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Polymeric microspheres as stabilizing anchors for oligonucleotide delivery to dendritic cells

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Abstract

The aim of this study is to evaluate a novel microspheric vector for delivery of oligonucleotides (ODN) into dendritic cells (DC). A requirement of decoy-based modulation of transcriptional activities in DC is that the ODN would have to accumulate inside the cell. Using an ex vivo DC culture model, we demonstrate that anionic microspheres (MS) coated with an ornithine/histidine-based cationic peptide (O10H6) is an effective carrier of short ODN. This method does not disrupt the colloidal nature of the microspheric particles. The MS provide stabilizing effect on DNA and O10H6 complexation. Accumulation of ODN in DC is greatly enhanced with the surface modified MS. Taken together, these data demonstrate that the self assembly system of MS_{O10H6} is an effective delivery vehicle for DNA-based modulation of DC functions.
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1. Introduction

Dendritic cells (DC) are the most potent antigen presenting cells known and play pivotal roles in maintaining immune homeostasis (reviewed in [1]). Genetic manipulation of this leukocyte subset has been employed to up-regulate or down-regulate effector functions of lymphocytes (reviewed in [2]). One such strategy of skewing immature DC into down-regulatory phenotypes is by diminishing binding of nuclear factor kappa B (NF-κB) transcription factors to response elements in the nucleus (reviewed in [3]). This can be accomplished by delivering oligonucleotides (ODN) encoding NF-κB consensus binding sites as decoys into DC. Accumulation of the decoys in the cytosolic space suppresses co-stimulatory activities and maturation of DC [4–7].

One strategy of altering NF-κB-dependent antigen presentation in vivo is by exposing ex vivo cultured DC to high concentration (up to 10 μM) of ODN decoys before injecting into animals [5]. This ex vivo approach is laborious and requires individualized protocol in clinical settings. Direct in vivo administration of decoys, however, is not feasible because the upper limit of local concentration of ODN attainable would likely to be below nano- or pico-molar. Unprotected DNA injected would be significantly degraded prior to reaching DC scattered in the body. Therefore, effective interference of NF-κB activities in DC in vivo would require the decoy to be loaded on protective carriers to assure sufficient accumulation in cells.

In a previous study we have demonstrated that a peptide which consists of ornithine and histidine repeats (O10H6) was effective in delivering plasmid DNA into

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JAWSII cells (a mouse DC line) and murine bone marrow-derived DC in vitro [8]. The ornithine/histidine peptide was shown to bind to DNA and was less toxic to DC than a corresponding lysine-based peptide [8]. Ornithine is a non-natural amino acid with a basic side chain ($-CH_2-CH_2-NH_2$) that is protonated at physiological pH. Poly ornithines have been shown to condense plasmid DNA and facilitate cell transfection in vitro [8–10]. The rationale for including histidine is to exploit the endosomal lysis capability of the imidazole group to allow DNA to escape from lysosomal degradation [11–16]. One limitation of this construct, however, is that condensates of DNA and cationic peptides are generally not stable in biological fluids and likely to dissociate prior to reaching target cells in vivo.

In the current study, we have devised a vector for ODN containing NF-κB consensus binding sequence by coating sub-micron sized carboxylate polystyrene microspheres (MS) with O10H6. The surface modified MS (MS$_{O10H6}$-ODN, cationic) is resistant to digestion/disruption by serum and heparin. Importantly, efficient accumulation of ODN in splenic CD11c+ DC was achieved with nanomolar concentration of the DNA. These data pave the way for further investigation of this microspheric vector in intact animals.

2. Methods and materials

2.1. Preparation of microsphere DNA complexes

The peptide $O_{10}H_6$ was custom synthesized by Sigma-Genosys (Houston, TX) custom synthesis service. Crude peptides were purified by HPLC ($>90\%$) and their identities were confirmed by liquid chromatography-mass spectrometry. The peptides were aliquot in sterile double distilled water (ddH$_2$O) and stored at $-80\,^{\circ}$C until use. Oligonucleotides containing the NF-κB binding sites were custom synthesized by Alpha DNA (Montreal, Quebec, Canada). Annealing of sense (5$'$-AGGGACTTTCCGCTGGGGACTTTCC-3$'$) and anti-sense (5$'$-TCCCTGAAAGGCGACCCCTGAAAGG-3$'$) strands of the decoy was performed in annealing buffer (10 mM Tris, 50 mM NaCl, 1 mM EDTA) by heating the ODN to 100 $^{\circ}$C for 2 min followed by slow cooling (over 60 min) to room temperature.

