Liposomal delivery of ferrous chlorophyllin: A novel third generation photosensitizer for in vitro PDT of melanoma

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Background: Cutaneous melanoma (CM) has substantially increased among Caucasian populations in the past few decades. This increased the number of CM deaths throughout the world. Pigmentation of melanoma reduces the efficacy of photodynamic therapy (PDT). Third generation photosensitizers (PSs) are characterized by improved targeting to the diseased tissue and reduced systemic side effects. This study is directed towards synthesis and characterization of liposomes encapsulating sodium ferrous chlorophyllin (Fe-CHL) and assessing its efficacy as a PS in PDT of melanoma.

Methods: Phenylthiourea (PTU) was used as a melanin synthesis inhibitor. PDT has been applied on de-pigmented melanoma cells using liposomes-encapsulated Fe-CHL. Cell death mechanisms after PDT were evaluated.

Results: Treatment of melanoma cells with 200 μM of PTU for 48 h provided 49.9% melanin inhibition without significant cytotoxicity. Transmission electron microscope (TEM) results proved an increase in the cellular uptake of liposomes by increasing incubation period from 6 to 24 h via endocytosis with preferential accumulation in the mitochondria and the nucleus. Following de-pigmentation, PDT was applied resulting in L50 of 18.20 and 1.77 μM after 24 and 48 h incubation with liposomes-encapsulated Fe-CHL respectively and exposure to 56.2 J/cm² monochromatic red laser of wavelength of 652 nm. Mechanism of cell death of Fe-CHL mediated PDT was found to be a combination of both apoptosis and necrosis.

Conclusions: Liposomes could be efficiently employed as a potential sustained release delivery system in the Fe-CHL-mediated PDT of de-pigmented melanoma.

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1. Introduction

Melanomas are cancers that develop from melanocytes which produce melanin, the brown pigment that gives skin its colour. They can arise in melanocytes present in many organs; however, the most common type is the CM [1]. CM has the highest potential to metastasize in all body organs and represents a life-threatening disease [2]. Its incidence per year reaches 50 per 100,000 among light-skinned Caucasians. Countries with high incidence of melanoma include Australia, New Zealand, USA, Denmark and Canada [3]. In men, melanoma most commonly occur on the trunk followed by the back, while in women, prevalence of melanoma in the lower extremities is more common than on the trunk [4].

Current therapeutic approaches for melanomas include surgical resection, chemotherapy, immunotherapy, PDT and others [5]. Melanoma, especially melanotic melanoma, is a resistant tumour to various treatment strategies including PDT. The reasons for such a resistance include; optical interference by melanin pigment especially in the region of light of wavelengths from 500 to 600 nm and its antioxidant effect where it can act as a free radical and reactive oxygen species (ROS) scavenger inside the cells [6–8]. There have been several approaches to overcome melanoma resistance to PDT, among which de-pigmentation has received much consideration. Several approaches could be employed to reduce the pigmentation
PDT is a potential non-invasive treatment modality that combines a chemical entity named PS, a light source and oxygen molecules [10]. PSs when activated by non-thermal light of a specific wavelength matching their absorption spectrum produce highly reactive hydroxyl radical (‘OH) and singlet oxygen (1O2) species that can induce tumour destruction via direct cytotoxicity (apoptosis or necrosis) [11], vascular damage [12], and activation of the immune response [13].

PSs are classified into first, second and third generations. Chlorophyll derivatives and dyes belong to the second generation of photosensitizers. They can absorb light at longer wavelengths, and significantly reduce side effects of skin photosensitization due to the rapid clearance when compared to first generation photosensitizers that are porphyrin-based (e.g. hematoporphyrin) [14]. Conjugating a targeting component, such as an antibody directed against the tumour antigens, to the PS allows it to localize, accumulate and bind selectively at the diseased site. An alternative approach would be to use an optimized Nano carrier such as a liposome or targeted Nano species. These targeting approaches have led to the third-generation PSs [14–16].

