Cross-Linked Microparticles as Carriers for the Delivery of Plasmid DNA for Vaccine Development

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Plasmid DNA was directly encapsulated into biocompatible polymer microparticles via radical polymerization in an inverse emulsion system. Acrylamide-based microspheres 0.2−1 μm in diameter were prepared using an acid-cleavable difunctional monomer. Retention of the DNA payload at physiological pH with complete release under acidic conditions at lysosomal pH was demonstrated. By trapping the plasmid DNA within the cross-linked microparticle, enzymatic degradation was prevented when exposed to serum nucleases. For vaccine development, these delivery vehicles were also investigated for their ability to generate immune responses when delivered to phagocytic cells of the immune system. Encapsulated plasmid DNA demonstrated immunostimulatory activity in macrophages, leading to cytokine secretion of IL-6 with a response ~40-fold higher than that achieved with DNA alone.

INTRODUCTION

Recent advances in polymeric drug delivery have focused on the encapsulation of biomolecules, such as protein, peptide, and DNA antigens, in microgel and hydrogel systems (*1*−*3*). DNA is a promising therapeutic for use in gene therapy applications (*4*) but may also be applicable as a coadjuvant for protein-based vaccines to stimulate the immune system (*5*, *6*). It is well-known that bacterial DNA is recognized by vertebrate immune systems as foreign and elicits a response, such as the release of cytokines or antimicrobial agents (*7*−*10*). Characteristics of bacterial DNA responsible for this activity are the higher frequency of CpG dinucleotides within the sequence as compared to vertebrate DNA, as well as the presence of unmethylated cytosine bases (*11*, *12*). Both plasmid DNA and short oligonucleotides have been shown to elicit an immune response in vertebrate systems (*12*−*15*); the incorporation of unmethylated bases is accomplished for oligonucleotides using the standard cytosine monomer during solid-phase chemistry, and for plasmid DNA through bacterial transformation. The sequence of these antigens can be selected to either provoke or prevent an immune response as desired (*14*−*17*). Delivery of immunostimulatory DNA results in the release of inflammatory triggers such as IL-6, IL-12, TNF-α, and IFN-γ. These cytokines are crucial in the maturation and activation of T cells and dendritic cells (*18*).

Encapsulation of plasmid DNA provides protection from nuclease degradation in the serum and can aid in directing its delivery to target cells, phagocytes. Current delivery vehicles for DNA-based therapeutics include cationic liposomes, lipoplexes, and polymer complexes (*19*−*31*). Many of these systems require the use of cationic polymers to entrap plasmid DNA via an electrostatic interaction with the negatively charged backbone, in addition to a neutral component to mitigate the toxicity effects. However, by using a cross-linked microparticle, we are able to encapsulate plasmids physically, rather than electrostatically, and without the use of polycations, which also allows this delivery system to be compatible with biomolecules of other charged states such as proteins. Previously, much attention has been focused on biodegradable systems, such as poly(lactic-*co*-glycolic acid), which release DNA via hydrolysis of the polymer chains (*32*−*37*). The ester backbone of these microparticles readily degrades under basic conditions and much more slowly under physiological pH. As the particles circulate throughout the body, a slow release of the encapsulated payload is observed. In contrast, we chose to design a polymer-based system that would exhibit minimal degradation in the serum at pH 7.4 until the particles encounter an acidic environment. Once internalized in lysosomal compartments of the desired cell type, the particles would rapidly degrade and deliver the encapsulated antigen.

Taking advantage of the pH differential between serum (pH 7.4) and the lysosome (pH 5.0), we have recently developed a new strategy for the delivery of protein-based vaccines using an acid-degradable microbead (*38*, *39*). Given their size of roughly 500 nm in diameter, the cross-linked microparticles should not be readily taken up by most cells. Instead, phagocytic cells such as macrophages and dendritic cells, which play a key role in the activation and maturation of the immune system, selectively ingest the microparticles, delivering the particle and its encapsulated payload to a lysosomal compartment within the cell. Cross-linked with a pH sensitive linker, the polymer microbead degrades in the acidic environment, resulting in an increase in osmotic pressure within the compartment and leading to lysosomal disruption, thus providing cytoplasmic delivery of the encapsulated biomolecule intact. Protein delivery using this microbead system has been successful at demonstrating class I antigen presentation with macrophages through the activation of cytotoxic T cells, a necessary step in vaccine development.

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Therefore, it would be beneficial to combine the T cell activation ability of immunostimulatory DNA with these protein microparticles to further enhance the effectiveness of protein vaccines. Here, we extend the versatility of our acid-sensitive polymer microparticle by investigating the encapsulation and release of plasmid DNA in macrophages and examining the immunostimulatory effects as a next step in microparticle vaccine strategies.

MATERIALS AND METHODS

Reagents and Materials. β-Galactosidase reporter plasmid (*p*SV-β-gal vector, Promega, Madison, WI) was amplified by bacterial transformation with DH5α (Invitrogen, Carlsbad, CA) competent cells in Luria broth (Sigma, St. Louis, MO) and purified using a Qiagen Mega kit (Qiagen, Valencia, CA). Double-stranded DNA quantification assays were performed using PicoGreen (Molecular Probes, Eugene, OR). Absorbance and fluorescence readings were taken on a SPECTRAmax 190 and a SPECTRAmax Gemini XS, respectively, microplate reader (Molecular Devices, Sunnyvale, CA). Macrophage cell lines RAW 309.1CR and RAW 264.7 and 293T kidney cells (ATCC, Manassas, VA) were prepared by the University of California Tissue Culture/Media Facility by plating at the density indicated and then incubating overnight at 37 °C and 5% CO_2 before use.

