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Screening of Nanoparticle Embryotoxicity Using Embryonic Stem Cells

Luisa Campagnolo, Ivana Fenoglio, Micol Massimiani, Andrea Magrini, and Antonio Pietroiusti

Abstract

Due to the increasing use of engineered nanoparticles in many consumer products, rapid and economic tests for evaluating possible adverse effects on human health are urgently needed. In the present chapter the use of mouse embryonic stem cells as a valuable tool to in vitro screen nanoparticle toxicity on embryonic tissues is described. This in vitro method is a modification of the embryonic stem cell test, which has been widely used to screen soluble chemical compounds for their embryotoxic potential. The test offers an alternative to animal experimentation, reducing experimental costs and ethical issues.

Keywords: Nanoparticles, ENP, Engineered nanoparticles, Nanomaterials, Embryo, In vitro test, Mouse embryonic stem cells

Electronic supplementary material

The online version of this chapter (doi:10.1007/7651_2013_11) contains supplementary material, which is available to authorized users.

1 Introduction

Over the last two decades the introduction of nanotechnology and the use of engineered nanomaterials (ENMs) have brought considerable progress in a number of industry, medicine, and basic research fields. Developing new nanomaterials with enhanced physicochemical properties has thus attracted great interest from the industrial and scientific community. In this respect, identification of challenges that nanomaterials may pose to public health and the environment has become a concern. Many data have been published demonstrating adverse effects of engineered nanoparticles (ENPs) on different cell types in vitro and in vivo (1–3). Most of the studies have focused on the respiratory and immune systems and only very recently possible adverse effects on mammalian embryonic development

have been investigated for a limited number of ENPs. Indications that certain nanoparticles might negatively interfere with embryonic development may be inferred from studies on the development of the zebrafish embryo (4, 5), a useful model to study molecular mechanisms underlying embryonic development; fish and mammal development, however, can only be compared for limited aspects. The need for information on embryotoxic effects of ENPs in mammals has stimulated studies to identify in vitro model systems to rapidly screen different ENPs, and also to identify physicochemical properties that might be modulated to limit such effect.

A few years ago, an in vitro test using mouse embryonic stem (mES) cells was developed to evaluate embryotoxicity of chemical compounds (6, 7). Such method has been validated by the European Committee for the Validation of Alternative Methods (ECVAM) and is currently used in the pharmaceutical industry. By using two stable cell lines (NIH3T3 and mES cells, representing differentiated and embryonic tissue, respectively) the embryonic stem cell test (EST) aims to identify the concentrations of a tested compound that inhibit by 50 % the proliferation of the two cell lines and the differentiation of mES cells. An algorithm eventually integrates the three values, called IC_{503T3}, IC_{50mES}, and ID_{50}, to infer an evaluation of embryotoxicity for the compound.

Only few attempts have been reported in recent years to in part apply the EST to the evaluation of silica, cobalt ferrite, and gold nanoparticles (8, 9). We have recently published data on the use of the complete EST protocol to evaluate the embryotoxic potential of single-walled carbon nanotubes (SWCNTs). An essential difference with the two above-mentioned studies was that to validate the EST as a test that might be used to reliably predict in vivo effects of ENPs, we additionally performed parallel in vivo experiments on pregnant mouse females that did demonstrate a correspondence between the in vitro and in vivo results (10).

Few issues need to be addressed when applying the EST to nanomaterials. For any tested material, determination of elemental composition (including trace elements, size, shape, solubility, surface coating, and charge) is of primary importance. The techniques used for such physicochemical characterization largely depend on the kind of nanoparticle and therefore a detailed description of methods cannot be done here. This information is however essential for the correct interpretation of the EST results.

A crucial concern is the preparation of nanoparticle suspensions. Nanoparticles need to be uniformly dispersed in the medium and the suspension needs to be stable enough to allow dosage. Adsorption of medium components, in particular proteins, at the surface of nanoparticles may alter the repulsive forces existing among particles, thus causing either particle agglomeration or stabilization of the suspension (11–13). The formation of agglomerates has to be possibly avoided. Thus, analysis of nanoparticle dispersion in culture medium and sonication prior to use are key

2 Materials

2.1 Cell Cultur
Equipment and
Preparation of M

factors. In addition, interaction of nanoparticles with the cell medium components might alter medium composition due to (1) partial dissolution of the nanoparticles or (2) adsorption of small molecules or proteins at the surface of the nanomaterials. In this respect, since among the different components of cell culture medium, proteins are those having the largest affinity for the surface, depletion of proteins, leading to possible cell toxicity, needs to be also controlled.