Chlorophyll derivatives include chlorophyllin metal complexes (M-CHL), where (M= Fe, Mg or Cu). They have lots of advantages such as good water solubility, excellent photosensitization at excitation of long wavelength in the region 650–670 nm. Such wavelength is characterized by good tissue penetration [16], high affinity to biomolecules, and the unique advantage that the excited states are long-lived enough (even a few microseconds under oxygen saturated conditions). These chlorophyll derivatives can generate several kinds of ROS, more obviously, 1O2 molecules and to a lesser extent, ‘OH under visible-light irradiation. The ROS production potential of these derivatives is in the order of Fe-CHL > Mg-CHL > Cu-CHL. Consequently, Fe-CHL displays the strongest photodynamic activity [17].

Liposomes are Nano vesicles that can encapsulate drugs and deliver them for the treatment of diseases [18]. Liposomes are bio-compatible Nano carriers composed of a uni- or multilamellar lipid bilayer membrane with an aqueous inner core [19]. Various methods of preparation are available to synthesize liposomes of different sizes and lamellarity namely dry lipid film, the so-called dehydration/rehydration method, solvent injection and high-pressure homogenization. Liposomes produced by the dry lipid film method are superior due to their reported high encapsulation efficiency (EE) [20]. They can encapsulate and stabilize PSs in either the lipid bilayer or the aqueous core. They enhance skin penetration and localization of the PS upon the topical application or prolong circulatory time for the systemic administration [18,21]. In this work, they were studied as a delivery system for the PSs to pave the road for future in vivo and clinical applications of PDT in treatment of melanoma.

2. Materials and methods

2.1. Quantification of melanin expression level in B16-F10 melanoma cells

Quantification of melanin inhibition by PTU was preceded by spectral analysis of the inhibitor. Then, melanin level was evaluated according to a modified protocol of Sharma et al., 2011 [9]. A number of 1 × 10^5 cells per well of B16-F10 melanoma cell line (ATCC; CRL-6475) were seeded in 3 ml/well of DMEM growth medium in 6-wells plate, and incubated under standard culture conditions (5% CO2 & 37 °C). On the next day, concentrations of 100, 150 and 200 μM of PTU (Sigma Aldrich, Germany, grade ≥ 98%), diluted in DMEM low serum-supplemented medium (5% FBS) were added to the cells and incubated for 2 or 3 days. Incubation with PTU was followed by aspiration of the medium containing the inhibitor, washing with phosphate buffer saline (PBS), and finally cell harvesting in order to quantify the amount of melanin in the PTU inhibited cells. Every 1 × 10^6 cells/ml were re-suspended in 200 μl of 1 M NaOH and solubilized by heating at 100 °C for 30 min. Samples were then centrifuged at 23510g for 20 min at room temperature and the absorbance of the supernatants were photometrically quantified at 405 nm in the multi-well plate reader (Victor 3V-1420, Perkin Elmer, USA), and followed by calculation of melanin concentration with reference to a standard calibration curve of a commercially-available melanin from Sepia officinalis.

2.2. Preparation of liposomes and determination of encapsulation efficiency (EE)

Liposomes were prepared by dehydration/rehydration method using Cholesterol (Sigma grade ≥ 99%) and L-alpha-Phosphatidyl choline from Soybean Type IV-S, ≥ 30% (enzymatic). Different formulae of phosphatidylcholine (PC): cholesterol (Chol) molar ratios at (10:1, 10:3, 10:5 & 10:10) were dissolved in 10 ml methanol followed by solvent evaporation in a rotary evaporator (Büchi, EVA411024) at 60 °C under vacuum and maximum speed rotation for 1 h in order to ensure complete evaporation of the solvent that would yield a thin lipid film. The lipid film was then rehydrated by 10 ml of PBS for preparation of plain liposomal formulae (PLFs) or PBS containing different amounts (1, 2, 2.5, 5 and 10 mg) of Fe-CHL (MWT = 677.7 gm/mol – Shandong Guangtongbao Pharmaceuticals, China) for the medicated liposomal ones (MLFs) and reattached to the rotary evaporator at a minimum speed for another 1 h in order to allow the formation of liposomes. Later, the suspension was sonicated in a water bath sonicator for 3 min in order to reduce the size of the formed liposomes [18].

