TECO GmbH, Neufahrn, Germany) according to the manufacturer's protocol in a Coatron M1

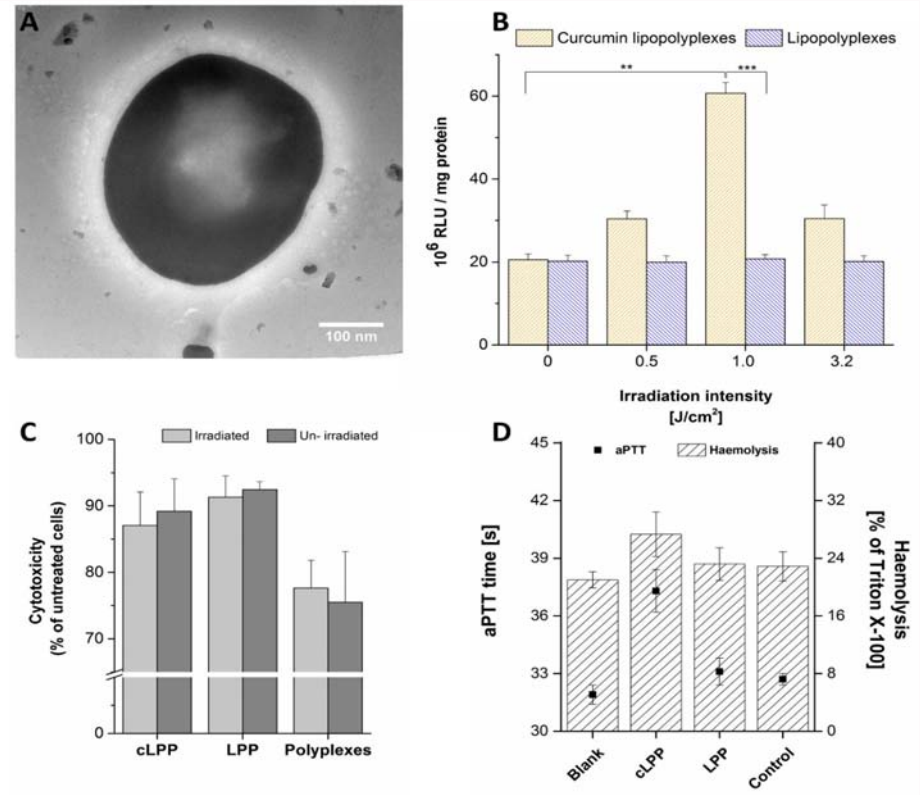

Figure 2: A.) Transmission electron micrograph of a curcumin lipopolyplex; scale bar 100 nm. B.) Photochemical internalisation in SK-OV-3 cells with curcumin lipopolyplexes and lipopolyplexes without curcumin as a control. Irradiation intensity of 0 indicates dark i.e. un-irradiated cells. Values are presented as the mean ± SD (n = 3) and statistical differences (two-tailed Student's t-test) are denoted as "**" p <0.01, "***" p <0.001. C.) MTT cytotoxicity assay of lipopolyplexes with and without curcumin in SK-OV-3 cells at an irradiation intensity of 3.2 J/cm^2. D.) Activated partial thromboplastin time test (in seconds; left y-axis) and haemolysis assay (as % of haemolysis caused by Triton™ X-100; right y-axis) of lipopolyplexes with and without curcumin. Blank indicates plasma in case of aPTT test and blood in case of haemolysis assay, control represents isotonic NaCl.

coagulation analyser (TECO GmbH). Isotonic NaCl and plasma were used as controls.

