Available online at www.sciencedirect.com
ScienceDirect
ELSEVIER
International Journal of Pharmaceutics 321 (2006) 143–154
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Uptake characteristics of NGR-coupled stealth PEI/pDNA nanoparticles loaded with PLGA-PEG-PLGA tri-block copolymer for targeted delivery to human monocyte-derived dendritic cells

Stanley Moffatt *, Richard J. Cristiano

Department of Genitourinary Medical Oncology, Laboratory of Experimental Therapeutics, University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030, United States

Received 14 February 2006; received in revised form 3 May 2006; accepted 4 May 2006
Available online 10 May 2006

Abstract

We have investigated the *in vitro* uptake, toxicity, phenotypic consequences and transfection efficiency of a stealth NGR/PEG/PDBA-coupled-SHA-PEI/pDNA targeting polyplex loaded with PLGA-PEG-PLGA tri-block copolymer in human monocyte-derived dendritic cells (DCs). Modification with PEG effectively shielded and reduced non-specific phagocytosis by immature DCs to approximately 20%. Coupling the NGR cell-specific peptide to the PEGylated polyplex (NGR/PEG/PDBA-SHA-PEI/pDNA) however resulted in specific and enhanced phagocytosis in DCs without any observable toxicity at the optimum concentration of 0.25% of the copolymer. DNase treatment had no effect on DNA integrity in the encapsulated polyplex. Confocal microscopy confirmed intracellular localization of the targeting NGR/PEG/PDBA-SHA-PEI/pDNA microparticles, resulting in more enhanced uptake of the radiolabeled plasmid DNA and approximately 5- and 10-fold increase over the control tri-block Pluronic F68 copolymer and the non-targeting polyplex, respectively. More importantly, phagocytosis of the targeting microparticles neither altered the functionality of immature DCs nor the phenotypic expression of DC-specific cell surface molecules, CD80, CD86, CD40 and CD54 (ICAM-1), suggesting that uptake of the targeting microparticles by themselves did not induce DC maturation. Taken together, these results suggest that PLGA-PEG-PLGA encapsulation of this stealth targeting polyplex has no negative effects on key properties of immature DCs and should pave the way for targeting DCs for vaccination purposes.

Keywords: Polyethyleneimine (PEI); PLGA-PEG-PLGA; Polyethylene glycol (PEG); Phenyl(di)boronic acid (PDBA); Salicyl hydroxamic acid (SHA); NGR peptide; Dendritic cells; Targeted delivery

1. Introduction

Dendritic cells (DCs) are potent antigen presenting cells with a unique capacity to induce an immune response and have thus been identified to play a crucial role in DNA vaccination (Coombes and Mahony, 2001). Antigen uptake and processing by DCs triggers a cascade of signaling network resulting in activation and maturation of DCs, phenotypically characterized by increased expression of CD40, ICAM-1, CD80, CD86 costimulatory molecules, MHC molecules, secretion of cytokines (e.g. IL-12) and chemokines (e.g. CCL19 and CCL22). DCs display 10–100 times more MHC molecules than other antigen presenting cells (APCs) and hence offer a powerful tool to manipulate the immune system (Banchereau and Steinman, 1998) for DNA vaccination. However, inherent difficulty in transfecting DCs, in combination with low numbers of DCs found in the epidermis (Bergstresser et al., 1980) is considered to be one of the major bottlenecks of this vaccination approach.

Gene delivery systems using non-viral polymeric carriers have received much attention because they have conceivably far less safety problems. Emphasis has been placed on microparticulate DNA vaccine delivery systems formed between DNA and polycations due to the inherent capability of DCs to efficiently phagocytose particles in the micrometer range (Thiele et al., 2001; Foged et al., 2002). Polycations including synthetic polymers (Mumper et al., 1996), lipids (Felgner et al., 1987) and polymer–lipid hybrids (Han et al., 2001) have been utilized in these formulations to improve non-viral gene delivery. Previous

* Corresponding author. Tel.: +1 713 563 4765; fax: +1 713 745 2245.
E-mail address: stanley-moffatt@excite.com (S. Moffatt).

0378-5173/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2006.05.007

studies have shown that PLGA microparticles are readily phagocytosed by monocyte-derived DCs *in vitro* (Jilek et al., 2004). In other common approaches, non-ionic polymers consisting of tri-block copolymers of polyethyleneoxide and propyleneoxide (PEO-PPO-PEO) pluronics have been used to enhance gene delivery (Lemieux et al., 2000). While encapsulation of DNA in biodegradable PLGA microparticles leading to protection and controlled release of DNA seems to be an attractive strategy for gene delivery, there is a paucity of information on extensive characterization and formulation of PLGA-encapsulated nanoparticles for gene delivery to DCs.

Previous studies have shown that drug release from PLGA microspheres is very low due to the hydrophobicity of both drug and PLGA (Mu and Feng, 2001; Feng et al., 2004). It is therefore conceivable that incorporation of a hydrophilic segment such as poly(ethylene glycol) (PEG) into the hydrophobic PLGA chain forming the tri-block copolymer PLGA-PEG-PLGA would greatly facilitate the drug release. Indeed, earlier studies (Deng et al., 1990; Li et al., 2000) have demonstrated that this tri-block copolymer increases the release rate of hydrophilic proteins. Another potential advantage provided by the hydrophilic PEG would be improvement of the biocompatibility of the delivery vehicle. This is because most of the biological environment is hydrophilic in nature and biocompatibility appears to be correlated directly with the degree of hydrophilicity that a surface exhibits (LaPorte, 1997). Consequently, we utilized PLGA-PEG-PLGA in the present study to encapsulate the PEGylated polyplex to yield a stealth gene delivery vehicle for evaluation of transfection efficiency in DCs.

Phagocytic cells such as DCs recognize injected polymeric particles as foreign materials and remove them quickly and efficiently from the blood stream. The non-specific nature of the phagocytosis by DCs represents one of the main hurdles that need to be overcome in order to achieve selective targeting of particulates to selected cells. PEG surface coatings have been investigated for a wide variety of biomedical applications where their immobilization on surfaces have been shown to decrease protein adsorption (Norman et al., 1993; Kenausis et al., 2000) and the non-specific uptake by phagocytes (Norman et al., 1993; Luck et al., 1998).

We recently reported a novel formulation of a salicyl hydroxamic acid (SHA)-derivatized PEI/pDNA and a PEGylated PDBA linkage for enhanced *in vivo* transfection (Moffatt et al., 2005; Moffatt et al., 2006a,b). In an attempt to use this coupling strategy for DNA vaccination, the first aim of the present study was to investigate if the disulphide-bridged NGR (Asparagine-Glycine-Arginine) domain-containing CNGRC cell-specific peptide coupled to the PEGylated polyplex (NGR/PEG/PDBA-SHA-PEI/pDNA) would enhance receptor-mediated phagocytosis, since NGR receptors are over-expressed on the surface of immature phagocytic DCs (Arap et al., 1998; Dong et al., 2000). Secondly, because the availability of functionalized PEG-PLGA permits the preparation of target-specific nanoparticles by conjugation of cell surface ligands, this study was undertaken to investigate the effect of PEGylating the surface of the encapsulated microparticles containing the derivatized SHA-PEI/pDNA on alleviating non-specific phagocytosis by DCs. We also performed a comparative evaluation of the *in vitro* uptake, toxicity profile and functional characteristics of immature DCs transfected with a control Pluronic F68-encapsulated and PLGA-PEG-PLGA-encapsulated NGR/PEG/PDBA-SHA-PEI/pDNA targeting polyplex. Even more importantly, we examined the influence of the encapsulated targeting polyplex on the phenotypes of immature DCs and compared it to the matured stage upon LPS challenge. Our results reveal this coupling strategy and delivery vehicle as a potent and efficient platform for targeting particulate vaccine delivery systems to professional antigen presenting cells like immature DCs, without any significant adverse effects on the phenotypes and functionality of the cells.