Liposomes were then separated from un-encapsulated Fe-CHL by centrifugation of 1 ml liposomal suspension at 23510 × g for 3 h at 4 °C. The supernatant was then collected and the pellet was washed with 1 ml of PBS then centrifugation was repeated under the same conditions to separate the liposomes from the washing buffer. The total amount of un-encapsulated Fe-CHL was determined using a previously constructed calibration curve of Fe-CHL at a wavelength of 397 nm [21]. The EE for the PS in the liposomes was calculated from the following formula:

\[ \text{EE} = \frac{([\text{initial PS added to liposomes–un-encapsulated PS}]/\text{initial PS added to liposomes}) \times 100}{ } \]

2.3. Physical characterization of the liposomes

2.3.1. Size and zeta potential

The liposomal suspension of all prepared formulae was diluted 10 times in PBS and triplicate samples were measured for their size in Zetasizer (Malvern, Nano-ZS) at a wave length 633 nm of He–Ne laser source at 25 °C while zeta potential was assayed for one of the batches of the reproducible formulae [22].

2.3.2. In vitro drug release

An amount of liposomes containing 0.5 mg of Fe-CHL (predetermined by EE) in PBS were placed in a Spectra/Por cellulose acetate (CE) dialysis tubing (Spectrum labs, 131342) of 20 kDa molecular weight cut off (MWCO). The bag was tightly sealed by plastic clips and immersed in 5 ml PBS as release medium and placed in a shak-
ing water bath adjusted at 100 rpm and 37 °C. 5-ml samples were withdrawn after 1, 2, 4, 6, 24 and 48 h and replenished with fresh buffer incubated at 37 °C. The amounts of Fe-CHL in the withdrawn samples were determined spectrophotometrically using a standard calibration curve of Fe-CHL at a wavelength of 397 nm.

2.3.3. Transmission electron microscope (TEM)

The liposomal suspension of the MLF with PC: Chol molar ratio of 10:5 was diluted 10 times in PBS and adsorbed on a surface of a copper grid. This was followed by staining with 2% phosphotungstic acid (PTA) for 30 s. The specimens were examined by TEM (JEOL, JEM 1400) operated at 80 kV [23].

2.4. Evaluation of photodynamic effect of liposomal Fe-CHL

Sterilization of the prepared liposomes was performed by filtration through sterile CE membrane syringe filters (CHM, SCA20025K-S) of 0.2 μm (200 nm) pore size before being applied on the cells [24]. A number of 1 × 10^3 of melanoma cells were seeded in a 96-wells plate and incubated overnight then treated with 200 μM of PTU for 2 days. This was followed by incubation of the cells with liposomes-encapsulated Fe-CHL diluted to the desired concentration in DMEM low serum-supplemented medium (5%FBS) at 5% CO_2 and 37 °C for either 24 or 48 h. Cells were then subjected to monochromatic red laser (Diode laser – Biolitec, 1091-G, Germany) of 652 nm wavelength, and 200 mW/cm² power density, providing 56.2 J/cm² light dose.

Cell viability was determined using MIT colorimetric assay (WST-1 premix cell proliferation assay – Takara, Germany), measured at a wavelength of 450 nm. Cellular toxicity resulting from PDT effect was calculated using the following formula (specified by the kit protocol); % Cell viability = [(Absorbance of treated cells – absorbance of blank)/(Absorbance of control cells – Absorbance of blank)] × 100. It is worth to mention that neither the applied light dose nor the concentration of the PS had a sole cytotoxic effect on the melanoma cells.

2.5. Evaluation of cell death pathways

2.5.1. TEM investigations

TEM analysis was performed in order to prove that liposomes were incorporated into the cells and to localize them in different cellular organelles. A comparison was made at different time points to detect the optimum incubation period for the highest cellular uptake of the nanovesicles. Also, post-PDT mechanism of cell death was evaluated.