Polyacrylamide Microparticle Synthesis. Microparticles were prepared by inverse microemulsion polymerization using a modification of a previously published procedure (*38*). β-Galactosidase reporter plasmid was dissolved in 400 μL of 300 mM sodium phosphate buffer pH 7.4 along with acrylamide (100 mg, 1.4 mmol), an acid labile cross-linker *N*,*N*′-bisacryloylbis(2-aminoethoxy)-[4-(1,4,7,10-tetraoxaundecyl)phenyl]methane (*38*) (**2**) (25 mg, 0.052 mmol, 3.6 mol %), and potassium persulfate (6 mg, 0.022 mmol) as the initiator. The organic phase consisted of 4 mL of hexane containing 120 mg of a 3:1 weight ratio of Span 80 (sorbitan monooleate) and Tween 80 (poly(ethylene glycol)−sorbitan monooleate). The emulsion was prepared by sonicating the two phases for 30 s using a Branson Sonifier 450 with an output setting of 2 and a duty cycle of 40%. Polymerization initiated by the addition of 25 μL of *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TMEDA) to the stirred mixture was continued for 10 min at room temperature before recovering the microparticles by centrifugation. The particles were washed twice with hexane to remove residual surfactant and then with acetone and finally dried under vacuum overnight. The particles were analyzed by scanning electron microscopy using a WDX ISI-ds130C instrument (Microspec Corporation, Inc.) at 15 kV.

Nondegradable beads encapsulating plasmid DNA were prepared in similar fashion, using the same wt % of cross-linker. For this set of microparticles, the aqueous phase consisted of acrylamide (100 mg, 1.4 mmol), *N*,*N*′-methylenebisacrylamide (25 mg, 0.162 mmol), and 0.50 mg of the β-galactosidase reporter plasmid in 400 μL of 300 mM sodium phosphate buffer pH 7.4 along with initiating species.

Determination of Plasmid DNA Loading. The plasmid DNA-loaded particles were washed by dispersing the particles in 300 mM sodium phosphate buffer at pH 7.4 at a concentration of 5 mg/mL using a series of sonication and vortex steps. The particles were collected as a pellet by centrifuging for 10 min. The wash supernatant was removed by pipet, and an equivalent volume of acidic buffer (pH 5.0, 300 mM acetic acid) was added to maintain the concentration. The sample was then heated overnight at 37 °C, and the hydrolyzed solution was analyzed for the concentration of double-stranded DNA using PicoGreen. All loadings were determined in triplicate.

pH Dependent Release. Plasmid-loaded microparticles were washed with 300 mM sodium phosphate pH 7.4, as described above, at a concentration of 5 mg/mL. The wash supernatant was removed, and either sodium phosphate buffer, pH 7.4, or acetic acid buffer, pH 5.0, was added, and the particle solutions were incubated at 37 °C. After the desired time point, the beads were collected by centrifugation and the supernatant removed. Any remaining particles were hydrolyzed at 37 °C overnight in acidic buffer, and both the supernatants and the hydrolyzed solutions were analyzed for the concentration of double-stranded DNA. All samples were performed in triplicate. The percent release was defined as the ratio of the concentration of DNA in the hydrolyzed solution compared to that found in both the hydrolyzed and supernatant solutions.

Microparticle Toxicity. RAW 309.1CR macrophages were plated at a cell density of 5×10^4/well in a 96-well format. Microparticles with DNA loadings of 0 (empty beads), 0.88, 2.14, and 4.29 μg plasmid DNA/mg bead were suspended in Dubelcco's Modified Eagle Medium (DMEM) at a concentration of 10 mg/mL. An aliquot of the bead solution was added to the cells, along with DMEM containing 10% fetal bovine serum (FBS) to give a final volume of 100 μL with an overall concentration of 5% FBS and 0.5 to 5 mg of beads/mL. After being incubated overnight at 37 °C and 5% CO_2, the supernatant was aspirated, and the cells were washed three times with DMEM to removed any remaining beads from the wells. To each well, 100 μL of DMEM + 10% FBS was added, and the cells were incubated for an additional 24 h. Toxicity was measured using the MTT assay (*40*); briefly, the supernatant was aspirated, and 100 μL of DMEM + 10% FBS along with 20 μL of a 5 mg/mL solution of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) in medium were added to each well. The cells were incubated for 4 h at 37 °C and 5% CO_2, after which time the supernatant was removed, and the MTT crystals were dissolved in 200 μL of dimethyl sulfoxide (DMSO). The purple solutions (10 μL) were further diluted with 190 μL of DMSO and 25 μL of a glycine buffer (0.1 M glycine, 0.1 M NaCl, pH 10.5). The concentration of MTT was determined by measuring the UV absorbance at 570 nm.

Nuclease Stability of Encapsulated Plasmid. Microparticles with a loading of 2.14 μg DNA/mg bead were suspended in DMEM + 10% FBS to give a final concentration of 5 mg of beads/mL. The solutions were incubated at 37 °C for the desired time (0, 0.5, 1, and 2 h), and then the microparticles were collected by centrifugation. The supernatant was decanted, and the pelleted beads were washed with 300 mM sodium phosphate buffer pH 7.4 to remove the color from residual medium, as well as any fragmented DNA. Finally, the microparticles were incubated overnight at 37 °C in 300 mM acetic acid buffer pH 5.0 to fully release any remaining plasmid DNA. The stability for a given time point was calculated as the concentration of double-stranded DNA in the hydrolyzed beads compared to the concentration determined at 0 h. DNA released from the exposed beads was analyzed by agarose gel electrophoresis.

