Ecotoxicity of a potential drug nano-formulation: PAMAM-dendrimer and minocycline

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Introduction

The varied composition, size, and shape of nanomaterials (1-100 nm size range) offer numerous exciting possibilities for the development of new industrial, biomedical, electronic products and have the potential to stimulate the global economy.[1] On the darker side, the marked research efforts presently deployed to develop novel applications with nanomaterials will eventually lead to some releases in the aquatic environment likely via urban industrial/municipal point sources of pollution. This is clearly cause for concern as recent studies suggest that adverse health[2,3] and environmental[4,5] effects are linked to nanoproduct pollutants.

In the biomedical field, drug delivery systems combining nano-dendrimers (as a platform for delivery) and specific guest molecules (*e.g.* pharmaceuticals) are being investigated for efficient treatment of diseases (*e.g.*, cancer, inflammation, cardiac and microbial problems).[6] While characteristics (molecular size, shape, dimension, density, polarity, flexibility, solubility, drug carrying capacity, etc.) of dendrimers will vary based on their construction,[7] cationic dendrimers are among the most common candidates in terms of pharmaceutical development and they are being considered for the drug delivery of anti-microbials such as minocycline.[8] In this study, we investigated the ecotoxicity of three PAMAM dendrimers (artificial macromolecules with tree-like structures, described in the methods section, and of minocycline, individually and in combination by conducting toxicity tests with microorganisms representing different levels of biological organization. Our objectives are to obtain preliminary information on the potential hazard of PAMAM dendrimers and minocycline.

Materials and Methods

Three PAMAM (poly-amidoamine) dendrimers, made up of a 1,4-diaminobutane core, were purchased from Sigma Chemical Co., USA. We specifically studied PAMAM Generation 2, 4 and 5 dendrimers, characterized by 16 (G2), 64 (G4) and 128 (G5) NH_2 surface groups, respectively.[6] The antibiotic minocycline (MC) was purchased from Sigma Chemical Co., USA. Characteristics of bioassays conducted to assess dendrimers and MC toxicity are highlighted in Table 1.[9-12] References listed in this table can be consulted for more ample details on testing procedures. Measurement endpoints generated with the bioassays for individual substances tested were determined with statistical methods and software recommended for each procedure. For interactive toxicity testing (*e.g.*, dendrimer G4 and MC), the experimental approach employed is described in the following section.

Results and Discussion

Classifying bioassay data as a result of toxicity tests conducted in Table 1, according to EU-Directive 93/67/EEC,[13] offers some estimate of hazard potential for the PAMAM dendrimers and MC studied (Table 2). These comparative bioassay responses indicate that the spectrum of toxicity encompasses all cut-off classes (*i.e.*, from *harmful* to *extremely toxic*) for dendrimers G2, G4 and/or G5 and from *not toxic* to *extremely toxic* for MC. Clearly, the wide range of sensitivity justifies the continued use of representative species within test batteries to properly appraise the toxic potential of PAMAM dendrimers, since responses can be biological level-, test procedure- and endpoint specific (Table 2). Phototrophic test systems (*i.e.*, algal and LuminoTox assays) and the Hydra assay appear particularly sensitive to the toxic effects of dendrimers G2, G4 and G5, as all of their responses, barring one (LuminoTox response for G2), fall into the *very toxic* to *extremely toxic* category. Expectedly, the antibiotic MC showed greater toxicity in bioassays with microorganisms (algal and bacterial tests) and subcellular photosynthetic enzyme complexes (PECs of the LuminoTox test) as their responses were all generated in the *toxic* and *extremely toxic* classes compared to *not toxic* and *harmful* classes in the fish, hydra and micro-invertebrate (*T. platyurus*) bioassays. In light of this initial toxicity data, bioassay batteries comprised of the LuminoTox, algal and hydra tests should be used for future determination of the toxic potential of PAMAM dendrimeric nanomaterials due to their high sensitivity.