A modified TEM analysis protocol of El Maghraby et al. [25] was applied as; a number of 1.5 × 10^4 cells per well were incubated overnight in 6-wells plate and de-pigmented for 2 days with 200 μM of PTU then incubated with 20 μM of liposomes-encapsulated Fe-CHL in DMEM for 6, 12 and 24 h. Cells from each well were collected and washed, then fixed with 300 μl of 2.5% glutaraldehyde for 1 h. This was followed by, rinsing 3 times in PBS, then post fixation staining in osmium tetroxide (2% in PBS) for another 1 h. Dehydration of the stained cells was applied in alcohol, followed by infiltration with Epon resin. Micromtome sections were prepared at approximately 500–1000 μm thickness, stained with toluidine blue, and examined by ICC50 HD Leica camera. Ultra thin sections (Leica, Ultracut UCT) prepared at approximately 50–70 nm thickness were stained with uranyl acetate and lead citrate, then the carbon-coated 400 mesh Copper grids were examined by a transmission electron microscope (JEOL JEM 1400), at the candidate magnifications. Electron micrograph images were captured by; a CCD camera (Optronics, AMT), of 1632 × 1632 pixels format as a side mount configuration, and of an acquisition 1394 fire wire board. Similar conditions were applied to cells treated with the LC_{50} conditions of the liposomal preparations and exposed to 56.2 J/cm² to evaluate the cellular mechanism of cell death after PDT application.

2.5.2. FACS analysis

Flow cytometry was applied in order to determine the apoptotic and necrotic melanoma cells after PDT application. Fluorescent markers of fluorescein isothiocyanate (FITC)-Annexin V and Ethidium Homodimer III (Ethd-III) from (Biotium, USA) for detection of apoptosis and necrosis were used respectively. A number of 2–3 × 10^6 cells/ml treated with the LC_{50}–PDT conditions were re-suspended in 1 ml of 1X binding buffer diluted in distilled water, then 5 μl of FITC-Annexin V or Ethd-III were added to 100 μl of cell suspension and incubated at room temperature for 15 min in dark. Finally, 400 μl of 1X binding buffer was added to the cell suspension and fluorescence was measured within 1 h of staining.

2.6. Statistical analysis

Statistical analysis was applied using the GraphPad Prism 5.0 software. Results were collected from 3 independent experiments and data were expressed as the mean ± standard error of the mean (SEM), using One-way ANOVA Tukey’s Multiple Comparison Test, and 95% confidence interval levels. Data were considered significant at P value < 0.05, with very highly significant difference (***)) when P<0.001, highly significant difference (**) when P=0.001 to 0.01, and significant difference (*) when P=(0.01–0.05).

![Fig. 1. The effect of different PTU concentrations on the percentage of melanin expression. B_{50} F_{50} melanoma. cells treated with (0, 100, 150 and 200 μM) for 2 or 3 days. Asterisks indicate significant difference in melanin level of PTU treated cells compared to untreated ones. (*)& Very highly significant difference, (***) highly significant & (****) Very highly significant.](image1)

![Fig. 2. Encapsulation efficiency (EE) at different PC: Chol molar ratios. The used PC:Chol ratios are (10:1, 10:3, 10:5 & 10:10). ns = not significant.](image2)
3. Results

3.1. Melanin expression level

The spectral analysis of PTU revealed a single peak at 249 nm. Quantification of melanin inhibition using 100, 150 & 200 μM of PTU, which proved to have no cytotoxic effect at 2 and 3 days incubation periods was performed. Statistical analysis of melanin concentration obtained from PTU-treated cells, showed significant decrease (0.01 < P < 0.05) in percentage of melanin to 60.96% ± 6.51 and 58.79% ± 10.85, at 100 and 150 μM of PTU respectively, and a highly significant decrease (0.001 < P < 0.01) at 200 μM of PTU to 50.11% ± 5.74, at 2-day incubation as compared to un-treated cells. Meanwhile, all the concentrations of PTU (100, 150 and 200 μM) showed very highly significant decrease (P < 0.001) in percentage of melanin concentration to 61.76% ± 2.12, 54.89% ± 5.95 and 53.60% ± 1.15 respectively at 3 days of incubation (Fig. 1).

