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Self-Assembly of Cell–Microparticle Hybrids**

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The self assembly of building blocks into more complex structures has attracted increasing attention for use in the fabrication of higher order devices and structures.^[1,2] Most published reports pertaining to the assembly of building blocks focus on the self-assembly of synthetic structures^[2–5] via physical, covalent, or biological interactions.^[6,7] For example, rods and spheres have been assembled onto substrates and with each other using electrostatic interactions or interactions involving DNA strands.^[8–10] Indeed, combining biological and synthetic materials has become increasingly important for tissue engineering, advanced drug delivery, and the development of intelligent biosynthetic devices.^[11–15]

Here, we demonstrate the self-assembly of biological-cell–synthetic-microparticle hybrids using biotin–avidin interactions. The biotin–avidin linkage is one of the strongest known biological interactions and has been utilized for the directed assembly of spheres and rods.^[16–21] Here, we demonstrate that cells can be readily surface functionalized with biotin and that microparticles can be engineered with a biotin-enriched surface. The biotinylated microparticles have then been bound to the biotinylated cell surface with avidin as a bridging protein. This approach works well for both adherent and non-adherent cells.

The overall strategy for the preparation of cell–microparticle hybrids is schematically depicted in Figure 1a. First, a biotinylated poly(lactic acid)–poly(ethylene glycol)–biotin (PLA–PEG–biotin, molecular weight (M_w) of 23 400) copolymer has been synthesized by reacting *N*-hydroxysuccinimide (NHS)-biotin with the amine terminus of bifunctional α -amine- ω -hydroxy-PEG.^[22] The α -amine- ω -hydroxy-PEG has been prepared by reducing α -amine- ω -carboxylic acid-PEG (Nektar Therapeutics) in a 1 M tetrahydrofuran–borane mixture (Sigma). The appearance of a ¹H NMR peak at 7.8 ppm (amide proton) and a triplet resonance at 2 ppm (methylene from biotin group alpha to the amide) have been used to confirm the attachment of biotin to bifunctional PEG. Lactide (Purac Biochem bv) has then been graft polymerized

onto the hydroxyl terminus of α -biotin- ω -hydroxy-PEG with stannous 2-ethyl hexanoate as the initiator (Sigma). A molecular weight of 23 400 has been deduced for the polymer by comparing the integral ratios of the lactide signals with the integral ratio of the fixed-molecular-weight PEG signal. Gel permeation chromatography (GPC) analysis has been used to confirm the purity of the PLA–PEG–biotin.

In the next step, PLA–PEG–biotin microparticles have been prepared using a double-emulsion solvent-evaporation approach.^[23] This method utilizes three distinct phases, an inner water phase wherein the relevant proteins or drugs are entrapped, an intermediate organic phase composed of the polymer/methylene chloride solution, and an outer water phase containing an emulsifying agent. These microparticles can be loaded with a wide variety of drugs, proteins, or fluorescent molecules for imaging applications.^[23] The particle size of these microparticles has been determined to be 1.4 μ m from light scattering measurements using a Zetasizer Nano ZS instrument. PLA–PEG copolymers display hydrophobic–hydrophilic characteristics that give rise to a PEG-enriched surface upon organization into microparticles.^[24] The PEG spacer also increases the biotin–avidin binding efficiencies.^[25] We have used a partially hydrolyzed polymer PVA (88 %, Sigma), derived from the copolymerization of poly(vinyl acetate) and poly(vinyl alcohol) that is optimal for the preparation of microparticles.^[23,26] PVA binds to the surface of the microparticles through interpenetration of PVA and PLA–PEG molecules upon removal of the organic solvent from the interface. However, previous surface plasmon resonance (SPR) studies have shown that significant avidin immobilization still occurs with masking of up to ca. 30 %. This suggests that there are still sufficient binding sites remaining on the microparticle surface.^[27] By carefully controlling the PVA concentrations and stirring rates, we have prepared PLA–PEG–biotin microparticles with well-defined size distributions and drug- or protein-release profiles.^[23,26]

The microparticles have been incubated with an excess of tetramethylrhodamine-conjugated avidin (500 μ g mL⁻¹, Molecular Probes) and washed using deionized water. The washing process consists of several sequential centrifugation and resuspension cycles. Incubation of the microparticles with excess avidin prevents aggregation of the microparticles because of the paucity of free biotin sites.

