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Chlorophyll *a* in cyclodextrin supramolecular complexes as a natural photosensitizer for photodynamic therapy (PDT) applications

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ABSTRACT

Chlorophyll *a* (Chl *a*), an amphipathic porphyrin, was employed as natural photosensitizer for photodynamic therapy applications. Due to its lacking solubility in water and high tendency to aggregate, Chl *a* was included into different modified cyclodextrins (CDs) to form stable water-soluble supramolecular complexes. To achieve this aim, 2-Hydroxypropyl-β-cyclodextrin (2-HP-β-CD), 2-Hydroxypropyl-γ-cyclodextrin (2-HP-γ-CD), Heptakis (2,6-di-*o*-methyl)-β-cyclodextrin (DIMEB) and Heptakis(2,3,6-tri-*o*-methyl)-β-cyclodextrin (TRIMEB) were used. The chemical physical properties of Chl *a*/CD complexes in cellular medium were studied by means of UV–Vis absorption spectroscopy. Results demonstrated the good aptitude of 2-HP-γ-CD, and more particularly of 2-HP-β-CD, to solubilize the Chl *a* in cell culture medium in monomeric and photoactive form. Then, Chl *a*/2-HP-β-CD and Chl *a*/2-HP-γ-CD complexes were evaluated *in vitro* on human colorectal adenocarcinoma HT-29 cell line, and cytotoxicity and intracellular localization were respectively assessed. Further tests, such as phototoxicity, ROS generation, intracellular localization and mechanism of cell death were then focused exclusively on Chl *a*/2-HP-β-CD system. This complex exhibited no dark toxicity and a high phototoxicity toward HT-29 cells inducing cell death *via* necrotic mechanism. Therefore, it is possible to affirm that Chl *a*/2-HP-β-CD supramolecular complex could be a promising and potential formulation for applications in photodynamic therapy.

1. Introduction

Photodynamic therapy (PDT) is a non-invasive and highly selective clinical treatment for several pathological conditions such as cancer, infectious diseases, age-related macular degeneration, psoriasis and autoimmune disorders [1–5]. PDT relies on three components: a non-toxic drug known as photosensitizer (PS), visible light able to match the absorption spectrum of the PS, and endogenous molecular oxygen. The PS, localized in a specific tissue/cell, is photoactivated by light with an appropriate wavelength. In presence of endogenous molecular oxygen, in its ground-state triplet ($^3O_2$, $^3\Sigma_g^-$), the excited PS induces the production of Reactive Oxygen Species (ROS) through photochemical processes [6,7]. ROS trigger cellular responses such as an irreversible photooxidative damage to lipids, DNA and proteins with consequent cell death through several ways, including apoptosis and necrosis or autophagia [8].

The most common PSs used in PDT are cyclic tetrapyrrole molecules and their analogues, such as porphyrins, chlorins, bacteriochlorins, phthalocyanines, and expanded porphyrins, which present intense absorption bands in the red and NIR region of the spectrum, where the maximum light depth penetration into biological tissues is obtained [9].

The natural porphyrin Chlorophyll *a* (Chl *a*) has the features of a good PS: is a pure compound with constant composition, is characterized by high values of triplet state yield, high quantum yield of singlet oxygen production and efficient energy-transfer for the formation of $^1O_2$ [10,11]. Therefore, several derivatives of Chl *a* are currently in use or in advanced clinical trials [12,13], or are investigated for their possible application in PDT [14]. In this study, we evaluated the PDT performance of the natural Chl *a*. The use of this molecule could be more advantageous than the semisynthetic ones in terms of biocompatibility and costs [12]. Effectively, we have already demonstrated that the photoactivated natural Chl *a* is able to induce the production of ROS such as H_2O_2, superoxide radical anion ($^{\bullet}O_2^-$) and $^1O_2$ [15–17].

