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Sub-micron gold particles are superior to larger particles for efficient Biolistic® transformation of organelles and some cell types

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Summary

Stable transformation of maize, *Chlamydomonas reinhardtii*, and *Saccharomyces cerevisiae* nuclear genomes, as well as *C. reinhardtii* chloroplasts and *S. cerevisiae* mitochondria was achieved by particle bombardment with the PDS-1000/He instrument using plasmid DNA coated onto gold or tungsten microparticles. Results demonstrate that the kind and size of microparticles are important factors in determining the efficiency of transformation. Maize callus is transformed approximately five-fold more efficiently with 0.6 µ gold particles than with 1 µ gold particles. Nuclear transformation of *S. cerevisiae* is over ten-fold more efficient using 0.6 µ gold particles than with 1 µ gold particles. Yeast mitochondrial transformants, which arise at a very low frequency using 0.6 µ gold particles, were not found using larger gold particles or M5 tungsten particles. Nuclear transformation of *Chlamydomonas* was 2.5-fold more efficient with 0.6 µ gold particles than with either 1 µ gold or M10 tungsten particles. Using 0.6 µ gold particles, the transformation efficiency of *Chlamydomonas* chloroplasts was 2.5-fold higher than with M10 tungsten particles and 4-fold higher than with 1 µ gold particles.

Introduction

Biolistic technology, or particle bombardment, is a physical method of introducing DNA into cells. In theory, all cells should be transformable by this method. The technique was originally developed to transform monocotyledonous plants, and has since been used to transform numerous species of both mono- and dicotyledenous plants (Klein, *et al.*, 1990). Particle bombardment has also been used to transform a wide variety of tissue culture cells and animal organs (Johnston & Tang, 1993). Additionally, the technique has been used to transform bacteria and subcellular organelles (Boynton & Gillham, 1993, 1996; Butow & Fox, 1990; Smith, *et al.*, 1992).

Transformation of all these different cell types requires optimization of the physical parameters used in bombardment (Sanford, *et al.*, 1993). The parameters which have the greatest effect on transformation efficiency include the vacuum in the bombardment chamber, the distance the particles travel before striking the target cells, and the size and density of the particles used in bombardment. Of less importance are the helium pressure, the gap distance (the distance between the rupture disk and the macrocarrier), and the macrocarrier travel distance. For most applications, a helium pressure of about 1,100 psi, a gap distance of 5–10 mm, and a macrocarrier travel distance of about 8 mm are near optimal. Each of these parameters affects the particle velocity and each interacts with the others. The greater the particle velocity, the more likely the particle will penetrate the cell; this is particularly important when using small particles. The chamber vacuum affects the velocity of the microparticles by reducing the drag on the particles as they travel toward the target tissue: the higher the chamber vacuum, the less the particles will be decelerated. The distance between the stopping screen and the target tissue has several effects on the transformation efficiency. The longer the travel distance, the greater the spread of the particles over the target and the less pronounced is the helium shock wave striking the cells. On the other hand, a longer travel distance results in reduced particle speed and a decreased likelihood that a particle will penetrate the target.

Because the likelihood of a subcellular-sized particle penetrating a cell is proportional to the kinetic energy of the particle, materials of high density have generally been preferred for use in biolistic technology. These compounds include tungsten, platinum, and gold. Of these, gold is favored because it is biologically inert and because spherical gold particles can be produced in a narrow size range. Physical parameters of gold particles are presented in Table 1. Note the nearly five-fold increase in the number of particles per gram for the 0.6 µ gold relative to the 1.0 µ gold. The importance of particle size and density on transient expression has been demonstrated for a variety of cell systems (Sanford, *et al.*, 1993). Here we present data to demonstrate that for stable expression in several biological systems, particle bombardment is more efficient using 0.6 µ gold particles than using particles of larger size.

Table 1. Physical parameters of gold microparticles

Particle diameter (µ)	Surface area (µ²)[1]	Mass (pg)[2]	Number of particles (particles/g)	Relative to 1 µ particles		
				Surface area	Mass	Particle number
0.6	1.1	2.2	4.6×10^{11}	0.4	0.2	4.6
1.0	3.1	10.1	9.9×10^{10}	1.0	1.0	1.0
1.6	8.0	41.4	2.4×10^{10}	2.6	4.1	0.2

