Nanotoxicity: Threat posed by nanoparticles

Nanotechnology is the convergence of engineering and molecular biology, leading to the development of structures, devices and systems that have novel functional properties with size ranging between 1 and 100 nm. Nanotechnology has tremendous potential to change and improve many sectors of the economy, including consumer products, healthcare, transportation, energy and agriculture. It is estimated that over 200 consumer goods that are already available consist of nanomaterials. Nanoparticles (NPs) are present in some sunscreens, cosmetics, toothpastes, sanitary-ware coatings, silicon chips and even in food products. Worldwide investment on nanotechnology is on the rise[1,2] and the trend is expected to continue over the next decade.

While the tremendous positive impacts of nanotechnology are widely publicized, potential threats or risks to human health and the environment are just beginning to emerge[3]. With limited information available for support, critics are presenting a number of concerns on the devastating effects of nanotoxicity on human health and the environment[4–6]. Several aspects of societal implications of nanoscience and nanotechnology in developing countries have been discussed in a recent article[7]. Detailed studies on the long-term effects of NPs are the need of the day to overcome or reduce possible threats. Simultaneous agglomeration, sedimentation and diffusion at physiologically relevant concentrations should be taken into account while conducting quantitative studies on the uptake of NPs into biological systems, to assess the corresponding risks on human health[8]. In addition, ecological risk assessment is essential to understand environmental implications of nanomaterials. The fate of nanomaterials in aqueous environment is controlled by many biotic/abiotic processes such as solubility/dispersability, interactions between the nanomaterials and natural/anthropogenic chemicals in the ecosystem.

Outburst of nanomaterial research will certainly pump a lot of NPs to the environment, which will ultimately lead to the so-called nanoparticle pollution. To date, there are no detailed studies on the mechanism of transport and biodegradation or association of NPs with biological materials that may eliminate nanomaterials. Processes that control transport and removal of NPs in water and wastewater are yet to be investigated to understand the fate of NPs. Studies on the effect of NPs on plants and microbes are also rare. Alumina NPs are commonly employed in scratch-resistant transparent coatings and sunscreen lotions that provide transparent-UV protection. However, a recent study reported that alumina NPs led to phytotoxicity by retarding the growth of root in five plant species (corn, cucumber, soybean, cabbage and carrot) of significant economic value[6]. In another study, toxicity of fullerene-C_{60} (an engineered NP) in two aquatic species, *Daphnia* and *Pimephales* elevated lipid peroxidation (LPO) in brain, significantly increased LPO in gill, and resulted in significant increase in expression of genes related to the inflammatory response and metabolism (mostly the CYP2 family)[9]. In contact with water, C_{60} spontaneously forms a stable aggregate (nano-C_{60}) with nanoscale dimensions (d = 25–500 nm). Prokaryotic exposure to these aggregates even at relatively low concentrations is growth-inhibitory (≥0.4 ppm) and decreases the rate of aerobic respiration (4 ppm)[10]. The latter clearly demonstrates the environmental fate, distribution and biological risks associated with engineered nanomaterials and advocates the need for a model to address not only the engineered nanomaterials, but also that of the aggregated form generated in aqueous media[10].

Studies on the effect of NPs on human health have also gained momentum recently. According to a recent estimate, about 1.5–2 million human deaths every year worldwide could be attributed to indoor air pollution[11]. Toxicologists suggest that the NP component of particulate air is most potent and likely to be responsible for adverse health effects. Whether the inhaled carbon NPs translocate directly into the circulation in humans or not, is a conflicting issue. A study suggests that inhaled carbon NPs are capable of rapid translocation into the circulation[12]. On the contrary, a conflicting report suggests that inhaled carbon NPs remain within the lung up to 6 h after inhalation, without passing to systemic circulation[13]. In a recent study[14], suitability of mouse spermatogonial stem cell line as a model system to assess nanotoxicity was evaluated in the male germline *in vitro*. Light microscopy, cell proliferation and standard cytotoxicity assays revealed the effect of different types of NPs on these cells. The results of these experiments revealed concentration-dependent toxicity for all types of NPs tested, whereas the corresponding soluble salts had no significant effect. In the assay conditions, silver NPs were the most toxic, while molybdenum trioxide (MoO_3) was least toxic. The toxicity of copper NPs (23.5 nm) was assessed *in vivo* based on LD_{50}, morphological changes, pathological examinations and blood biochemical indices of experimental mice[15]. Grave nanotoxicity was attributed to several factors; for instance, huge specific surface area, ultrahigh reactivity and exceeding consumption of H^+.