The principal vector for delivering the ODN was carboxylate polystyrene MS (Polysciences Inc., Warrington, PA) coated with $O_{10}H_6$ (MW = 2250). The MS have an average diameter of 0.1 micron (μm) with less than 10% variance. We have worked with these MS in a previous study aimed at delivering plasmid DNA to DC via the tumor necrosis factor receptor system [17]. The delivery system in the current study was formulated as follow: 3 μl of the MS (2.5% w/v; 4.55×10^{13} particles ml^{-1}) were first coated with 200 μg of $O_{10}H_6$ (gentle shaking for 2 h at RT) in 300 μl of ddH$_2$O. The resulting positively charged particles (denote as MS$_{O10H6}$) were then equilibrated with DNA (18.7 ng) for 30 min at RT. Unbound DNA and peptides were removed by membrane filtration with a Nanosep device (10 K molecular weight cutoff) (Pall, East Hills, NY) centrifuged at 500 g for 5 min at room temperature. MS were recovered and added to 3.0×10^6 cells in 1.5 ml OptiMEM media (Invitrogen, Carlsbad, CA), resulting in a final DNA concentration of 1.55 nM.

2.2. Particle size and zeta potential analysis

A Nicomp 380 ZLS analyzer (Particle Sizing Systems, Santa Barbara, CA) was used to determine particle size distribution and zeta potential of the MS complexes. The mean particle size was measured using suspensions (0.5 ml) of plain MS or MS coated with O10H6 and ODN (MS$_{O10H6}$-ODN). Filtered mixture containing MS$_{O10H6}$-ODN (1.37×10^{11} MS particles), O10H6 (200 μg), and DNA (18.7 ng) were diluted 10 times with ddH$_2$O and analyzed in 1 ml disposable glass tubes. Radius was calculated using the Stokes–Einstein Equation based on diffusion coefficient of the particles in the medium. Data were integrated over 10 min in duplicate experiments and were test fit for Gaussian and non-Gaussian functions. The instrument operates on the dynamic light scattering principle, capable of detecting particles size in the range of 1 nm to 5 μm. Zeta potential measurement was performed with samples diluted 30 times (with ddH$_2$O) integrating data over 10 min.

2.3. Gel electrophoresis

O10H6-ODN and MS$_{O10H6}$-ODN were incubated with 10% FBS at 37 $^{\circ}$C for 24 h followed by addition of low molecular weight heparin sodium (Eikins-Sinn Inc., Cherry Hill, NJ) (5 U/μl) 30 min (room temperature) prior to loading in sample reservoir. DNA was visualized by ethidium bromide staining. Titration of O10H6 in MS and ODN complexation was performed by varying the concentration (20 ng → 20 μg) of O10H6 in fixed amounts of MS and DNA. Samples were analyzed in 1% agarose gel after incubation with 5 U/μl of low molecular weight heparin to liberate DNA bound via ionic interaction. Migration of DNA was modeled using a four-parameter sigmoidal dose–response function $y = (a + (b - a))/(1 + ((c/x)^{\mathrm{d}})$ based on the Levenberg–Marquardt method.

2.4. Isolation of CD11c+ dendritic cells from mouse splenic population

Six to eight weeks old female (certified virus free) BALB/c mice (H-2$^{\mathrm{d}}$) were used to obtain the DC (Hilltop Lab Animals, Inc., Scottsdale, PA). Mice were housed in the Duquesne University Animal Care Facility and all experimentations were handled in accordance with the institutional animal care policy. Splenic cells were harvested from mice and red blood cells were removed with a hypotonic buffer. Cells were then washed extensively with RPMI media before incubating with anti-CD11c magnetic beads (Miltenyi Biotec, CA) in labeling buffer (PBS containing 2 mM EDTA). Bead-coated cells were washed with separation buffer (PBS, 2 mM EDTA, 0.5% Bovine serum albumin) and passed through a MiniMACS column (Miltenyi Biotec, Auburn, CA). Cells expressing CD11c in the eluted population was confirmed by flow cytometry to be greater than 90% (data not shown).

For DNA uptake studies, freshly isolated DC were exposed to either ODN alone (1.55 nM) or ODN complexed with MS_{O10H6} (MS_{O10H6}-ODN) for 2 h at 37 °C 5% CO_2. For CD86 analysis, transfected DC were exposed to lipopolysaccharides (LPS) (Alexis Biochemicals) isolated from *Escherichia coli* (055:B5) for 2 h before staining with anti-CD86 monoclonal antibody conjugated with PE (BD Pharmingen). Cells were then washed with PBS and analyzed using a Beckman Coulter EPICS XL bench-top flow cytometer. At least 10,000 events were sampled for each group. Data were analyzed using one-way ANOVA with confidence level of 90% considered as statistically significant.