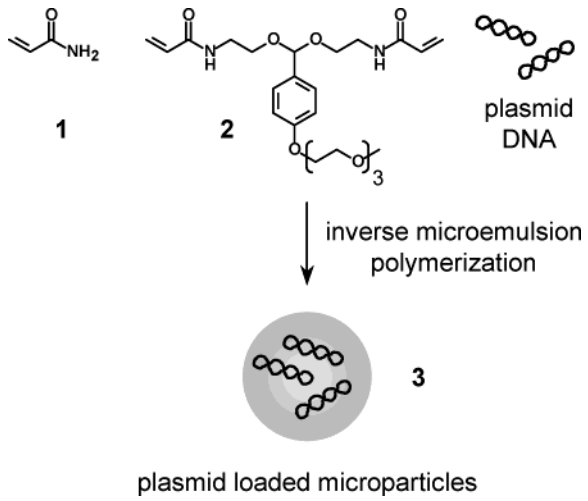
Fluorescence Microscopy. Microscopy studies were performed with both degradable and nondegradable, dextran-encapsulated microparticles. For degradable

beads, fluorescein-labeled dextran (5 mg, FITC-dextran, MW 70 kDa, Molecular Probes) was encapsulated using the inverse emulsion polymerization procedure described above using the acid sensitive cross-linker, *N*,*N*′-bisacryloylbis(2-aminoethoxy)-[4-(1,4,7,10-tetraoxaundecyl)phenyl]-methane (*38*). FITC-dextran microparticles were also prepared using 25 mg of *N*,*N*′-methylenebisacrylamide, a nondegradable cross-linker. Scanning electron micrographs of both samples showed spherical beads of similar size to the DNA-loaded microparticles. RAW 264.7 cells were prepared in a 24 well plate with cover slips at a density of 3×10^5 cells/well. The beads were suspended in DMEM + 10% FBS (5 mg/mL), and the solution delivered to the cells with additional medium to give a final volume of 1 mL and a final concentration of 0.25 mg beads/mL. After incubation overnight, the supernatant was aspirated, and the cells were washed five times with DMEM. DMEM + 10% FBS (1 mL) was then added to the cells, and they were allowed to grow for another 24 h. The cells were washed five times with phosphate-buffered saline (PBS, Gibco) and fixed to the cover slips by the addition of 1 mL per well of a paraformaldehyde solution. The cells were incubated for 10 min at room temperature and then washed twice with PBS. To prepare the paraformaldehyde solution, 1.5 g of paraformaldehyde was dissolved in 20 mL of H_2O and 20 μL of 10 M NaOH that was previously equilibrated to 56 °C. The solution was cooled to room temperature, and 25 mL of H_2O and 5 mL of 3 M sodium phosphate were added. The resulting solution was brought up to pH 10.5 using 10 M NaOH, sterilized by passing it through a 0.22 μm filter, and stored at 4 °C until needed. Micrographs were taken with a Nikon Eclipse TE300 inverted microscope, equipped with a Nikon Plan Fluor 40×/0.60 objective and the appropriate excitation/emission filters (494 nm/519 nm).

Transfection Studies. Plasmid-encapsulated beads (60 mg) were hydrolyzed by incubating them overnight in 3 mL of 300 mM acetic acid pH 5.0. The resulting plasmid was recovered by first passing the hydrolyzed solution through a Nap-25 column (Amersham, Piscataway, NJ) that had been equilibrated with water. Fractions displaying a UV absorbance at 260 nm were reduced in volume and then isolated again using a Qiagen Tip-20. 293T kidney cells were plated at a density of 6×10^4 cells/well in a 24-well plate. To each well, 500 μL of DMEM + 10% FBS was delivered along with 100 μL of a solution containing either original or isolated plasmid DNA (0.25 or 0.5 μg total) in OPTI-MEM (Gibco), in which the DNA had been complexed with 5 μg of Lipofectamine 2000 (Invitrogen) for 15 min. Control wells contained either medium alone or DNA without transfection agent. The cells were incubated overnight at 37 °C and 5% CO_2. The cells were aspirated, and 400 μL of Z buffer (*41*) was delivered to each well and incubated for an additional 6 h. The supernatant was transferred to a 96-well plate, and β-galactosidase activity was determined by monitoring the UV absorbance of the hydrolyzed phenolate at 570 nm. The Z buffer was made according to literature (*41*): 100 μM 2-mercaptoethanol, 9 mM $MgCl_2$, 0.125% NP-40 (Sigma), and 0.15 mM sodium chlorophenol red β-galactopyrandoside (CPRG, CalBioChem, San Diego, CA) in PBS.

IL-6 ELISA. A 96-well plate of RAW 264.7 macrophages at a density of 2×10^4 cells/well was incubated overnight with 100 μL of a 5 mg/mL solution of microparticles in DMEM + 10% FBS. Microparticles with DNA loadings of 0 (empty beads), 0.88, 2.14, 4.29 μg plasmid DNA/mg bead made with degradable and nondegradable cross-linkers were analyzed. Control wells consisted of untreated cells and cells with plasmid DNA alone. The media was diluted 5-fold and analyzed using an IL-6 ELISA kit (Pierce, Rockford, IL). Absorbance values were measured at $\lambda = 450$ nm and corrected using $\lambda = 550$ nm as a background reference. IL-6 concentrations were calculated using a standard curve.

Scheme 1. Synthesis of Plasmid-Loaded Microparticles



RESULTS AND DISCUSSION

Synthesis of Polyacrylamide Microparticles. Microparticles prepared using inverse microemulsion polymerization (*42*, *43*) were successfully loaded with plasmid DNA. The β-galactosidase reporter plasmid (pSV-β-gal, Promega, 6820 bp) was dissolved in aqueous buffer along with acrylamide **1** and 3.6 mol % of a bisacrylamide acetal cross-linker **2** (Scheme 1). This cross-linker was previously shown to be readily incorporated with acrylamide under free radical polymerization conditions to produce protein-loaded microparticles (*38*). The aqueous phase was dispersed in hexane containing 3% (w/v) surfactants (Span 80/Tween 80) using sonication. These neutral surfactants do not coordinate the phosphate backbone of DNA, as would ionic surfactants, thus resulting in disruption of the inverse emulsion. The radical polymerization was initiated using potassium persulfate and TMEDA, effectively trapping the plasmid inside the cross-linked beads, **3**. Following the synthesis, plasmid-loaded particles **3** were washed with excess solvent and dried under vacuum, with yields ranging from 50 to 85%. While these polymerization conditions were mild enough for protein encapsulation as shown previously, further analyses were performed to validate the stability and integrity of the encapsulated plasmid DNA.