Here we report a protocol for the assessment of ENP embryotoxicity using the EST, including the basic chemical determination, which is needed prior to the biological experiments.

2 Materials

All media and medium supplements are purchased from Lonza (Basel, Switzerland) and are endotoxin free. To guarantee that all tests are performed with the same batch of serum, thus reducing experimental variability and allowing comparison between results, serum batches—prescreened for supporting either stemness or differentiation—are purchased in amounts sufficient to cover the prospected experiments and stored at −80 °C until use, to guarantee that all tests are performed with the same batch of serum, reducing experimental variability and allowing comparison of the results. Leukemia-inhibiting factor (LIF) is purchased from Immunological Sciences (Rome, Italy).

All disposable cell culture supplies (plates, flasks, pipettes, tubes) are purchased from Corning Inc. (NY, USA) and have been tested for supporting proliferation of undifferentiated mES cells.

2.1 Cell Culture Equipment and Preparation of Media

1. Cell lines: mES D3 clone, representing the embryonic tissue, and the fibroblast cell line NIH3T3, representing the differentiated tissue, are purchased from the American Type Culture Collection (Manassas, VA, USA) and are stored in liquid nitrogen upon arrival.
2. Preparation of primary mouse embryonic fibroblasts (MEFs): Stereomicroscope; tissue culture hood; benchtop centrifuge; mouse embryos at day 13 or 14 of gestation; PBS; watchmaker forceps; trypsin–EDTA solution; scalpels.
3. MEF medium: For 500 ml of medium mix 435 ml DMEM with 50 ml heat-inactivated fetal calf serum (FCS), 10 ml 1 M Hepes, and 5 ml of a 5,000 U/ml penicillin–5 mg/ml streptomycin solution. Store at 4 °C.
4. mES media:
   (a) mES pluripotency medium: For 500 ml of medium, mix 400 ml of DMEM with 75 ml of heat-inactivated FCS, 10 ml of 1 M Hepes, 5 ml of 10 mM NEAA, 1 ml of 55 mM β-mercaptoethanol, 5 ml of 200 mM L-glutamine,

and 5 ml of 5,000 U/ml penicillin–5 mg/ml streptomycin solution. Store at 4 °C. Before use, transfer 10 ml of medium in a conical tube and add 10 µl of LIF (100 U/µl), to make the ES cell medium.

(b) mES proliferation medium: It is the same as the pluripotency medium without the final addition of LIF.

(c) mES differentiation medium: For 500 ml of differentiation medium, mix 424 ml DMEM with 50 ml heat-inactivated FCS, 10 ml 1 M Hepes, 5 ml 10 mM NEAA, 1 ml 55 mM β-mercaptoethanol, 5 ml 200 mM L-glutamine, and 5 ml of 5,000 U/ml penicillin–5 mg/ml streptomycin solution. Store at 4 °C.

5. NIH3T3 cell medium: For 500 ml mix 430 ml DMEM with 50 ml heat-inactivated FCS, 10 ml of 1 M Hepes, 5 ml of 200 mM L-glutamine, and 5 ml of 5,000 U/ml penicillin–5 mg/ml streptomycin solution. Store at 4 °C.
6. Gelatine solution: To make 500 ml of 0.7 % gelatine, weigh 3.5 g of cell culture-tested gelatine type A (C1890, Sigma Chemical Company, St. Louis, MO, USA) and add the powder to a clean bottle containing 500 ml of freshly made 18 MΩ distilled water; immediately autoclave and store at room temperature.

2.2 Proliferation Assays

Cell Proliferation Reagent WST-1 (Roche Diagnostics, Indianapolis, IN, USA). ELISA reader.

3 Methods

3.1 Preparation of Nanoparticle Suspensions

1. Weight 4 mg of nanoparticles and add 2 ml of a suitable solvent (stock solution) which needs to be determined depending upon the kind of material. In general, for hydrophilic nanoparticles (uncoated metal oxides or nanoparticles coated with charged or hydrophilic coatings) water or cell medium (DMEM) containing FCS (between 5 and 10 %) may be used. PBS generally decreases the stability of the suspension and therefore should be avoided. In the case of hydrophobic nanoparticles (unfunctionalized carbon-based or polymeric nanoparticles, nanoparticles coated with hydrophobic materials) medium containing FCS gives in some cases good results. Ethanol or a mixture of water/ethanol, DMSO, or surfactant like Tween 80 may also be used to improve dispersion.
2. Sonicate the stock solution for 10–30 min (bath sonicator, 40 W). Alternatively, a probe sonicator (100 W) may be used. In this case sonicate at a potency of 50 % for 1–2 min by maintaining the suspension on ice.

