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

C. Blaise, F. Gagne, J. Auclair, D. Maysinger, P. Sutthivaiyakit
1Aquatic Contaminants Research Division, Environment Canada, Montréal, QUE, Canada; 2Department of Pharmacology and Therapeutics, McGill University, QUE, Canada; 3Department of Chemistry and Center of Excellence for Innovation in Chemistry, Kasetsart University, Bangkok, Thailand

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 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 numerous exciting possibilities for the development of new industrial, biomedical, electronic products and have the potential to stimulate the global economy.1

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) 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 1.3-12 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, this 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., algae 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 (algae 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.

The experimental approach employed to assess interactive toxicity testing of PAMAM dendrimers with MC is illustrated in Figure 1 using 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 as 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-

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

| Trophic level     | Toxicity test                                      | Assessment endpoint                                           | Measurement endpoint | Reference                                      |
|-------------------|---------------------------------------------------|---------------------------------------------------------------|----------------------|------------------------------------------------|
| Decomposer        | Bacterial test V. fischeri (Microtox toxicity test) | Acute sublethal light inhibition (after a 15-min exposure)   | 15 min-IC25          | Environment Canada, 19929                      |
| Primary producer  | Algal test (Pseudokirchneriella subcapitata microplate assay) | Chronic sublethal growth inhibition (after a 72-h exposure) | 72 h-IC25            | Blaise and Vasseur, 2005                        |
| Phototrophic assay| LuminoTox assay with PECs*                        | Inhibition of photosynthetic efficiency                       | 15 min-IC25          | 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                         |
| Secondary consumer| Fish cell test (rainbow trout primary hepatocyte test) | Acute cytotoxicity (after a 48-h exposure)                    | 48 h-TEC°            | Gagné, 2005                                    |

*PECs, photosynthetic enzyme complexes isolated from spinach leaves; °TEC (threshold effect concentration) for cytotoxicity as manifested by a significant reduction in cell viability = (NOEC x 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/EEC13 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 0.060 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.

References

1. Kharu A, Ivask A. Mapping the dawn of nanocotoxicological research. Accounts Chem Res 2013;46:823-33.
2. Nuñez-Anita RE, Acosta-Torres LS, Vilar-Pineda J, Martínez-Espinosa JC, de la Fuente-Hernández J, Castaño VM. Toxicology of antimicrobial nanoparticles for prosthetic devices. Int J Nanomedicine 2014;9:3999-4006.
3. Turkez H, Sönmez E, Di Stefano A, Mokhtar YI. Health risk assessments of lithium titanate nanoparticles in rat liver cell model for its safe applications in nanopharmacology and nanomedicine. Cytotechnology 2014 [In press].
4. Blaise C, Gagné F, Férard JF, Eullaffroy P. Ecotoxicity of selected nano-materials to aquatic organisms. Environ Toxicol 2008;23:591-8.
5. Gagné F, Gagnon C, Blaise C. Aquatic nanotoxicology - a review. Res Trends Curr Topics Toxicol 2008;4:1-14.
6. Cheng Y, Wang J, Rao T, He X, Xu T. Pharmaceutical applications of dendrimers: promising nanocarriers for drug delivery. Frontiers Biosci 2008;13:1447-71.
7. Cheng Y, Xu Z, Ma M, Xu T. Dendrimers as drug-carriers: applications in different routes of drug administration. J Pharmaceut Sci 2008;97:123-43.
8. Svenson S. Dendrimers as versatile platform in drug delivery applications. Eur J Pharm Biopharm 2009;71:445-62.
9. Environment Canada. Biological test method: toxicity test using luminescent bacteria (Photobacterium phosphoreum). Environmental Protection Series, Report EPS 1/RM/24. Ottawa: Conservation and Protection, Environment Canada; 1992. pp 61.
10. Blaise C, Vasseur P. Algal microplate toxicity test. In: Blaise C, Férard JF,(eds. Small-scale freshwater toxicity investigations, Vol. 1. Dordrecht: Springer; 2005. pp 137-179.
11. Blaise C, Kusui T. Acute toxicity assessment of industrial effluents with a microplate-based Hydra attenuata assay. Environ Toxicol Water Qual 1997;12:53-60.
12. Gagné F. Acute toxicity assessment of liquid samples with primary cultures of rainbow trout hepatocytes. In: Blaise C, Férard JF,(eds. Small-scale freshwater toxicity investigations, Vol. 1. Dordrecht: Springer; 2005. pp 453-472.
13. CEC (Commission of the European Communities). Technical guidance document in support of commission directive 93/67/EEC on risk assessment for new notified substances. Part II, Environmental Risk Assessment. Luxembourg: Office for official publications of the European Communities; 1996.
14. Wang W, Lampi M, Huang X, Gerhardt, Dixon G, Greenberg B. Assessment of mixture toxicity of copper, cadmium and phenanthrenequinone to the marine bacterium Vibrio fischeri. Environ Toxicol 2009;24:166-77.
15. Kungolos S, Emmanouil C, Tsiridis V, Tsiropoulos N. Evaluation of toxic and interactive toxic effects of three agrochemicals and copper using a battery of microbistests. Sci Total Env 2009;407:4610-15.