3.2. Physical characterization of liposomes

3.2.1. Encapsulation efficiency (EE)

Statistical analysis of the results examining the effect of varying the PS content on EE proved that increasing the amount of PS from 1 to 10 mg (representing 0.01–0.1%) in the liposomal formula of PC: Chol molar ratio of 10:5 has no significant effect (P > 0.05) on the percentage of EE as indicated by 91.11% ± 3.11, 90.58% ± 1.22, 86.12% ± 4.79, 81.69% ± 3.83 and 81.45% ± 2.56, for 1, 2, 2.5, 5 and 10 mg PS respectively.

It is also worth to mention that results showed an EE of 69.65% ± 4.59, 81.45% ± 2.56 and 82.15% ± 1.49 with no significant difference (P > 0.05) between the MLFs of different molar ratios of PC: Chol equal to 10:3, 10:5 and 10:10 respectively, at 10 mg (0.1%) of the PS. However, the formula with 10:1 molar ratio showed significantly lower EE (56.17% ± 5.79) than the formulae with 10:5 and 10:10 molar ratios and no significant difference to the formula with 10:3 molar ratio (Fig. 2). Therefore, the two formulae (MLF1 &

![](image)

Fig. 3. The release profile of free & liposomes-encapsulated Fe-CHL over 48 h in PBS (pH=7.4). Two formulae were investigated; MLF1 and MLF2 of 10:5 & 10:10 PC: Chol molar ratios respectively.

![](image)

Fig. 4. TEM micrographs of the formula of choice (10:5 PC: Chol). Unilamellar morphology and size distribution for the PLF1 plain liposomes (a&c), and the MLF1 medicated ones containing 0.1% of PS (b&d).
MLF2) corresponding to 10:5 and 10:10 molar ratios respectively were nominated for further examination to guarantee good EE.

3.2.2. Vesicular size and zeta potential

The dynamic light scattering (DLS) analysis of different formulae showed a uni-modal (100%) size distribution pattern in all formulae with an average size ranging from 49.92 to 123.70 nm and 54.30 to 108.48 nm for plain and medicated liposomes respectively. Statistical analysis for the effect of incorporation of the large-sized PS molecules in the liposomes showed no significant difference \( (P > 0.05) \) in vesicular size at any of the tested formulae. On the other hand, zeta potential was found to be in the range of \(-15.8\) to \(-17.7 \text{ mV}\) for plain liposomes, and \((-21.9\) to \(-32.8 \text{ mV}\) for medicated ones. Analysis of the polydispersibility index (PDI) by Malvern zetasizer software V711 for all samples, exhibited monodispersibility index of \(\leq 0.7\) \(\) (Table 1).

3.2.3. In vitro drug release

Release studies were performed to evaluate the PS release rates from MLF1 & MLF2 over time and compare them to the diffusion of the PS alone in the release medium. Results showed a gradual abrupt increase in the cumulative release of free Fe-CHL during 6 h then slower release reaching 84.82% \( \pm \) 5.71 towards the end of the 48 h duration. While the cumulative released percentage...
of liposome-encapsulated Fe-CHL from the two formulae showed very highly significant drop when compared to the free form with no significant difference between the two formulae corresponding to 10.24% ± 0.91 and 7.21% ± 0.76 for MLF1 and MLF2 respectively (Fig. 3).

3.2.4. Transmission electron microscope (TEM)

Transmission electron micrographs of the plain liposomes of the selected formula with PC: Chol molar ratio of 10:5 (PLF1) (Fig. 4a&c) showed darkly stained vesicles with unilamellar morphology and particle size range of 59 nm–149 nm diameters. Similarly, MLF1 micrographs (Fig. 4b&d) exhibited unilamellar morphology, but were lightly stained with the PTA negative stain and showed particle size in the range of 25.4–80.3 nm diameter. This size distribution is in accordance with those obtained by the DLS.