2.5. Confocal imaging

Cells were grown on cover slips (Fisher Scientific, Pittsburgh, USA), washed with PBS, and incubated with MS_{O10H6}-ODN for 2 h at 37 °C. As described above, the ODN was labeled with fluorescein. After the incubation period, cells on the cover slip were washed in PBS and fixed in 4% paraformaldehyde for 15 min at 4 °C. Cells were then washed twice in PBS and once in water, and mounted in anti-fade mounting medium (MOWIOL 4-88, CalBiochem, DARM-STADT, Germany). Imaging data were collected using an inverted Leica TCS SP-2 spectral microscope (Leica Micro-systems AG, Wetzlar, Germany) with an argon/helium laser and FITC filter cube. Cells were identified with a 63X oil immersion objective. Images were averaged from twelve scans collected and were processed using ADOBE Photoshop.

3. Results

MS are particles fabricated from synthetic polymers and stabilized as dispersed colloidal systems. They have been used as carriers of nucleic acids for the delivery of plasmids or ODN molecules into mammalian tissues [18]. DNA can be entrapped in the polymer matrix, or passively adsorbed on the surface of polymeric particles [19–33]. For the surface adsorption approach, one method employed was to coat the microspheres with low molecular weight molecules (e.g. CTAB, DEAE-Dextran, cationic lipids), to mediate DNA binding. In this format, condensation of nucleic acid secondary structures results from formation of ion pairs between the DNA and cationic species pre-adsorbed on the particle surface.

In devising an ODN delivery system optimal for dendritic cells, we assessed the adsorption method using O10H6 to modify the surface of carboxylate MS (a schematic diagram of the construct is shown in Fig. 1a). We first studied the size and charge of the particles formed from assembly of MS, O10H6, and DNA (MS_{O10H6}-ODN). Prior to the analysis, the complex suspension was centrifuged through a Nanosep filtration device to remove unbound peptide and DNA. A Nicomp 380 ZLS analyzer (Particle Sizing Systems) was used to determine the hydrodynamic diameter distribution and surface potential (ζ) of MS_{O10H6}-

ODN using a dynamic light scattering technique. Consistent with the manufacture's specification, plain MS (Polysciences, Inc.) had an averaged diameter close to 100 nm with low variability (standard deviation = 5.6 nm; Fig. 1b). After assembly with O10H6 and ODN, the average particle size was determined to be 118 nm (standard deviation = 38.7 nm) conforming to a Gaussian distribution ($\chi^2 = 0.29$) (Fig. 1c). All MS_{O10H6}-ODN particles were found to be in sub-micron size (< 500 nm), suggesting that the particles remained in a dispersed system. Using flow cytometry, it was also revealed that the particles had a unimodal light scattering property (Fig. 1d), suggesting that a population of particles with similar external and internal complexities.

Zeta potential analysis revealed that the MS_{O10H6} particles carried a surface charge of + 30.1 mV. This is a reversal of the surface potential of plain MS (−30.9 mV). Addition of ODN onto MS_{O10H6} did not alter the ζ potential (+ 31.2 mV). This is because a relatively small amount of ODN (18.7 ng) was added to a large amount (200 μg) of O10H6 in the complex. In essence, the particle size and ζ potential analyses revealed that the complexation procedure resulted in colloidal dispersion stabilized by surface potential repulsion.

3.1. MS enhances stability of DNA complexation

Other groups have shown that DNA molecules adsorbed on polymeric particles were protected from de-stabilizing elements in biological fluid [19,33]. In this work, electrophoretic analysis also revealed that MS significantly stabilize the DNA/O10H6 condensates. Fig. 2 (upper panel) shows that, when heparin (5 U/μl) was added to the sample wells, DNA was released freely from O10H6-ODN and ODN but retarded in MS_{O10H6}-ODN (as evident by the second ban). This shows that MS conferred resistance to charge destabilization in the complex. The lower panel of the same figure shows that MS increases resistance of the complex from destabilization by serum incubation (10% FBS, 24 h at 37 °C). In this case, intact DNA was released by prolonged incubation (0.5 h at room temperature) with heparin (5 U/μl,). Serum was used to test the ability of MS_{O10H6} to protect the DNA from enzymatic digestion and protein destabilization. ODN complexed with MS_{O10H6} (MS_{O10H6}-ODN) remained largely intact (with DNA retained in loading wells). DNA complexed with O10H6 without MS were degraded or dissociated from the complex (Fig. 2 lower panel).

