Published in final edited form as:

Nat Protoc. 2014 February ; 9(2): 233–245. doi:10.1038/nprot.2014.002.

Engineering cells with intracellular agent-loaded microparticles to control cell phenotype

James A Ankrum^{1,2}, Oscar R Miranda^{1,2}, Kelvin S Ng^{1,2}, Debanjan Sarkar³, Chenjie Xu⁴, and Jeffrey M Karp^{1,2}

¹Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts, USA

²Harvard-MIT Division of Health Sciences and Technology, Harvard Stem Cell Institute, Cambridge, Massachusetts, USA

³Department of Biomedical Engineering, University at Buffalo, The State University of New York, Buffalo, New York, USA

⁴Division of Bioengineering, School of Chemical and Biomedical Engineering, Nanyang Technological University, Singapore

Abstract

Cell therapies enable unprecedented treatment options to replace tissues, destroy tumors and facilitate regeneration. The greatest challenge facing cell therapy is the inability to control the fate and function of cells after transplantation. We have developed an approach to control cell phenotype *in vitro* and after transplantation by engineering cells with intracellular depots that continuously release phenotype-altering agents for days to weeks. The platform enables control of cells' secretome, viability, proliferation and differentiation, and the platform can be used to deliver drugs or other factors (e.g., dexamethasone, rhodamine and iron oxide) to the cell's microenvironment. The preparation, efficient internalization and intracellular stabilization of ~1- μ m drug-loaded microparticles are critical for establishing sustained control of cell phenotype. Herein we provide a protocol to generate and characterize micrometer-sized agent-doped poly(lactic-co-glycolic) acid (PLGA) particles by using a single-emulsion evaporation technique (7 h), to uniformly engineer cultured cells (15 h), to confirm particle internalization and to troubleshoot commonly experienced obstacles.

© 2014 Nature America, Inc. All rights reserved.

Correspondence should be addressed to J.M.K. (jeffkarp.bwh@gmail.com).

AUTHOR CONTRIBUTIONS J.A.A., C.X., D.S., and J.M.K. conceived of the protocol and its initial applications. J.A.A., O.R.M. and K.S.N. optimized the protocol. J.A.A., O.R.M., K.S.N. and J.M.K. wrote the manuscript. J.A.A. designed and prepared all figures.

COMPETING FINANCIAL INTERESTS The authors declare competing financial interests: details are available in the online version of the paper.

Reprints and permissions information is available online at <http://www.nature.com/reprints/index.html>.

INTRODUCTION

The success of exogenous cell therapies depends on the fate, function and viability of cells after transplantation. Controlling the phenotype and engraftment of cells after transplantation is crucial for the success of cell-based therapies. Unlike the exquisite control that one can exert over cells in a culture dish, once cells are transplanted they are entirely at the mercy of the biological milieu and behave differently depending on their location. The lack of control of transplanted cells leads to variability in cell function and ultimately poor therapeutic outcomes^{1,2}.

Both allogeneic and autogenic cell-based therapies are prone to variability because of heterogeneity within and between cell populations that can be affected by differences in donors, isolation techniques and culture mediums. For example, the propensity of embryonic stem cells and induced pluripotent stem cells (iPSCs) to differentiate into specific lineages has been shown to vary markedly within and between cell lines³. Variation in the glucose sensitivity of transplanted pancreatic islets can lead to a failure to restore insulin independence⁴. In addition, mesenchymal stem cell (MSC) differentiation efficiency down osteogenic, chondrogenic or adipogenic lineages is strongly influenced by the MSCs' tissue of origin⁵. Furthermore, the ability of MSCs to secrete growth factors, chemokines and cytokines in response to inflammatory stimuli and suppress activated T cells varies considerably between donors^{2,6}. Specifically, MSC secretion of vascular endothelial growth factor⁶, a primary mediator of MSCs' angiogenic potential, and production of indoleamine 2,3-dioxygenase², a primary mediator of MSCs' immunomodulatory potential, vary depending on the donor from which the MSCs are isolated. Thus, there is a need to develop methods to polarize MSCs toward therapeutic phenotypes to maximize their therapeutic potency regardless of their source. Although small-molecule drugs have the ability to influence MSC phenotype *in vitro*⁷⁻¹⁰, applying preconditioning regimens to transplanted cells has been substantially limited given that they typically activate signal transduction pathways only for short durations and thus the induced effects do not persist after transplantation.

Development of particle-engineered cells

To maximize potency, establish stable control of cell phenotype and longitudinally track cell distribution after transplantation, we developed a technique for engineering cells with intracellular agent-loaded microparticles¹¹. By using an osteogenic differentiation assay, we demonstrated the ability of internalized dexamethasone-loaded microparticles to stimulate uniform differentiation of MSCs¹¹. Furthermore, drug released from particle-engineered cells into the microenvironment induced the differentiation of unmodified neighboring and distant cells in a paracrine-like and endocrine-like manner (see Sarkar *et al.*¹¹ for a detailed report). In addition to establishing control over MSC differentiation, we observed that the efficiency of MSC particle internalization was dependent on the size (assessed by dynamic light scattering (DLS) and confocal microscopy), as well as on the surface properties of PLGA microparticles¹¹.

PLGA microparticles were formed from PLGA with a carboxylic acid end group, resulting in particles with a negative ζ -potential. The surface properties of particles was then modified

through adsorption of polycationic polymers (poly-L-lysine, PLL) or chemical conjugation of antibodies or lipids to the particle surface by using N-hydroxysuccinimide-biotin chemistry (EX-Link NHS-Biotin, Thermo Scientific)¹¹. Confocal microscopy was used to evaluate the efficiency of particle uptake by MSCs. MSCs more efficiently internalized antibody-coated or positively charged particles over negatively charged particles (ζ -potential measured by Zetasizer)¹¹. Although multiple cell types efficiently internalize nanoparticles, substantial particle leakage through exocytosis has been documented^{12–16}. In addition, nanoparticles (<200 nm) typically exhibit lower drug loading and faster release compared with larger microparticles, limiting sustained control of cell phenotype. In contrast to nanoparticles that can be quickly exocytosed or cellular backpacks that are designed to remain on the cell surface^{17,18}, we discovered that particles ~1 μ m in diameter remained internalized within MSCs for several weeks.

To demonstrate the utility of this approach as a platform, we recently adapted it to enable longitudinal tracking of MSCs after transplantation. Tracking the location, engraftment and distribution of cells after transplantation is crucial for evaluating the success of cell-based therapies. Although iron oxide nanoparticles have been used to track cells by MRI, low iron content per cell and nanoparticle exocytosis prevented detailed and longitudinal monitoring of a cell's location. To overcome these limitations, iron oxide nanoparticles were encapsulated within ~1- μ m PLGA microparticles, resulting in substantially enhanced iron oxide loading and increased r_2 relaxivity of MSCs¹⁹. In addition, the enhanced residence time of microparticles within MSCs enabled cells to be detected by MRI for >12 d compared with only 4–6 d for nanoparticle-engineered MSCs (see Xu *et al.*¹⁹ for a detailed report).