Since one of the drawbacks for the use of Chl *a* in PDT is its high hydrophobicity [18], the use of supramolecular chemistry is an advantageous approach for the preparation of drug delivery carriers for

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hydrophobic drugs [19–22]. Furthermore, the non-covalent nature of supramolecular interactions between drugs and their host molecules allows to obtain a reversible and dynamic supramolecular complexation [23,24] permitting the unbound drugs to enter cells [25].

Cyclodextrins (CDs) based supramolecular complexes have already shown their ability to encapsulate many drug molecules for biomedical or pharmaceutical applications [26].

Therefore, in this study we aimed to investigate the effectiveness of some CDs in Chl *a* solubilizing into aqueous biological environment, making it available for therapeutic purposes.

CDs are cyclic oligosaccharides, with a toroidal shape, presenting an inner hydrophobic cavity and an external hydrophilic surface [27]. These characteristics allow them to solubilize hydrophobic molecules through the formation of inclusion complexes [28–30], improving stability, therapeutic efficiency, and bioavailability of guest molecules, and enhancing the permeation of poorly soluble drugs through biological barriers [31–34].

Despite extensive applications of CDs in pharmaceutical technology [35,36], the exact interaction mechanisms between cyclodextrin and cell membrane, and thus their effects on drug permeability, are difficult to determine. One early paper reported that CDs act as permeation enhancer by simply carrying hydrophobic drugs through the aqueous solution to the lipophilic surface of membranes [37]; on the other hand, Réti-Nagy et al. [38] recently demonstrated that some fluorescently labelled CDs and the complexed drugs were transported into cell cytoplasm *via* endocytosis; whereas molecular dynamic simulations assessed that, at least β-CDs spontaneously diffuse into membranes, but do not cross them [39].

In the last years, cyclodextrin inclusion complexes with natural and synthetic porphyrins have received great attention [40,41]. However, the inclusion complexes stability and the aptitude of CDs to solubilize hydrophobic molecules into aqueous solutions depend on different factors, such as sample preparation methods, substituent moieties on CDs rim, or medium composition [42]. Thus, it is important to characterize each condition, defining the appropriate formulations to prepare suitable delivery systems for photodynamic therapy.

In the present study, the inclusion complexes of natural Chl *a* with four functionalized cyclodextrins, *i.e.* 2-Hydroxypropyl-β-cyclodextrin (2-HP-β-CD), 2-Hydroxypropyl-γ-cyclodextrin (2-HP-γ-CD), Heptakis (2,6-di-*o*-methyl)-β-cyclodextrin (DIMEB), and Heptakis(2,3,6-tri-*o*-methyl)-β-cyclodextrin (TRIMEB) were studied. CDs ability to host Chl *a* and solubilize it in cellular medium was estimated by means of UV–Vis absorption spectroscopy. Moreover, cytotoxic and phototoxic effects of Chl *a*/2-HP-β-CD and Chl *a*/2-HP-γ-CD complexes, Chl *a* cellular uptake, PS intracellular localization, and mechanism of cell killing were investigated using HT-29 colorectal cancer cells. Due to the high worldwide incidence and aggressiveness of colorectal cancer, great interest has been paid on PDT as possible therapeutic treatment, and several studies have addressed this topic using HT-29 cell line [43,44].