Results and Discussion

The structure of the cLPP could be visualised using TEM show a spherical structure with a size of ~ 200 nm (Figure 2A) which was within the size range for efficient cellular uptake [27]. The size of the cLPP was consistent with that of the size obtained by DLS measurements (195 nm ± 6.2 nm). The zeta potential of the cLPP performed using laser Doppler velocimetry was found to be +8.6 mV ± 1.7mV. Taking into consideration the time required for endocytosis to occur (1h - 4h), irradiation was performed at time intervals of 1h, 2h and 4h [28]. Substantial results could only be obtained after an incubation time of 4h, therefore, subsequent irradiation experiments were carried out after 4h. Evaluation of the PCI experiments showed a dramatic improvement in the luciferase expression of irradiated SK-OV-3 cells incubated with cLPP (Figure 2B). This effect was pronounced at an irradiation fluence of 1 J/cm^2 which is seen as an optimal dosage in the experiments. However, at higher irradiation doses, this effect diminishes pointing towards photo bleaching of the curcumin, there by making it unavailable for further energy level transitions which is a key for photochemical reactions [29]. The absence of a similar effect in case of lipopolyplexes without curcumin confirms that the PCI was dependent upon the photosensitiser curcumin present inside the lipopolyplexes. Similarly, no improvement in the transfection efficiency was seen in un-irradiated (dark) experiments, pointing towards an activation of curcumin only on the presence of LED light at 457 nm. The known mechanism of action of photochemical internalisation is the energy level transition during which generation of singlet oxygen occurs. This singlet oxygen is responsible for the oxidising different cellular organelles [30]. Due to its short lifetime and short range of action, only cellular targets close to the photosensitiser are oxidised by the generation of singlet oxygen [31].

No considerable increase in cytotoxicity was observed between irradiated and dark cells incubated with LPPs with or without curcumin (Figure 2C). This might be due to the limited range and limited lifetime of the singlet oxygen generated during the photochemical reactions [29]. To get a deeper insight into the biocompatibility of our delivery system, we have performed haemocompatibility studies. Haemocompatibility studies also serve as a crucial link between *in vitro* and *in vivo* studies, the data whereof could be utilised to customise the dosage in an *in vivo* setup. Haemolysis assay gives the measure of damage to the erythrocytes, by measuring the amount of haemoglobin released. Only a slight increase in the haemolytic potential was observed in case of cLPP compared to LPP (Figure 2D). Similar was the case for aPTT test wherein only a slight increase (5s) in coagulation time could be noticed (Figure 2C). The coagulation time of the plasma tested was found to be 32s ± 0.1s, values between 30s - 40s are considered normal, with values above 50s having clinical significance. It is worth mentioning that for the haemocompatibility tests, 10x concentration of the PCI experiments was considered. The haemolysis effect observed with lipopolyplexes is very low and taking

into consideration, the ratio (v/v) of erythrocytes to injected delivery vehicles in the human body, is negligible. A PTT results suggest that the cLPPs and did not interfere with the coagulation pathway.

Conclusion

The multicomponent system in this study makes use of the proven lipopolyplexes in addition with curcumin for enhancing gene delivery via photochemical internalisation using a novel LED irradiation device. The use of curcumin loaded liposomes along with PEI/DNA polyplexes brings in the beneficial properties of the two systems together resolving issues related to bioavailability, biocompatibility and transfection efficiency. The PCI experiments in SK-OV-3 cells have shown at least a threefold increase in the transfection efficiency. The delivery system and the irradiation dose required for PCI were relatively non-toxic to the cells. Haemocompatibility studies have revealed the system to biocompatible, thereby making way for its use *in vivo*. Previous PCI studies for gene delivery used co-delivery of photosensitisers and gene delivery vehicles or by chemically linking the photosensitiser to the delivery vehicle. In our study, however, we have presented a system capable of introducing both the photosensitiser and genetic material together. This multi-component system has the potential to bring together photodynamic therapy and gene therapy opening new doors in the field of combination therapies for cancer treatment. Optimising this system for use against therapeutic targets *in vitro* and *in vivo* in chorioallantoic membrane model would be of our prime research focus in future.

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Contribution

Shashank Reddy Pinnapireddy: Optimisation of transfection conditions, haemocompatibility studies, electron microscopy.

Lili Duse: Optimisation of irradiation parameters, cytotoxicity studies, manuscript drafting.

Dena Akbari: Lipopolyplex preparation, photo-enhancement studies.

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