3.2. O10H6 bridges MS and DNA

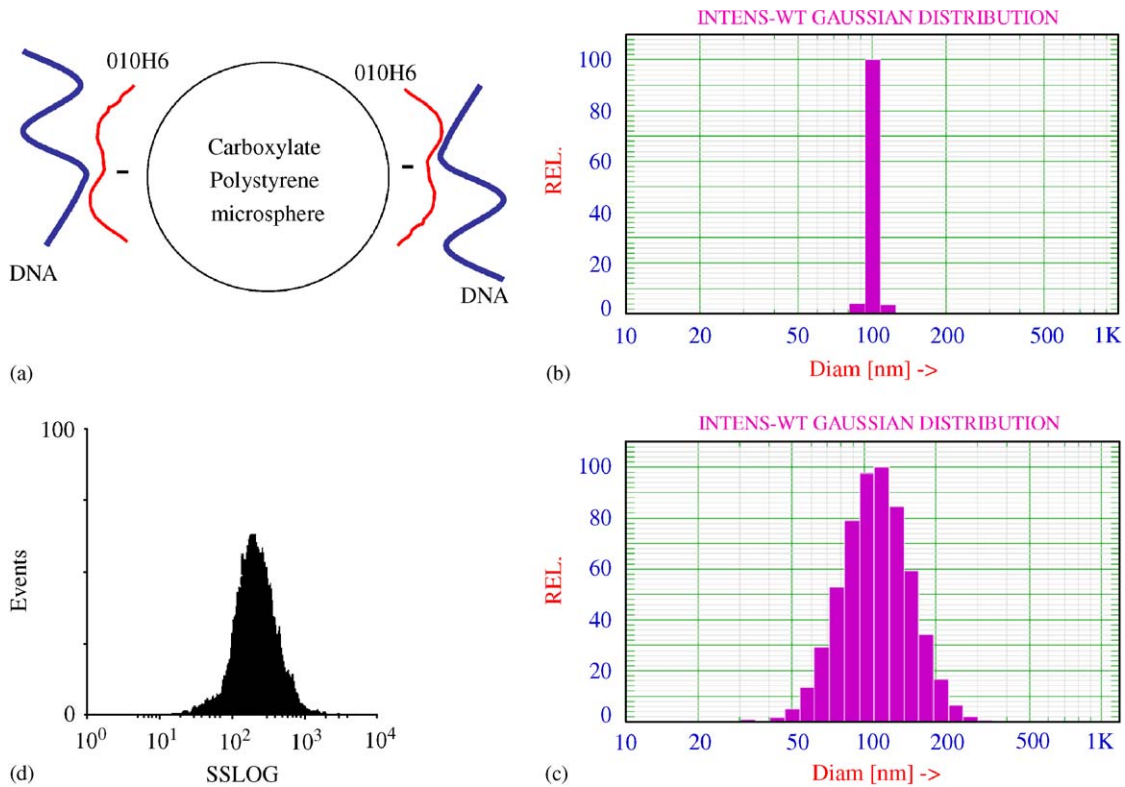We next determined the extent in which O10H6 interacts with both the MS (carboxylate) and the

Fig. 1. (a) A schematic diagram depicting a self assembly system of microspheres (MS), O10H6, and ODN. Particle size was analyzed using a Nicomp 380 ZLS instrument. Measurement was performed on (b) suspensions of plain microspheres, and (c) microspheres coated with O10H6 and ODN (MS$_{O10H6}$-ODN). MS$_{O10H6}$-ODN contains 1.37×10^{11} MS particles, 200 μg of O10H6, and 18.7 ng of DNA in 300 μl of ddH$_2$O. The instrument operates on the dynamic light scattering principle, detecting particle size in the range of 1 nm to 5 μm. Intensity-weighted distribution integrated over 10 min of each sample is shown. Data shown is representative of at least five experiments independently performed. Average particle size of MS$_{O10H6}$-ODN was determined to be 118 nm with a standard deviation of 38.7. (d) Side scattering property of MS$_{O10H6}$-ODN was determined using a Beckman Coulter EPICS XL flow cytometer.

negatively charged DNA. Electrophoretic analysis revealed that the complexation depends on the concentration of O10H6 in the construct (Fig. 3a). As the concentration of O10H6 increased, the amount of DNA liberated from the complex decreased. Using a dose–response function (Fig. 3b), the half-maximal amount required to impede dissociation of DNA from the MS (in the absence of serum) was determined to be 0.45 μg/1.37×10^{11} MS. Bound DNA began to migrate from the MS complex between 0.5 and 1 μg of O10H6. Based on this we estimated the range of N:P ratio in MS$_{O10H6}$-ODN to be 39–78:1. Zeta potential analysis confirmed that the surface charge is near zero with 1 μg of O10H6 in the complexation (data not shown). As expected, in the absence of O10H6, MS were not associated with the DNA (Fig. 3a). These data indicate that while inclusion of MS enhances complex stability, the component critical to assembly of the complex is the cationic peptide.

