

PAMAM dendrimers internalizes quickly in microalgae and cyanobacteria causing toxicity and oxidative stress

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1. Introduction

Poly(amidoamine) (PAMAM) dendrimers are hyper-branched polymeric, nanoscale molecules with exceptional properties that make them attractive for a variety of biomedical and technological applications [1]. Dendrimers are considered “perfect” polymers due to their symmetry, and are classified according to their “generation” “G”, which accounts for the number of “layers” of polymer forming the dendrimer. Each generation doubles molecular weight and surface functional groups. Furthermore they are susceptible of a variety of surface functionalizations. Despite their promising applications, they have been found to be toxic to mammalian cells depending on generation and surface functionalization and their possible adverse effects for aquatic life, and especially for microalgae are largely unknown. In the present work we chose generation G2, G3 and G4 native –NH₂ (cationic) and NH-C-(CH₂OH)₃ (-OH) (anionic) surface functionalized PAMAM dendrimers in order to study the dependency of polyamidoamine (PAMAM) dendrimer toxicity on generation and surface functionalization. As model organisms we chose a green microalga (*Chlamydomonas reinhardtii*) and a cyanobacterium (*Anabaena* PCC7120). We have applied a multi-method approach to get insight into the toxic mechanisms of action of PAMAM dendrimers on both *C. reinhardtii* and *Anabaena* sp. PCC 7120 including physicochemical characterization of PAMAM dendrimers in culture media, and different physiological and cell biology techniques.

2. Materials and methods

Materials and physicochemical characterization. Amine- and hydroxyl terminated G2, G3 and G4 PAMAM ethylenediamine core dendrimers were used (Sigma-Aldrich). The size distribution of nanoparticles was obtained using dynamic light scattering (DLS). Zeta potential was measured via electrophoretic light scattering. **Growth inhibition experiments** were performed with *C. reinhardtii* and *Anabaena* sp. PCC 7120 following the standard OECD TG 201. **Detection of reactive oxygen species (ROS):** DCF was used as indicator of intracellular ROS formation. C4-BODIPY was used for evaluating lipid peroxidation. **Internalization studies:** PAMAM-Alexa Fluor 488 conjugates were prepared following the standard protocol (A30006, Molecular probes). The Alexa Fluor 488 reactive dye has a tetrafluorophenyl (TFP) ester which reacts efficiently with primary amines. Anti-Alexa fluor 488 Rabbit IgG Fraction (A-11094, Molecular probes), was used in order to discriminate surface bound and truly internalized dendrimers. Fluorescence studies were performed by **flow cytometry** and **confocal microscopy**. Ultrastructure alterations were studied by **transmission electron microscopy (TEM)**.

3. Results and discussion

3.1. Toxicity of PAMAM dendrimers in cyanobacteria and microalgae

All the cationic dendrimers (native –NH₂) proved toxic to both the green alga and the cyanobacterium. G2 and G3 Anionic dendrimers (-OH surface functionalized) were nontoxic, however, G4-OH proved toxic for both organisms. When toxicity is referred to mass concentration (mg/L), cationic dendrimers showed similar toxicity, apparently irrespective of generation (size). However, considering the large differences in molecular weight of the tested dendrimers, concentrations expressed on a molar basis revealed a clear relationship between dendrimer generation and toxicity for both organisms.

3.2. Toxicity of PAMAM dendrimers correlated with oxidative stress

Increasing evidences indicate that nanoparticles in general can generate reactive oxygen species (ROS) and subsequently oxidative stress which might eventually lead to cell damage and cell death [2]. When the ability of the tested anionic and cationic dendrimers to elicit oxidative stress was evaluated by fluorometry, flow cytometry and confocal microscopy we found that the strong differences in toxicity between anionic and

cationic PAMAM dendrimers correlated with alterations in the ROS metabolism in both organisms. Figure 1 showed, as an example, ROS induction kinetics along the experimental lapse time (0 h-72 h) of *Anabaena* exposed to G2-OH and G2-NH₂. Interestingly, neither DCF fluorescence (general ROS indicator), nor Bodipy fluorescence (lipid peroxidation) co-localized with photosynthetic structures of both organisms even when lipid peroxidation was observed in *C. reinhardtii* based on flow cytometry analysis, suggesting that the photosynthetic machinery is neither affected nor the origin of the observed oxidative stress which is in disagreement with previous studies [3, 4].

3.3. PAMAM dendrimers were internalized very fast and presented low retention times in cell envelopes.

We made a time course of dendrimers internalization. In the cyanobacterium, the three dendrimers were quickly taken up (80% of G2 and G3 and 100% of G4 after 10 min). In the green alga, dendrimer uptake was slower with 100% uptake after 2 h, G4 dendrimer uptake was slightly quicker than the other two dendrimers. The experiments with the antiAlexa antibody showed that in both organisms the Alexa Fluor-dendrimer conjugates were largely internalized even at the shorter time assayed (10 min). Interestingly, similar to the ROS results, no co-localization of Alexa fluor488 and photosynthetic membranes was found supporting the hypothesis that oxidative stress is neither affecting nor coming from the photosynthetic machinery. Furthermore, dendrimers were found to target mitochondria producing mitochondrial peroxidation. It has been found that dendrimers are internalized in different animal and human cell systems[5]; however, to our knowledge, this is the first time that PAMAM dendrimer internalization is confirmed in algae and cyanobacteria.

Conclusions

Cationic (-NH₂) PAMAM dendrimers presented a generation-dependent increasing toxicity in both organisms. Anionic (-OH) PAMAM of generation G2 and G3 were non toxic, however, G4-OH presented a similar level of toxicity to G4-NH₂ in both organisms. Internalization of PAMAM dendrimers was observed by the first time in microalgae and cyanobacteria. Internalization was very fast (after 10 min of exposure) and with low retention time in cell envelopes of both organisms. Toxicity correlated with oxidative stress and dendrimer internalization. However, the photosynthetic machinery seemed to be unaffected, and most probably was not involved in oxidative stress.

4. References

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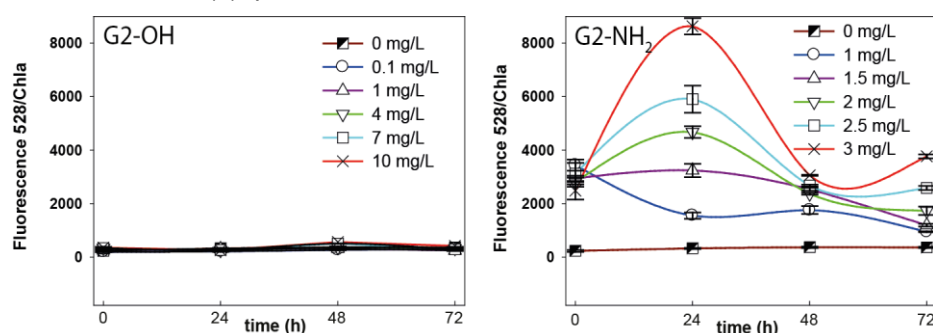


Figure 1. ROS induction kinetics (DCF fluorescence 488/528nm) in *Anabaena* PCC7120 exposed to increasing concentrations of anionic (-OH) and cationic (-NH₂) G2 PAMAM dendrimers along the experimental lapse time (72h).

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