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Nanobiolistics: New Generation Transfection System for Animals and Plants

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Abstract

The potentiality of genetic engineering is well recognized in recent times in creating genetic modifications so that desirable traits can be incorporated by transferring and integrating an alien gene within the genome of an organism. Several methods of biomolecule delivery are reported, of which the biolistic delivery of biomolecular cargoes to animal and plant cells is well established through the use of nucleic acid-coated gold and tungsten microparticles, bombarded with high velocity, driven by helium gas. However, the major drawback of this technology is considerable mechanical injury and tissue damage due to large-sized microprojectiles that makes further propagation or regeneration of cells difficult. In addition, the method is also not suitable for transforming isomorphic small cells or sub-cellular organelles like chloroplast and mitochondria. These problems can be circumvented by the advanced nanobiolistic method, where nanoparticles of smaller dimensions are used as nanoscale biolistic carriers which can penetrate the cells and deliver drugs or biomolecules like nucleic acids and proteins to living systems. This minireview discusses the biolistic delivery of nanoparticles to animal and plant systems as a promising tool to minimize tissue damages, while maintaining similar transformation efficiency like the micro-scale counterparts, thereby highlighting the prospect of broad-scale implementation of this technique for cargo delivery.

Keywords: Nanobiolistics; Nanoparticles; Animal and plant cells; Transfection; Biolistic method

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Introduction

One of the major challenges in genetic engineering is the proper transfer and integration of nucleic acids into living cells. Nanotechnology has emerged as a major field in medicines and as tool for drug and DNA delivery in animal and plant cells using nanoparticles, allowing manipulation at sub-cellular level, *via* bypassing the biological barriers. Nanobiotechnology involves the intersection of biology and nanotechnology so that the latter can be applied to several fields like molecular biology, biochemistry, medicine and healthcare, synthetic biology and agriculture. The field of nanomedicine utilizes nanomaterials to generate drug delivery systems to specific cell types so that the dose and side effects of drugs can be regulated [1]. The nanoparticles are found to be responsive to a number of physical factors like temperature, redox, pH and presence of cellular enzymes. Engineered nanoparticles as nanocarriers of fertilizers, pesticides, herbicides and desirable genes, can be introduced in plants at specific sites for their controlled release. Gold, magnetic iron oxide and silica nanoparticles are the chief nanoparticles for protein and drug delivery, and tumor therapy [2]. Hybrid gold/drug nanoparticles have actually been introduced for targeted therapy. Gold nanoparticles can bind to a wide range of organic and inorganic molecules; they have low toxicity with strong and tunable optical absorption. Silver nanoparticles also find considerable application for nucleic acid and protein delivery into animal and plant systems, conferring protection to DNA from nuclease attack. It is also important to study the interaction between nanomaterials and animal or plant cells, since any toxic or adverse effects on cellular systems will lead to the failure of such approach of cargo delivery.

Nanoparticles

The particles with dimensions between 1-100 nm are referred to as nanoparticles. Based on their origin, they can be grouped into (i) natural nanoparticles occurring in environment in the form of volcanic dust, lunar dust, mineral composites, and also isolated from plant extracts, etc., (ii) waste or anthropogenic nanoparticles, originating from man-made activities like industrial processes, diesel exhaust, coal combustion, welding fumes, etc., and (iii) engineered nanoparticles or nanomaterials, like metal-based particles (nanogold, nanozinc, etc.), dendrimers (nano-sized polymers built from

branched units), carbon-based materials (carbon nanotubes) and composite nanoparticles of various morphologies like sphere, rod, tube and prism. The action of nanoparticles depends on their chemical composition, size and/or shape [3]. The small size of nanoparticles and their highly tunable chemical and physical properties have facilitated nanoparticle engineering to bypass biological barriers and even nanoparticle localization in subcellular domains of CHO and HeLa cells. The nanoparticles serve as biocompatible and non-cytotoxic vectors capable of transporting a range of biomolecules, *viz*., small molecules, DNA, siRNA, miRNA, proteins and ribonucleoproteins to biological cells. The shape, tensile strength, functionalization, charge and aspect ratio have been tuned for efficient intracellular cargo delivery to the animal and plant systems. The DNA delivery into cells is mostly carried out by nanoparticles made of carbon nanotubes, calcium phosphate, gold, silica, strontium phosphate, magnetite, manganese phosphate, magnesium phosphate and especially Mesosporous Silica Nanoparticles (MSNs) with 3 nm pore size. Davis et al. (2010) [4] delivered siRNA to human melanoma tumor cells by designing a polymeric nanoparticle with a human transferring protein-targeting ligand and polyethylene glycol on the external surface of nanoparticle. The MSNs were used to encapsulate drug molecules and neurotransmitters enabling their slow and controlled release in neuroglial cells [5]. Following initial adsorption of nanoparticles on cell membrane, they are internalized by endocytosis, dissolved by the acids present in the endosomal vesicle and cytoplasm. The DNA-loaded nanoparticles attach to the surface of the nucleus where the import occurs. Although nanoparticle-mediated delivery is difficult in plants due to the challenge imposed by the cell wall, understanding the mechanism of biomolecule delivery in animals provides a blueprint for extrapolating the technique to plant systems by controlling the size of the nanoparticles to traverse the cell wall, tuning charge and surface properties to carry diverse cargo, and greater breadth in utility across plant species. The internalization challenge caused by the plant cell wall is usually overcome by using a mechanical aid to penetrate the cell wall, such as the use of a gene gun in biolistic transformations.