3.3. Cellular uptake of liposomes

TEM investigation of melanoma cellular uptake and differential accumulation of the nanovesicles from MLF1 after 6, 12, and 24 h incubation showed increased accumulation by time particularly in nuclear chromatin, the cytoplasm, and in the mitochondria. Cellular uptake of the nanovesicles occurred by endocytosis as indicated by existence of pseudopodia & liposome-containing endosomes.

TEM analysis of cells incubated for 6 h with the PS-containing liposomes (Fig. 5a&b) showed most of the liposomes located at the outer surface of the cells. However, some nanovesicles were uptaken and observed in the cytoplasm, nucleoplasm and nuclear chromatin. Also, liposome-containing endosomes were observed pointing out to endocytic uptake mechanism.

At 12 h incubation (Fig. 5c&d), melanoma cells promoted endocytosis as evidenced by the increase in the number of pseudopodia, and liposome-containing endosomes. Similar to the 6 h incubation, distribution of liposomes in the cellular organelles was observed where liposomes could be seen in the cytoplasm, nucleoplasm and nuclear chromatin. Early evidence of accumulation of liposomes in mitochondria could also be observed at this period of incubation.

Meanwhile, at 24 h incubation (Fig. 5e&f), B16 F10 melanoma cells showed maximum density of liposomes and preferential accumulation in the mitochondria, nucleoplasm and nuclear chromatin and less accumulation of liposomes in the cytoplasm. Envelopment of liposomes by the nuclear envelope has been also observed.

3.4. PDT effect of Fe-CHL liposomes

It is worth to mention that dark and light toxicity experiments showed no significant (P > 0.05) cellular toxicity induced by any of the utilized concentrations of the PS or light doses respectively, using MTT assay as compared to control non-exposed cells (data not shown).

Cellular toxicity induced by liposomes-encapsulated Fe-CHL-mediated PDT was evaluated by MTT assay at a light dose of 56.2 J/cm² after 24 and 48 h incubation.

Treatment of B16 F10 melanoma cells with MLF1 after 24 h incubation induced a significant decrease (0.05 < P < 0.01) in cell viability at 5 μM being (81.58% ± 3.39), and a very highly significant decrease (P < 0.001) at 10 and 20 μM indicated by (58.10% ± 1.45 & 52.29% ± 3.63) respectively. Meanwhile, after 48 h of incubation a highly significant decrease (0.001 < P < 0.01) in cell viability was shown (52.54% ± 2.58 & 39.57% ± 8.07%) at 1 & 5 μM respectively. While at 10 and 20 μM, a very highly significant drop (P < 0.001) to (14.43% ± 3.43 & 9.34% ± 0.98) was recorded respectively. The lethal concentrations that caused 50% melanoma cell death (LC50) were indicated to be 18.20 and 1.77 μM at 24 and 48 h incubation respectively (Fig. 6).

3.5. Mechanisms of melanoma cell death

Cellular mechanisms of melanoma cell death were investigated by TEM where de-pigmented melanoma was treated with the LC50 conditions of the formula of choice (MLF1) for 24 h followed by exposure to a light dose of 56.2 J/cm². Results of TEM examination proved the presence of necrotic, apoptotic, and late apoptotic (secondary necrotic) mechanisms in PDT-mediated melanoma cell death (Fig. 7).

Necrotic B16 F10 melanoma cell death was evident by the observation of cell swelling and disruption of cell membrane with the absence of most organelles (Fig. 7a). However, apoptotic cell death appeared in the form of cytoplasmic vacuolization, pyknosis (chromatin condensation at the nuclear periphery), mitochondrial vacuolization, with membrane intactness and early signs of blebbing (Fig. 7b). Meanwhile, disruption of the cell membrane at some points indicates the existence of late apoptotic (secondary necrotic) cell death (Fig. 7c).

Investigation of possible molecular mechanisms underlying melanoma cell death via application of LC50–PDT conditions using fluorescence associated cell sorter (FACS) analysis confirmed the presence of both apoptotic and necrotic pathways. The relative fluorescence (after compensation of auto-fluorescence of unlabeled cells) of the FITC-Annexin V and Ethidium Homodimer III as apoptotic and necrotic molecular markers respectively represented on the x-axis and the cell count on the y-axis (Fig. 8A).