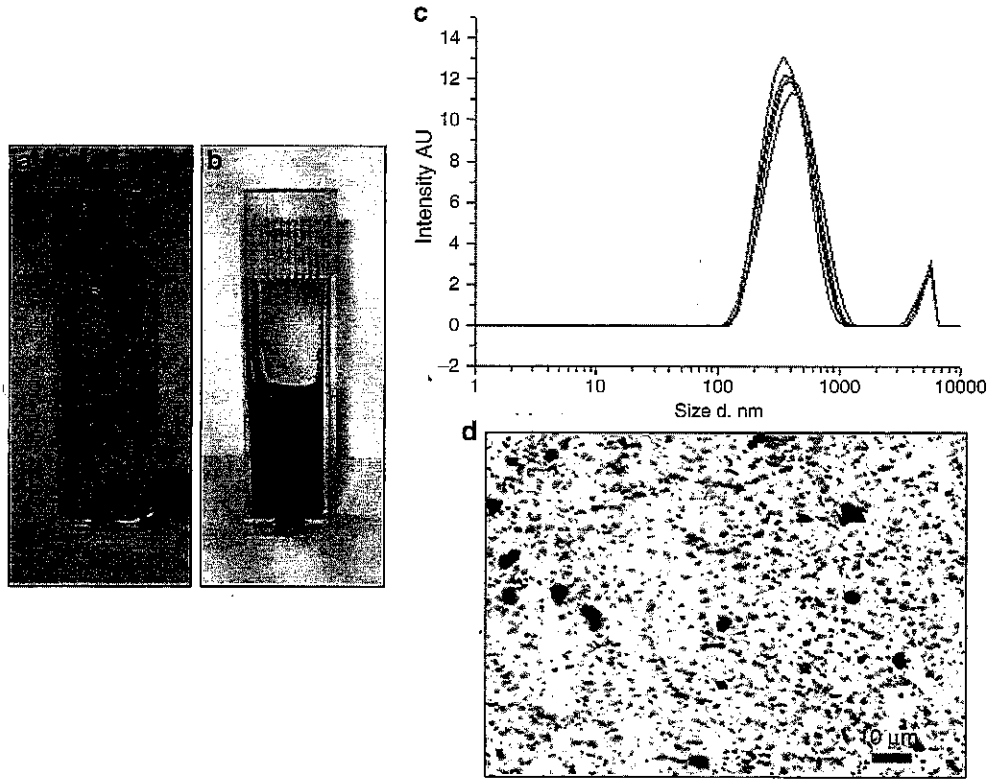

Fig. 1 Example of a nanoparticle suspension: Dispersion of carbon nanotubes. (**a**) Carbon nanotubes in PBS; (**b**) carbon nanotubes in culture medium + 10 % FCS; (**c**) measurements of agglomerate size (DLS) in the suspension shown in (**b**). The *curves* correspond to five measurements over a period of 15 min; (**d**) optical micrographs of the suspension shown in (**b**)

3. Add 10 ml of ES or NIH3T3 medium to 1, 0.5, 0.05, 0.005, and 0.0005 ml of stock solution (final concentrations 200, 100, 10, 1, and 0.1 µg/ml) and repeat the sonication. Use the prepared suspensions within few minutes.

3.2 Analysis of Nanoparticle Dispersion in Culture Media

1. Transfer 1.5 ml of the suspension in a disposable cuvette and analyze the size of aggregates (upper limit resolution of 1 µm) by dynamic light scattering (DLS) technique (see Note 1). Perform several measurements on the same suspension to evaluate its stability during the time needed for seeding the cells (Fig. 1).

3.2.1 Dissolution of the Nanoparticles

1. Incubate 2 ml of the suspension prepared as in Section 3.1 at 37 °C.
2. After 10 days, centrifugate the suspension at >13,000 × *g* to remove nanoparticles from the medium.
3. Collect the supernatant and pass it through a 100 nm acetate cellulose disposable filter.

4. Add to the collected supernatants 10 ml of a mixture 1:1 of H_2SO_4/HNO_3 and heat by using a water bath at 100 °C until the solutions become transparent.
5. Analyze the solutions diluted up to 100 ml with doubly distilled water by inductively coupled plasma atomic emission spectrometry (AE-ICP).