We have shown that particle formulations containing dexamethasone, rhodamine or iron oxide remain stable within MSCs for >18 d, making this a useful platform for prolonged exposure to small molecules¹¹ and simultaneous longitudinal tracking of a cell's location¹⁹. Furthermore, particle-engineered MSCs maintain their phenotype after cryopreservation, thus enabling off-the-shelf control of MSC phenotype¹¹.

Nuances and limitations of the particle engineering platform

Previously, we have shown the flexibility of the platform through cell internalization of particles encapsulated with hydrophobic small molecules, rhodamine 6G and dexamethasone, as well as iron oxide nanoparticles^{11,19}. Drug loading and release kinetics can be tuned for specific applications by modifying the particle synthesis protocol through changing the composition and molecular weight of the polymer (e.g., 10-kDa PLGA degrades faster than 50-kDa PLGA, and it will result in a faster drug release). Each new batch of particles must be analyzed for drug loading and release profile as described in this protocol.

Although we anticipate that adaptation of the platform to other hydrophobic drugs will be straightforward, encapsulation and delivery of hydrophilic molecules including peptides, proteins, DNA and RNA have yet to be optimized for this platform and will require substantial modification and optimization of the protocol. Hydrophilic small molecules can be adapted to the platform by modifying particle synthesis. For example, co-solvents, such as methanol or trifluoroethanol, or double-emulsion techniques can be used to enhance the

encapsulation of hydrophilic small molecules in microparticles^{20–22}. Thus, iteration of particle formulation strategies should enable adaptation of the particle-engineered MSC platform to hydrophilic drugs.

Over the past 20 years, numerous strategies have been developed to influence the kinetics of drug release from polymeric particles by modifying the choice of solvent, initial drug loading and the molecular weight and composition of the polymer. We anticipate that these strategies can readily be adapted to this protocol to tune release kinetics to continuously release drugs over days to weeks and potentially even months depending on the application.

In addition to small molecules, many biological agents including proteins, RNA and DNA have been used to control a cell's phenotype, including its expression of cell surface receptors, secretome and differentiation^{23–27}. Although techniques for delivering these agents have been established, substantial challenges remain and care must be taken not to damage the structure of the molecules. Secondary and tertiary structures may be damaged during particle synthesis, owing to exposure to organic solvents and high-intensity agitation, or upon sorting to acidic lysosomes after particle endocytosis.

Although PLGA nanoparticles have been reported to undergo endolysosomal escape to deliver genes and siRNA^{28–31}, achieving efficient intracellular delivery without inducing cytotoxicity remains a challenge³². In addition, if the goal is to deliver the agent to an extracellular target, as in the case of growth factors that bind to cell surface receptors, the agent must be able to transverse the plasma membrane via diffusion or active transport. Therefore, although this platform can be easily adapted to accommodate a variety of agents, a molecule's structure, target and susceptibility to degradation should be considered.

In addition, we anticipate that the platform could potentially be adapted to accommodate the use of particles made from other materials such as alginate, which may be desirable for encapsulating hydrophilic or sensitive molecules such as peptides or proteins.

With these considerations in mind, the protocol herein will serve as a guide for successfully establishing nonviral transient control over locally or systemically administered cells to develop more effective cell-based therapies (Fig. 1).

MATERIALS

REAGENTS

Particle preparation

- 50:50 poly(DL-lactic-co-glycolic)-COOH (PLGA), i.v. 0.15–0.25 g dl⁻¹ (Lactel Absorbable Polymers, cat. no. B6013-1)
- 50/50 poly(DL-lactic-co-glycolic)-COOH (PLGA), i.v. 0.55–0.75 g dl⁻¹ (Lactel Absorbable Polymers, cat. no. B6013-2)
- Dichloromethane (DCM) (Sigma-Aldrich, cat. no. 270997-100ML)

! CAUTION DCM is an eye and skin irritant and harmful if swallowed. Use proper personal protective equipment (PPE) and always work in a chemical fume hood.

- Poly(vinyl alcohol) (PVA), M_w 9,000–10,000, 80% hydrolyzed (Sigma-Aldrich, cat. no. 360627-25G)
- Filtered water (Milli-Q water or Sigma-Aldrich, cat. no. W4502-1L)
- Glass scintillation vials with polyvinyl-lined caps (VWR, cat. no. 66010-267)
- Rhodamine 6G (Sigma-Aldrich, cat. no. 252433-250MG)
- Poly-L-lysine hydrochloride, $MWCO >30,000$ Da (Sigma-Aldrich, cat. no. P9404-25MG)
- Pasteur pipette (Fisher Scientific, cat. no. 13-678-4A)
- Pasteur pipette rubber bulbs (Sigma-Aldrich, cat. no. Z111597-12EA)
- Cell strainer, 40- μ m (Fisher Scientific, cat. no. 22-363-547)
- Steriflip 0.22- μ m vacuum filter, 50 ml (Millipore, <http://www.millipore.com/>, cat. no. SCGP00525)
- Transfer pipette (VWR, cat. no. 16001-180)
- Aluminum foil (VWR, cat. no. 89068-734)
- Disposable capillary cell (ζ -potential) (Malvern, cat. no. DTS1061)
- Square cuvette, 12 mm (DLS) (Malvern, cat. no. DTS0012)
- Methanol (Sigma-Aldrich, cat. no. 34860-4X4L-R)
- DMSO (Sigma-Aldrich, cat. no. 472301-100ML)

Cell engineering

- Human mesenchymal stem cells at 70% confluence (<http://medicine.tamhsc.edu/irm/msc-distribution.html>); examples of cells that have been used so far are discussed in the ANTICIPATED RESULTS; we have not yet encountered cells that could not be engineered with particles

! CAUTION Human cell lines may harbor pathogens. Use proper PPE, adhere to all institutional ethics guidelines and handle cells in a BSL-2-certified biosafety cabinet.

- T25 culture flask (VWR, cat. no. 29185-300)
- MEM- α (Invitrogen, cat. no. 12561-072)
- FBS (Atlanta Biologicals, cat. no. S11550)
- Penicillin-streptomycin (Invitrogen, cat. no. 15140-163)
- L-Glutamine (Invitrogen, cat. no. 25030-081)
- PBS without calcium chloride and magnesium chloride (PBS-/-; Sigma-Aldrich, cat. no. A00475)

Analysis

- 20-kDa-MWCO dialysis tubing (Fisher Scientific, cat. no. 08-607-068)
- Paired standard and weighted dialysis closures (Spectrum Labs, cat. no. 132749)
- Fluorodish glass-bottom dish (World Precision Instruments, cat. no. FD35-100)
- Vybrant DiO cell-labeling solution (Invitrogen, cat. no. V-22886)

! CAUTION This solution contains the organic solvent and carcinogen dimethylformamide. Wear proper PPE and handle it with caution.