The isolated microbeads were analyzed in the dry state using scanning electron microscopy (Figure 1). Ranging from 0.2 to 1 μm in diameter, plasmid-loaded microparticles displayed similar surface characteristics to beads that contained no DNA. This size is appropriate for targeting delivery to macrophages, which readily ingest particles of 0.5−1 μm (*44*).

Microparticle Loadings and DNA Integrity. The concentration of plasmid DNA in the microparticles was determined by first washing the microparticles in 300 mM sodium phosphate at pH 7.4. A small amount of plasmid DNA is detected in this washing step, corresponding to the removal of DNA that is physisorbed to the surface of the microparticle. The sample was then heated overnight at 37 °C in an acidic buffer to fully

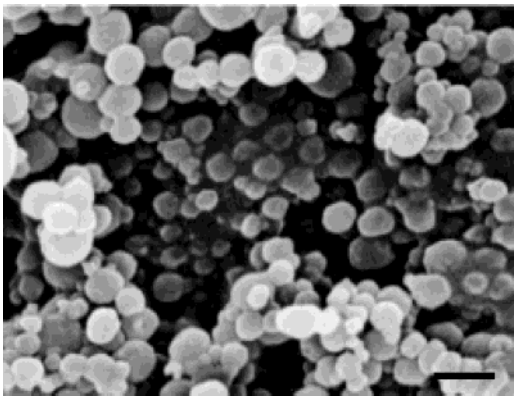


Figure 1. Scanning electron micrograph of plasmid-loaded microparticles. 15 kV, 20 000 ×. Scale bar represents 500 nm.

Table 1. Plasmid DNA Loading in Microparticles

initial DNA loading (μg/mg polymer)	observed loading (μg/mg polymer)	encapsulation efficiency, %
2.0	0.88 ± 0.15	44
4.0	2.14 ± 0.15	53
8.0	4.29 ± 0.17	54

hydrolyze the acetal cross-links, releasing linear polyacrylamide chains, a tri(ethylene glycol)-functionalized benzaldehyde, and plasmid. The concentration of double-stranded DNA in the acidic buffer was determined using PicoGreen (Table 1).

An initial loading of 2.0 μg/mg of plasmid DNA on a 125 mg scale was used in the synthesis of the acid-degradable microparticles. After the beads were washed and isolated, a loading of 0.88 μg of DNA/mg of polymer was determined, giving an encapsulation efficiency of 44%. Increasing the concentration of plasmid in the polymerization mixture to 4.0 and 8.0 μg/mg produced higher DNA loadings within the resulting microparticles. While the encapsulation efficiency remained fairly constant, the amount of DNA found encapsulated in the particles roughly doubled for each 2-fold increase in the initial concentration, with the largest loading being slightly higher than those reported with PLGA particles of comparable size (*36*, *37*).

Having successfully encapsulated plasmid DNA in microparticles, we next sought to verify that the structure of the released DNA remained intact. Should the DNA be degraded into smaller fragments during the course of the polymerization procedure, the fragments could diffuse out of the microparticle prior to phagocytosis and hydrolysis. Also of interest is preserving the tertiary structure of the plasmid. Possible detrimental conditions prevailing during the microparticle polymerization and subsequent acidic degradation include sonication to form the emulsion, reactions involving free radicals, and the use of a treatment at pH 5.0, 37 °C, for 18 h. In other microparticle formulations, plasmid DNA has been shown to be sheared during sonication in addition to being susceptible to cleavage and degradation during homogenization or drying steps (*37*, *45*, *46*). Therefore, DNA recovered from our polyacrylamide microparticles after acidic cleavage of the acetal cross-linkers was analyzed by agarose gel electrophoresis to investigate its integrity (Figure 2). The original plasmid DNA, prepared by bacterial transformation, is shown in lane 2; the upper band corresponds to the open circular form of isolated plasmid DNA and the lower to the supercoiled structure, as it is more compact and therefore migrates faster through the agarose gel. After the polymerization and hydrolysis steps, the plasmid DNA is composed mostly

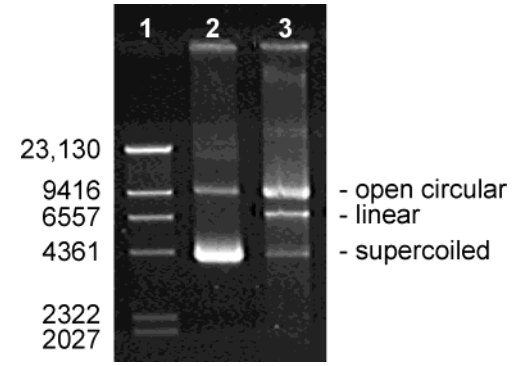


Figure 2. Analysis of released DNA by agarose gel electrophoresis. Lane 1: Lambda Hind III ladder, fragment size (bp) given to the left. Lane 2: original plasmid DNA. Lane 3: DNA recovered from microbeads after acid hydrolysis. Conditions: 1% agarose, 75 V for 1 h, stained with ethidium bromide.

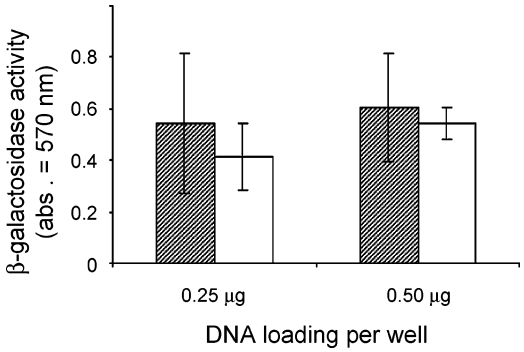


Figure 3. Transfection of 293T Cells. β-Galactosidase activity was measured as the absorbance of hydrolyzed CPRG for control (shaded) and recovered DNA (open), complexed with Lipofectamine 2000.

of the open circular form, with some linear and supercoiled structures as also seen in lane 3. A double restriction digest of each of these samples yielded identical linear fragments, another indication that the double-stranded DNA remained unchanged during the polymerization procedure and that only the tertiary structure is affected by the processing conditions. While the tertiary structure of plasmid DNA has not been studied with respect to its immunostimulatory efficiency, it has been shown that open circular and supercoiled DNA have roughly equivalent transfection capabilities (*47*, *48*).