3.2.2 Adsorption of Proteins

1. Incubate nanoparticles in ES or NIH3T3 medium and collect supernatant as reported in Section 3.2.1.
2. Measure total protein content in the medium before and after incubation with nanoparticles using the bicinchoninic acid (BCA) assay (biuret method).

3.3 Preparation of γ-Irradiated Mouse Embryonic Fibroblasts

1. Isolate embryos, place them in a culture dish filled with PBS, and wash them. Perform all the following steps in a tissue culture hood.
2. Transfer embryos in a clean dish. Under the dissection microscope, use forceps to remove head and internal organs from embryos, leaving only the carcasses.
3. Wash carcasses twice with PBS, and place them in a clean dish with a few milliliters of 0.5 % trypsin–EDTA (enough to cover the embryos).
4. Using a scalpel, mince carcasses into small pieces; for ten embryos this step should take approximately 15 min. Add 2 ml more of trypsin–EDTA, mix with the tissue, and incubate for 30 min at 37 °C.
5. In the meanwhile prepare MEF medium.
6. Remove the dish from the incubator and add 10 ml of MEF medium. Transfer the suspension to a 50 ml conical tube and dissociate the tissue by vigorous pipetting using a 10 ml serological pipette.
7. Allow large fragments to settle down by gravity and gently transfer the supernatant to a new tube.
8. To the tube containing the fragments add 10 ml of MEF medium and pipette again. Repeat this three more times.
9. Combine all supernatants so that at the end of the procedure, a tube containing about 40 ml of cell suspension should be obtained. Plate cells in T165 flasks considering one embryo per flask.
10. After 2 days cells should reach confluence and can be frozen with DMSO (two vials from each flask). Store cells in liquid nitrogen. These vials are the stock of MEF cells and are used to prepare the γ-irradiated feeder layer.

3.4 Culture of
Cells

11. To make γ-irradiated MEFs, thaw one vial of primary MEFs from liquid nitrogen, transfer the suspension to a 15 ml conical tube, and centrifuge for 10 min at 120 × *g* to pellet the cells.
12. Remove supernatant, resuspend the pellet in 6 ml MEF medium, split the volume in three T165 flasks containing 30 ml of MEF medium, and incubate.
13. When at confluence (it usually takes 3 days) harvest cells and plate them in nine T165 flasks.
14. After an additional 3–4 days (or when at confluence) harvest once more the cells and plate them in twenty-seven 150 mm maxiplates.
15. Wait for cells to reach the confluence and then trypsinize each dish with 2 ml of 0.25 % trypsin–EDTA. Combine cell suspensions from all dishes in a 50 ml tube and centrifuge for 10 min at 120 × *g*.
16. Remove supernatant and resuspend pellet in 45 ml MEF medium. Irradiate cells with 30 Gy using a γ-irradiation source.
17. Add 5 ml DMSO, split in 0.5 ml aliquots in freezing vials, and store at −80 °C. Each vial is sufficient to make a feeder layer for a T75 flask.

3.4 Culture of mES Cells

All the following procedures are carried in a cell culture hood.

1. Gelatinize a T25 flask with 3 ml of a 0.7 % gelatine solution and incubate it for 15 min at 37 °C.
2. In the meanwhile, thaw at 37 °C an aliquot of γ-irradiated mouse fibroblasts and transfer to a 15 ml conical tube containing 4.5 ml of DMEM; centrifuge at 120 × *g* for 5 min, discard the supernatant, and resuspend the pellet in 1 ml MEF medium.
3. Remove excess gelatine from the flask and add 4.5 ml MEF medium. Transfer 0.5 ml of the MEF cell suspension to the flask. Place the flask in the incubator (37 °C, 5 % CO_2) for 30 min.
4. After about 20 min, quickly thaw mES cells from liquid nitrogen; transfer the suspension to a 15 ml conical tube prefilled with 4 ml DMEM. Centrifuge for 5 min at 120 × *g*. Remove supernatant and suspend the pellet in 5 ml ES medium.
5. Remove from the incubator the T25 flask and check for the presence of adhered MEF cells under an inverted microscope. At this time most MEFs should appear as small round, dark gray cells with a central nucleus attached to the flask, and it generally takes a day for them to spread completely and assume the typical fibroblast phenotype.

6. Aspirate the medium from the flask and add the ES cell suspension. Incubate at 37 °C in an atmosphere of 5 % CO_2. ES cells are usually subcultured every 2–3 days; medium is replaced everyday.