2. Materials and methods

2.1. Materials

PLGA-PEG-PLGA tri-block copolymer (MW = 4200; PEG MW 1450) was obtained from MacroMed Inc. (Sandy, UT, USA); PEG (MW 3000) was from Sigma and PDBA-x-NHS was obtained from Prolinx Inc. (Seattle, WA, USA). The cell-specific CNGRC and the control CARAC peptides were gifts from Drs. Arap and Pasqualini (MD Anderson Cancer Center, Department of Genitourinary Medical Oncology). Branched polyethylenimine (MW 25000) was purchased from Aldrich (Milwaukee, WI, USA). Pluronic F68 (MW 8400) was obtained from BASF (Parsippany, NJ, USA). RPMI medium and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). Petri dishes (Costar, Cambridge, MA); Glutamine, 1000 U/ml penicillin–streptomycin (Life Technologies, Gaithersburg, MD); GM-CSF (1000 U/ml) and IL-4 (200 U/ml) were obtained from Schering Plough Research Institute (Kenilworth, NJ, USA); 2 units/μl TURBO DNase™ (Ambion, Inc., Austin TX); Alexa Fluor 488, 4′-6-diamidino-2-phenylindole (DAPI) and PicoGreen quantification assay kit (Molecular Probes, Eugene, OR). Propidium iodide (PI) and FITC-annexin V were from Becton Dickinson (Mountain View, CA); BCA protein assay kit (Pierce, Rockford, IL, USA) and the [α-^{32}P]dATP radioactive label kit were from Amersham (Piscataway, NJ, USA). Lipopolysacharide (LPS) (1 μg/ml, *Escherichia coli* 0.55:B5) was purchased from Sigma.

2.2. Methods

2.2.1. Establishment of DCs from human peripheral blood mononuclear cells (PBMCs)

After mixing whole blood from healthy donors (>10 participants) with Ficoll-Hypaque, the mononuclear cells was collected after centrifugation and plated in petri dishes for 1 h at 37 °C to remove non-adherent cells. After washing with PBS, adherent cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 2 mM glutamine, 1000 U/ml penicillin–streptomycin, 800 U/ml GM-CSF and 500 U/ml IL-4. The culture medium was changed every other day with 300 μl of fresh medium containing 2400 U of GM-CSF and 1500 U of IL-4. The detached cells, the main population of CD1a$^+$ cells, were used for experiments after culture for 7 days. During this period of time, the exam-

ination of cell type-specific markers in a gated population of established DCs consistently showed CD14 (0%); CD3 (0%); CD19 (1%); and CD56 (2%). Consistently, more than 95% of the cells in the gated region expressed CD1a and more than 95% of monocyte-derived DCs obtained under the present experimental conditions were pure as analyzed by flow cytometry (data not shown). 1.5×10^5 DCs were cultured at 37 °C in growth medium in a humidified 5% CO_2 atmosphere.

2.2.2. Preparation and size analysis of microparticle formulations

Formation of the PEI derivative in which 5% of the primary amine groups of PEI was replaced with SHA (SHA-PEI), was done by Prolinx Inc., (Seattle, WA), using 25 kDa PEI (Aldrich Chemical Company, Inc., Milwaukee, WI, USA). A working solution of 10 mM SHA-PEI (pH 7.4) was made in endotoxin-free water and stored at 4 °C. Generation of NGR/PEG/PDBA and SHA-PEI/pDNA (Moffatt et al., 2005) as well as PEG/PDBA (Moffatt et al., 2006a) polyplex have been adequately described previously. Different concentrations of either Pluronic F68 or PLGA-PEG-PLGA copolymer dissolved in PBS (0, 0.1, 0.25, 0.5 and 1%, w/v) was then added to the PEGylated polyplex and the incubation continued for an additional 15 min at room temperature (RT). The hydrodynamic radii of the polyplex alone or PEGylated polyplex in the presence and absence of PLGA-PEG-PLGA or control copolymer were measured by dynamic light scattering, and ζ potentials by a Zetasizer 3000HS (Malvern, Southboro, MA). The measurements were performed according to the manufacturer's instructions with the following settings: temperature, 25 °C; scattering angle, 90°; analysis mode. Data were interpreted using the CONTIN software (Malvern Instruments, Malvern, England).

2.2.3. Confocal microscope and scanning electron micrograph examinations

DCs were transfected with Pluronic F68- or PLGA-PEG-PLGA-encapsulated NGR/PEG/PDBA-SHA-PEI/pDNA polyplex. Cells were collected at 24 h after transfection and fixed in 4% paraformaldehyde in 0.1 M PBS, pH 7.4, for 30 min. For immunofluorescence microscopy, microparticles were first labeled with rhodamine–albumin (5 μg/ml) (Sigma–Aldrich, St. Louis, MO) for 2 h, co-cultured with DCs for 16 h at either 4 or 37 °C, fixed with 1% formaldehyde, permeabilized with Triton X-100 (0.1%) and then washed gently with PBS. DCs containing labeled microparticles were further labeled with a rhodamine–phalloidine diluted 1:10 in 2% bovine serum albumin (BSA) in PBS for 2 h to delineate the actin cytoskeleton, and then washed thoroughly to remove non-adherent or extracellular particles before incubation for 60 min in the dark. DCs were then stained with Alexa Fluor 488 and 4′-6-diamidino-2-phenylindole (DAPI), according to manufacturer's instructions. The samples were washed twice for 5 min each with PBS before mounting on cover slips for fluorescence microscopy (Axiovert, Zeiss, Germany; excitation 546/emission 590, beam splitter 580). Deconvolution analysis was performed with Openlab Deconvolution Software (Improvision, Lexington, MA). The morphologies of particles were assessed by scanning electron microscopy using an AMR-1000 at 10 kV with a gold–palladium conductive coating after 24 h post culture.

2.2.4. Cell viability assay

DCs were seeded at a density of 2×10^4 cells/well in 96-well plates. Cells were incubated for 24 h at 37 °C in a CO_2 incubator with 1 ml growth medium prior to transfection with the targeting polyplex either alone or with different concentrations of copolymer. Medium was replaced with fresh medium on the day of transfection before adding polyplex formulations in a total volume of 40 μl at 37 °C. Apoptotic cells were evaluated by using propidium iodide (PI) or FITC–annexin V staining according to the manufacturer's instructions. The early apoptotic cells were evaluated by FITC–annexin V binding where cells were washed in cold annexin V buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$) prior to treatment with FITC–annexin V for 15 min at 4 °C.

2.2.5. FACS analysis of DC phenotype

DCs were analyzed on a FACScan flow cytometer (Becton Dickinson) after staining with the following mAbs according to the manufacturer's instructions: FITC-labeled anti-CD80, FITC-labeled anti-CD40, FITC-labeled anti-CD86 and FITC-labeled anti-CD54 (ICAM-1) (BD Biosciences Pharmingen, San Jose, CA). The respective isotype controls were FITC-labeled mouse IgG1, IgG1, IgG2a and IgG1 (Pharmingen). For maturation, cells were challenged with 5 μg/ml LPS 48 h before antibody labeling.

2.2.6. DNA internalization and uptake studies

Plasmid DNA was labeled with [α-^{32}P]dATP (400 Ci/mmol, 10 mCi/ml). 1×10^5 cells/ml growth medium was cultured for 24 h. Labeled DNA complexed with SHA-PEI and encapsulated in PLGA-PEG-PLGA was added to the cells and further incubated for 3 h at either 4 or 37 °C. After extensive washing in PBS and cell lysis, cellular uptake of DNA was measured with a liquid scintillation counter. The amount of internalized DNA was expressed as a percent radioactivity by relating cells with the PLGA-PEG-PLGA formulation and ones with either PEI/pDNA or SHA-PEI/pDNA complexes alone.

2.2.7. Encapsulated DNA integrity and release profile

NGR/PEG/PDBA-SHA-PEI/pDNA polyplex containing different concentrations of PLGA-PEG-PLGA copolymer were dispersed in 100 μl of 10 mM Tris–HCl buffer (pH 8.0) and 80 μl of 10 mM $MgSO_4$ in water. Consequently, 1 μl of 2 units/μl TURBO DNase™ I in 0.9% NaCl was added and incubated for 30 min at 37 °C. After digestion, microparticles were washed three times with 200 μl of fresh Tris–EDTA buffer and the DNA was extracted and quantitated. DNA release was determined by suspending 0.25% (w/v) of either Pluronic F68 or PLGA-PEG-PLGA in a total volume of 3 ml of PBS, pH 7.4, containing either SHA-PEI-complexed empty plasmid, SHA-PEI/pDNA, PEG/PDBA-SHA-PEI/pDNA or NGR/PEG/PDBA-SHA-PEI/pDNA. The vials were fixed in a 37 °C shaker, samples withdrawn at regular time intervals over a 7-week period and replaced with fresh medium. In all

oligonucleotide analysis, DNA was extracted and quantitated by PicoGreen assay.

2.2.8. DC transfection analysis

Isolation and purification of pCMVβgal plasmid DNA (pDNA) containing the *Escherichia coli* βgal gene under the control of the cytomegalovirus enhancer and promoter for transfection studies were performed as described previously (Moffatt et al., 2005). Approximately 1.5×10^5 cells were plated in 12-well plates (Falcon, Becton Dickinson) in the appropriate medium 24 h prior to transfection. Microparticle formulations containing 6 μg pDNA in a total volume of 40 μl were incubated in 1 ml of serum-free medium for 3 h at 37 °C in a CO_2 incubator. After the initial incubation, 1 ml medium was withdrawn, replaced with an equal amount of growth medium and cells incubated for an additional 24 h at 37 °C. Expression and quantitation of βgal from transfected cells was monitored for 28 days using a Galacto-light chemiluminescent reporter assay (Tropix), as previously described (Moffatt et al., 2005).