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The experimental approach employed to assess interactive toxicity testing of PAMAM dendrimers with MC is illustrated in Figure 1using Hydra test data as an example. Briefly, starting with test concentrations of 1.5 mg/L for G4 and 50 mg/L for MC, their individual EC50s were determined to be 1.25 and 15.2 mg/L, respectively (Figure 1A and B). Each EC50 was then expressed in % v/v and then transformed to toxic units (TU), where TU=100% v/v÷EC50 endpoint, respectively yielding TU values of 1.2 and 3.29 for G4 and MC (Figure 1A and B). Next, the interactive mixture was made up of a 1:1 mix of 3 mg/L of G4 and 100 mg/L of MC from which, following the same transformation protocol as above, a combined G4 and MC EC50 of 2.28 TUs was obtained (Figure 1C). From the three types of interaction results possible (Figure 1D), it stands that G4 and MC together display antagonism as their combined toxicity, where TUs=2.28 with 95% confidence intervals between 1.75-3.0, is significantly less than the sum of their individual toxicities, where TUs=4.49 with 95% confidence intervals between 3.83-5.29 (Figure 1E).

Other interactive bioassays conducted with the same protocol as above (*data not shown*) demonstrated antagonism with the algal test (G2+MC) and micro-crustacean test (G4+MC), additivity with the fish cell test (G5+MC), and synergism with the bacterial test (G4+MC). Such variable responses resulting from mixtures have been reported in the literature. For example, *V. fischeri* co-toxicity of Cu/PAH was shown to be dependent on the

ratio of concentrations of each chemical in the mixture and synergism, antagonism and additivity were observed with different combinations of Cu and PAHs.[14] Again, the interactive effects of Cu and agrochemicals varied depending on the test species (*V. fischeri*, *P. subcapitata*, *D. magna*) as well on the chemicals investigated and their respective concentrations.[15]

Conclusions

PAMAM dendrimers (G2, G4, G5) proved toxic to all of the taxonomic groups represented by the bioassays and the span of toxicity responses ranged from 0.082 mg/L (*P. subcapitata* IC25: G2) to 31.8 mg/L (*V. fischeri* IC25: G2). The most sensitive responses were generated by phototrophic (algae, LuminoTox) and *H. attenuata* toxicity tests which justifies their inclusion in future bioassay batteries aimed at determining the toxic potential of dendrimeric nanomaterials. Expectedly, MC was more toxic toward phototrophic (algae, LuminoTox) and bacterial (*V. fischeri*) species. Initial interac-