The microparticles have been imaged by fluorescent microscopy. The PLA–PEG–biotin microparticles are strongly fluorescent because of the rhodamine conjugated to the avidin. This confirms that the microparticles have been surface functionalized with avidin (Fig. 1d). Control microparticles

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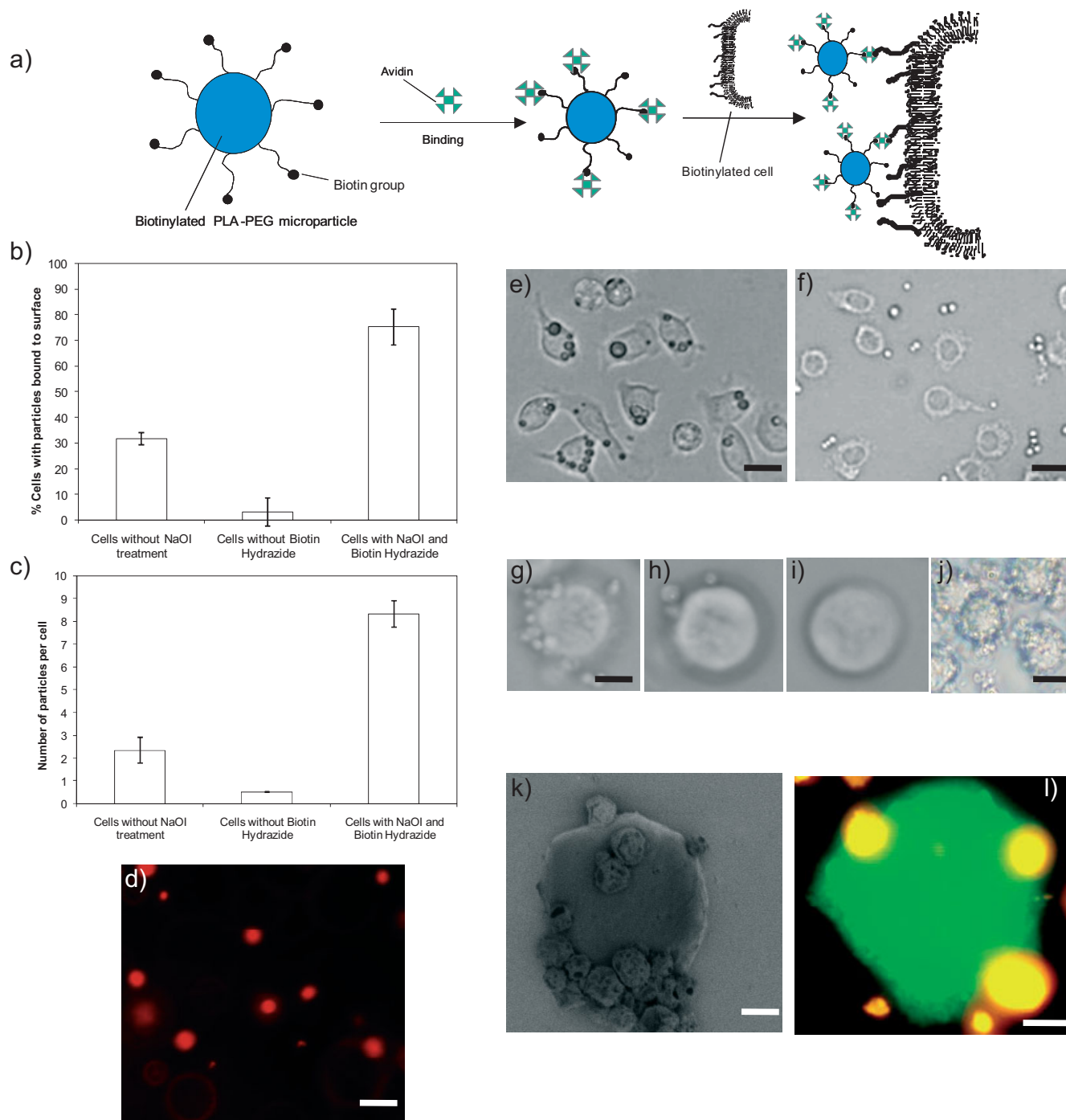