Not all NPs are dangerous. The toxicology and biodynamics of silica NPs investigated in a mice model revealed that silica NPs were not toxic and can be used *in vivo*[16]. Results generated from the protocol developed by an insurance company for the purpose of calculating insurance premium for chemical manufacturers revealed that the relative environmental risk from manufacturing five nanomaterials (single-walled carbon nanotubes, bucky balls (C_{60}), one variety of quantum dots, alumoxane NPs and nano-titanium dioxide) was comparatively lower than common industrial manufacturing processes[17]. This study should not be misunderstood to promote the manufacture of these nanomaterials without detailed assessment of environmental and human risks. Besides the workers in the manufacturing wing, others who get exposed (e.g. occupational health nurses) to NPs should be aware of the potential risks and possible means to avoid health risks[18]. There is a need to identify specific regulatory regimes to protect personnel involved in the production and use of NPs for cosmetic, medical and agricultural purposes.

In nanotechnology, we have a unique opportunity to test hazards and control risks as and when the technology develops. At present, safety assessment protocols for products possessing engineered NPs are poorly structured. However, at the current pace of research, we may need several years or decades to clearly

establish the health and environmental risks from engineered nano-scale particles. Although research on the adverse effects of NPs on human health is progressing rapidly, environmental fate of NPs is still in its infancy. Before unknowingly dumping a huge amount of dangerous nanomaterials into the environment, we need to investigate the solubility and degradability of engineered NPs in soils and waters, to establish baseline information on their safety, toxicity and adaptation of soil and aquatic life. Development of novel NPs must be followed by the assessment of their potential risks on life and environment, and possible remedial measures.

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A modified freeze–thaw method for efficient transformation of *Agrobacterium tumefaciens*

There is a great potential for genetic manipulation of crop and medicinal plants to enhance productivity through increasing pest and microbial disease resistance and environmental stress tolerance, and also for studying gene function and regulation of physiological and developmental processes[1]. Transgenic plants that transmit the introduced trait to progeny generations are produced using various DNA delivery methods such as particle acceleration[2], electroporation[3] and polyethylene glycol permeabilization of protoplasts[4]. However, most commonly used method for obtaining transgenic plants is by the *Agrobacterium tumefaciens*-mediated transformation[5,6]. *Agrobacterium* can transfer DNA to a remarkably broad group of organisms – numerous dicot and monocot angiosperm species[5], gymnosperms[6] and fungi, including yeast[7], ascomycetes[8] and basidiomycetes[9]. Recently, *Agrobacterium* was reported to transfer DNA to human cells[10].

The general process for manipulating genes to be transferred into the genome of plant cells is carried out in two phases. First is the cloning of the desired gene into binary vector and transforming the same into *Escherichia coli*. Secondly, the binary vector construct is mobilized from *E. coli* to *Agrobacterium* by triparental mating[11], or direct introduction of the genetically engineered binary vector construct into *Agrobacterium* by electroporation[12] or freeze and thaw method[13]. Triparental mating requires at least five to seven days in order to determine the successful mobilization into *Agrobacterium* and is confined to strains harbouring plasmids that carry the *mob* gene[11]. Electroporation is faster and more efficient than triparental mating, but requires special equipment. Till date, the transformation frequency reported by freeze–thaw method in *Agrobacterium* has remained low[13–15]. Here we describe a simple and reliable protocol for competent cell preparation and efficient transformation by freeze–thaw method in *A. tumefaciens* LBA4404.

A. tumefaciens strain LBA4404 (pAL 4404) was procured from Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, India. The binary vector used for transformation was the pGreen-CaCPK2 (calcium-dependent protein kinase isoform 2 cDNA isolated from *Cicer arietinum* L.) construct, with the neomycin phosphotransferase (*npt*II) gene as a selectable marker (confers to kanamycin resistance). *A. tumefaciens* strain LBA4404 was streaked out on a LB[16] plate containing 1 mg/l rifampicin and grown at 28°C overnight. A single colony was used to inoculate 3 ml of LB medium containing 1 mg/l rifampicin and grown overnight at 28°C, shaking at 160 rpm. A 50 ml of LB medium with 1 mg/l rifampicin was inoculated with 0.5 ml (1/100th volume) of the overnight culture and grown in an incubator-shaker at 28°C at 160 rpm to obtain cell densities of 0.3, 0.4, 0.5 and 0.6 at OD_{600}. The cultures were chilled on ice for 15 min and the cells were then harvested by centrifugation at 3000 rpm for 5 min at 4°C, resuspended in 10 ml of sterile ice-cold 50 and 100 mM $MgCl_2$ solutions, and incubated on ice for 1 h. After another centrifugation step as above, the resulting pellet was resuspended in