Biolistic Method

The high-velocity microprojectiles were first demonstrated to deliver exogenous nucleic acids to living cells during late 1980s by J.C. Sanford at the Cornell University. The term biological ballistics or biolistics was proposed since the process involved the bombardment of nucleic acid-coated metal micro-particles within tissues [6]. The particle gun instrument was invented by Dennis McCabe at Agracetus in 1986 where a high-voltage electric shock was utilized to convert a water droplet into a shock wave to drive DNA-coated gold microprojectiles into plant tissues. The biolistic technique for delivering indicators using a commercial gene gun (Bio-Rad, Helios Gene Gun System, #165-2431) was first described by Gan et al. (2000) [7]. They propelled 1.3 μm particles coated with carbocyanine dyes for a distance of 1-2 cm into the exposed tissue. In order to protect the tissues from the shockwaves and particle clusters, membrane filters were placed between the specimen and gun. Since then, this method has proved useful to transfer DNA into prokaryotic and eukaryotic organisms ranging from bacteria, algae, fungi, animals and particularly in plants.

The biolistic or gene gun method involves the use of mechanical force or high velocity to drive the accelerated metal microparticles in presence of the pressurized pulse of helium, the inert gas. The nucleic acid or genetic cargo is coated with tungsten or more commonly gold microparticles (also called microcarriers) followed by shooting the microparticles into target tissues with high velocity driven by helium pump, rupturing the cell wall and/or cell membranes [8]. These particles must be non-toxic, non-reactive, and smaller than the target cell. The loading of DNA onto the particles, the particle size, and the timing of delivery and the velocity of acceleration are the important determining factors governing the efficiency of this method. The final results and expression of the introgretted gene also depend on the number of DNA-coated beads delivered, and on the degree to which the particles are coated with DNA. The biolistic machiene consists of three main components: (i) a rupture disk, (ii) macrocarrier (holding microcarrier particles), and (iii) stopping screen. The rupture disk is a membrane that bursts at a critical pressure of accelerated helium gas, creating a shock wave that propels the macrocarrier towards the cells. The stopping screen functions to retard the momentum of the macrocarrier, allowing the genetic cargo-loaded microcarriers to pass and penetrate the tissues [9]. Biolistic method facilitates transformation of nuclear, plastidal and mitochondrial genomes due to the nonspecific localization of genetic cargo, with high transformation efficiency. In case of plant systems, this method enables transformation of recalcitrant tissues where *Agrobacterium tumefaciens*-mediated transformation is quite difficult [10]. Kettunen et al. (2002) [11] used this same technique to deliver fluorescent calcium indicators into neural tissue, demonstrating a non-deleterious delivery method for functional dyes. Biolistic delivery of vital dyes has also been used to study cellular processes and morphology in various retinal cell types [12,13].