2.2.9. Phagocytosis studies

Approximately 5×10^6 DCs cultured on cover slips in 24-well plates were first washed gently with growth medium and fixed with 3% paraformaldehyde for 20 min at RT. Microparticles containing the various polyplex formulations were added after gently washing out medium before incubating cells for 6 h at 37 °C. Due to the weak adherence of DCs to the cover slips, washing steps were performed with extreme caution. Approximately 300 μl of medium was then added for better visualization before photography of phagocytosed cells.

2.2.10. Statistical analyses

All data were processed and analyzed by Sigma-Plot 8.0 software (SPSS, IL). The statistical significances were evaluated by *t*-test of the software and $P < 0.05$ was considered significant.

3. Results

3.1. Establishment of DCs from human PBMCs

DCs were propagated from culturing adherent human PBMCs in the presence of GM-CSF and IL-4. Detached cells were removed at different time points, stained with anti-CD1a or isotype-matched control mAb and analyzed in a flow cytometer. As shown in Fig. 1A, after culturing for 7 days, more than 95% of the gated monocyte-derived DC population expressed CD1a. In contrast, the expression of CD14 was barely detectable after 7 days of culture (Fig. 1B). All experiments in the current study were performed with DCs cultured for 7 days, at which time point DCs routinely represented 75–95% of the total cell population.

3.2. Size analysis and uptake characteristics of microparticles by immature DCs

In the present study, there was no detectable change in the sizes (125.4–132 nm) and the surface ζ potential (45.8 ± 0.68 mV) of SHA-PEI complexes in the presence of 0.25% PLGA-PEG-PLGA. There was also no observable difference in morphology between PLGA-PEG-PLGA- and Pluronic F68-encapsulated particles in DCs (data not shown). The interaction of DCs with the microparticles was studied by fluorescence microscopy. After incubation at 37 °C, most DCs were observed to be associated with microparticle formulations (Fig. 2A–C). To verify this, deconvolution analysis of acquired images confirmed that the particles were actually localized intracellularly (Fig. 2G), thus excluding the possibility that the more efficient delivery of encapsulated polyplex to the DCs was due to cell surface-adherent microparticles creating high local concentrations at the cell membrane. DCs were also imaged at later time points, and engulfed particles were still visible in cells 48–72 h after loading (data not shown). However, if DCs were incubated at 4 °C (Fig. 2D–F), no particles were visible in association with the cells, suggesting that the uptake of particles was an energy-dependent process. These data show that the PEGylated SHA-PEI/pDNA polyplexes loaded on PLA-PEG-PLGA microparticles, and having diameters of less than 5 μm, are preferentially and avidly phagocytosed by professional antigen presenting cells (APCs).

3.3. Effect of encapsulated microparticle uptake on viability, phenotype and function of immature DCs

One of the major concerns about the uptake of microparticles by DCs is that it may cause cytotoxicity or disrupt DC function. We hence investigated viability, maturation and function of immature DCs following culture with empty or loaded microparticles. Immature DCs were co-cultured with Pluronic F68 or PLGA-PEG-PLGA (0.25%, w/v) containing various polyplex formulations overnight and the degree of cell death was measured by annexin V binding using empty microparticles as controls. There was no significant cell death at the optimized concentration of 0.25% of the PLGA-PEG-PLGA copolymer, and cell death was also not significantly different from DCs co-cultured with the control Pluronic F68 copolymer (Fig. 3A). Furthermore, there was no observable difference in cytotoxicity between targeting and non-targeting polyplexes, and between PEGylated and unPEGylated complexes (data not shown) suggesting the usefulness of PEGylation in minimizing the steric hindrance in order to improve overall polyplex bioavailability. To assess the influence of encapsulating the targeting polyplex on DC maturation, we measured the expression of the DC surface markers, CD80, CD40, CD86 and ICAM-1 on immature and mature DCs with or without the targeting microparticles. As shown in Fig. 3B, immature DCs bearing microparticles exhibited a similar DC phenotype to those without any loading. The stimulation by LPS to convert immature DCs to matured stage induced significant upregulation CD80, CD40, CD86 and ICAM-1 which was also comparable to that observed without any loading**.** Similar results were obtained with the unPEGylated polyplex (data not shown). These results indicate that the microparticle uptake did not influence DC phenotype and their ability to mature. Moreover, under the present experimental conditions, the uptake of the encapsulated microparticles did not by

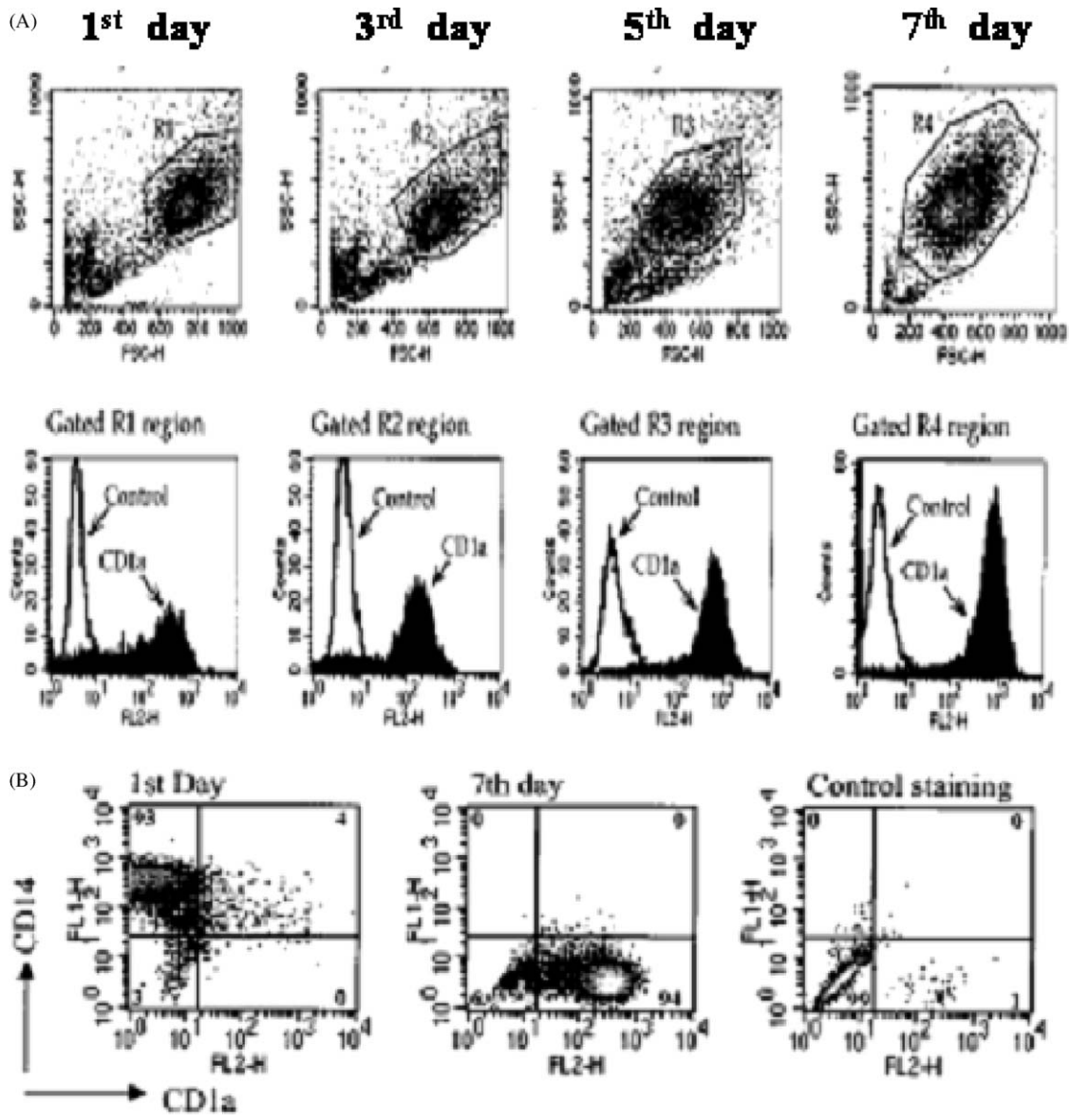


Fig. 1. Generation and identification of DCs from human peripheral blood. (A) Whole blood was obtained from healthy donors. After mixing with Ficoll-Hipaque and centrifugation, the layer containing mononuclear cells was collected. The adherent mononuclear cells were maintained in complete medium containing IL-4 and GM-CSF. After culturing for 1, 3, 5 and 7 days, the detached cells were stained with CD1a or isotype-matched control mAb. (B) The first day cells (adherent) and the seventh day cells (detached) were stained with both CD1a and CD14 isotype-matched control mAb. The gated areas represent the monocyte-derived DC populations. Each density plot or histogram is generated using at least 10^4 events.