- Hoechst stain (Invitrogen, cat. no. H3570)
- Neutral-buffered formalin, 10% (vol/vol) (Sigma-Aldrich, cat. no. HT501128)

! CAUTION Formalin contains formaldehyde, a carcinogen and eye, respiratory and skin irritant. Wear proper PPE and handle it in a chemical fume hood.

EQUIPMENT

Particle preparation

- Scale, Mettler Toledo X5105 DualRange
- Glass beaker, 50 ml
- Magnetic stir bar, 0.5-inch
- Stir plate, Corning PC-420D
- Probe sonicator, Misonix Sonicator 3000 with microtip
- Tissue homogenizer, Omni International Tissue Master 125 with 7-mm probe
- Clamp stand
- Centrifuge, Eppendorf 5430 centrifuge
- Mini-centrifuge
- Lyophilizer

Particle characterization

- Zetasizer, Malvern Instruments, ZEN 3690
- Fluorescence microscope, Nikon Eclipse TE2000U
- Benchtop flow cytometer, Accuri C6
- Confocal microscope, Zeiss 700

REAGENT SETUP

PLL solution—Dissolve 4 mg of PLL into 40 ml of filtered distilled water to make a 0.01% (wt/vol) PLL solution. Store the solution at 4 °C for up to 3 months.

PROCEDURE

Preparation of microparticles ● TIMING 7 h

▲ **CRITICAL** An overview of this stage of the procedure is shown in Figure 2.

- 1| Dissolve 200 mg of PVA in 20 ml of water to make a 1% (wt/vol) PVA solution. Add a stir bar and place it on a magnetic stir plate for 1 h to allow for complete dissolution. Note: concentrations of PVA as low as 0.2% have also been used. (PVA stabilizes the emulsion and prevents the particles from aggregating into larger particles.)

▲ **CRITICAL STEP** PVA can aggregate and adhere to the bottom of the beaker. The position of the stir bar should be periodically adjusted to free aggregates from the beaker surface and to ensure consistent generation of PVA solution.

? TROUBLESHOOTING

- 2| Add 50 mg of PLGA into a 10-ml glass scintillation vial.
- 3| Add 1 mg of rhodamine 6G dye (or a small molecule of choice) into the vial.
- 4| In a chemical fume hood, add 2 ml of DCM to the glass vial containing PLGA/rhodamine.

! **CAUTION** DCM is an eye and skin irritant and is harmful if swallowed. Use proper PPE and always work in a chemical fume hood.

▲ **CRITICAL STEP** DCM will dissolve most plastics; use a glass syringe or glass Pasteur pipette to avoid contamination of the polymer solution.

▲ **CRITICAL STEP** DCM is an organic solvent with a low boiling point. Cap the vial to avoid evaporation and loss of volume.

▲ **CRITICAL STEP** The concentration of polymer in the organic solvent is crucial for determining the final particle size. Reducing the concentration of the polymer in the solvent will result in smaller-diameter particles, whereas increasing the concentration will increase the particle diameter.

■ **PAUSE POINT** A 1- to 2-h break is acceptable at this point, with the vials capped and stored in a fume hood at room temperature (20 °C).

- 5| When the 1% (wt/vol) PVA solution is completely dissolved, filter it through a 0.2-μm vacuum filter into a clean 50-ml glass beaker.

▲ **CRITICAL STEP** The solution will foam and undergo a twofold increase in volume once homogenization begins. Use a 50-ml glass beaker to avoid overflow.

- 6| Place the beaker of PVA solution on ice and allow it to chill to 4–8 °C.

- 7| When the PLGA/rhodamine solution is completely dissolved, probe-sonicate it for 10 s at 10–12 W to ensure even distribution of the small molecule among the polymer chains.

▲ CRITICAL STEP Wash the probe sonicator with acetone and ethanol and dry it completely before use to avoid contamination of particles.

? TROUBLESHOOTING

- 8| Secure the tissue homogenizer with a clamp stand over an ice bucket.
- 9| Place the beaker of PVA in an ice bucket and position the homogenizer probe so that the probe is submerged but not in contact with the glass surface (e.g., 0.5 cm).
- 10| Turn the tissue homogenizer to 35,000 r.p.m. (highest speed on a Tissue Master 125).

! CAUTION Follow the manufacturer's safety instructions in the product manual.

- 11| Use a glass Pasteur pipette to add PLGA solution to the PVA solution dropwise while homogenizing.

! CAUTION Use safety glasses as part of your PPE to avoid a splash hazard.

▲ CRITICAL STEP The solution will foam and undergo a two-fold increase in volume during mixing. Use a 50-ml glass beaker to avoid overflow.

▲ CRITICAL STEP When you are adding PLGA solution to the PVA solution, avoid dripping PLGA onto the homogenizer probe or wall of the beaker.

- 12| Homogenize the mixture for 2 min to create a single emulsion.

? TROUBLESHOOTING

- 13| Turn off the homogenizer and remove it from the beaker.

! CAUTION Disconnect the homogenizer from the energy source before removing it.

- 14| Move the particle suspension to a stir plate in a chemical fume hood and add a 0.5-inch magnetic stir bar.

- 15| Set the stir plate to 300 r.p.m.

- 16| Cover the particle suspension with aluminum foil perforated with 10–20 holes to allow for evaporation of the organic solvent in a chemical fume hood.

- 17| Allow 4–5 h for complete evaporation of the organic solvent.

▲ CRITICAL STEP Incomplete evaporation of the solvent will result in particle aggregation and loss of microparticles in subsequent steps. To test, take 200 μ l of the sample and centrifuge it at 1,000g for 5 min at room temperature.

Particles should easily resuspend into a single-particle suspension in distilled water by triturating with a 1-ml pipette.

▲ CRITICAL STEP Excessive evaporation time will lead to breakdown of particles owing to hydrolysis and gradual loss of dye or drug loading.

- 18| Transfer the particle suspension to 15-ml centrifuge tubes and centrifuge them at 1,000g for 5 min at room temperature.

▲ CRITICAL STEP Excessive centrifugal forces can cause an aggregation of particles that can be difficult to disperse.

- 19| Remove the supernatant and gently resuspend it in 10 ml of distilled water by using a transfer pipette.
- 20| Repeat the wash process twice.
- 21| After the third wash, resuspend the particles in 1 ml of distilled water.
- 22| Filter the suspension through a 40-µm cell strainer to remove large particulates and aggregates.

? TROUBLESHOOTING

- 23| Use 1 ml of fresh distilled water to wash the cell strainer and collect additional particles.
- 24| Transfer the particle suspension to 2-ml centrifuge tubes.
- 25| Remove 20 µl of particle suspension for characterization.
- 26| Freeze the particle suspension at –80 °C and lyophilize it for 24 h.

■ PAUSE POINT The particles can be frozen overnight.