3.5 Cell Proliferation Experiments

3.5.1 Proliferation of mES Cells

1. When mES colonies are about 80 % confluent, trypsin–EDTA the culture in order to obtain a single-cell suspension.
2. Plate the cells on a gelatinized 10 cm culture plate containing 7 ml of mES medium and place it in the incubator for 20–30 min, to remove the more adherent MEFs.
3. Collect the culture supernatant, centrifuge it at 120 × *g* for 5 min, and resuspend the pellet in 2 ml of mES proliferation medium. Carefully count cells with a hemocytometer, ignoring possible contaminating MEFs that appear much larger than mES.

3.5.2 Proliferation of NIH3T3 Cells

4. Using mES proliferation medium, dilute the cell suspension to 10,000 mES/ml, pipette 50 µl per well of a 96-well plate that has been previously gelatinized, and place in the incubator to allow cells to adhere to the plate (see Note 2).
5. After 2 h, add to each well 150 µl of control medium or medium containing the nanoparticles under evaluation that have been previously sonicated as reported in Section 3.1. Do so following the scheme in Fig. 2: In each well at the periphery of the plate add mES proliferation medium. In columns 2, 6,

3.5.3 Cell Viability

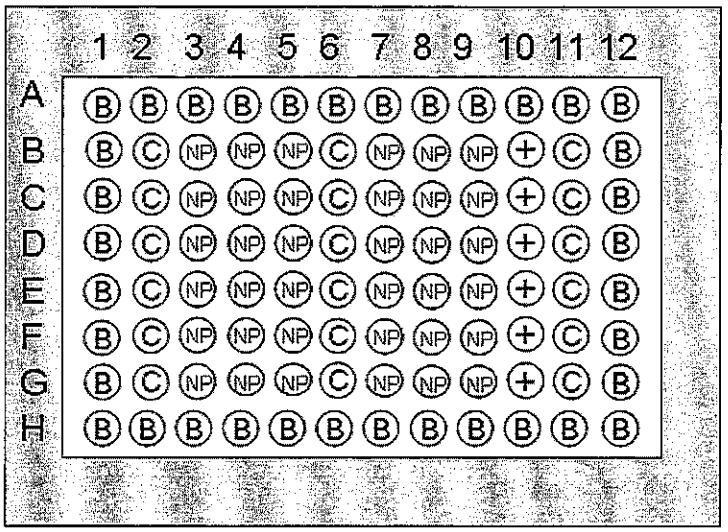

Fig. 2 Preparation of a 96-well plate for the proliferation assay. (*B*) Blank made with the culture medium; (*C*) cells grown in the presence of culture medium and the ENP suspension medium (vehicle control); (*+*) cells grown in the presence of a proven embryotoxic compound (positive control); (*NP*) cells grown in the presence of ENPs

3.6 mES Cell Differentiation Experiments

and 11 add proliferation medium containing the nanoparticle suspension vehicle (use the amount present in the highest ENP concentration tested). In columns 3–5 and 7–9 add proliferation medium containing ENP at different concentrations. In a first pilot experiment use a range of concentrations between 0.1 and 100 µg/ml, from which the test concentrations for the following experiments can be extrapolated. In column 10 add mES proliferation medium containing a known embryotoxic compound, e.g., 0.086 µg/ml 5-Fluorouracil.

6. Following the scheme previously used, replace medium at days 3 and 7 of culture. After 10 days of culture, perform the WST-1 colorimetric assay.

3.5.2 Proliferation of NIH3T3 Cells

1. Plate 500 NIH3T3 cells suspended in 50 µl 3T3 medium in each well of a 96-well plate and let them adhere for 2 h.
2. Add the ENPs under study diluted in 150 µl of the same medium at the desired concentration. Initially test a concentration range between 1 and 200 µg/ml (see Note 3).
3. On day 6 of culture, perform the colorimetric assay, using the cell proliferation reagent WST-1, as detailed in the following section.

3.5.3 Cell Viability Tests

1. Thaw the WST-1 reagent (see Note 4). Mix in a tube 9.8 ml of culture medium and 980 µl of the WST-1 reagent.
2. Remove medium from the 96-well plate.
3. Wash wells twice with 150 µl PBS.
4. Dispense 110 µl of the diluted WST-1 solution in each well.
5. Place the plate in the incubator for 2 h.
6. Remove the plate from the incubator, shake it for 1 min on a shaker, and read the absorbance against a background control using a microplate reader (Bio Rad Microplate Reader 3550) at 450 nm, with reference wavelength at 655 nm.
7. Analyze data and present them as percentages relative to control (mean ± standard error).