3.3. MS enhances uptake of ODN in DC

To determine the ability of MS$_{O10H6}$ to enhance ODN accumulation in DC, we used CD11c+ cells freshly isolated from splenocytes of BABL/c mice as the target. Splenic leukocytes contain a heterogeneous mix of interdigitating and marginal DC with differential phagocytic activity, antigen presenting capacity, and turnover rate [34]. Thus, we chose to use splenic DC rather than bone marrow-derived DC (as we have previously done in the O10H6 work [8]) to assess the delivery system in a more diverse phenotypes of DC population pre-existed in vivo. Since all mouse DC subsets identified thus far express CD11c on their cell surface [34], isolation was performed by coating bulk splenic cells (after removal of erythrocytes) with anti-CD11c magnetic beads followed by separation on a MiniMACS column (Miltenyi Biotec, CA). The enrichment was highly efficient (>90%; data not shown), resulting in cultures with minimal contamination with other leukocytes. The antigen presenting capacity of the isolated cells had been confirmed in mixed leukocytes cultures with allogeneic H-2^b (C57BL/6 mice) T lymphocytes as the responders (data not shown).

Enriched DC were then exposed to ODN, O10H6-ODN, MS-ODN, or MS$_{O10H6}$-ODN for 2 h at 37 °C in serum free OptiMEM media. The enhanced stability of MS$_{O10H6}$-ODN in serum/heparin led us to experiment
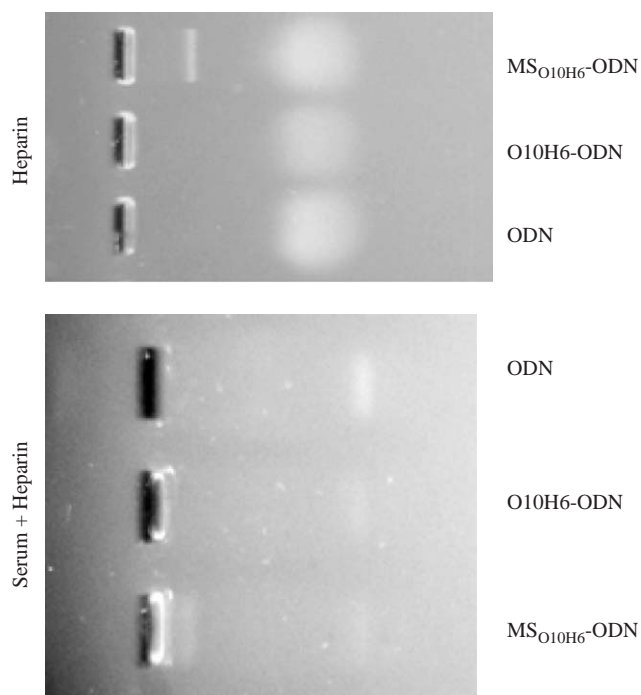
Fig. 2. Electrophoretic analysis of DNA binding to MS_{O10H6} in 1% agarose gel. Upper panel: complexes were disrupted with $5\,U/\mu l$ of low molecular weight heparin. Lower panel: complexes were incubated with 10% FBS at $37\,^{\circ}C$ for 24 h. DNA was released from the complexes by addition of heparin $(5\,U/\mu l)$ for 30 min at room temperature prior to loading in sample reservoir. ODN alone without serum was included as the control. DNA was visualized by ethidium bromide staining. A representative of three experiments is shown.