To verify the activity of the plasmid DNA, the transfection ability of the recovered plasmid was evaluated using 293T kidney cells. Control DNA, as well as plasmid recovered from hydrolyzed microparticles, was complexed with Lipofectamine 2000, a commercially available transfection agent. From the agarose gel analysis described above, the isolated plasmid is mostly in the open circular form, whereas the original sample of DNA is mostly supercoiled (Figure 2). Both of these structures are capable of undergoing transcription in cells, but not, however, the linear species that are present in the recovered plasmid (*47*, *48*). Figure 3 depicts the transfection results of the DNA complexes with 293T kidney cells. Untreated cells or wells with DNA alone showed no β-galactosidase activity. For DNA−Lipofectamine assemblies, there is a minor decrease in the transfection ability of the isolated plasmid compared to the original DNA vector, most likely due to the presence of a small amount of linear plasmid in the isolated DNA sample. Encapsulated DNA is capable of transfection, as only the tertiary structure of the plasmid is altered by the polymerization and hydrolysis procedures. The inverse

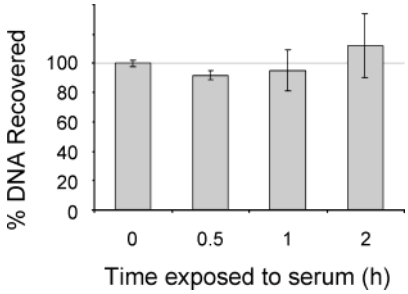


Figure 4. Nuclease stability of plasmid-encapsulated microparticles.

microemulsion polymerization technique described herein is compatible with both DNA and proteins (*38*), making possible future studies in vaccine delivery involving the encapsulation of biomolecules that vary by function (specific T cell activation, immunostimulation, or antisense) and classification (protein, DNA, or RNA).

Nuclease Stability of Encapsulated Plasmid. Stability of the microparticles at pH 7.4 is also important as the polymer scaffold functions to protect the encapsulated plasmid. Within 5 min of exposure to serum nucleases, plasmid DNA begins to break down rapidly and is completely degraded within 1 h (*49*); at pH 7.4, however, the DNA does not diffuse out of the microparticle as demonstrated in the release experiments. The pores of the microparticle are small enough to keep the plasmid DNA encapsulated until the delivery vehicle reaches the acidic environment of the phagolysosome. The serum stability of the plasmid microparticles was investigated to determine whether nucleases could enter the microparticle and degrade the DNA. To demonstrate that the microparticles actually protect the encapsulated DNA, the beads were exposed to serum nucleases by combining them with Dubelcco's modified Eagle medium containing 10% fetal bovine serum at 37 °C (Figure 4). The amount of DNA remaining in the beads was analyzed. Any degradation of the plasmid by the nucleases present in this medium would result in small, linear fragments. Since short DNA sequences cannot be retained, they would readily diffuse out of the particles. However, no appreciable decrease in the concentration of double-stranded DNA was observed with the plasmid-encapsulated particles and thus, no degradation of the DNA was observed over a period of 2 h. Additionally, when analyzed by agarose gel electrophoresis, no change was detected for the DNA recovered from microparticles that had been exposed to serum nucleases.

The toxicity of the beads was tested in RAW 309 macrophages. The cells were >65% viable for all samples tested, even at concentrations as high as 5 mg of beads/mL. There were minimal toxicity differences between polymer microparticles and plasmid-loaded particles. While polyacrylamide itself is nontoxic, the effects of the aldehyde byproducts of the degradation have yet to be examined (*50*).

pH Dependent Release of Plasmid Encapsulated Microparticles. The pH dependence of DNA release from the microparticles was evaluated by incubating the microbeads at 37 °C in pH 7.4 sodium phosphate buffer or pH 5.0 acetic acid buffer. After the desired time point, the beads were collected by centrifugation and the supernatant analyzed for DNA (Figure 5). At physiological pH, there was an initial burst release of 10%, indicating that some of the DNA is not fully encapsulated within the beads. Additional incubation at neutral pH showed no further release of DNA since the pores of the

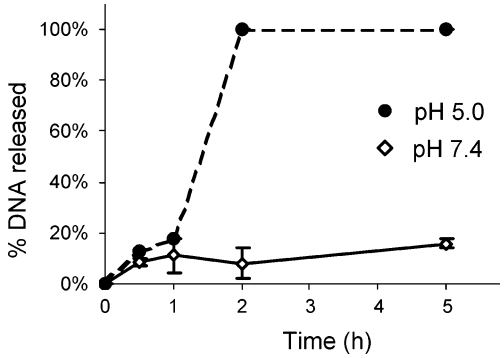


Figure 5. pH dependent release of plasmid DNA from microparticles at 37 °C.