Nanobiolistic Transfection of Animal Cells

Because of their ability to cross biological membranes, protect and release several cargoes and undergo multifaceted targeting, the nanoparticles are valuable materials for targeted intracellular biomolecule delivery and controlled release in mammalian systems. O'Brien and Lummis (2011) [14] developed nanobiolistic transfection system using three different types of animal cells and tissues. Gold-nanoparticles of 40 nm dimensions were synthesized, followed by addition of spermidine and DNA. While adding calcium chloride, the mixture was vortexed, followed by centrifugation to collect the supernatant. The gold pellets were re-suspended and converted into bullets by placing them in Tefzel tubing, rotated to ensure even spread of gold particles. Human embryonic kidney cells grown on glass cover slips in plates were biolistically transfected with the nanoparticle projectiles using 50 psi pressure at a distance of 1 cm, and then fixed with 4% paraformaldehyde (PFA), counterstained with diamidino-2-phenylindole (DAPI). Adult mouse ear tissues were likewise shot using a gas pressure of 75 psi at a distance of 5 mm. They were fixed in 4% PFA, whereas brain slices were transfected using a gas pressure of 50 psi at a distance of 10 mm, and counterstained with DAPI. The method caused lesser tissue damages in all the cases, and the efficiency of transfection was similar to that of microprojectiles. The use of nanoparticles caused 30% less damaged HEK293 cells and <10% of damaged nuclei for mouse ear tissue, as compared to >20% in microparticle-transfected samples. Both spermidine and calcium chloride appeared to be crucial for successful transfection. The small size of the particles permitted them to transfect regions of cells that are not efficiently transfected with larger particles. In another work of Arsenault and O'Brien (2013) [15], organotypic brain slices from mice were transformed with nanobiolistic method, and the cell survival was checked *via* LDH assay, a terminal deoxynucleotide transferase dUTP nick end labeling (TUNEL) assay and propidium iodide (PI),

which clearly concluded decreased cell and tissue damages with nanoparticles as compared to microprojectiles. Chitosan and poly-γ-glutamic acid nanoparticles, ranging in size from 150-250 nm, prepared through ionic gelation method, were used by Lee et al. (2008) [16] to deliver GFP-encoding plasmids for transdermal delivery in mice. In another approach, the 250 nm particles were composed of a poly (D,L-lactic-co-glycolic acid) (PLGA) core and glycol chitosan (GC) shell. The particles were loaded with GFP plasmids and targeted at Langerhans cells of mice epidermis. Though not strictly within the canonical size range of 'nanoparticles', they led to the release of DNA cargo in a pH-responsive manner. Chitosan polymeric nanoparticles, 150-270 nm, were also used for low-pressure biolistic delivery of *GFP* reporter genes, a plasmid encoding *β-galactosidase* genes, and a Japanese encephalitis virus DNA vaccine to mice *via* transdermal bombardment [17].

Roizenblatt et al. (2006) [18] developed a nanobiolistic technique for loading functional indicators into living neurons of mouse retinal cells. Silver-nanoparticles, having dimensions of 80 nm, in the form of silver spheres were spread on a glass slide. They were immersed in an aqueous solution containing dextran-conjugated fluorescent probe. After scraping the NPs from glass slides, they were used to fill the gun cartridge and propelled under 250 psi of pneumatic pressure, from a distance of 10 cm through a filter which was located 2.5 cm over the specimen. After washing the chamber with a suitable buffer, fixation was performed using 4% glutaraldehyde for 15 min. Upon penetration of the coated particles within the cells, the dye was released which spread uniformly throughout the cytoplasm giving a strong fluorescence. This technique proved to be helpful to stain rapidly and efficiently the whole layers of cells inside the retina tissue retaining their viability, without causing significant cellular damages, as compared to larger particles in a conventional biolistic method. The higher surface area to volume ratio of smaller nanoparticles enabled delivery of larger amounts of dye for the same particle mass. However, there are limitations of non-selective staining of cells as it is impossible to target individual cells with this technique, as well as ensuring adequate depth of penetration through the overlying medium and tissue.