Fig. 3. Titration of O10H6 in MS and ODN complexation. (a) Varying concentration $(20\,ng \rightarrow 20\,\mu g)$ of O10H6 was added to fixed amounts of MS and DNA. Samples were analyzed in 1% agarose gel after incubation with $5\,U/\mu l$ of low molecular weight heparin to liberate DNA bound via ionic interaction. (b) Migration of DNA was modeled using a four-parameter sigmoidal dose–response function $y = (a + (b - a))/(1 + ((c/x)^d)$ fit using the Levenberg–Marquardt method $(r^2 = 0.98)$. A range of N:P ratio between 39–78:1 was calculated. This was based on estimating the number of anionic sites available on MS calculated from the amount $(0.5–1\,\mu g)$ of O10H6 to initiate DNA release from the complexes (Fig. 3). Zeta potential analysis confirmed that surface charge of the complex is near zero within $0.5–1\,\mu g$ of O10H6 (data not shown).

with a low concentration (1.55 nM in final cell culture volume) of ODN for the in vitro delivery. A saturating amount of O10H6 (200 μg) was used to coat the MS $(1.37 \times 10^{11}$ particles) to obtain particles with positive ζ potential to ensure stability of the colloidal system in the cell culture environment. In preparing MS_{O10H6}-ODN, free (unbound to MS) ODN and peptides were removed from the solution by membrane filtration as a final step prior to adding to cells.

In three independent experiments conducted, we found MS_{O10H6} to be the most efficient in delivering ODN into DC compared to the O10H6, MS, and ODN alone groups. In a representative experiment, 51% of DC had taken up the ODN in MS_{O10H6}-ODN-treated DC whereas only 11.7% of cells were positive in the ODN alone group (Fig. 4b and e). MS_{O10H6}-ODN delivery also resulted in higher uptake than O10H6-ODN and MS-ODN treatments (Fig. 4c, d and e). Collective analysis of mean fluorescent intensity (MFI) from all experiments conducted showed cells exposed to MS_{O10H6}-ODN had approximately four fold higher uptake than in DC exposed to "naked" ODN (Fig. 4f). MS_{O10H6}-ODN delivery also resulted in significantly higher DNA uptake than MS-ODN, confirming that the cationic peptide is an essential component of the
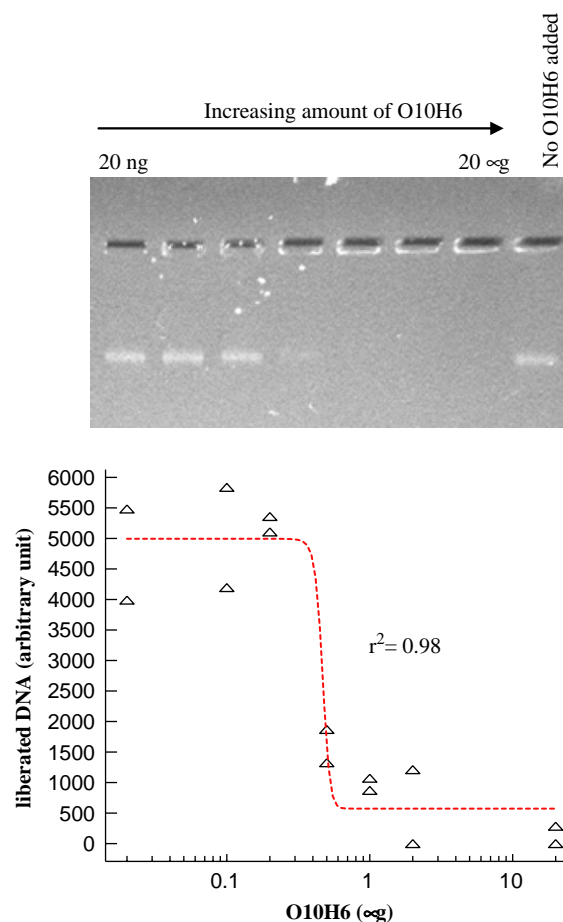
delivery. $O_{10}H_6$-ODN-exposed DC had lower uptake compared to MS_{O10H6}-ODN.