[1] Assuming all spherical particles

[2] Density of gold = 19.3 g/cc

Methods and Results

Stable transformation of Type I callus of *Zea mays*. Maize Type I callus (Wan, *et al.*, 1995) was subjected to osmotic pretreatment by placing the tissue on high sucrose media for 4 hr. prior to bombardment. Tissues were bombarded in the PDS-1000/He with 1 µ or 0.6 µ gold particles coated with pBC17 (Goff, *et al.*, 1990), a plasmid which encodes genes for the C1 regulatory gene and the *B-Peru* allele of the *B* regulatory gene in the maize anthocyanin biosynthetic pathway. When expressed together, these genes activate this pathway and give rise to pigmented cells. Bombardment conditions were as follows. Each plate was shot twice. Each shot contained 0.06 mg or 0.5 mg of 0.6 µ gold particles, or 0.5 mg of 1 µ gold particles, and all particles were coated with 0.33 µg of plasmid pBC17; 16 target tissue pieces were placed in a ring on a 100 mm plate; 3 plates were shot per treatment; a stainless steel 200 x 200 wire mesh screen (McMaster-Carr, cat. no. 9236T11) was inserted at Level 1 as a post-launch baffle (Russell, *et al.*, 1992) to reduce cell damage and the tissue sections were placed at Target Level 2; the bombardment chamber was reduced to 28" Hg vacuum and cells were bombarded at a helium pressure of 650 or 900 psi.

Transient expression and target tissue damage were qualitatively assessed 4 days after gene delivery. Generally, bombardment with 1 mg of 1 µ gold particles produced relatively high levels of transient expression as judged by observation of pigmented cells. The level of transient expression in cells bombarded with 0.6 µ gold particles was dependent on the amount of gold used in bombardment. Reducing the amount of gold eight-fold, from 1 mg to 0.12 mg, resulted in an eight-fold decrease in the number of gold particles and in a proportional decrease in the number of transiently expressing cells; plates bombarded with 1 mg of 0.6 µ gold particles had more transiently-expressing anthocyanin cells than did plates bombarded with 1 mg of 1 µ particles. Tissue damage was less in cells bombarded with 0.6 µ gold than in tissue bombarded with the same amount of 1 µ gold. Although the presence of the wire mesh did not affect the number of cells that transiently express anthocyanin, it did reduce tissue damage and resulted in higher numbers of stable events. Results of stable transformation are presented in Table 2. Transformation was assessed by counting the number of individual multi-celled red-pigmented sectors present after 6 weeks of growth on non-selective media. Stable sectors are derived from cells which have been transformed, divide, and continue to express the anthocyanin gene in the daughter cells. Each stable sector has the potential of growing into a mature plant expressing the transforming gene. Compared to callus tissue bombarded with 1 µ gold, four- to eight-fold more red sectors were present in tissue bombarded with 0.6 µ gold, although in one experiment eight-fold less gold (and hence, eight-fold fewer gold particles) was used than in the other experiment (see Table 2). These results indicate that the level of transient expression is not always an accurate indication of the level of stable expression. Since the experiments summarized in Table 2 were carried out at different times and with different callus culture lines, the absolute numbers of transformants should not be compared between Experiment 1 and Experiment 2.

Table 2. Anthocyanin-expressing stable sectors in maize callus produced by bombardment with 0.6 µ or 1 µ gold particles.

Microparticles	Stable Sectors[1]	
	Expt 1[2]	Expt 2[3]
1 µ gold	0.7 ± 1.0	1.7 ± 0.5
0.6 µ gold	5.3 ± 0.3	6.3 ± 0.5

[1] Each value indicates the average number (plus or minus standard deviation) of stable sectors in maize callus found on each of three plates following bombardment.

[2] 0.5 mg 1 µ gold/bombardment or 0.06 mg 0.6 µ gold/bombardment

[3] 0.5 mg gold/bombardment

Stable transformation of Chlamydomonas. *Chlamydomonas reinhardtii* with an arginine-requiring point mutation in the *ARG7* gene encoding argininosuccinate lyase (strain CC-48) or a non-photosynthetic, acetate-requiring deletion mutation in the *atpB* gene (strain CC-373) were used for assaying nuclear and chloroplast transformation, respectively. For nuclear transformation, CC-48 cells were bombarded with plasmid P-389 (pARG7.8; Debuchy, *et al.*, 1989) containing the wild-type *ARG7* argininosuccinate lyase gene from *C. reinhardtii*; for chloroplast transformation, CC-373 cells were bombarded with plasmid P-437 (*Bam*H I chloroplast fragment 10 cloned into pBluescript KS+) containing the wild type *atpB* gene from *C. reinhardtii.* Bombardment conditions were as follows (Boynton & Gillham, 1993): 0.6 mg of 0.6 µ or 1 µ gold or M10 tungsten (average size = 0.7 µ) particles were coated with 1.5 µg of CsCl-purified P-389 (for nuclear transformation) or P-437 plasmid (for chloroplast transformation); 4 x 10^7 log-phase cells in 0.1% top agar were spread on 100 mm agar plates; 10 plates were shot for each experimental condition; cells were placed at Target Level 2; the bombardment chamber was reduced to 26" Hg vacuum and cells were bombarded at a helium pressure of 1,300 psi. Following bombardment, cells from each plate were re-spread onto two plates of selective media: nuclear transformants were re-plated onto arginine-free media and chloroplast transformants were re-plated onto acetate-free media.