itself induce phenotypic maturation of DCs. Integral to this, it was essential to assess the effect of polyplex uptake on APC function by measuring the ability of DCs to stimulate allogeneic T cells following incubation with microparticles (0.25%, w/v) with or without various polyplex formulations. Fig. 3C shows that the degree of T cell proliferation elicited by DCs co-cultured with microparticles alone was both insignificant and identical to that of DCs co-cultured with polyplex with empty plasmid DNA. While there was a robust stimulation of T cells, there was also no significant difference ($P < 0.02$) between the DCs co-cultured with the encapsulated targeting polyplex and the non-targeting polyplex. Again, there was no significant difference ($P < 0.02$) between Pluronic F68- and PLGA-PEG-PLGA-loaded polyplex and T-cell stimulation index (data not shown). These results suggest that the uptake of NGR/PEG/PDBA-SHA-PEI/pDNA loaded with PLGA-PEG-PLGA microparticles is not toxic to DCs and perturbs neither their maturation state nor their ability to stimulate T cells *in vitro*.

3.4. DNA integrity and uptake studies

An essential study was to examine the protection of the microparticle-loaded DNA from enzymatic degradation upon exposure to DNase. Fig. 4A shows the structure of free and PLGA microparticle-encapsulated pDNA in the targeting polyplex after exposure to DNase enzyme. Gel electrophoresis of DNase-treated free pDNA (lane 4) shows complete degradation of the pDNA. On the other hand, PLGA encapsulation of pDNA

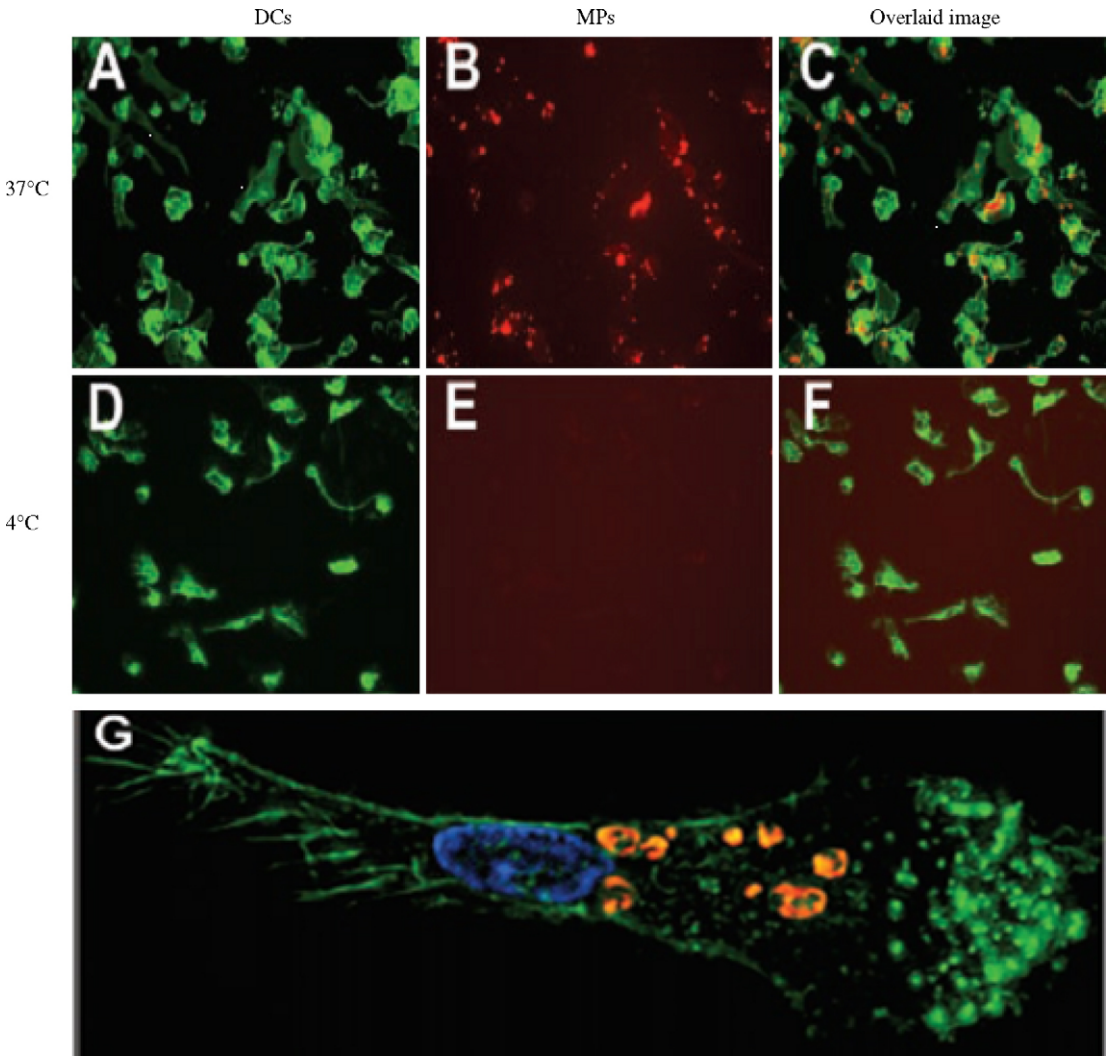
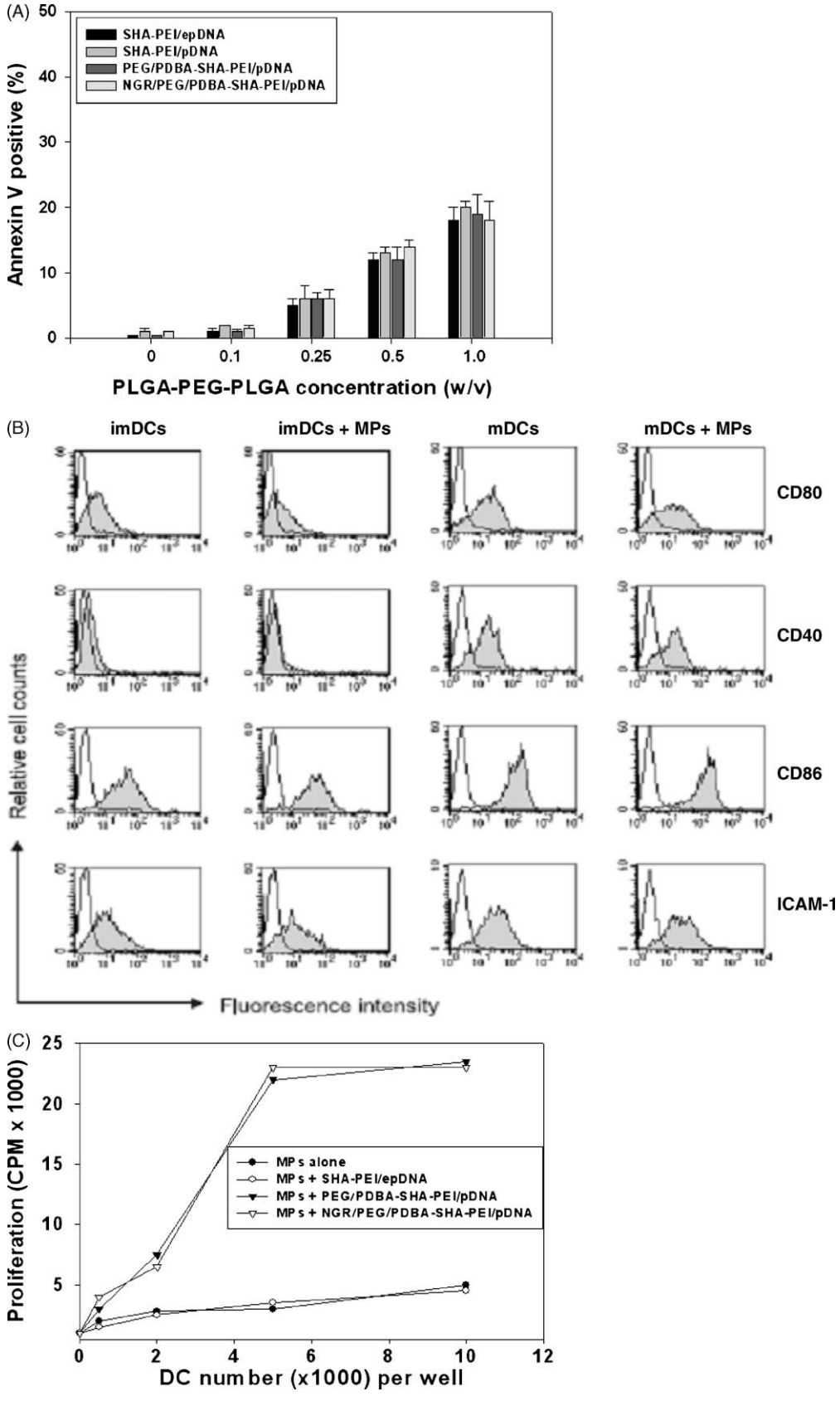