Nanobiolistics in Plant Systems

Unlike the animal systems, nanoparticle-mediated DNA delivery in plants is rather complicated due to the challenge imposed by the plant cell wall, which exclude particles larger than 5-20 nm, although 50 nm nanoparticles have been shown to be cell wall-permeable. The uptake of nanoparticles and their transport is also limited by pore diameters, size exclusion limits for different tissues and organs, and additional barrier created by plasma and nuclear membrane (especially where cytosolic and nuclear localization studies need to be performed) [19]. Nanoparticle charge and shape also influences translocation through cell membrane and therefore need to be optimized for efficient nanobiolistic procedure. Moreover, cationic nanoparticles, by virtue of their better binding with the negatively charged cell membrane, are internalized faster and more efficiently than anionic nanoparticles [20]. The regeneration potential also varies widely across species, genotype and even within an individual plant depending on the developmental stage of the explant tissues. The most commonly used nanoparticles for nanobiolistic delivery are MSNs and gold nanoparticles. The MSNs contain a highly porous structure that permits internal loading of biomolecules like DNA, RNA and proteins, and subsequent biolistic delivery to plant tissues. Nanoparticle-DNA codelivery was first demonstrated in *Nicotiana benthamiana* cotyledons through biolistic approach using 100-200 nm gold-capped honey-comb like MSNs, of 3 nm pore size, coated with plasmid [21]. The MSN was loaded with plasmid DNA containing the Green Fluorescent Protein (GFP) under the control of constitutive promoter, and the ends were capped with gold nanoparticles. The optimal coating ratio for DNA/MSN was 1:10 (w/w), so that a stable complex was formed between DNA and MSN, without any free DNA after incubation for two hours. Following biolistic delivery, uncapping of gold nanoparticles released the chemical inducer, β-estradiol in the presence of dithiothreitol in regeneration media and activated the transient GFP expression after 36 hours of incubation of DNA with MSNs. MSNs became effective when pore-capped with 10-15 nm gold nanoparticles, linked by amide coupling, probably due to the larger hybrid particle density. However, MSNs alone were non-functional due to their very low mass density. While DNA-coated gold MSN produced 32±11 GFP-fluorescent foci per cotyledon, standard 0.6 μm AuNP bombardment produced 73±24 GFP-fluorescent foci per cotyledon. Martin-Ortigosa et al. (2012a) [22] delivered active GFP and FITC conjugated BSA coupled with 600 nm gold nanoparticles (10 nm diameter) to onion epidermal cells, tobacco leaves and teosinte leaves, showing efficient delivery of proteins with hydrodynamic radii of several nanometers. In another study by Martin-Ortigosa et al., (2012b) [23], the introduction of DNA and MSNs with 10 nm pore size and 600 nm diameter was optimized by plating gold multiple times onto the surface of MSNs, including the pore walls, in order to increase the surface loading of gold and hence the particle mass density. The drastic improvement of NP delivery was demonstrated when the particles were combined with 0.6 μm gold particles during bombardment. Gold plating technique allowed more gold due to the amount of surface area capable of plating, as compared to the number of pores that could be capped per MSN. Although gold plating lowered the MSN porosity and cargo capacity, it eliminated the disadvantages of smaller size and density, as well as the need for synthesis of gold nanoparticles, attachment to pore entrances and subsequent uncapping for releasing the encapsulated molecules. The use of calcium chloride and spermidine, routinely used in gold or tungsten microprojctile bombardment, highly facilitated the adsorption, complexation and coating of DNA with MSN surface, regardless of the surface charge, that could be efficiently bombarded into various tissues like onion epidermal cells, maize and tobacco leaves to show high expression efficiency. Twice-bombarded tissues at higher pressure (1350 or 1550 psi) and smaller target distances (4 cm) showed more transient expression of fluorescent proteins than the cells bombarded once or at lower pressure (650 psi) and longer target distances (10 cm) in tobacco leaves and maize immature embryos. Gold MSNs were also used by Martin-Ortigosa et al. (2014) [24] for biolistic co-delivery of GFP DNA and Cre-recombinase enzyme-protein for gene editing. This work justified the importance of nanobiolistic approach to produce precisely modified non-transgenic plants by DNA-free methods. Several research groups have proposed that the size of the nanoparticles is a major determinant for DNA delivery. Mortazavi and Zohrabi (2018) [25] observed that plasmid delivery to rice embryogenic calli by bombardment with 50, 100, 600 and 1000 nm gold nanoparticles resulted in similar levels of transgene integration across all carrier sizes. Okuzawi et al. (2013) [26] found that 300 nm gold particles showed better efficiency than 600 nm gold particles and slightly less effective than 70 nm gold particles for plastid transformation in tobacco, which implicated the importance of nanobiolistics in the transformation of even the subcellular

organelles.

Conclusion

Biolistic delivery is a powerful and popular biotechnology tool where the traditional carriers like micron-sized gold or tungsten particles cause variable levels of tissue and cell injuries, along with gene rearrangements, and are almost unsuitable for mitochondrial and plastid transformation. The newly evolved area of nanobiolistics provide cargo delivery platform which has largely facilitated genetic transformation, drug delivery and imaging of tissues, since it causes less cellular damages, increased loading capacity and ability to target more cell types and transform smaller targets, while maintaining similar transformation efficiency levels. The sub-cellular organelle transformation, co-delivery or controlled and targeted release of cargo, *viz*., DNA, proteins and small molecules, and protection of cargo from cellular metabolism are amongst the other advantages. However, there are still scopes of further optimizing the technique by analyzing the effect of nanoparticles on different cell and tissue types, effect of particle size, shape, charge and stiffness on the efficiency of delivery. The issues of biocompatibility of nanoparticles and nucleic acid protection also need to be examined carefully. Overall, the exploration of nanocarriers for biomolecule delivery remains a nascent field, with much potentiality in near future for the disciplines like plant and animal biotechnology to derive both transient and stable gene expression, and for genome editing.

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