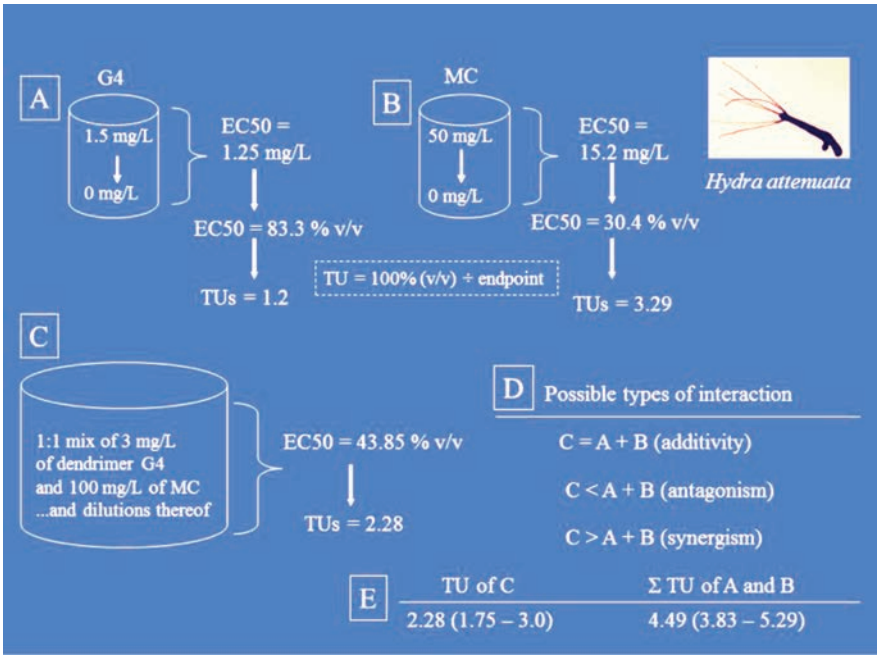

Figure 1. Experimental approach: PAMAM dendrimer G4 and minocycline (MC) interactive toxicity testing with the Hydra assay (see text for details).

Table 1. Characteristics of the small-scale bioassays used in this study.

Trophic level	Toxicity test	Assessment endpoint	Measurement endpoint	Reference
Decomposer	Bacterial test *Vibrio fischeri* (Microtox® toxicity test)	Acute sublethal light inhibition (after a 15-min exposure)	15 min-IC25	Environment Canada, 1992[9]
Primary producer	Algal test (*Pseudokirchneriella subcapitata* microplate assay)	Chronic sublethal growth inhibition (after a 72-h exposure)	72 h-IC25	Blaise and Vasseur, 2005[10]
Phototrophic assay	LuminoTox assay with PECs*	Inhibition of photosynthetic efficiency	15 min-IC20	Lab_Bell Inc., http://www.lab-bell.com
Primary consumer	*Thamnocephalus platyurus* micro-crustacean test (ThamnoTox kit assay)	Acute lethality (after a 24-h exposure)	24 h-LC50	Microbiotests Inc., http://www.microbiotests.be/
Secondary consumer	Cnidarian test (*Hydra attenuata* assay)	Acute sublethality indicated by morphology changes (after a 96-h exposure)	96 h-EC50	Blaise and Kusui, 1997[11]
Secondary consumer	Fish cell test (rainbow trout primary hepatocyte test)	Acute cytotoxicity (after a 48-h exposure)	48 h-TEC°	Gagné, 2005[12]

*PECs, photosynthetic enzyme complexes isolated from spinach leaves; °TEC (threshold effect concentration) for cytotoxicity as manifested by a significant reduction in cell viability = $(NOEC \times LOEC)^{1/2}$, where NOEC=no observed effect concentration and LOEC=lowest observed effect concentration.

Table 2. Toxicity classification of dendrimers (G2, G4, G5) and minocycline (MC) based on European Union Commission Guideline 93/67/EEC[13] and the most sensitive bioassay measurement endpoint values (LCx/ECx/ICx, etc.).

Chemical	Extremely toxic (<0.1 mg/L)	Very toxic (0.1-1 mg/L)	Toxic (1-10 mg/L)	Harmful (10-100 mg/L)	Not toxic (>100 mg/L)
G2	A*	H	F, T	B, L	-
G4	-	A, H, L	B, F, T	-	-
G5	-	A, H, L	F, T	B	-
MC	A	-	B, L	F, H	T

Toxicity tests: A, algal assay; B, bacterial assay; F, fish cell assay; H, Hydra assay; L, LuminoTox assay; T, ThamnoTox assay. *Example: for dendrimer G2, the green alga *P. subcapitata* gave the most sensitive response (72 h IC25=0.082 mg/L with lower and upper 95% confidence intervals of 0.069 mg/L and 0.097 mg/L, respectively), thereby placing this compound in the *extremely toxic* category.

tion experiments with dendrimers (G2, G4, G5) and MC demonstrate that mixture effects (antagonism, additivity, synergism) are trophic level dependent. The results suggest that these nanoproducts can be considered hazardous to aquatic life. In real life situations, risk to aquatic species will depend on quantities discharged to surface waters, on chemical interactions and on their bioaccumulation/biomagnification potential.

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