Quantitative determination of the percentage of apoptotic or necrotic cells (Fig. 8B) showed on one hand, a highly significant
Fig. 7. Transmission electron micrographs showing cellular mechanisms of B16 F10 melanoma cell death. Tumour cells were treated with LC50 conditions of MLF1-encapsulated Fe-CHL for 24 h. Necrotic cell death (a), apoptotic (b), and late apoptotic (c) cell death are indicated. B = membrane blebbing, Cm = intact cell membrane, Cm(d) = disrupted cell membrane, Mv = mitochondrial vacuole, Nm(d) = disrupted nuclear membrane, Nv = nuclear vacuole, PK = pyknosis and V = cytoplasmic vacuole.

(0.001 < P < 0.01) increase represented by 51.0% of LC50–PDT treated cells expressing the necrotic cell death markers. On the other hand, a very highly significant (P < 0.001) increase in percentage of cells expressing the apoptotic cell death markers indicated by 73.2% as compared to untreated cells. It should be noted that there is an overlap between the percentages of apoptotic and necrotic cells indicating the presence of late apoptotic (secondary necrotic) cells.

4. Discussion

Melanotic melanoma, is a resistant tumour to various treatment strategies including PDT Such a resistance could be attributed to the presence of melanin pigment [6–8]. Phenyli thiourea has been used as a melanin synthesis inhibitor that causes partial de-pigmentation of melanoma cells which was found to increase susceptibility of melanoma to PDT [9].

Melanin assay was performed to evaluate the level of melanin inhibition in melanoma cells after incubation with 100, 150 & 200 µM PTU for either 2 or 3 days. The maximum reduction in melanin expression was achieved at conditions of 200 µM of PTU for 2 days rather than 3 days incubation period, where the percentage of melanin was 50.11% ± 5.74. This can be explained on the basis of the mechanism of melanin inhibition by PTU. PTU is a reversible inhibitor of tyrosinase enzyme – the key enzyme in melanin synthesis – where longer incubation periods might give a chance for the regain of tyrosinase activity by the cells or lead to elimination of PTU from the cells. Similar inhibition patterns were achieved by Sharma et al., 2011 [9] where regain of tyrosinase enzyme was evident after 5 days of incubation when compared to 2 days of incubation.

Liposomes have been used as carriers to enhance selective uptake of chemotherapeutics into tumour cells and minimize cellular uptake by the healthy tissue [26]. They were synthesized by dehydration/rehydration method reported to produce high percentage of EE [21]. Cholesterol is incorporated in the formulae because it is known to increase compactness and stability of liposomes and their retention and EE of added solutes [27].

Analysis of the vesicular size and zeta potential of formulated liposomes with molar ratios of PC: Chol equal to 10:1, 10:3, 10:5 and 10:10 yielded uni-modal distribution of particle size. While, analysis of zeta potential proved the stability of the MLFs since it is reported that nanoparticles suspensions with zeta potential above +20 mV or below –20 mV are considered stable because they have sufficient electrostatic repulsion to remain in suspension [28]. Also, it was observed that addition of PS increases suspension stability of
the MLFs when compared to their respective PLFs as observed with the more negative zeta potential values, this could be explained as follows; PS encapsulation is likely to cause changes in the orientation of the PC head groups at the surface of liposomes thus imparting a more electronegative charge at the vesicular surface [29]. It was also found that the MLF2 exhibited highest value for zeta potential indicating that increasing the PC: Chol content from 10:1 to 10:10 prominently increases suspension stability, an effect that is not observed at lower contents of cholesterol. Therefore, the two formulae with the highest cholesterol molar ratios 10:5 and 10:10 were chosen for further analysis to guarantee high entrapment efficiency.

The release results were in agreement with the results of other studies in which liposomes showed sustained release of the loaded drugs. This is because the lipid-rich composition of liposomes is suggested to produce a depot effect for the drug molecules [22]. The presence of high cholesterol synergizes this effect by tightening the liposomal walls hindering the fast release of encapsulated drugs [30]. Release results of the two formulae showed no significant difference in the cumulative released percentage of the PS. However, the MLF2 was excluded based on being less reproducible as well as small vesicular size where smaller-sized nanovesicles possess higher surface energy than bigger-sized nanovesicles that might impart more instability [31].