Fig. 2. Fluorescence microscopy of DCs cultured with NGR-targeting polyplex/microparticles. Human DCs were incubated for 1 h at 37 °C (A–C) or 4 °C (D–F) with NGR/PEG/PDBA-SHA-PEI/pDNA-loaded PLGA-PEG-PLGA microparticles containing rhodamine–lactalbumin (red), washed extensively, and then stained to demarcate the actin cytoskeleton (green). Panels show DCs (A and D), microparticles (B and E), overlaid images (C and F) or deconvolution fluorescence microscopy of single DC containing rhodamine–lactalbumin microparticles after incubation at 37 °C (G). Actin cytoskeleton is stained green and the nucleus blue.

in its free form or as a complex with SHA-PEI (lanes 5 and 6) was able to retain the structural integrity of pDNA after exposure to DNase. These data further suggest that gene delivery to DC by way of PLGA-PEG-PLGA encapsulation is an effective strategy of protecting the loaded DNA. Besides the uptake of the PEGylated targeting complexes, equally important was to investigate if the PLGA-PEG-PLGA aided the intracellular trafficking of DNA. Internalization of the complexes was examined by using ^{32}P-labelled plasmid DNA. The level of plasmid DNA uptake increased by 3-fold in the presence of 0.25% PLGA-PEG-PLGA at 37 °C (Fig. 4B) relative to unencapsulated DNA with no significant DNA uptake at 4 °C, further confirming the observation that, the cellular endocytosis process is also suppressed at this temperature. Relative DNA uptake was calculated by relating the radioactivity of the groups with PLGA-PEG-PLGA formulation to that of the group without the formulation.

Fig. 3. Effect of poplyplex/microparticle formulations on DC viability, phenotype and function. (A). Apoptosis of DCs loaded with encapsulated polyplex formulations and cultured for 24 h was assessed by FITC–annexin staining and analyzed by flow cytometry. Background apoptosis of cells cultured in medium alone was subtracted. Data are representative of two separate experiments with DCs from different donors. (B) Cell surface expression of DCs after 48 h in culture with microparticle formulations. Immature DCs were activated to matured stage by stimulation with LPS. Immature DCs alone (imDCs); immature DCs with the targeting polyplex/microparticles (imDCs + MPs); mature DCs alone (mDCs) and mature DCs with targeting polyplex/microparticles (mDCs + MPs). Results are representatives of four experiments with different donors. (C). Ability of DCs to stimulate allogeneic T cells following culture with PLGA-PEG-PLGA microparticles alone (MPs alone; closed circles); SHA-PEI + empty pDNA (MPs + SHA/PEI/epDNA; empty circles); PEGylated SHA-PEI/pDNA polyplex (MPs + PEG/PDBA-SHA-PEI/pDNA; closed triangles) or with PEGylated targeting polyplex (MPs + NGR/PEG/PDBA-SHA-PEI/pDNA; empty triangles) was assessed by [^{3}H] thymidine incorporation. For reasons of clarity, error bars were not plotted in (C). Results are triplicate experiments from three different donors cultured for 5 days with the indicated number of DCs per well and standard deviations ranging between 1 and 5%.


(A)
SHA-PEI/epDNA
SHA-PEI/pDNA
PEG/PDBA-SHA-PEI/pDNA
NGR/PEG/PDBA-SHA-PEI/pDNA
Annexin V positive (%)
0
10
20
30
40
50
0
0.1
0.25
0.5
1.0
PLGA-PEG-PLGA concentration (w/v)
(B)
imDCs
imDCs + MPs
mDCs
mDCs + MPs
CD80
CD40
CD86
ICAM-1
Relative cell counts
Fluorescence intensity
(C)
Proliferation (CPM x 1000)
0
5
10
15
20
25
0
2
4
6
8
10
12
DC number (x1000) per well
MPs alone
MPs + SHA-PEI/epDNA
MPs + PEG/PDBA-SHA-PEI/pDNA
MPs + NGR/PEG/PDBA-SHA-PEI/pDNA

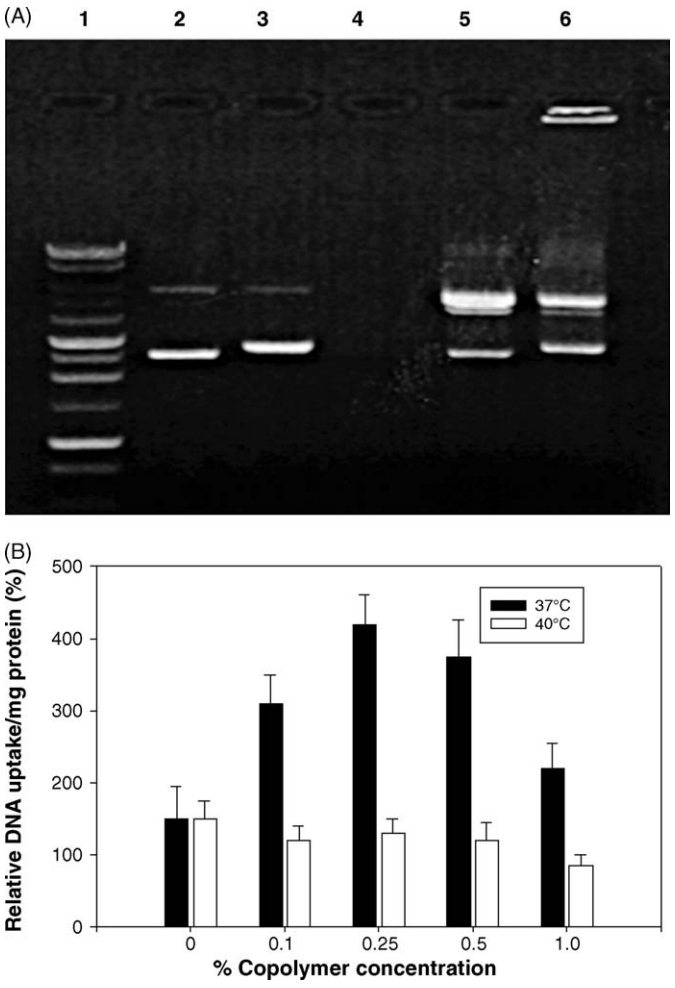


Fig. 4. (A) The effect of DNase on unencapsulated and encapsulated pDNA. Lane 1: 1 kb MW ladder; lane 2: unencapsulated free pDNA standard; lane 3: unencapsulated SHA-PEI/pDNA polyplex; lane 4: DNase treated free unencapsulated pDNA; lane 5: DNase treated encapsulated pDNA; lane 6: DNase treated encapsulated SHA-PEI/pDNA polyplex. (B) Relative cellular uptake of DNA as a function of PLGA-PEG-PLGA concentration at 37 °C (black bars) and 4 °C (white bars). Plasmid DNA was labeled with ^{32}P and complexed with SHA-PEI. Cellular uptake of DNA performed after 24 h. Data are representative of three separate experiments ± mean S.D.

3.5. Kinetic analysis of DNA release profile

Selected microparticle formulations were tested for the release of DNA *in vitro*. A typical release pattern of hydrophilic molecules encapsulated by biodegradable PLGA results in two distinguishable phases of an initial burst release and a second gradual release as a result of gradual degradation of the copolymer. The burst release of Pluronic F68-encapsulated targeting polyplex was about 32% (Fig. 5A) and that by PLGA-PEG-PLGA was found to be approximately 12% (Fig. 5B) within the first week of the entire 7-week duration of incubation. The second release phase of pDNA from the polyplex was more gradual with PLGA-PEG-PLGA than the control Pluronic F68 copolymer. Inclusion of PEG in the formulation decreased the release rate but was elevated upon NGR coupling for both copolymers.