For nuclear transformation, arg^+ colonies appeared within 7 days post-bombardment, while photosynthetic colonies resulting from chloroplast transformation were visible 3–5 days post-bombardment. In these experiments, final scoring of all transformants was performed 13–14 days after bombardment. Results of several bombardments are summarized in Table 3. Both nuclear and chloroplast transformation efficiencies were on average 2.5-fold higher using 0.6 µ gold particles compared to using M10 tungsten particles. In a single experiment, nuclear transformation of *Chlamydomonas* was about two-fold lower using 1 µ gold particles than using 0.6 µ gold particles, while the level of *Chlamydomonas* chloroplast transformation was four-fold lower using 1 µ gold particles than using 0.6 µ gold particles.

Table 3. Transformation of *Chlamydomonas* using gold or tungsten microparticles.

Microparticles	Nuclear transformation[1]			Chloroplast transformation[2]	
	Expt 1	Expt 2	Expt 3	Expt 1	Expt 2
0.6 µ gold	302	329	747	846	761
1 µ gold	180	n.d.[3]	n.d.[3]	204	n.d.[3]
M10 tungsten	218	145	142	275	361

[1] Values represent the average number of ARG^+ colonies on each of ten plates following bombardment of CC-48 cells with microparticles coated with plasmid P-389.

[2] Values represent the average number of photosynthetic colonies on each of ten plates following bombardment of CC-373 cells with microparticles coated with plasmid P-437.

[3] n.d. = not determined.

Stable transformation of yeast mitochondria. A derivative of *Saccharomyces cerevisiae* strain MCC109 (*MATα ade2-101 ura3-52 kar1-1*) lacking mitochondrial DNA [*rho*^{o}] was used for assaying nuclear and mitochondrial transformation. Cells were bombarded with particles carrying the *URA3* gene on plasmid YEp352 and a plasmid carrying intron 2 from the *COXI* gene (either pJVM161 or pJVM164; Moran, *et al.*, 1995) and Ura^+ transformants were selected on media lacking uracil. Mitochondrial transformants were identified by mating the [*rho*^{o}] Ura^+ nuclear transformants with a respiration-deficient mutant (*S. cerevisiae* strain 161) containing a deletion within intron 2 of the *COXI* gene (Zimmerly, *et al.*, 1995) and screening for progeny capable of respiring on glycerol-containing media. Recombination between the defective portion of the *COXI* gene present in *S. cerevisiae* strain 161 and the *COXI* gene from the plasmid present in the mitochondria of the transformant restores respiratory growth. Bombardment conditions were as follows: 0.6 mg of gold or M5 tungsten (average size = 0.4 µ) were coated with a mixture of 8 µg of *COXI* mitochondrial plasmid DNA and 1.6 µg of YEp352 plasmid; 2 x 10^8 cells were spread on 100 mm agar plates; 5 plates were shot for each experimental condition; the bombardment chamber was reduced to 28" Hg vacuum and cells were bombarded at Target Level 2 at a helium pressure of 1,100–1,300 psi.

Nuclear transformation was quantitated by counting Ura^+ transformants 5 days post-bombardment following growth of transformed cells on uracil-deficient media. Ura^+ transformants were then analyzed for mitochondrial transformation by mating to *S. cerevisiae* strain 161 on glucose-containing media (Anziano & Butow, 1991; Butow, *et al.*, 1996). Mitochondrial transformants were identified by replica plating these colonies onto media containing glycerol. Results are summarized in Table 4. Nuclear transformation is quite efficient using either 0.6 µ gold particles or M5 tungsten to deliver the DNA; there is a greater than 10-fold decrease in the number of transformants when 1 µ gold particles are substituted for 0.6 µ gold particles. In these experiments, mitochondrial transformants were only isolated when sub-micron sized gold particles were used. While mitochondrial transformants have been isolated using larger tungsten particles, results with those particles were extremely erratic in our hands, even in terms of nuclear transformation. Similar results were found when bombardment was performed with the Ura^+ and *COXI* sequences on a single plasmid (results not shown).

Table 4. Transformation of *S.cerevisiae* strain MCC109 using gold or tungsten microparticles.

Microparticles	Ura^+ transformants[1]	COXII transformants[2]
M5 tungsten	2,000–3,000	0
1 µ gold	200–300	0
0.6 µ gold	3,000–6,000	0.6[3]

[1] Values represent the approximate number of Ura^+ colonies on each of five plates from one experiment (M5 tungsten) or six experiments (1 µ or 0.6 µ gold) following bombardment with the the various particles.