2. Materials and methods

2.1. Chemicals

Chlorophyll *a* (Molecular Formula: $C_{55}H_{72}MgN_4O_5$; Molecular Weight: 893.5) was extracted and purified from fresh leaves of spinach using the method of Omata and Murata [45]. Ethanol and all organic solvents were purchased from Sigma-Aldrich® (now Merck, Darmstadt, Germany) and used without further purification. 2-Hydroxypropyl-β-cyclodextrin (2-HP-β-CD), 2-Hydroxypropyl-γ-cyclodextrin (2-HP-γ-CD), Heptakis(2,6-di-*o*-methyl)-β-cyclodextrin (DIMEB), Heptakis (2,3,6-tri-*o*-methyl)-β-cyclodextrin (TRIMEB), 2′,7′-dichlorofluorescein diacetate (DCFDA), and ABCG2 inhibitor Fumitremorgin C (FTC) were obtained from Sigma-Aldrich® (now Merck, Darmstadt, Germany). Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), Trypsin and Penicillin-Streptomycin were purchased from EuroClone® (EUROCLONE S.p.A., Pero, MI, Italy). 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT), Lyso Tracker® Green DND-26, Mito Tracker® Green FM, ER Tracker™ Green, and FITC Annexin V/Dead Cell Apoptosis Kit were obtained from Life Technologies (Thermo Fisher Scientific Inc., Waltham, MA, USA). Pierce™ BCA Protein Assay Kit was obtained from Thermo Fisher Scientific (Thermo Fisher Scientific Inc., Waltham, MA, USA).

2.2. Preparation of Chl a/CD inclusion complexes

Stock solutions of Chl *a*, 2-HP-β-CD, 2-HP-γ-CD, DIMEB and TRIMEB were prepared dissolving the appropriate quantities in ethanol. Chl *a*/CD inclusion complexes, at desired concentrations, were obtained mixing an opportune volume of Chl *a* stock solution with a specific volume of each CD stock solutions. These solutions were maintained in agitation for 15 min at room temperature by means of an ultrasonic homogenizer.

Then, ethanol was dried under nitrogen flow and the obtained dehydrate Chl *a*/CD complexes were re-dissolved in appropriate volume of cell culture medium. In this way, only the fully formed complexes passed in aqueous solutions, while the free dehydrate Chl *a* remained on the bottom of vials. All experiments were conducted keeping the CDs concentration one hundred-fold greater than that of the PS. All procedures were finished in dark conditions to avoid light exposure of the light-sensitive Chl *a*.

2.3. Chemical physical characterization of Chl a/CD solutions

The stability of Chl *a*/CD inclusion complexes in cell culture medium, in the presence or in the absence of 5% heat inactivate fetal bovine serum (FBS), was analyzed by spectroscopy. To study the photostability of the PS, solutions containing the supramolecular complexes were exposed to light of an artificial neon lamp, for 30 min. A lamp emitting a broad white light source (400–700 nm) with a power surface density of 60 mW/cm^2 was used.

UV–Vis absorption spectra were recorded using the Varian Cary 5000 UV–Vis-NIR Spectrophotometer (Varian Inc., now Agilent Technologies Inc., Santa Clara, CA, USA) and fluorescence measurements were conducted by means of Varian Cary Eclipse Fluorescence Spectrophotometer (Varian Inc., now Agilent Technologies Inc., Santa Clara, CA, USA).

2.4. Cell line and culture conditions

Human colorectal adenocarcinoma HT-29 cell line (ATCC® HTB-38) was used. The cells were maintained in DMEM supplemented with 5% FBS and 2% penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 in air. 24 h after seeding at a density of 3.0×10^4 cell/well in 96-well plates, cells were washed twice with phosphate buffered saline (PBS) and then incubated at 37 °C in media containing different concentrations of the molecules under investigation, in the presence or in the absence of 5% FBS.

2.5. Cytotoxicity test

The possible dark toxicity of Chl *a*, of the four studied CDs and of their respective Chl *a*/CD supramolecular complexes was investigated. Cells in 96-well plates were incubated in serum-free (-FBS) DMEM in which were separately dissolved the only Chl *a* (1.0×10^{-5} M) in 1% ethanol, the different CDs (1×10^{-3} M) and Chl *a*/CD inclusion complexes (Chl *a* 1.0×10^{-5} M/CD 1.0×10^{-3} M). The incubation was carried for fixed times (1, 2, 3 and 4 h) in the dark. After that, the medium was replaced with complete growth medium without phenol red and the plates were further incubated for 24 h. The cell viability was measured by means of the colorimetric MTT assay. Furthermore, cytotoxicity of Chl *a*/2-HP-β-CD and Chl *a*/2-HP-γ-CD inclusion