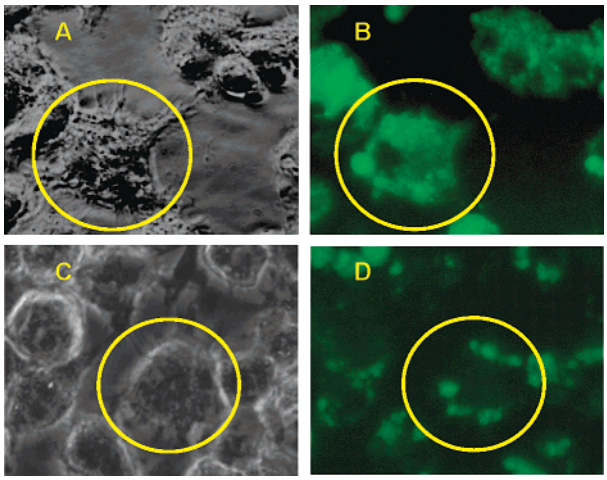


Figure 6. Fluorescence microscopy of macrophages. Phase contrast and fluorescence images of dextran-loaded microparticles with a degradable (A, B, respectively) and a nondegradable (C, D) cross-linker.

cross-linked microparticles are too small to allow for diffusion of the plasmid out of the beads. Presumably then, the microparticles would not release the DNA payload until the microparticle hydrolyzed in an acidic environment.

Under acidic conditions, however, full release of the DNA is achieved within 2 h at pH 5. The hydrolysis kinetics of acetal cross-linkers similar to **2** have been measured previously with a half-life of 24 h at pH 7.4 and only 5 min at pH 5.0 (*39*). The release of plasmid from the microbeads is slower, as would be expected given their incorporation in a cross-linked network.

Fluorescence Microscopy Studies. A key issue with DNA delivery is its distribution within the cellular environment. To examine where the payload is delivered in a macrophage, polyacrylamide microparticles were prepared encapsulating fluorescently (FITC) labeled dextran, MW 70 kDa. Dextran was labeled instead of DNA because fluorescent tags for DNA may dissociate and intercalate into cellular DNA, giving misleading results as to the location of the encapsulated biomolecule. Particles with degradable and nondegradable cross-linkers were prepared and added to RAW 264.7 macrophages. After incubation overnight, the cells were examined by phase contrast mode and with fluorescein excitation (Figure 6). Cells alone showed no autofluorescence. In contrast, cells treated with beads containing the acid sensitive cross-linker showed a diffuse fluorescence in the cytoplasm, (B), indicating that the acid labile linkage degraded in the lysosome and released the fluorescently labeled payload into the cytoplasm with no

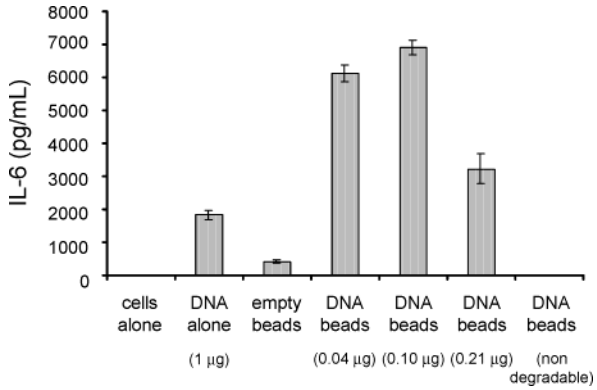


Figure 7. Interleukin-6 ELISA. Below each bar in the graph is the total mount of plasmid DNA added per well.

migration of the FITC-dextran to the nucleus. Nondegradable beads made with *N*,*N*′-methylenebisacrylamide as the cross-linker showed a much more punctate fluorescence (D), indicating that the FITC-dextran remained localized in the lysosome of the cell. The nondegradable beads are readily phagocytosed by the macrophages, but are sequestered in the lysosome as the particles possess no synthetic switch to initiate lysosomal escape.

IL-6 ELISA. Differences between bacterial and vertebrate DNA are recognized by vertebrate immune systems such that bacterial DNA will invoke a general immune response that can be characterized by the secretion of interleukins, radicals, and prostraglandins. The cytosine residues of bacterial DNA are not methylated as they are in vertebrates. In addition, there is a 6-fold increase in the percentage of CG motifs in bacterial DNA, typically ∼1.2% in vertebrate and 6−7% in bacterial plasmids (*15*). The β-galactosidase plasmid sequence used in these studies contains 6.7% of the CpG dinucleotide and therefore should be capable of inducing an immune response in macrophages.

This response is mediated by a Toll-like receptor, TLR9, which is thought to be located within the lysosome or on the cell surface (*51*−*53*). Receptor binding of TLR9 on the cell surface can only be accomplished by DNA that is located on the outside of the particle, as there is negligible release observed at neutral pH in the extracellular environment. However, if the TLR9 is recruited to the lysosome, then an immunostimulatory response should be observed when plasmid encapsulated microparticles are delivered to cells, as it has been discussed above that the DNA will be released when it encounters the acidic environment of the phagolysosome. Upon interaction of the encapsulated plasmid with TLR9, macrophages secrete IL-6 to mediate the growth and differentiation of T cells, a vital component of vaccine treatment.

To examine the immunostimulatory properties of the plasmid-loaded microparticles, RAW 264.7 macrophages were incubated overnight with 1 μg of naked plasmid or 50 μg of plasmid microbeads of various loadings. Nondegradable beads with an initial loading of 2.0 μg/mg of plasmid DNA were also tested. It was not possible to determine the true loading of these microparticles, as they are not capable of acidic degradation so the DNA cannot be recovered. Assuming a similar encapsulation efficiency as with the degradable system, the final loading of these beads should correspond to the medium-loaded beads in which 0.10 μg of plasmid were delivered per well. The supernatant was analyzed for interleukin-6 (IL-6) production using an ELISA kit (Figure 7). Beads encapsulating plasmid DNA successfully induced the secretion of IL-6, with a general trend of higher loaded plasmid beads producing larger amounts of IL-6. The maximum response is observed when just 0.1 μg of encapsulated DNA is delivered per well using beads with a loading of 2.14 μg/mg and resulted in an IL-6 response of 6890 pg/mL, which is 3.75 times that when 1 μg of DNA alone was added. Treatment with more highly loaded particles, 0.2 μg of DNA per well, did not produce an increased cytokine response, indicating that a response to DNA loading had reached a maximum. Untreated cells secreted a minimal amount of IL-6. Furthermore, negligible IL-6 concentrations were measured when unloaded beads were delivered to the cells, indicating that the polymeric microparticles themselves do not induce IL-6 production. With nondegradable particles, there was minimal immunostimulation as indicated by the ELISA results. Because the plasmid DNA is trapped inside the beads, it is not available to be recognized by Toll-like receptors either on the cell surface or within the lysosomal compartment. Only when the microparticles are capable of releasing the encapsulated DNA inside the lysosome is an enhanced response observed.