[2] Values represent the average number of mitochondrial transformants on each plate in six experiments bombarding five plates per experiment.

[3] Between 0 and 10 mitochondrial transformants were isolated from each experiment of five plates each.

Conclusions

Results presented here show that particle size is an important factor for optimizing Biolistic transformation. Maximum stable transformation of cellular organelles (chloroplasts and mitochondria) and certain cell types (*Chlamydomonas*, yeast, and maize callus) occurs using sub-micron sized gold particles. Smaller particles are probably more efficient than larger particles for Biolistic transformation in these systems because they cause less cell damage, resulting in better survival. Organelles could be irreversibly destroyed upon impact with large particles. In the case of maize callus, while cells bombarded with both 0.6 µ and 1.0 µ gold particles transiently express equivalent levels of anthocyanin four days post-bombardment, cells bombarded with the smaller particles gave rise to many more stable transformants, most likely because they sustained less cell damage which could be more easily repaired.

Advantages of gold microparticles include their uniform size and shape and their biological inertness. In contrast, tungsten microparticles are irregular in shape and heterogeneous in size, can be toxic to cells, are subject to surface oxidation that can alter DNA binding, and, over time, can catalytically degrade DNA bound to them (Sanford, *et al.*, 1993). Thus, because of higher transformation efficiency of gold particles, greater variability in results using tungsten, and the need for special precautions in particle preparation, handling, and transformation procedures when using tungsten, gold particles are superior to tungsten particles for Biolistic applications. 0.6 µ gold microparticles can be used with the Yeast Optimization Kit (catalog number 170-3100) to introduce the novice to the principles of particle bombardment.

The size of small particles may be determined by several methods, including molecular sieving, sedimentation, light scattering, electrical sensing, optical sensing, and electron microscopy. Accurate size analysis of gold microparticles is particularly difficult because of their high density and their potential to agglomerate. Most suppliers of gold powder determine particle size by sedimentation techniques. This procedure has the advantage of analyzing a very large number of particles as well as being a rapid and relatively simple procedure. However, sedimentation analysis does not distinguish between individual and agglomerated particles, and particle diameter is estimated assuming perfectly spherical particles. While electron microscopy is more labor intensive, it permits visual determination of the particle shape and allows direct measurement of the diameter of individual particles. Therefore, non-spherical batches of gold particles can be excluded from further assay and agglomerated particles can be eliminated from the size calculation

for maximum consistency between samples. Figure 1 shows scanning electron micrographs of gold and tungsten particles representative of the those sold by Bio-Rad. Also shown are size distributions for the four sets of particles used in these experiments. The contrast between the two types of particles in terms of the size and shape is particularly evident. Scanning electron microscopy has been used as a part of the quality control procedure to determine the average size, the variation in size, and the sphericity of Bio-Rad gold particles since 1993. Information on the average particle diameter, the standard deviation of the diameter, and the aspect ratio (the ratio of the long axis of the particle to the short axis of the particle—a measure of sphericity) is provided with every lot of gold microparticles. Additionally, quality control includes a biological assay using the particles in bombardment and measuring transient or stable expression. Together, these assays are designed to assure lot-to-lot consistency of gold particles for Biolistic applications.

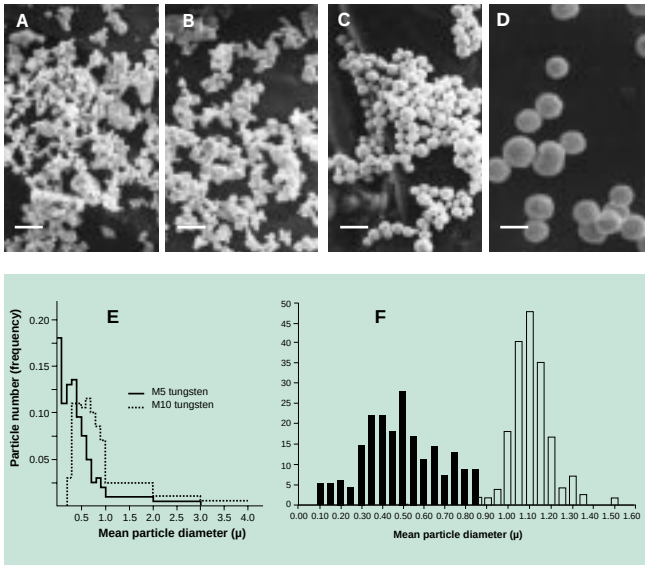

Fig. 1. Scanning electron micrographs of (A) M5 tungsten, (B) M10 tungsten, (C) 0.6 µ gold and (D) 1 µ gold particles. The bar indicates 1 µm. (**E**) The size distribution of the tungsten particles was determined by sedigraphic analysis. (**F**) The size distribution of the gold particles was determined by electron microscopy.

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