Figure 1. a) Schematic depiction of the self-assembly of microparticle–cell hybrids, b) mean percentage of human embryonic kidney 293 (HEK293) cells with PLA-PEG-biotin microparticles adhering to the cell surface, c) mean number of microparticles binding per HEK293 cell, d) fluorescence microscopy image of PLA-PEG-biotin microparticles surface engineered with rhodamine-labeled avidin (Olympus BX40 555/580 nm, scale bar = 4.5 μm), e) light microscopy image of poly(lactic acid)-poly(ethylene glycol)-biotin (PLA-PEG-biotin) microparticles bound to avidinylated HEK293 cells (scale bar = 25 μm), f) PLA-PEG-biotin microparticles incubated with control HEK293 cells without any biotinylation (scale bar = 5 μm), g) HEK293 cells in suspension treated with NaIO₄ and biotin hydrzide and self-assembled with PLA-PEG-biotin microparticles (scale bar = 30 μm), h) HEK293 cells in suspension treated with biotin hydrzide alone and self-assembled with PLA-PEG-biotin microparticles, i) control HEK293 cells in suspension that have not been treated with biotin hydrzide do not self-assemble upon incubation with PLA-PEG-biotin microparticles, j) typically non-adherent EG7 tumor cells treated with NaIO₄ and biotin hydrzide efficiently assemble with PLA-PEG-biotin microparticles (scale bar = 10 μm), k) SEM image of self-assembled microparticle–cell hybrids (scale bar = 1.5 μm), l) fluorescence microscopy overlay image of HEK293 cells transfected with green fluorescent protein and assembled with PLA-PEG-biotin microparticles loaded with rhodamine 123 (Olympus BX40 555/580 nm, 494/518 nm, scale bar = 1.5 μm).

without rhodamine–avidin binding do not show any fluorescence response.

In the next step of this process, human embryonic kidney 293 (HEK293, American Type Culture Collection (ATCC)) cells have been biotinylated. This has been achieved by converting native sialic acid residues on the cell surfaces into non-native aldehydes using a mild NaIO_4 solution. Sialic acid is a ubiquitous terminal cell surface monosaccharide group with amplified expression in many cancers.^[28] The aldehyde groups have been reacted with biotin–hydrazide to produce biotinylated cells. To achieve this objective, HEK293 cells have been grown to 60–65 % confluence in 12-well plates. Cell culture media in the wells have been removed and replaced with freshly warmed media and further incubated for 1 h. Subsequently, the cells have been washed twice with phosphate buffered saline (PBS) and incubated with a 1 mM solution of NaIO_4 in cold PBS for 20 min in the dark at 4 °C. The HEK293 cells have been washed with PBS at pH 6.5 supplemented with 0.1 % bovine calf serum (BCS) at room temperature. Next, the cells have been incubated with a 0.5 mM solution of biotin hydrazide (Sigma) in PBS (pH 6.5) for 90 min at room temperature. Then, the cells have been washed twice with PBS solution at pH 7.4 supplemented with 0.1 % BCS. After washing, the cells have been incubated with 1 mg mL^{-1} of avidin-saturated microparticles for 20 min at 4 °C. Subsequently, the cells have been again washed twice with PBS (pH 7.4) prior to imaging by light microscopy (Olympus BX40). The degree of biotinylation on the surface of the HEK293 cells has been determined using the 2-(4-hydroxyazobenzene)benzoic acid (HABA)/avidin assay to be $(3.6 \pm 0.45) \times 10^9$ biotin moieties per cell. The cell viability has been determined using trypan-blue measurements. Biotin-functionalized cells demonstrate a viability of 89.7 % as compared to 97.51 % for untreated cells. Figure 1e shows an image of PLA–PEG–biotin microparticles that have been incubated with HEK293 cells surface functionalized with avidin/biotin. The results of the control experiment in Figure 1f shows that the microparticles do not bind as effectively to control HEK293 cells that have not been treated with biotin hydrazide. For control HEK293 cell samples that have not been treated with biotin hydrazide, it has been observed that the microparticles readily settle in areas where the non-confluent cells have not spread. The ability to specifically bind particles to non-confluent cells using receptor-mediated interactions has significant potential for improving in vitro drug and gene delivery. For example, biotinylated nanoparticles loaded or complexed with plasmid DNA could potentially significantly enhance the transfection efficiencies of avidin–biotin surface-engineered cells. To demonstrate that cell–microparticle hybrids can be prepared in solution, the cells have been trypsinized and treated with NaIO_4 and biotin hydrazide as described above. Avidinylated PLA–PEG–biotin microparticles (1 mg mL^{-1}) have been self-assembled with the biotinylated cells (1×10^5) by gently pipetting the two solutions into a single vial. Control experiments have also been performed wherein the cells are treated in exactly the same manner except for treatment with NaIO_4 .