3.6. In vitro transfection and phagocytosis of DCs

While the encapsulated PEGylated targeting polyplex presented a more controlled release of DNA, it was also important to examine the effect of PEGylation and encapsulation on the transfection efficiency of SHA-PEI/pDNA. About 6 μg of pDNA was used in all the formulations for transfection and the expression of βgal was monitored after 28 days. Whereas no significant difference ($P < 0.01$) in transfection efficiency was observed between SHA-PEI/pDNA and PEI/pDNA polyplexes (data not shown), there was approximately 5-fold and 10-fold increase in transfection efficiency of NGR/PEG/PDBA-SHA-PEI/pDNA encapsulation over that of the control Pluronic F68 copolymer and the non-targeting polyplex respectively after 28 days (Fig. 6A and B). The initial levels of transfection for the control copolymer (up to seven days) was observed to be higher than that of the PLGA-PEG-PLGA copolymer, but comparatively lower for the remainder of the experimental period. This agrees with the initial DNA burst release results obtained previously with the control copolymer. There was significant ($P < 0.03$) enhancement of gene expression at the end of the culture period with PLGA-PEG-PLGA encapsulation. Interestingly, the transfection efficiency of the non-targeting PEGylated polyplex was dramatically lower and reduced to approximately 50% with Pluronic F68 encapsulation and 85% for PLGA-PEG-PLGA as compared to the targeting PEGylated polyplex. Moreover, there was no enhancement of gene expression using SHA-PEI either alone or complexed with an empty plasmid DNA as controls, thereby excluding the possibility of any additive effects from the backbone polymer without DNA (data not shown). While polyplex without PEGylation were non-specifically phagocytosed (Fig. 6C), PEGylation efficiently reduced phagocytosis by DCs ($P < 0.03$) to about 20% of the average number of phagocytosed particles observed with unPEGylated particles (Fig. 6D). Coupling of the NGR targeting motif to the PEGylated polyplex however abolished the repellent nature of PEGylation and led to a cell-specific phagocytosis (Fig. 6E). This data also suggest that ligand-specific phagocytosis can be obtained while efficiently abolishing non-specific phagocytosis by inclusion of PEG in the targeting polyplex. Furthermore, the level of phagocytosis by coupling a control non-specific peptide, CARAC, was not significantly different from that of the PEGylated non-targeting polyplex (data not shown). Taken together, these results indicate that PLGA-PEG-PLGA can be used as a more effective copolymer than the Pluronic F68 for enhancing gene delivery, and that inclusion of the cell-specific NGR peptide in the PEGylated polyplex mediates efficient and specific phagocytosis leading to enhanced gene transfection in DCs.

4. Discussion

Our previous studies (Moffatt et al., 2005; Moffatt et al., 2006a,b) have extensively characterized and exploited the interaction between PDBA and SHA molecules as high affinity binding components to couple NGR-functionalized PDBA to SHA-derivatized PEI/pDNA via a PEG linker, allowing for a self-assembling linkage between PEG-PDBA and SHA-

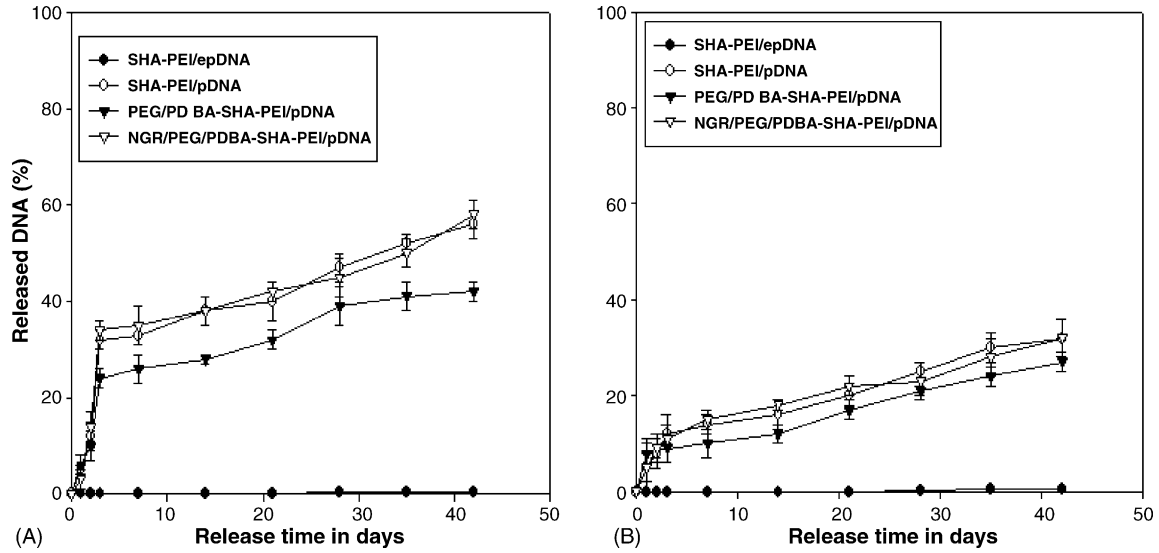


Fig. 5. *In vitro* release of encapsulated DNA. The kinetics of DNA release from (A) Pluronic F68- and (B) PLGA-PEG-PLGA-encapsulated formulations containing either empty plasmid DNA (SHA-PEI/epDNA), plasmid DNA (SHA-PEI/pDNA), non-targeting PEGylated pDNA (PEG/PDBA-SHA-PEI/pDNA) or with targeting PEGylated pDNA (NGR/PEG/PDBA-SHA-PEI/pDNA) over an extended period of 7 weeks. Release studies were performed in PBS at pH 7.4 and samples were analyzed and quantitated for double-stranded DNA. Data are represented as ± mean S.D. for three different experiments.

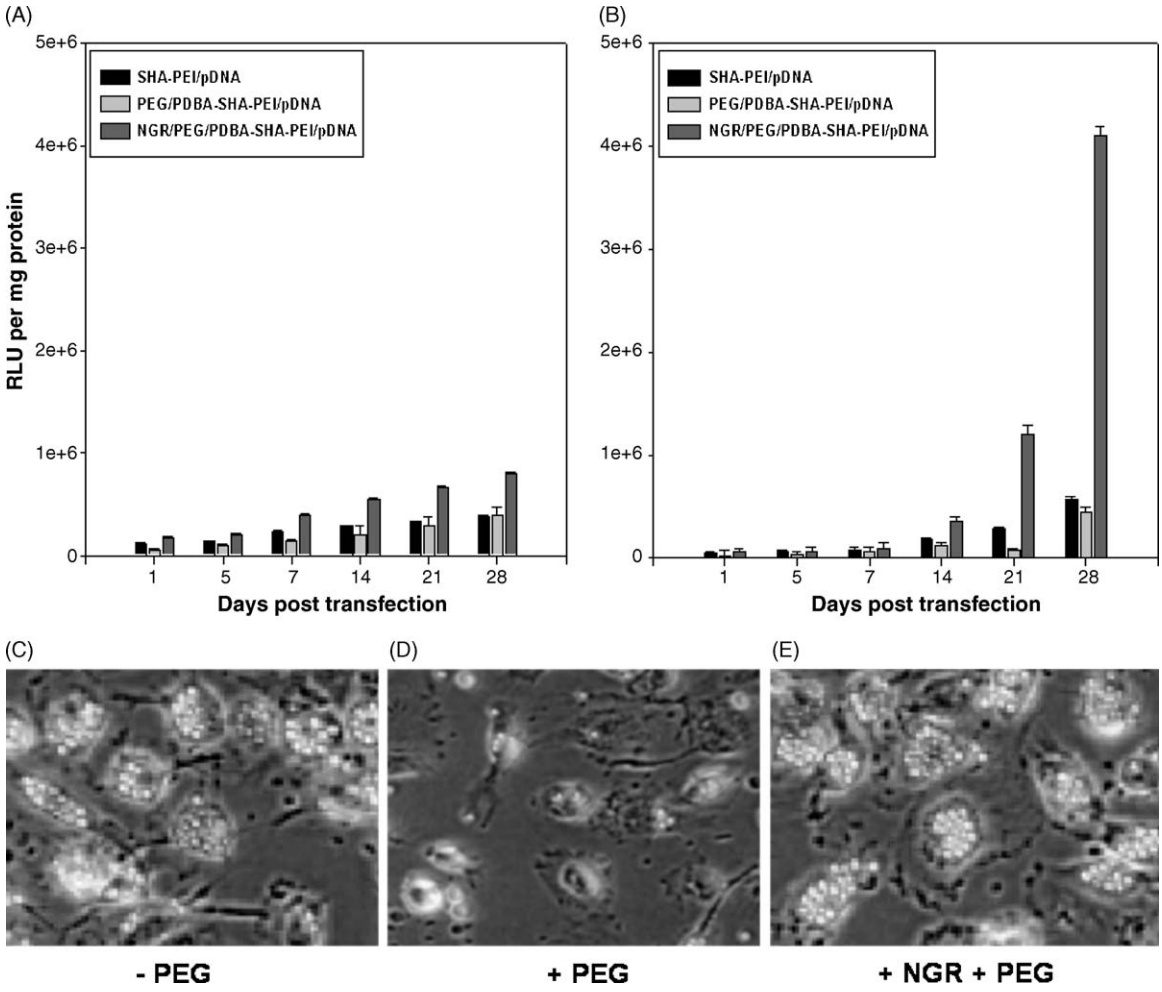


Fig. 6. Effect of copolymer type on the transfection efficiency of PEGylated or unPEGylated SHA-PEI/pDNA polyplexes in human DCs. Cells were transfected with either (A) Pluronic F68- or (B) PLGA-PEG-PLGA-encapsulated polyplex and the kinetics of gene expression was monitored over 28 days. Data are representative of three independent experiments ± mean S.D. Phagocytosis of encapsulated formulations containing (A) unPEGylated SHA-PEI/pDNA alone (B) non-targeting/PEGylated polyplex and (C) targeting/PEGylated polyplex. Pictures were taken after 6 h of DC co-culture at 37 °C.