Preservation of microparticles ● TIMING 24 h

- 27| Store the lyophilized particles in 2-ml centrifuge tubes at –80 °C. Seal the lids with Parafilm to prevent moisture contamination that can degrade particles.

■ PAUSE POINT Particles can be frozen for at least 6 months.

Characterization of microparticles ● TIMING 1.5 h

- 28| Add 10 µl of particle suspension to 1 ml of distilled water in a cuvette.
- 29| Mix well and insert the mixture into the Zetasizer to measure the hydrodynamic diameter and polydispersity index of the PLGA microparticles through DLS.

? TROUBLESHOOTING

- 30| Transfer 20 µl of diluted particle suspension to a clean glass slide.
- 31| Use a fluorescence microscope at ×40 magnification to visualize particles and to confirm particle size and polydispersity. Scanning electron microscopy (SEM) can also be performed to confirm the size distribution and assess the surface morphology.

▲ CRITICAL STEP The presence of large particles or debris can cause errors in DLS measurements.

? TROUBLESHOOTING

- 32| Dilute 2 μ l of concentrated particle suspension in 1 ml of distilled water.
- 33| Add the diluted particle suspension into a disposable capillary cell and measure ζ -potential with a Zetasizer. PLGA-COOH should generate particles with a ζ -potential of approximately -40 mV.

▲ CRITICAL STEP Excessive particle concentration and high ion concentrations (e.g., cell medium) can cause the electrodes on the capillary cell to burn, resulting in inaccurate measurements.

? TROUBLESHOOTING

- 34| Quantify drug loading and determine the encapsulation efficiency as described in Box 1. Quantify the release kinetics as described in Box 2.

Box 1

Quantification of drug loading and encapsulation efficiency

The concentration of drug and duration of MSC exposure to small molecules is crucial to controlling the phenotype. For example, protocols to induce differentiation of MSCs *in vitro* typically rely on multiple days of continuous activation of signal transduction pathways by select agents included within the medium. With the particle engineering approach, the drug loading and release kinetics can be altered by modifying the particle synthesis protocol by changing the specific composition and molecular weight of the polymer, by altering the concentration of the drug or by using co-solvents to aid in dissolution of the small molecule in the polymer solution. Quantification of drug loading, encapsulation efficiency and release kinetics should be iterated until a formulation with desirable characteristics is generated. Drug loading is the mass fraction of a particle that is composed of drug and calculated by Equation 1. Encapsulation efficiency describes the fraction of drug incorporated into particles compared with the total amount of drug that was added during particle synthesis, and it is calculated by Equation 2.

Quantification of drug loading and encapsulation efficiency

1. Weigh 2 mg of particles into each of three 1.5-ml centrifuge tubes.
2. Collect dry particles into the bottom of the tube by performing a quick spin-down (2,000g, 1–2 s) in a mini-centrifuge.
3. Two methods can be used to solubilize the drug contained in the particles: dissolving the particle (polymer and drug) in a solvent such as DMSO or swelling the particle to allow release of the drug into solution. The swelling method maintains the PLGA as a solid, and typically the majority of drug can be separated from the drug in a methanol solution; however, this method should only be used if the encapsulated drug has high solubility in methanol.

A. Dissolving the particles

- i. Add 0.5 ml of DMSO to particles and allow the particles to completely dissolve. The DMSO solution can then be analyzed directly by spectrophotometry.

B. Releasing the drug by the swelling method

- i. Add 0.5 ml of methanol to swell the particles and release small molecules into solution. Particles will clump together and the release will be rapid. To ensure complete release, incubate the mixture on a shaker at 37 °C for 1 h.
 - ii. Centrifuge the solutions at 2,000g for 5 min at room temperature to pellet debris, and collect the supernatant into labeled tubes.
4. Analyze the sample by HPLC according to the absorbance spectrum of the small molecule. Prepare standard solutions of the small molecule in methanol (alternative solvents may be required depending on the solubility of the small molecule) for calibration. Include a control generated from blank particles (i.e., without the small molecule).
 5. Determine drug loading and encapsulation efficiency by using Equations 1 and 2, respectively, where C_R is the drug concentration of the release medium, V_R is the volume of release medium, m_{mp} is the mass of microparticles and m_D and m_{PLGA} are, respectively, the mass of drug and mass of PLGA initially added during particle synthesis.

$$\text{Drug Loading: \%DL} = \frac{C_R V_R}{m_{mp}} \times 100 \quad (1)$$

$$\text{Encapsulation Efficiency: \%EE} = \frac{C_R V_R / m_{mp}}{m_D / m_D + m_{PLGA}} \times 100 \quad (2)$$

Box 2**Quantification of drug release**

Release of small molecules from microparticles *in vitro* can be determined by using dialysis as previously described^{33,34}. Although release kinetics of drug from particles internalized within cells is influenced by the intracellular environment (e.g., the presence of enzymes or altered pH), the simplified dialysis system is an important tool that can provide insight into the release kinetics and it should highlight relevant trends and pitfalls including excessive burst release and incomplete release. The procedure below assumes that the maximum drug concentration in the release medium will remain an order of magnitude below the drug's solubility limit; elevated drug concentrations will reduce the rate of dissolution of the drug from the particle³⁵.

Procedure

1. Prepare a 10 mg ml⁻¹ particle suspension in PBS.
2. Pipette 200 µl of solution into a 2-inch section of 20-kDa-MWCO dialysis tubing clamped with a weighted closure.
3. Carefully close the second end of the dialysis tubing with an unweighted closure.
4. Load two additional tubings for replicates (for $n = 3$).
5. Place the loaded tubings within 50-ml centrifuge tubes. Add 40 ml of PBS-/- to each tube and cap securely.
6. Place the tubes in a rack on an orbital shaker at 37 °C. At each time point, collect 1 ml from the outer fluid phase and store it in a labeled centrifuge tube. Replace it with an equal volume of fresh PBS-/-.
7. The samples can be frozen at -80 °C until analysis.
8. The samples may need to be diluted with methanol or another solvent before analysis to reach a detectable drug concentration within the linear range of the calibration curve.
9. Determine cumulative release by using Equation 3 where CR_t is the cumulative drug release at sample time ' t ', C_t is the drug concentration of the sample at time ' t ', V_R is the volume of release medium, C_i is the drug concentration at sample time ' i ' and V_r is the volume removed at each sample time.

$$\text{Cumulative release at time 't': } CR_t = C_t V_R + \sum_{i=0}^{t-1} C_i V_r \quad (3)$$

10. Release medium should also be applied to cells to assess the bioactivity of the released agent to ensure that the agent was not damaged during encapsulation.
11. The intracellular concentration of the drug at specific time points can also be determined by washing MSCs that contain internalized particles with PBS, followed by a PBS solution containing 0.1% (vol/vol) Triton X-100 to lyse the cell membranes¹¹. The drug within the solution can then be analyzed to determine the intracellular drug concentration by using HPLC as described above.

Microparticle surface charge modification ● TIMING 3 h

▲ **CRITICAL** Sample data for this stage of the procedure are shown in Figure 3.