The results of the control experiments show a two- to three-fold reduction in the percentage of cells binding microparticles and a four-fold reduction in the number of particles bound per cell (Fig. 1b and c). Control experiments where the cells have not been treated with biotin hydrazide indicate limited or no assembly of microparticle–cell hybrids (Fig. 1f and i). This confirms that the self-assembly process arises from specific biotin–avidin receptor-mediated interactions (Fig. 1b, c, e, g, k, and j). Alternative approaches for the assembly of microparticle–cell hybrids that would bypass the surface engineering of cells include the use of tumor cells transfected to present biotin and then assembled with avidinylated microparticles. For example, Weissleder and co-workers have recently demonstrated that tumor cells can be genetically engineered to present a recombinant reporter protein incorporating a biotin acceptor peptide between a *N*-terminal signal sequence and a transmembrane domain.^[29] However, limitations to this approach include a reduced capacity to subsequently transfect the biotin-presenting cell for genes encoding therapeutic, fluorescent, or antigenic proteins.

To demonstrate the potential of our cell–microparticle hybrids in dual synthetic–biological drug and protein delivery applications, we have transfected non-adherent EL4 cells and adherent HEK293 cells with green fluorescent protein (GFP). Microparticles loaded with rhodamine 123 have been prepared using the double-emulsion solvent-evaporation technique and assembled with GFP-expressing cells using the biotin–avidin interaction. Figure 1l shows a fluorescent overlay image of rhodamine-labeled microparticles efficiently assembled onto HEK293 cells expressing GFP. To demonstrate that this process is compatible with non-adherent cells transfected with antigenic proteins, EG7 cells (ATCC, 1×10^5) have been engineered with biotin using the same procedure as described above for the HEK293 cells. The EG7 cells have been derived from the murine T-cell lymphoma EL4 cell line transfected with cDNA for a model protein antigen, ovalbumin. Figure 1j shows that when the biotinylated EG7 cells are incubated with avidinylated PLA–PEG–biotin microparticles, cell–microparticle hybrids are readily constructed.

In summary, we have demonstrated for the first time the preparation of synthetic biodegradable microparticle–biological-transfected-cell hybrids. The microparticles can easily be loaded with proteins, immunostimulatory molecules, or growth factors.^[23,26] Here, we have shown that this assembly process can be carried out with cells that have been transfected to produce fluorescent or antigenic proteins. This cell–microparticle hybrid system therefore has significant potential for multifunctional drug delivery applications. We are currently investigating irradiated tumor cells that have been transfected with granulocyte-macrophage colony-stimulating factor (GM-CSF) and self-assembled with microparticles loaded with immunostimulatory molecules such as CpG oligonucleotides as a new and potent vaccine for cancer. Cell–microparticle hybrids also have significant potential as building blocks for scaffolds in tissue engineering, and for the

design of intelligent biological–synthetic hierarchical structures.

Experimental

Synthesis of PLA–PEG–Biotin: α -hydroxy- ω -amine PEG (1 g) was dissolved in a mixture of acetonitrile (2 mL, Aldrich), methylene chloride (1 mL, Aldrich), and Et₃N (80 μ L, Aldrich). After the addition of NHS–biotin (0.250 g, Sigma), the reactants were stirred overnight under argon. Subsequently, the reaction was worked-up by the slow addition of diethyl ether (40 mL, Aldrich) to precipitate the polymer. The polymer was reprecipitated from hot isopropyl alcohol (70 °C, Aldrich). The reprecipitated polymer (350 mg) was then dried azeotropically and left under vacuum. Lactide (2 g, Purac Biochem bv) was added to biotin–PEG–OH (0.35 g) and diluted with 10 mL toluene and Sn(oct)₂/toluene (0.1 g in 1 mL). The reaction mixture was then brought to reflux at 110 °C for 4 h under argon. The product was precipitated from a dichloromethane (DCM) solution into a cold stirring solution of diethyl ether and isolated by vacuum filtration. The final product was characterized by GPC and ¹H NMR spectroscopy.