3.6 mES Cell Differentiation Experiments

1. Harvest mES cells (obtained as in Section 3.5.1) and plate them on a gelatinized 10 cm plate containing 7 ml of mES medium; incubate at 37 °C for 20–30 min.
2. Collect and count cells in the supernatant, and centrifuge the suspension at 120 × *g* for 5 min. Discard supernatant and suspend the pellet in differentiation medium in order to have 37,500 cells in 20 µl.
3. Fill one 1 ml tube for every ENP concentration to be tested with 950 ml differentiation medium; add 30 µl of ENP suspension at the appropriate concentrations (test the same

concentration range used for the mES cell proliferation assays). Add 20 µl of the cell suspension and mix by pipetting, and then aliquot the obtained suspension on the lid of a 10 cm plate making forty to fifty 20 µl drops. With a gentle but firm movement invert the lid with the drops and place it to cover the bottom part of the plate containing 10 ml of deionized water. Place the plate in the incubator.

4. After 3 days prepare a number of 6 cm Petri dishes equivalent to the number of 1 ml tubes previously prepared, and fill each of them with 5 ml differentiation medium containing each of the ENP concentration under test.
5. Remove from the incubator one plate with a given concentration of ENPs, gently remove and invert the lid, and with the help of a dissection microscope, collect all the drops, which now contain small spheres of aggregated cells, the embryoid bodies (EBs). Transfer all the EBs in a 6 cm plate containing differentiation medium and the correspondent ENP concentration. Put in the incubator. Repeat the same procedure for all dishes.
6. After 2 days, prepare two 24-well plates for each 6 cm dish by putting in each well 1 ml of differentiation medium containing the correspondent concentration of ENPs. Transfer one EB per well.
7. After 5 additional days of culture, using an inverted microscope evaluate the presence of beating cell areas in each EB, reflecting the differentiation of contractile cardiomyocytes (see Video 1 in supplementary material). Identify by a dose–response curve the concentration of ENPs that inhibits by 50 % the formation of contracting EBs (ID_{50}) (see Note 5).

EST algorithm

The obtained IC_{50} and ID_{50} values are introduced in the following algorithm:

Function I: $5.92\ \lg(IC_{503T3}) + 3.50\ \lg(IC_{50mES}) - 5.31(IC_{503T3} - ID_{50}/IC_{503T3}) - 15.27$

Function II: $3.65\ \lg(IC_{503T3}) + 2.39\ \lg(IC_{50mES}) - 2.03(IC_{503T3} - ID_{50}/IC_{503T3}) - 6.85$

Function III: $-0.125\ \lg(IC_{503T3}) - 1.92\ \lg(IC_{50mES}) + 1.50\ (IC_{503T3} - ID_{50}/IC_{503T3}) - 2.67$

According to the standard classification applied to chemical compounds from EST data, ENPs are classified into three classes:

Class 1, non-embryotoxic, if I > II and I > III.

Class 2, weakly embryotoxic, if II > I and II > III.

Class 3, strongly embryotoxic, if III > I and III > II.

4 Notes

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4 Notes

1. DLS measurements, although give a good estimation of nanoparticle dispersion, underestimate the amount of small aggregates.
2. Accuracy of pipetting when performing the proliferation assays is fundamental to reduce variability. In proliferation assays always prepare suspensions and solutions for at least two additional wells, to correct for pipetting errors and avoid ending up without enough solution for the last wells. We have experimented that the use of a p100 instead of a multichannel pipette gives better results. The multichannel pipette helps during the PBS washing steps.
3. Consider that 3T3 cells are generally less sensitive to factors perturbing culture conditions than mES; thus, when planning experiments with 3T3, it is advisable to test a concentration range ten times higher than that used for mES cells.
4. To evaluate cell viability and proliferation, several cell proliferation reagents can be used. As a general rule, when investigating the cytotoxic effect of nanoparticles, possible interference of the nanoparticles themselves with the colorimetric assay used needs to be assessed.
5. Results from the differentiation experiments are to be discarded if in the control plate less than 75 % of EBs acquire a contractile phenotype.

Electronic Supplementary Material

Below is the link to the electronic supplementary material.

7651_2013_MOESM1_ESM.avi
Video 1. Visualization of contracting EBs at the end of the differentiation experiments (AVI 15,590 kb)

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Abstract

Self-assembled p
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1 Introducti