- 35| Measure 5 mg of lyophilized particles into a 1.5-ml centrifuge tube.
- 36| Perform a quick spin-down in a mini-centrifuge (2,000g for 1–2 s) to minimize the loss of particles that may stick to the surface of the tube.

- 37| Add 1 ml of 0.01% (wt/vol) PLL solution and gently resuspend the particles.
- 38| Shake the suspension at 37 °C for 2 h to allow for adsorption of PLL onto the surface of particles.
- 39| Add 10 µl of PLL-modified particle suspension to 1 ml of distilled water.
- 40| Measure the ζ -potential as in Step 33.

? TROUBLESHOOTING

■ **PAUSE POINT** PLL-modified particles can be frozen at –20 °C for 6 months.

Engineering cells with microparticles ● TIMING 14–18 h

- 41| Grow MSCs to 70–80% confluence in a T25 flask.
▲ **CRITICAL STEP** Incubating cells with particles at lower confluence will result in excessive amount of free particles in solution and particles adhered to the flask surface.
- 42| Prepare particle-laden medium by diluting 0.3 mg of PLL-modified particles in 1 ml of MEM- α with 1% (vol/vol) FBS, 1% (vol/vol) penicillin-streptomycin and 1% (vol/vol) l -glutamine.
- 43| Probe-sonicate at 1–3 W, pulsed for 10 s, to ensure that particles are uniformly dispersed in solution.
- 44| Add the suspended particles to 2 ml of 1% (vol/vol) FBS-supplemented MEM- α medium to create 3 ml of 0.1 mg ml^{–1} particle suspension.
- 45| Wash MSCs with PBS-/- three times.
- 46| Add particle-laden medium to MSCs and incubate overnight (e.g., >12 h).
▲ **CRITICAL STEP** Shorter incubation times will result in particle association with the cell membrane; however, internalization of particles may not be complete.
- 47| Aspirate the spent medium and wash the flask three times with PBS-/- at room temperature to remove free particles.

? TROUBLESHOOTING

- 48| Add 10% (vol/vol) FBS medium or split and proceed with downstream analysis or experiments.

Characterization of the internalization of microparticles

- 49| After engineering MSCs with microparticles, it is crucial to analyze the cells to ensure efficient internalization. Poor uptake of particles will result in nonuniform exposure of cells to the encapsulated agent and reduce the concentration and duration in which the particles are able to control the cell phenotype. Described here are techniques to assess the uniformity of

microparticle uptake within the cell population, the presence of free microparticles and subcellular location of microparticles.

Although flow cytometry and fluorescence microscopy (option A) are useful in determining ideal microparticle formulations and incubation conditions that maximize association of microparticles with cells, these techniques cannot easily distinguish between membrane-bound and intracellular microparticles, and thus additional techniques such as confocal microscopy are required to assess this. To examine internalization of microparticles within cells, MSCs can easily be analyzed by confocal microscopy (option B). The technique described in option B has been developed to fix cells in a spherical morphology, rather than monolayer cultures, to facilitate assessment of particle internalization. Other complementary techniques can be applied to confirm particle internalization including transmission electron microscopy (TEM). If performing TEM, particles should be formulated with iron oxide or gold nanoparticles to aid in the identification of PLGA particles within cells, as described by Xu *et al.*¹⁹ (Fig. 4).

A. Quantifying the uniformity and degree of microparticle association with cells

- i. After Step 48, MSCs can be analyzed by flow cytometry to determine the degree and uniformity of cell uptake of dye-loaded microparticles. Collect cells by washing them three times with 2 ml of PBS^{-/-} and incubating them for 3–4 min with trypsin or Accutase cell detachment solution.
- ii. Centrifuge the detached cells in a 15-ml conical tube at 300g for 5 min at room temperature to pellet the cells.
- iii. Resuspend the pellet in 1 ml of fresh culture medium and analyze it with a flow cytometer. Unmodified (native) MSCs and free microparticles serve as useful controls to determine cell gating and to set the threshold for background fluorescence. Fluorescence intensity of microparticle-engineered MSCs will rise with increased microparticle loading. Side scatter has also been observed to increase as a result of the increased granularity of the microparticle-loaded cells.
- iv. After analysis via flow cytometry, samples can be plated on glass slides and visualized with a fluorescence microscope to assess the relative number of free microparticles versus cell-associated microparticles (Fig. 4a).

? TROUBLESHOOTING

B. Confirming microparticle internalization

- i. Engineer MSCs with dye-loaded microparticles as described in Steps 41–48.

- ii. Coat a glass-bottom dish or chamber slide with 100 μ l of 20 μ g ml^{-1} fibronectin for 1 h to aid in rapid cell attachment.
- iii. Meanwhile, collect MSCs and resuspend them in medium supplemented with 5 μ l ml^{-1} Vybrant DiO membrane dye and 1 μ g ml^{-1} Hoechst nuclear dye.
- iv. Incubate the mixture on ice for 15 min. Aspirate fibronectin from the dish or slide, and add 100 μ l of cell suspension as a droplet on the fibronectin-coated spot.
- v. Carefully transport the dish or slide to a 37 °C incubator and incubate it for 5 min.
- vi. Use a microscope to examine cell attachment: the majority of cells should be attached but not spread on the culture surface.

▲ CRITICAL STEP Extended incubation after plating will lead to cell spreading, making it difficult to determine whether microparticles are intracellular or membrane associated.

- vii. Aspirate the liquid and replace it with 1 ml of 10% neutral-buffered formalin.
- viii. Fix cells for 5 min, wash them four times with PBS/- and analyze by confocal microscopy (Fig. 4b,c).

? TROUBLESHOOTING

? TROUBLESHOOTING

Troubleshooting advice can be found in Table 1.

● TIMING

Steps 1–26, day 1, preparation of microparticles: 7 h

Step 27, day 2, preservation of microparticles: 24 h

Steps 28–40, day 3, characterization of microparticles: ~4.5 h

Steps 41–48, day 4, engineering cells with microparticles: 14–18 h

Step 49, day 5, characterization of the internalization of microparticles: variable

Box 1, quantification of drug loading and encapsulation efficiency: 4–8 h

Box 2, quantification of drug release: 7 d

ANTICIPATED RESULTS

This protocol establishes a robust technique for controlled delivery of small molecules or other cargo intracellularly to an exogenous population of cells through the generation of drug-loaded microparticles (Figs. 1 and 2), followed by surface modification of

microparticles (Figs. 1 and 3) and functionalization of cells with microparticles (Figs. 1 and 4).