CONCLUSION

The flexibility of the inverse microemulsion polymerization allows for the encapsulation of both plasmid and protein to produce microparticles for vaccine development. The polymer scaffold serves to protect the DNA from nuclease degradation and prevents unwanted release at physiological pH. The particles are readily phagocytosed by macrophages and induce an immune response, resulting in IL-6 secretion. Knowing that the plasmid DNA released from the microparticles is capable of undergoing transfection and that, according to fluorescent microscopy studies using dextran-encapsulated microparticles, a bioactive payload is directed to the cytoplasm of macrophages, further improvements in the polymeric carrier are required to affect nuclear delivery, which would allow gene therapy applications to be accessible with this degradable microparticle system. In addition, further experiments are underway to develop multicomponent microparticles containing both protein for antigen-specific T cell activation and immunostimulatory DNA for general T cell stimulation for enhanced vaccine capabilities.

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LITERATURE CITED

(1) Prokop, A., Kozlov, E., Carlesso, G., and Davidson, J. M. (2002) Hydrogel-Based Colloidal Polymeric System for Protein and Drug Delivery: Physical and Chemical Characterization, Permeability Control and Applications. *Adv. Polym. Sci. 160*, 119−173.

(2) Galaev, I. Y., and Mattiasson, B. (1999) "Smart" Polymers and What They Could Do in Biotechnology and Medicine. *Trends Biotechnol. 17*, 335−340.

(3) Park, H., and Park, K. (1996) Hydrogels in Bioapplications. *ACS Symp. Ser. 627*, 2−10.

(4) Amara, R. R., Villinger, F., Altman, J. D., Lydy, S. L., O'Neil, S. P., Staprans, S. I., Montefiori, D. C., Xu, Y., Herndon, J. G., Wyatt, L. S., Candido, M. A., Kozyr, N. L., Earl, P. L., Smith, J. M., Ma, J.-L., Grimm, B. D., Hulsey, M. L., Miller, J., McClure, H. M., McNicholl, J. M., Moss, B., and Robinson, H. L. (2001) Control of a Mucosal Challenge and Prevention of AIDS by a Multi Protein DNA/MVA Vaccine. *Science 292*, 69−74.

(5) Merad, M., Sugie, T., Engleman, E. G., and Fong, L. (2002) in Vivo Manipulation of Dendritic Cells to Induce Therapeutic Immunity. *Blood 99*, 1676−1682.

(6) Davila, E., and Celis, E. (2000) Repeated Administration of CpG ODN with Peptide/Protein Immunization Results in Enhanced CTL Response. *J. Immunol. 165*, 539−547.

(7) Lipford, G. B., Heeg, K., and Wagner, H. (1998) Bacterial DNA as Immune Cell Activator. *Trends Microbiol. 6*, 496−500.

(8) Klinman, D. M., Yi, A.-K., Beaucage, S. L., Conover, J., and Krieg, A. M. (1996) CpG Motifs Present in Bacterial DNA Rapidly Induce Lyphocytes to Secrete Interleukin 6, Interleukin 12, and Interferon Gamma. *Proc. Natl. Acad. Sci. 93*, 2879−2883.

(9) Messina, J. P., Gilkeson, G. S., and Pisetsky, D. S. (1991) Stimulation of in Vitro Murine Lymphocyte Proliferation by Bacterial DNA. *J. Immunol. 147*, 1759−1764.

(10) Gilkeson, G. S., Grudier, J. P., Karounos, D. G., and Pisetsky, D. S. (1989) Induction of Anti-Double Stranded DNA Antibodies in Normal Mice by Immunization with Bacterial DNA. *J. Immunol. 142*, 1482−1486.

(11) Weiner, G. J. (2000) The Immunobiology and Clinical Potential of Immunostimulatory CpG Oligonucleotides. *J. Leukocyte Biol. 68*, 455−463.

(12) Sparwasser, T., Koch, E.-S., Vabulas, R. M., Heeg, K., Lipford, G. B., Ellwart, J. W., and Wagner, H. (1998) Bacterial DNA and Immunostimulatory CpG Oligonucleotides Trigger Maturation and Activation of Murine Dendritic Cells. *Eur. J. Immunol. 28*, 2045−2054.

(13) Yasuda, K., Ogawa, Y., Kishimoto, M., Takagi, T., Hashida, M., and Takakura, Y. (2002) Plasmid DNA Activates Murine Macrophages to Induce Inflammatory Cytokines in a CpG Motif-Independent Manner by Complex Formation With Cationic Liposomes. *Biochem. Biophys. Res. Commun. 293*, 344−348.

(14) Cho, H. J., Takabayashi, K., Cheng, P.-M., Nguyen, M.-D., Corr, M., Tuck, S., and Raz, E. (2000) Immunostimulatory DNA-Based Vaccines Induce CTL Activity by a T-Helper Cell-Independent Mechanism. *Nat. Biotechnol. 18*, 509−514.

(15) Krieg, A. M., Yi, A.-K., Matson, S., Waldschmidt, T. J., Bishop, G. A., Teasdale, R., Koretzky, G. A., and Klinman, D. M. (1995) CpG Motifs in Bacterial DNA Trigger Direct B−Cell Activation. *Nature 374*, 546−549.

(16) Shen, W., Waldschmidt, M., Zhao, X., Ratliff, T., and Krieg, A. M. (2002) Antitumor Mechanisms of Oligodeoxynucleotides with CpG and PolyG Motifs in Murine Prostate Cancer Cells: Decrease of NF−KB and AP-1 Binding Activities and Induction of Apoptosis. *Antisense Nucleic Acid Drug Dev. 12*, 155−164.