To confirm that ODN had indeed internalized into cells, we performed confocal image analysis of splenic adherent cells incubated with MS_{O10H6}-ODN at $37\,^{\circ}C$ or $4\,^{\circ}C$ for 2 h. Fig. 5 shows strong fluorescence signal in cells exposed to MS_{O10H6}-ODN at $37\,^{\circ}C$ but only weak signal when incubated at $4\,^{\circ}C$, suggesting an active uptake process. In addition, we have examined the biological activity of MS_{O10H6}-ODN in LPS-treated DC. Expression of CD86, a costimulatory molecule, in splenic CD11c+ cells exposed to MS_{O10H6}-ODN was only two-thirds in percent positively gated of untreated control while cells treated with ODN only
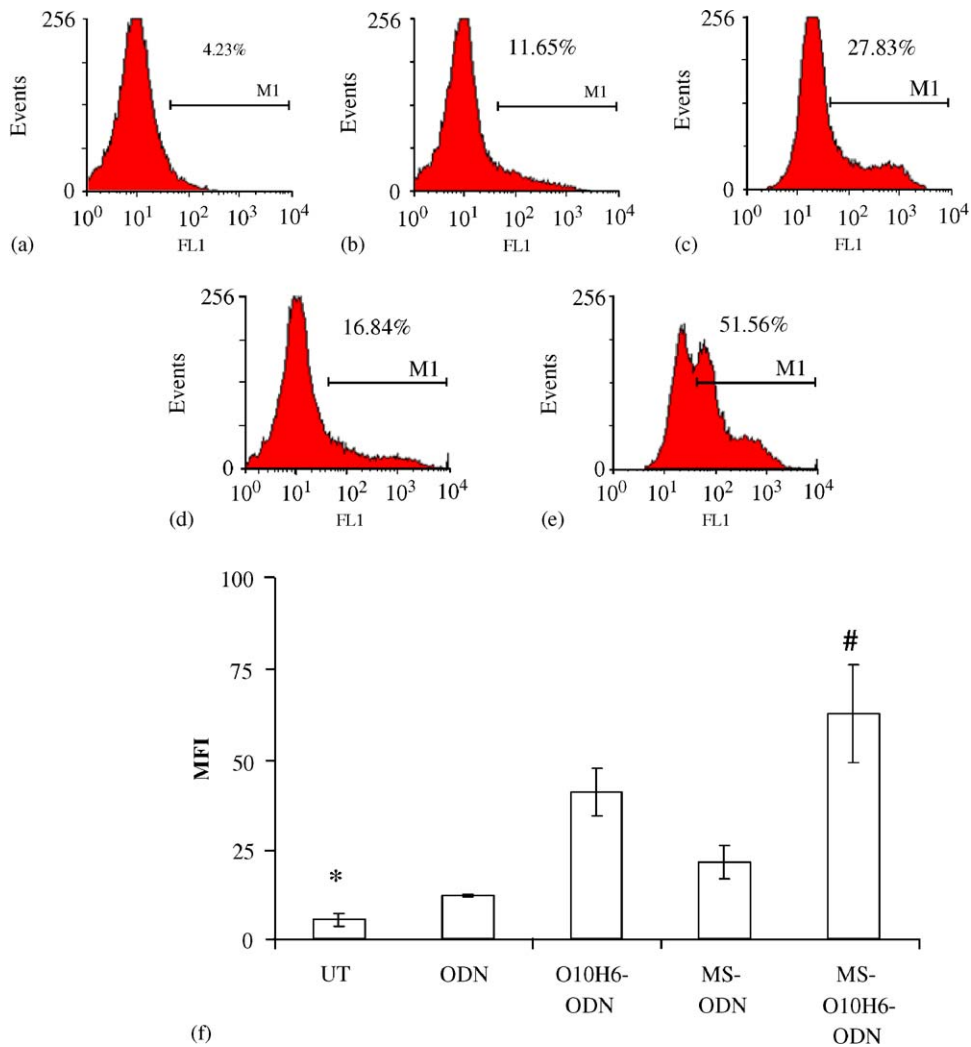
Fig. 4. Uptake efficiency of FITC-labeled ODN by various carriers was determined in CD11c+ DC cultures. DC were isolated from bulk splenocytes after removal of erythrocytes by passing cells coated with anti-CD11c magnetic beads through a MiniMACS column. Purified cells were then exposed to the respective construct for 2 h at 37 °C 5% CO_2 before washing with phosphate buffer saline and analyzed using a Beckman Coulter EPICS XL flow cytometer. Data were obtained from at least 10,000 events gating on live cells. Histograms of a representative experiment are shown (a)–(e): (a) control DC, (b) ODN, (c) O10H6-ODN, (d) MS-ODN, (e) MS_{O10H6}-ODN. (f) Data in bar graph represent averaged mean fluorescent intensity (MFI) calculated from three independent experiments. Errors represent standard error of mean (SEM). *Control group was DC cultured under the same conditions. #One-way ANOVA shows statistical significant difference based on 90% confidence interval between ODN and MS_{O10H6}-ODN and MS-ODN and MS_{O10H6}-ODN.

or O10H6-ODN remained the same. This suggests that MS_{O10H6}-ODN was able block signaling of the NF-κB pathway triggered by LPS.

4. Discussion

In the current study, we have devised a microspheric-based system for delivering ODN molecules in mammalian cells. The system is designed to take advantage of the combined effects of the stabilizing surface of anionic MS and O10H6, a DNA binding peptide compatible with DC [8]. The construct has the necessary physical characteristics with respect to size (nano scale) and charge (positive ζ potential) to facilitate cellular uptake of DNA. We observe a significant advantage of ODN accumulation in DC exposed to MS_{O10H6}-ODN compared to unprotected ODN. Significantly, ODN delivery with MS_{O10H6} is achieved with a low concentration (1.55 nM) of the decoy.