To demonstrate the flexibility of this platform to modify multiple cell types, rhodamine dye-loaded microparticles were generated and used to modify MSCs, MIN6 beta cells and macrophages. After lyophilization (Step 27), the ζ -potential of particles was modified by using FITC-labeled PLL (FITC-PLL) in accordance with Steps 35–40. Figure 3 shows representative fluorescence images and ζ -potential measurements of dye-loaded particles before and after modification with FITC-PLL. To prepare cells for particle modification, human MSCs, MIN6 beta cells and RAW 264.7 macrophages were plated in T25 flasks and allowed to grow to 70% confluence (Step 41). PLL-modified particles were then diluted in cell culture medium at a concentration of 0.3 mg ml^{-1} (Step 42).

To determine whether particles were in a single particle suspension, we placed $5 \mu\text{l}$ of particle-laden medium on a glass slide and analyzed it by fluorescence microscopy, which revealed the presence of large particle aggregates that cells would have difficulty internalizing (Fig. 5a).

Probe sonication (Step 43) was effective at dispersing particles back into a single particle suspension (Fig. 5b). The concentration of the particle suspension was then adjusted to 0.1 mg ml^{-1} by the addition of low-serum medium, and particle-laden medium was added to each flask (Steps 44–46). All cell types were incubated with the particle-laden medium overnight, washed, trypsinized and analyzed by flow cytometry and confocal microscopy according to Steps 47–49. For all cell types, cells without particles were used as controls, and debris and free particles were gated out with BD Accuri's CFlow analysis software (Fig. 6 shows representative gating for MSCs).

Flow cytometry, which measures the percentage of cells associated with particles but not necessarily particle internalization, revealed that MSCs, MIN6 beta cells and RAW 264.7 macrophages were all strongly associated with particles but to different extents, with $>94\%$ of MSCs and macrophages and 46% of MIN6 beta cells associated with particles (determined by an increase in the fluorescence intensity of the cells compared with unmodified controls) (Fig. 7). It should be noted that MIN6 beta cells tend to grow in clusters with some cells in the core and others on the periphery. These clusters were not disassociated before incubation with particle-laden medium. Therefore, it is possible that all MIN6 beta cells were not equally exposed to microparticles, resulting in increased variability in the extent of particle association. It is anticipated that the efficiency of MIN6 modification could be improved by dispersing MIN6 cells into a single-cell suspension and then plating the cells at a high density immediately before the introduction of the particle-laden medium.

To assess particle internalization by confocal microscopy, particle-modified MSCs, MIN6 beta cells and RAW 264.7 macrophages were plated on fibronectin-coated glass dishes and allowed to attach before formalin fixation (Step 49B). Cells were incubated until a monolayer of cells was observed by light microscopy to be attached to the dish. MSC attachment to the dish was observed 5 min after plating, whereas RAW 264.7 macrophages

and MIN6 beta cells took 10 and 40 min to attach, respectively. The outer membrane of cells was stained with DiO and the nuclei were stained with Hoechst in accordance with Step 49B.

Confocal microscopy of the cells not only confirmed observations from flow cytometry, revealing that nearly all of the MSCs and macrophages and half of the MIN6 beta cells were associated with particles, and also that particles were internalized by all cell types (Fig. 7).

The particle-engineered cell platform is highly tunable, and we anticipate that it can be adapted to a variety of applications such as promoting and accelerating engraftment of HSCs, enhancing glucose sensitivity of transplanted beta cells and maximizing the immunomodulatory function of MSCs (Fig. 8). Figure 8 also shows that molecules can be transported to the extracellular environment passively via diffusion or actively through exosomes or drug efflux pumps. This can be used to influence the cell's microenvironment, as we have previously demonstrated with dexamethasone¹¹.

Acknowledgments

This work was supported by US National Institutes of Health grant no. HL095722 to J.M.K. and by a Prostate Cancer Foundation Challenge Award to J.M.K. J.A.A. was supported by the Hugh Hampton Young Memorial Fellowship.

References

1. von Bahr L, et al. Long-term complications, immunologic effects, and role of passage for outcome in mesenchymal stromal cell therapy. *Biol Blood Marrow Transplant*. 2012; 18:557–564. [PubMed: 21820393]
2. François M, Romieu-Mourez R, Li M, Galipeau J. Human MSC suppression correlates with cytokine induction of indoleamine 2,3-dioxygenase and bystander M2 macrophage differentiation. *Mol Ther*. 2012; 20:187–195. [PubMed: 21934657]
3. Cahan P, Daley GQ. Origins and implications of pluripotent stem cell variability and heterogeneity. *Nat Rev Mol Cell Biol*. 2013; 14:357–368. [PubMed: 23673969]
4. Fomina-Yadlin D, et al. Small-molecule inducers of insulin expression in pancreatic alpha-cells. *Proc Natl Acad Sci USA*. 2010; 107:15099–15104. [PubMed: 20696901]
5. Da Silva Meirelles L, Chagastelles P. Mesenchymal stem cells reside in virtually all post-natal organs and tissues. *J Cell Sci*. 2006; 119 (Part 11):2204–2213. [PubMed: 16684817]
6. Zhukareva V, Obrocka M, Houle JD, Fischer I, Neuhuber B. Secretion profile of human bone marrow stromal cells: donor variability and response to inflammatory stimuli. *Cytokine*. 2010; 50:317–321. [PubMed: 20185331]
7. Wu W, Ye Z, Zhou Y, Tan WS. AICAR, a small chemical molecule, primes osteogenic differentiation of adult mesenchymal stem cells. *Int J Artif Organs*. 2011; 34:1128–1136. [PubMed: 22198598]
8. Suzuki Y, Kim HW, Ashraf M, Haider HK. Diazoxide potentiates mesenchymal stem cell survival via NF- κ B-dependent miR-146a expression by targeting Fas. *Am J Physiol Heart Circ Physiol*. 2010; 299:H1077–82. [PubMed: 20656888]
9. Dong Q, Yang Y, Song L, Qian H, Xu Z. Atorvastatin prevents mesenchymal stem cells from hypoxia and serum-free injury through activating AMP-activated protein kinase. *Int J Cardiol*. 2011; 153:311–316. [PubMed: 20832877]
10. Brey DM, et al. High-throughput screening of a small molecule library for promoters and inhibitors of mesenchymal stem cell osteogenic differentiation. *Biotechnol Bioeng*. 2011; 108:163–174. [PubMed: 20824673]