TEM analysis for the cellular uptake of the Fe-CHL encapsulating liposomes suggested that a 6 h incubation period with the liposomes is not sufficient for accumulation of liposomes inside the cells, as evidenced by the accumulation of most of them at the cell external surface. The mechanism of cellular uptake of the liposomes was verified at 12 h incubation period where pseudopodia and endosomal organelles were observed indicating that liposomes were up taken by means of endocytosis. This is in an agreement with the study of Bajoria et al. [32], where it was suggested that liposomes were internalized by trophoblast cells via an energy-dependent mechanism, most probably by endocytosis. Moreover, the 24 h incubation showed maximum cellular accumulation of liposomes with preferential localization on the nuclear chromatin, mitochondria and in the cytoplasm. All organelles were identified based on micrographs of different research studies [33–36].

Photodynamic effect on melanoma cells was found to increase gradually as the concentration of PS increases after both the 24 and 48 h incubation periods. This can be solely attributed to photodynamic effect because neither light not PS alone have induced any toxicity to the cells as confirmed by the control experiments (data not shown). Also, PTU was found to possess a single absorption peak at 249 nm which proves no interference in absorption of the irradiation light by the melanin inhibitor (data not shown). It was also obvious that the liposomal formula of the PS was more powerful at the longer incubation period as evidenced by the eighteen-folds decrease in the LC50 from 18.20 μM at 24 h incubation to 1.77 μM at 48 h. This could be explained by the fact that, cells need longer incubation periods for saturation with the liposomes, destabilization of the lipid bilayer and their lysis in the cellular lysosomes releasing the encapsulated contents, which follows endocytosis as proposed earlier from the cellular uptake results [37,38].

Evaluation of the mechanism of cell death following liposomal Fe-CHL mediated PDT, by TEM showed evidence of apoptotic, necrotic and late apoptotic (secondary necrotic) cell deaths in which the absence of phagocytes at the in vitro cell cultures causes eventually permealization and disruption of the cell membranes [39]. Apoptotic cells were generally shrunk, their cytoplasm became dense, and the organelles were tightly packed. In the nucleus, pyknosis (condensation of chromat at the nuclear membrane periphery) was observed. On the other hand, necrosis, which is considered to be a toxic process where the cell is a passive victim and follows an energy-independent mode of death, was also observed. Necrotic cells were characterized by various morphological changes including swollen or ruptured mitochondria, disruption of organelles membranes and eventual lysis of the cell membrane [40].

The obtained cellular mechanisms of cell death recorded by TEM were also confirmed by the flow cytometric analysis of the apoptotic (FITC-Annexin V) and necrotic (Ethidium Homodimer III) cell markers. Each of the green and red fluorescence of FITC-Annexin V and Ethidium Homodimer III respectively were individually assayed. Analysis of single-parameter histograms of FACS showed 51% necrotic cells, and 73% apoptotic ones when compared to control cells. These results included the late apoptotic cells, which were positive for both stains but were difficult to be quantified due to the difficulty of eliminating the effect of melanin auto fluorescence in the double-parameter histogram [41–43].

5. Conclusions

In conclusion, this work represents the potential for the success of application of Fe-CHL-mediated PDT in the treatment of de-pigmented melanoma using liposomal delivery systems. Such promising data encourages future outlook for in vivo application of CHL-mediated PDT in melanoma animal models utilizing different Nano vesicular formulations in order to evaluate their relative skin delivery and treatment efficacies.

Conflict of interest

All the authors confirm that the research work contained in this manuscript is completely original and unpublished. Authors also declare that they have no conflict of interest including any financial, personal or other relationships with other people or organizations.

Authors’ contribution

German University in Cairo (GUC) led the conception, design acquisition of all experimental work as well as the manuscript preparation. All authors read and approved the final manuscript.
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References