Preparation of PLA–PEG–Biotin Microparticles: PLA–PEG–biotin (50 mg) was dissolved in 5 mL of DCM. For rhodamine-loaded microparticles, 1 mg rhodamine 123 (Sigma) was also dissolved in 5 mL DCM. The polymer solution was then added to 500 μ L of a 1% (w/v) PVA (M_w : 250 000, 88% hydrolyzed, Sigma) solution and ultrasonicated for 30 s. The primary emulsion was then added to 50 mL of 1% (w/v) PVA solution and homogenized further for 3 min at 13 500 rpm. The emulsion was then left stirring overnight over a magnetic stirrer to allow DCM to evaporate and to enable the formation of microparticles. The average diameter of the microparticles was 1.4 μ m, as determined by measurements made using a Zetasizer NanoZS (Malvern Instruments) instrument.

Cell Culture: HEK293 cells and EG7/EL4 (CRL-2113/TIB-39) cells were obtained from ATCC (Manassas, VA). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) obtained from Gibco BRL (Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), streptomycin at 100 μ g mL⁻¹, penicillin at 100 U mL⁻¹, and 4 mM L-glutamine at 37 °C in a humidified 5% CO₂-containing atmosphere. The HEK293 cells were passaged at pre-confluence every 4 days in a 1:4 ratio using 0.25% trypsin. Fresh DMEM medium was replenished every 2 days during cell culture. The EG7/EL4 cells were passaged every alternate day in a 1:3 ratio by aspirating two-thirds of the medium and replacing with fresh medium.

Amplification and Purification of Plasmid DNA: GFP-plasmids (Clontech) were transformed to *Escherichia coli* DH5a and amplified in Terrific Broth media at 37 °C overnight at a shaking speed of 300 rpm. The plasmid was purified using an endotoxin-free Qiagen Giga plasmid purification kit (Qiagen, Valencia, CA) according to the protocol provided by the manufacturer. Purified pDNA was dissolved in saline, and its purity and concentration were determined by UV absorbance at 260 and 280 nm.

Transfection of HEK293 and EL4 Cells: HEK293 and EL4 cells were seeded into 24-well plates at a density of 8×10^4 cells per well 24 h before starting transfection. Each well of the 24-well plate was transfected with 0.5 mL reduced-serum Opti-MEM media (Gibco). Polyethyleneimine (PEI, 25 000 branched, Sigma)/pDNA complexes in a ratio of 5:1 comprising 5 μ g PEI in 40 μ L Opti-MEM and 1 μ g DNA in 40 μ L Opti-MEM were added to each well. After 4 h, the transfection media was removed and the cells were washed. After 2 days of further incubation in serum-containing media, the wells were washed with PBS and imaged live. The cells were then ready for use in microparticle–cell assembly experiments.

Scanning Electron Microscopy (SEM): Cell–microparticle hybrids were seeded onto a poly(L-lysine) coated (1 μ g cm⁻²) coverslip and fixed with 2.5% glutaraldehyde solution. After 1 h, the hybrids were

washed twice with a 0.2% sodium cacodylate buffer solution and dehydrated with 25, 50, 75, 95% (4 min each), and 100% ethanol (10 min each) solutions. The cell–microparticle hybrids were then treated with hexadimethylsilazane (HDMS) for 10 min and dried for 3 h. The samples were then sputter coated and visualized using SEM (Hitachi S4800).

HABA/Avidin Assay: This analysis was carried out using a spectrophotometer (Spectramax 384 plus, Molecular Devices, CA) at a fixed wavelength of 500 nm. 180 μ L of HABA/avidin reagent was added to a 96-well plate and three readings of absorbance were recorded with an average read time of 0.5 s. After the initial recording, 120 μ L of the supernatant collected following cell treatment with biotin hydrazide was added to each of the 96-well plates. Three sets of absorbance readings were recorded again after allowing 3 min for reaction. The absorbance decreased proportionately depending on the amount of biotin present on the cell surface because the biotin displaces HABA owing to its higher affinity for avidin. The changes in absorbance were used in conjunction with a calibration curve to calculate the extent of biotinylation of the cell surface.

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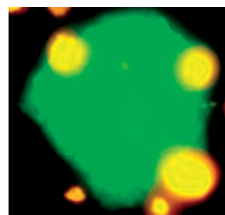
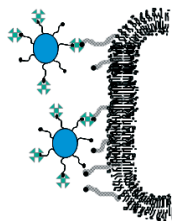
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COMMUNICATIONS

Self-Assembly

Synthetic-biological hybrids of biodegradable microparticles and transfected cells are assembled as schematically illustrated in the figure. The cells are surface functionalized with biotin and the particle surface is also engineered with biotin. Avidin is then used as a bridging protein to bind together the microparticles and the surface-functionalized cells.



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