11. Sarkar D, Ankrum J, Teo GSL, Carman CV, Karp JM. Cellular and extracellular programming of cell fate through engineered intracrine-, paracrine-, and endocrine-like mechanisms. *Biomaterials*. 2011; 32:3053–3061. [PubMed: 21262537]
12. Slowing II, et al. Exocytosis of mesoporous silica nanoparticles from mammalian cells: from asymmetric cell-to-cell transfer to protein harvesting. *Small*. 2011; 7:1526–1532. [PubMed: 21520497]
13. Jiang X, et al. Endo- and exocytosis of zwitterionic quantum dot nanoparticles by live HeLa cells. *ACS Nano*. 2010; 4:6787–6797. [PubMed: 21028844]
14. Panyam J, Labhasetwar V. Dynamics of endocytosis and exocytosis of poly(D,L-lactide-co-glycolide) nanoparticles in vascular smooth muscle cells. *Pharm Res*. 2003; 20:212–220. [PubMed: 12636159]
15. Jin H, Heller DA, Sharma R, Strano MS. Size-dependent cellular uptake and expulsion of single-walled carbon nanotubes: single particle tracking and a generic uptake model for nanoparticles. *ACS Nano*. 2009; 3:149–158. [PubMed: 19206261]
16. Chithrani BD, Chan WCW. Elucidating the mechanism of cellular uptake and removal of protein-coated gold nanoparticles of different sizes and shapes. *Nano Lett*. 2007; 7:1542–1550. [PubMed: 17465586]
17. Swiston A, Gilbert J, Irvine D, Cohen R, Rubner M. Freely suspended cellular ‘backpacks’ lead to cell aggregate self-assembly. *Biomacromolecules*. 2010:4920–4925.
18. Stephan MT, Moon JJ, Um SH, Bershteyn A, Irvine DJ. Therapeutic cell engineering with surface-conjugated synthetic nanoparticles. *Nat Med*. 2010; 16:1035–1041. [PubMed: 20711198]
19. Xu C, et al. Tracking mesenchymal stem cells with iron oxide nanoparticle loaded poly(lactide-co-glycolide) microparticles. *Nano Lett*. 2012; 12:4131–4139. [PubMed: 22769232]
20. Cohen-Sela E, Chorny M, Koroukhov N, Danenberg HD, Golomb G. A new double emulsion solvent diffusion technique for encapsulating hydrophilic molecules in PLGA nanoparticles. *J Control Release*. 2009; 133:90–95. [PubMed: 18848962]
21. Karnik R, et al. Microfluidic platform for controlled synthesis of polymeric nanoparticles. *Nano Lett*. 2008; 8:2906–2912. [PubMed: 18656990]
22. Thomas TT, Kohane DS, Wang A, Langer R. Microparticulate formulations for the controlled release of interleukin-2. *J Pharm Sci*. 2004; 93:1100–1109. [PubMed: 15067687]
23. Sawada M, Hayes P, Matsuyama S. Cytoprotective membrane-permeable peptides designed from the Bax-binding domain of Ku70. *Nat Cell Biol*. 2003; 5:352–357. [PubMed: 12652309]
24. Ieda M, et al. Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors. *Cell*. 2010; 142:375–386. [PubMed: 20691899]
25. Murry CE, Keller G. Differentiation of embryonic stem cells to clinically relevant populations: lessons from embryonic development. *Cell*. 2008; 132:661–680. [PubMed: 18295582]
26. Brenner S, et al. CXCR4-transgene expression significantly improves marrow engraftment of cultured hematopoietic stem cells. *Stem Cell*. 2004; 22:1128–1133.
27. Haider HK, Jiang S, Idris NM, Ashraf M. IGF-1-overexpressing mesenchymal stem cells accelerate bone marrow stem cell mobilization via paracrine activation of SDF-1 α /CXCR4 signaling to promote myocardial repair. *Circ Res*. 2008; 103:1300–1308. [PubMed: 18948617]
28. Panyam J, Labhasetwar V. Biodegradable nanoparticles for drug and gene delivery to cells and tissue. *Adv Drug Deliv Rev*. 2003; 55:329–347. [PubMed: 12628320]
29. Panyam J, Zhou WZ, Prabha S, Sahoo SK, Labhasetwar V. Rapid endo-lysosomal escape of poly(DL-lactide-co-glycolide) nanoparticles: implications for drug and gene delivery. *FASEB J*. 2002; 16:1217–1226. [PubMed: 12153989]
30. Patil Y, Sadhukha T, Ma L, Panyam J. Nanoparticle-mediated simultaneous and targeted delivery of paclitaxel and tariquidar overcomes tumor drug resistance. *J Control Release*. 2009; 136:21–29. [PubMed: 19331851]
31. Acharya S, Sahoo SK. PLGA nanoparticles containing various anticancer agents and tumour delivery by EPR effect. *Adv Drug Deliv Rev*. 2011; 63:170–183. [PubMed: 20965219]
32. Timko BP, et al. Advances in drug delivery. *Annu Rev Mater Res*. 2011; 41:1–20.

33. Kostanski JW, DeLuca PP. A novel *in vitro* release technique for peptide containing biodegradable microspheres. AAPS Pharm Sci Tech. 2000; 1:E4.
34. D'Souza SS, DeLuca PP. Methods to assess *in vitro* drug release from injectable polymeric particulate systems. Pharm Res. 2006; 23:460–474. [PubMed: 16400516]
35. Dokoumetzidis A, Macheras P. A century of dissolution research: from Noyes and Whitney to the biopharmaceutics classification system. Int J Pharm. 2006; 321:1–11. [PubMed: 16920290]

Figure 1.

Flow diagram for the particle engineering protocol. Generation and characterization of appropriately sized and charged particles are essential to achieve consistent particle internalization by cells. Drug activity, loading and release kinetics should be studied to determine the optimal particle characteristics for each application. If drug activity is lost, loading is too low or release kinetics are inappropriate for the intended application, adjustments to the particle formation protocol should be made and new particles should be generated. Once particles with desired characteristics have been formed, cells can be engineered with particles, characterized and used in downstream applications. The dashed lines represent iterative loops to follow whether poor particle internalization is observed: (i) particles are aggregated; (ii) particles have negative charge; or (iii) particles are too large to be internalized. White boxes represent steps involving only particles, whereas gray boxes represent cells in culture and may require additional lead time to expand cells to the appropriate confluence.

Figure 2. Generation of drug-loaded microparticles. **(a)** Schematic of single-emulsion evaporation technique. Particles are generated by dissolving PLGA and drug into DCM. Drug solution is then added dropwise to a stabilizing solution of PVA while homogenizing to create an emulsion. Particles are then allowed to solidify in suspension while the solvent evaporates. **(b)** Representative distribution of particle diameters generated by using this method with 0.15–0.25 g dl⁻¹ i.v. (green line) or 0.55–0.75 g dl⁻¹ i.v. (red line) PLGA. Inset is a representative SEM image of particles. Scale bar, 1 μm.

Figure 3. Surface modification of particles with PLL to enhance particle uptake. **(a,b)** Rhodamine-PLGA particles were imaged before **(a)** and after **(b)** surface modification with positively charged FITC-PLL. Scale bars, 5 μ m. **(c,d)** The FITC-PLL coating results in a shift in ζ -potential from -48 mV before coating **(c)** to +10 mV after coating **(d)**.

Figure 4.