Previous studies have shown complexes could form spontaneously between DNA and oligo-lysines [35–40] and oligo-ornithines [8–10] above a certain N:P ratio. Thus, complexes of O10H6-ODN may exist in the MS_{O10H6}-ODN dispersion due to exchange of O10H6 and ODN on and off of the MS. However, the data suggest that the equilibrium is shifted heavily towards the ternary form. First, the titration experiment of varying amounts of O10H6 on MS in complextion with DNA (Fig. 3) suggests the self-assembly process resulted in a single type of complex. If significant amount of duplex (or higher order)
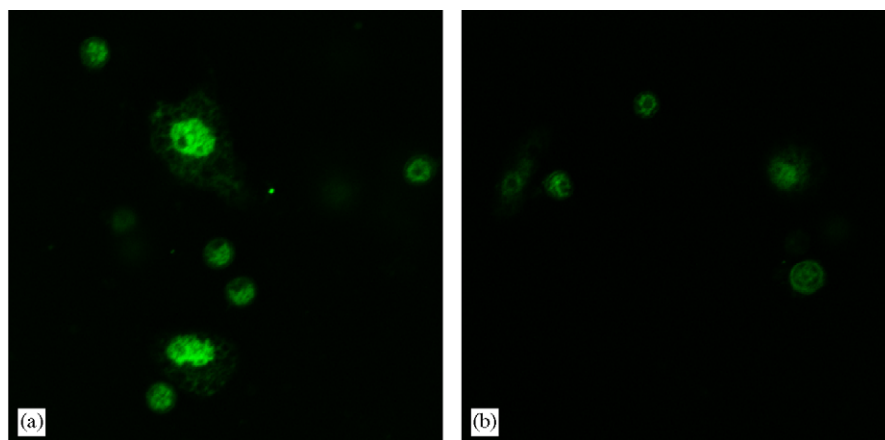
Fig. 5. Confocal microscopy image of ODN (FTIC) captured by splenic APC exposed to MS_{O10H6}-ODN. Adherent splenic cells were incubated with the complex for 2 h at (a) 37 °C or (b) 4 °C. Images were captured by a Lecia confocal microscope at 63X magnification.

of O10H6-ODN were present, charge disruption by heparin would not have exhibited as a single transition but biphasic (or multiphasic). Second, the unimodal size distribution in both Gaussian and non-Gaussian (data not shown) fits suggests the absence of significant quantity of O10H6-ODN in MS_{O10H6}-ODN because the dual complex would likely to have different scattering properties than the ternary complex. Third, side scattering data obtain from flow cytometry analysis of the particles without cells also revealed a unimodal population, suggesting that a single population of similar surface and internal complexities (Fig. 1d). Taken together, most probably MS_{O10H6}-ODN is the dominant element in the transfection experiments.

Enhanced delivery of DNA with MS may be explained by two mechanisms. One is the ability of the MS to stabilize the $DNA/O_{10}H_6$ condensates against serum and heparin disruption (Fig. 2). A possible second mechanism is that the uptake was driven by sedimentation of particles through the medium onto the cell monolayer. Luo and Saltzman [41,42] have demonstrated that faster rate of sedimentation of silica-anchored plasmid DNA increased residency time of the transfection particles on HeLa cells. Therefore, the observed enhanced delivery with MS_{O10H6} in the current study is likely due to the combined effects of increased localization of the particles on the cell surface and protection of the complex from dissociation in cell culture environment.

A potential advantage of MS_{O10H6}-ODN as a therapeutic delivery system is its inert and non-toxic nature. At the density used in this study, the MS do not activate or adversely affect the antigen presentation functions of DC (reviewed in [18]). This might be due to lowering of charge density of MS_{O10H6}-ODN by diffusing the protonated amines of O10H6 on the surface of the MS (theoretical surface area: $4\pi r^2 \times 1.37 \times 10^{11} = 2.4 \times 10^8 \, m^2$). Condensates of O10H6-ODN would have much higher charge density due to their limited surface area and more likely to

disrupt membrane lipid bilayer. The construct also allows considerable design flexibility because a wide range (over 2 log values) of O10H6 can be used to accommodate DNA of different sizes. We propose that this self assembly system may be effectively applied in delivering decoy ODN to modulate DC-mediated immune functions in vivo.

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