PEI/pDNA. We have successfully utilized this coupling strategy to generate a stoichiometrically defined modified polyplex with stability maintained under a wide variety of conditions for target-specific gene delivery both *in vitro* and *in vivo*.

As an extension of the use of this successful strategy for gene delivery, the aim of the present study was to determine how the uptake of the targeting NGR/PEG/PDBA-SHA-PEI/pDNA polyplex encapsulated in PLGA-PEG-PLGA tri-block copolymer would affect key functional and phenotypic properties of immature CD1a+ DCs prepared under conditions appropriate for clinical application as DNA vaccines. We found that none of these features of DCs *in vitro* were adversely affected by the uptake of PLGA-PEG-PLGA-encapsulated targeting polyplex. These findings further provide a proof-of-principle for the development of DC-specific gene delivery through this novel coupling strategy (patent pending approval). This strategy may lead to tremendous improvement in DC-based DNA vaccination.

The rationale of using microparticles as gene delivery vehicles is based on the selectiveness of phagocytes such as DCs to readily internalize particles in the micrometer range (Yasuhiko and Yoshito, 1988; Kanke et al., 1986). While encapsulation of DNA by PLGA microparticles have been reported, it was essential to examine the phagocytotic competency of DCs for the PEGylated NGR-coupled SHA-derivatized PEI/pDNA formulation. Since the cell surface characteristics of this new formulation was not known, and hence could very well have an effect on DC phagocytosis, it was equally important to document that the particles actually entered the cells upon transfection. Deconvolution microscopy confirmed their intracellular localization, thus excluding the possibility that efficient delivery of the encapsulated derivatized polyplex was due to cell surface adherent microparticles. The level of plasmid DNA uptake also increased by three-fold in the presence of 0.25% PLGA-PEG-PLGA at 37 °C relative to unencapsulated DNA with no significant DNA uptake at 4 °C, further confirming the observation that, the cellular endocytosis process is also suppressed at this temperature. Additionally, after detailed DNase analysis, the structural integrity of the encapsulated DNA was demonstrated to be unaltered. These results indicate that these microparticle formulations were taken up by phagocytosis.

With the current formulation, DNA was released from the particles with or without complexation to PEG/PDBA and NGR/PEG/PDBA but not from polyplex with empty plasmid DNA. There was a significantly delayed second release phase for the polyplex with PLGA-PEG-PLGA encapsulation. Microparticles containing only the PEG/PDBA hydrophilic elements released DNA at a lower pace than the SHA-PEI/pDNA alone but elevated upon NGR inclusion in the PEGylated microparticle formulation. This may presumably be due to the increased water uptake triggered by the hydrophilic groups in the polymer, as well as the cell-specific targeting, resulting in the increased number of DNA released per cell for the NGR-coupled polyplex. This data agrees with the DC-specific phagocytosis of the PEGylated complexes and is also in accordance with hydrophilicity-dependent release of DNA from encapsulated microparticles (Norman et al., 1993; Luck et al., 1998). In the current study, in-depth DNA decay examination was not performed but the overall release pattern over a 7-week period for the targeting microparticles was more controlled for PLGA-PEG-PLGA than the PEO-PPO-PEO copolymer.

Particle sizes smaller than 10 μm (Yasuhiko and Yoshito, 1988; Kanke et al., 1986) have been noted as a very crucial parameter for DNA-containing microparticles, which are expected to be taken up by phagocytic cells. The microparticle preparation process was therefore optimized to give the desired particle size distribution without compromising the structural and functional integrity of the pDNA. In the present study, there was no detectable change in the sizes and the surface ζ potential of SHA-PEI complexes in the presence of 0.25% PLGA-PEG-PLGA. The lower light scattering intensity for PLGA-PEG-PLGA micelles (ca. 455 nm) than that of SHA-PEI/pDNA polyplexes (172 nm) observed in this study could be due to the sterically repulsive PEG chains on the surface of PLGA-PEG-PLGA, minimizing their physical interaction with the complexes. Scanning electron microscopy showed no significant difference between polyplex encapsulated by either the control F68 Pluronic or PLGA-PEG-PLGA tri-block copolymer in DCs. One other major concern, however, in designing microparticulate systems for gene delivery, is whether the polymers would be toxic at optimized concentrations. In this study, toxicity was very minimal and insignificant at the effective concentration of 0.25% of the copolymer with the various microparticle formulations containing the derivatized PEI/pDNA after 24 h incubation, even though confocal microscopy revealed DCs to have phagocytosed a significant number of particles per cell.

Importantly, the functional properties and phenotype of immature DCs co-cultured with either PEG/PDBA-SHA-PEI/pDNA-microparticles or NGR/PEG/PDBA-SHA-PEI/pDNA-microparticles were not altered, further lending support to using this PEGylated targeting polyplex for DNA delivery to DCs. Additionally, since immature DCs inhibit, whereas mature DCs activate cytotoxic T-cell response (CTL), a prerequisite for their use as immunostimulatory cellular vaccines (Dhodapkar et al., 2001; Jonuleit et al., 2001), we examined the influence of the encapsulated microparticles on DC-specific phenotypes. As judged by cell surface staining of the maturation markers CD80, CD40, CD86 and ICAM-1, we observed the same upregulation of these markers for microparticle-loaded and unloaded DCs. The delivery of the polyplex by PLGA-PEG-PLGA microparticles also resulted in a robust stimulation of T cells *in vitro*, which was significantly greater than that caused by non-stimulating microparticles alone. Thus the enhanced T cell stimulation was not due simply to maturation of DCs, but probably increased and prolonged presentation of antigen from the PEGylated polyplex. This conclusion is drawn from the fact that DCs loaded with microparticles containing SHA-PEI-complexed empty plasmid barely stimulated T cells to proliferate. An interesting finding from this experimental design was the observation that the use of the control tri-block copolymer, Pluronic F68, or the underivatized PEI/pDNA yielded fairly similar results under similar conditions suggesting that neither

the microparticle by itself nor modification of PEI to yield SHA-PEI triggered proliferation of the T cells.

In this study, we were able to monitor reporter gene expression in DCs, albeit, more than a log lower than that obtained with non-phagocytic cells in our previous studies (Moffatt et al., 2005; Moffatt et al., 2006a,b). This could either be due to the effect of PLGA-PEG-PLGA encapsulation or the intracellular trafficking of the complexes in DCs even with the targeting polyplex. The observed increased gene expression with PLGA-PEG-PLGA encapsulation on day 28 may also be due to the faster rate of decay of the microparticles as compared to that of Pluronic F68. The release of DNA and proteins in PLGA systems is based on several factors, particularly, the physical and chemical state of the microsphere preparation. Very often, the more water the polymer matrix absorbs (in this case, PEG in the copolymer), the more rapid the protein release. Our first hypothesis to explain this increased gene expression on day 28 is that there is initially increased water uptake triggered by the PEG hydrophilic groups in the polymer which increases the load and hence a delay in cell trafficking of the polyplex to a point where the NGR targeting motif can be most effective. The importance of the water uptake kinetics during the release is also emphasized by our second hypothesis; the fact that water content typically affects the microenvironment inside the polymer by diluting and accelerating the release of encapsulated agents produced during degradation of the polymer. Previous studies to inhibit phagocytosis of di-block and tri-block copolymers of PLA/PLGA by PEGylation have resulted in the extension of circulation time by up to 6 h (Verrecchia et al., 1995) and inhibition of phagocytosis in the range of 20–55% which is comparable to that obtained with the PEG/PDBA conjugation in this study. In addition to the inhibition of non-specific phagocytosis, the coupling of the NGR cell-specific peptide, as opposed to a non-specific peptide, resulted in ligand-specific phagocytosis of NGR/PEG/PDBA-SHA-PEI/pDNA in DCs, restoring or even increasing the level to that obtained without PEGylation. This to our knowledge is the first delivery technology enabling cell-specific targeting of DCs with a tri-block copolymer encapsulating a PEI-based DNA polyplex.