Confirming cellular internalization of microparticles. (a) An inverted fluorescence microscope can be used to examine the association of cells and particles and the presence of free particles (arrows), but it cannot be used to conclusively determine whether particles have been internalized. The cell perimeter is outlined in the red fluorescence channel to distinguish particles that could possibly be internalized from those that are on the periphery. (b,c) Representative confocal microscopy images of MSCs with particle internalization at low (b) and high (c) efficiencies. Images represent a slice through the cell at the plane of the nuclei showing the presence of mostly outer membrane-associated particles (b) and numerous intracellular particles (c). Scale bars, 10 μ m. (d) MSCs modified with (black arrows) iron oxide nanoparticle-loaded PLGA particles examined with TEM imaging. By comparing the location of the particles with (red arrow) the cell membrane, TEM can be used to confirm internalization of particles. Particle diameters observed in TEM are a

function of the plane in which the image slice is taken, and they may be smaller than expected owing to PLGA dissolution during TEM sample processing. Scale bar, 500 nm.

Figure 5.

Troubleshooting particle aggregation to improve MSC uptake of particles. Particle aggregation can be caused by numerous factors, including high particle concentration during preservation, presence of excessive residual PVA or weak ζ -potential. Aggregation effectively reduces the concentration of particles in the medium that MSCs are capable of internalizing (leading to reduced internalization). **(a,b)** Shown are representative images of a 0.1 mg ml^{-1} particle suspension in medium before **(a)** and after **(b)** sonication. If particle aggregation is suspected, use a fluorescence microscope to confirm (if fluorescent dye was loaded in particles), and disassociate particle aggregates with a sonication probe or water-bath sonication. Scale bars, $10 \mu\text{m}$.

Figure 6.

Gating fluorescent particle-engineered MSCs in flow cytometry. **(a,b)** Forward scatter but not side scatter is preserved between unmodified (native) **(a)** and particle-engineered MSCs **(b)**. Increased particle internalization increases side scatter. To adequately measure the fluorescence of particle-engineered cells and not free particles or debris, gate (red box labeled P1) with a range of side scatter larger than the typical range for cells. Free particles and cell debris will cluster at the bottom left corner of the forward/side scatter plot (dashed red circle). Apoptotic cells may be excluded using appropriately labeled annexin V. **(c)** Fluorescent particle-engineered cells (red line) should show markedly higher fluorescence than unmodified (native) cells (black line).

Figure 7. Potential for universal applicability of the particle-engineered cell platform. (a–f) The particle engineering protocol was applied to human bone marrow MSCs (a,b), MIN6 beta cells (c,d) and RAW 264.7 macrophages (e,f). One day after particle engineering, cells were collected and analyzed by flow cytometry. All cells showed increased red fluorescence (FL2-A channel) (black lines) compared with unmodified controls (red lines). The percentage of cells with particles is stated in the upper right corner of each graph. To confirm particle internalization, confocal imaging was performed as described above. Images represent a slice through an MSC (b), a MIN6 beta cell (d) and a RAW 264.7 macrophage (f) at the plane of the nuclei showing internalization of particles. Scale bars, 10 μm. Green (DiO stain), membrane; red (rhodamine 6g), particles; blue (Hoechst), nuclei.

Figure 8.

Tailoring cells with intracellular depots for multiple applications. **(a)** A diverse toolbox of particles can be generated and used to engineer cells to track their location, to locally deliver drugs or to control cell phenotype including proliferation, viability, differentiation and secretome by targeting (i) intracellular targets, (ii) membrane-bound targets (in an autocrine-like manner) and (iii) extracellular receptors (in a paracrine-like manner). **(b)** Engineered cells can be transplanted locally or systemically. For example, drugs released from intracellular particles can be used to control the phenotype of the particle-modified cell by enhancing the MSC secretome **(b, top)** Alternatively, the platform can be used to locally deliver drugs to tissues where cells reside (where the cell is used as a delivery vehicle) **(b, bottom)**.

TABLE 1

Troubleshooting table.

Step	Problem	Possible reason	Solution
1	PVA is not dissolving	PVA is stuck to beaker	Use ultrasonic water bath to detach PVA clumps from beaker
7	PLGA is not dissolving	Large polymer pellets or high-molecular-weight PLGA take more time to dissolve	Use ultrasonic water bath to facilitate dissolution of polymer pellets
12	Solutions not mixing, two layers visible	Surface area of the beaker is too large	Use a beaker with a smaller diameter to reduce surface area
		Homogenizer is not adequately submerged	Lower the homogenizer so that the tip is submerged in PVA solution
	Emulsion overflows	PVA solutions have a tendency to foam	Reduce the PVA concentration to 0.25–0.5% (wt/vol)
		Beaker is too short	Use a taller beaker
22	Particle clumping	Solvent evaporation was not complete	Leave suspension on stir plate for an additional 2 h with adequate ventilation
		Centrifugal forces are too high	Reduce centrifuge speed and increase time to pellet particles
		Particles have a neutral charge due to excessive PVA coating	Wash with a larger volume of distilled water before initial spin or reduce the PVA concentration to 0.25–0.5% (wt/vol)
29	Repeat readings yield different results	Presence of large particles or settling debris	Filter the suspension with a cell strainer before measurement or allow large particles to settle out for 2 min, and then carefully collect the particles in suspension into a new tube
31	Presence of large particles	PLGA concentration or agitation speed were not adequate	Filter the particle suspension with a 5- μ m Supor Membrane using a 20-ml syringe
33	ζ -potential is near zero	Ion concentration is too high causing electrodes to burn	Dilute particle suspension and use a new capillary cell
		Excessive PVA residue on particle surface	Reduce the PVA concentration to 0.25–0.5% (wt/vol) or add an additional wash step before measurement
40	No change in ζ -potential after PLL modification	Inadequate adsorption of PLL onto the particle surface	Prepare a fresh batch of PLL and make sure that particles remain in suspension during modification
47	Excessive number of free particles remain after washing	Concentration of particle-laden medium was too high	Reduce the concentration of the particle-laden medium
		Particles added when cells were at low confluence	Replate the cells, allow them to attach for 20 min, and then wash with PBS to remove free particles
			Separate cells from free particles by Ficoll density gradient separation
			Wash cells with trypsin for 1 min to remove particles, then replace with normal full-serum medium
49	Poor or inconsistent particle loading in cells	Particles are too large	Reduce polymer concentration or increase agitation speed to generate smaller particles, and carefully verify the size distribution
			Reduce the size of the beaker used during emulsification so solution is more evenly and vigorously mixed
		Particles are clumping	Probe-sonicate to break up clumps and confirm with microscopy before adding to cells
			Excipients such as PEG, sorbitol, or mannose can be added before lyophilization at nontoxic concentrations
			Reduce particle concentration
		Particle suspension is not evenly distributed over cells; flask is tilted	Gently tilt flask front-to-back and side-to-side in incubator to evenly coat cells; do not swirl

Step	Problem	Possible reason	Solution
		Low particle fluorescence due to low dye loading	Check and increase dye loading
		Poor cell health	Check viability and morphology of MSCs before particle incubation and use fresh medium
			Check toxicity of drug and reduce drug loading accordingly
			Reduce particle concentration
			Reduce duration of particle incubation in 1% (vol/vol) FBS
			Check for microbial contamination