Another essential aim of the present study was to undertake a comparative analysis of SHA-PEI/pDNA and PEI/pDNA in conjunction with the tri-block copolymer to evaluate the effect on gene delivery to DCs. No observable and quantifiable difference was observed between the derivatized and underivatized PEI/DNA in gene delivery and effects on immature DCs. From the enhanced transfection levels attained with this particulate system, coupled with the high immuno-stimulatory potential of DCs, the strategy described in this study seems sufficient for targeting DCs to avoid non-specific phagocytosis. Furthermore, the essential characteristics of very low toxicity at a PLGA-PEG-PLGA concentration of 0.25% and biodegradability of this microparticle formulation would be added advantages for *in vivo* targeted gene delivery. Clearly, the use of PEGylation with this derivatized PEI-based targeting polyplex in combination with biodegradable PLGA-PEG-PLGA tri-block copolymer for targeted delivery to DCs *in vivo* is currently in progress and would constitute an essential study to validate this concept.

Acknowledgments

We are grateful to Dr. Randall Evans (Department of Molecular Hematology and Therapy) of the Flow Cytometry/Cell Sorting and Confocal Microscope/Image Analysis Core of the University of Texas MD Anderson Cancer Center for his invaluable technical assistance in confocal microscopy. This work was supported in part by NIH grant #5P30CA016672-29 supporting the core facility.

References

Arap, A., Pasqualini, R., Ruoslahti, E., 1998. Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model. Science 279, 377–380.

Banchereau, J., Steinman, R.M., 1998. Dendritic cells and the control of immunity. Nature 392, 245–252.

Bergstresser, P.R., Fletcher, C.R., Streilin, J.W., 1980. Surface densities of Langerhans cells in relation to rodent epidermal sites with special immunologic properties. J. Invest. Dermatol. 74, 77–80.

Coombes, B.K., Mahony, J.B., 2001. Dendritic cell discoveries provide new insights into the cellular immunobiology of DNA vaccines. Immunol. Lett. 78, 103–111.

Deng, X.M., Xiong, C.D., Cheng, L.M., Xu, R.P., 1990. Synthesis and characterization of block copolymers from D,L-lactide and poly(ethylene glycol) with stannous chloride. J. Polym. Sci. Polym. Lett. 13, 411–416.

Dhodapkar, M.V., Steinman, R.M., Krasovsky, J., Munz, C., Bhardwaj, N., 2001. Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells. J. Exp. Med. 193, 233–238.

Dong, X., An, B., Salvucci Kierstead, L., Storkus, W.J., Amoscatto, A.A., 2000. Modification of the amino terminus of a class II epitope confers resistance to degradation by CD13 on dendritic cells and enhances presentation to T cells. J. Immunol. 164, 129–135.

Felgner, P.L., Gader, T.R., Holm, M., Roman, R., Chan, H.W., Wenz, M., Nerthrop, J.P., Ringold, G.M., Danielsen, M., 1987. Lipofection: a highly efficient lipid-mediated DNA transfection procedure. Proc. Natl. Acad. Sci. U.S.A. 84, 7412–7417.

Feng, S.S., Mu, L., Win, K.Y., Huang, G.F., 2004. Nanoparticles of biodegradable polymers for clinical administration of paclitaxel. Curr. Med. Chem. 11, 413–424.

Foged, C., Sundblad, A., Hovgaard, L., 2002. Targeting vaccines to dendritic cells. Pharm. Res. 19, 229–238.

Han, S., Mahato, R.I., Kim, S.W., 2001. Water-soluble lipopolymer for gene delivery. Bioconjug. Chem. 12, 337–345.

Jilek, S., Zurkaulen, H., Pavlovic, J., Merkle, H.P., Walter, E., 2004. Transfection of a mouse dendritic cell line by plasmid DNA-loaded PLGA microparticles in vitro. Eur. J. Pharm. Biopharm. 58, 491–499.

Jonuleit, H., Schmitt, E., Steinbrink, K., Enk, A.H., 2001. Dendritic cells as a tool to induce anergic and regulatory T cells. Trends Immunol. 22, 394–400.

Kanke, M., Morlier, E., Geissler, R., Powell, R., Kaplan, A., DeLuca, P.P., 1986. Interaction of microspheres with blood constituents II: uptake of biodegradable particles by macrophages. J. Parent. Sci. Tech. 40, 114–118.

Kenausis, G.L., Voros, J., Elbert, D.L., Huang, N.P., Hofer, R., Ruiz-Taylor, L., Textor, M., Hubbell, J.A., Spencer, N.D., 2000. Poly(L-lysine)-g-poly(ethylene glycol) layers on metal oxide surfaces: Attachment mechanisms and effects of polymer architecture on resistance to protein adsorption. J. Phys. Chem. B 104, 3298–3309.

LaPorte, R.J., 1997. In: Laporte, R.J. (Ed.), Hydrophilic polymer coatings for medical devices: structure/properties, development, manufacture, and applications. Technomic Publishing Co, Lancaster, PA.

Lemieux, P., Guerin, N., Paradis, G., Proulx, R., Chityakova, L., Kabanov, A., Alakhov, V., 2000. A combination of poloxamers increases gene expression of plasmid DNA in skeletal muscle. Gene Ther. 7, 986–991.

Li, X., Deng, X., Yuan, M., Xiong, C., Huang, Z., Zhang, Y., Jia, W., 2000. In vitro degradation and release profiles of poly-DL-lactide-poly(ethylene glycol) microspheres with entrapped proteins. J. Appl. Polym. Sci. 1, 140–148.

Luck, M., Pistel, K.F., Li, Y.X., Blunk, T., Muller, R.H., Kissel, T., 1998. Plasma protein adsorption on biodegradable microspheres consisting of poly(D,L-lactide-co-glycolide), poly(L-lactide) or ABA triblock copolymers containing poly(oxyethylene)—influence of production method and polymer composition. J. Control. Release 55, 107–120.

Moffatt, S., Wiehle, S., Cristiano, R.J., 2005. Tumor specific gene delivery mediated by a novel/polyethylenimine/DNA polyplex targeting aminopeptidase N (CD13). Hum. Gene Ther. 16, 57–67.

Moffatt, S., Papasakelariou, C., Wiehle, S., Cristiano, R.J., 2006a. Successful in vivo tumor targeting of prostate specific membrane antigen with a highly efficient J591/PEI/DNA molecular conjugate. Gene Ther. 13, 761–772.

Moffatt, S., Wiehle, S., Cristiano, R.J., 2006b. A multifunctional PEI-based cationic polyplex for enhanced systemic p53-mediated gene therapy. Gene Ther., advance online publication, doi:10.1038/sj.gt.3302773.

Mu, L., Feng, S.S., 2001. Fabrication, characterization and in vitro release of paclitaxel (Taxil) loaded poly(lactic-co-glycolic acid) microspheres prepared by spray drying technique with lipid/cholesterol emulsifiers. J. Control. Release 3, 239–254.

Mumper, R.J., Duguid, J.G., Anwer, K., Barron, M.K., Nitta, H., Rolland, A.P., 1996. Polyvinyl derivatives as novel interactive polymers for controlled gene delivery to muscle. Pharm. Res. 13, 701–709.

Norman, M.E., Williams, P., Illum, L., 1993. Influence of block copolymers on the adsorption of plasma proteins to microspheres. Biomaterials 14, 193–202.

Thiele, L., Rothen-Rutishauser, B., Jilek, S., Wunderli-Allenspach, H., Merkle, H.P., Walter, E., 2001. Evaluation of particle uptake in human blood monocyte-derived cells in vitro. Does phagocytosis activity of dendritic cells measure up with macrophages? J. Control. Release 76, 59–71.

Verrecchia, T., Spenlehauer, G., Bazile, D.V., Murry-Brelier, A., Archimbaud, Y., Veillard, M., 1995. Non-stealth (poly(lactic acid/albumin)) and stealth (poly(lactic acid–poly ethylene glycol)) nanoparticles as injectable drug carriers. J. Control. Release 36, 49–61.

Yasuhiko, T., Yoshito, I., 1988. Effect of the size and surface charge of polymer microspheres on their phagocytosis by macrophages. Biomaterials 9, 356–362.