(17) Krieg, A. M., Wu, T., Weeratna, R., Efler, S. M., Love-Homan, L., Yang, L., Yi, A.-K., Short, D., and Davis, H. L. (1998) Sequence Motifs in Adenoviral DNA Block Immune Activation by Stimulatory CpG Motifs. *Proc. Natl. Acad. Sci. 95*, 12631−12636.

(18) Hirano, T. Interleukin-6 (1998) *The Cytokine Handbook* (Thomson, A., Ed.) 3rd ed., Chapter 8, Academic Press, San Diego.

(19) Akinc, A., Lynn, D. M., Anderson, D. G., and Langer, R. (2003) Parallel Synthesis and Biophysical Characterization of a Degradable Polymer Library for Gene Delivery. *J. Am. Chem. Soc. 125*, 5316−5323.

(20) Choi, J. S., MacKay, J. A., and Szoka, F. C., Jr. (2003) Low pH Sensitive PEG Stabilized Plasmid Lipid Nanoparticles: Preparation and Characterization. *Bioconjugate Chem. 14*, 420−429.

(21) Kihara, F., Arima, H., Tsutsumi, T., Hirayama, F., and Uekama, K. (2003) in Vitro and in Vivo Gene Transfer by an Optimized α-Cyclodextrin Conjugate with Polyamidoamine Dendrimer. *Bioconjugate Chem. 14*, 342−350.

(22) Kunath, K., van Harpe, A., Fischer, D., and Kissel, T. (2003) Galactose-PEI-DNA Compelxes for Targeted Gene Delivery. *J. Controlled Release 88*, 159−172.

(23) Arbós, P., Wirth, M., Arangoa, M. A., Gabor, F., and Irache, J. M. (2002) Gantrez AN as a New Polymer for the Preparation of Ligand-Nanoparticle Conjugates. *J. Controlled Release 83*, 321−330.

(24) Erni, C., Suard, C., Freitas, S., Dreher, D., Merkle, H. P., and Walter, E. (2002) Evaluation of Cationic Solid Lipid Microparticles as Synthetic Carriers for the Target Delivery of Macromolecules to Phagocytic Antigen-Presentation Cells. *Biomaterials 23*, 4667−4676.

(25) Gregoriadis, G., Bacon, A., Caparros-Wanderley, W., and McCormack, B. (2002) A Role for Liposomes in Genetic Vaccination. *Vaccine 20*, B1−B9.

(26) McAllister, K., Sanzani, P., Adam, M., Cho, M. J., Rubinstein, M., Samulski, R. J., and DeSimone, J. M. (2002) Polymeric Nanogels Produced Via Inverse Microemulsion Polymerization as Potential Gene and Antisense Delivery Agents. *J. Am. Chem. Soc. 124*, 15198−15207.

(27) Lee, M., Nah, J.-W., Kwon, Y., Koh, J. J., Ko, K. S., and Kim, S. W. (2001) Water-Soluble and Low Molecular Weight Chitosan-Based Plasmid DNA Delivery. *Pharm. Res. 18*, 427−431.

(28) Sakurai, F., Inoue, R., Nishino, Y., Okuda, A., Matsumoto, O., Taga, T., Yamashita, F., Takakura, Y., and Hashida, M. (2000) Effect of DNA/Liposome on the Physicochemical Characteristics, Cellular Uptake, and Intracellular Trafficking of Plasmid DNA. *J. Controlled Release 66*, 255−269.

(29) Uherek, C., and Wels, W. (2000) DNA-Carrier Proteins for Targeted Gene Delivery. *Adv. Drug Delivery Rev. 44*, 153−166.

(30) (1999) *Nonviral Vectors for Gene Therapy* (Huang, L., Hung, M.-C., Wagner, E., Eds.) Academic Press, San Diego.

(31) O'Hagan, D. T., Palin, K., Davis, S. S., Artursson, P., and Sjöholm, I. (1989) Microparticles as Potentially Orally Active Immunological Adjuvants. *Vaccine 7*, 421−424.

(32) Jang, J.-H., and Shea, L. D. (2003) Controllable Delivery of Non-Viral DNA from Porous Scaffolds. *J. Controlled Release 86*, 157−168.

(33) De Rosa, G., Quaglia, F., Bochot, A., Ungaro, F., and Fattal, E. (2003) Long-Term Release and Improved Intracellular Penetration of Oligonucleotide-Polyethylenimine Compelxes Entrapped in Biodegradable Microspeheres. *Biomacromolecules 4*, 529−536.

(34) Zhou, S., Liao, X., Li, X., Deng, X., and Li, H. (2003) Poly-D, L-Lactide-*co*-Poly(Ethylene Glycol) Microspheres as Potential Vaccine Delivery Systems. *J. Controlled Release 86*, 195−205.

(35) Perez, C., Sanchez, A., Putnam, D., Ting, D., Langer, R., and Alonso, M. J. (2001) Poly(Lactic Acid)-Poly(Ethylene Glycol) Nanoparticles as New Carriers for the Delivery of Plasmid DNA. *J. Controlled Release 75*, 211−224.

(36) Tinsley-Brown, A. M., Fretwell, R., Dowsett, A. B., Davis, S. L., and Farrar, G. H. (2000) Formulation of Poly(DL-Lactic-Co-Glycolic Acid) Microparticles for Rapid Plasmid DNA Delivery. *J. Controlled Release 66*, 229−241.

(37) Walter, E., Moelling, K., Pavlovic, J., and Merkle, H. P. (1999) Microencapsulation of DNA Using Poly(DL-Lactide-*co*-Glycolide): Stability Issues and Release Characteristics. *J. Controlled Release 61*, 361−374.

(38) Murthy, N., Xu, M. C., Schuck, S., Kunisawa, J., Shastri, N., and Fréchet, J. M. J. (2003) A Macromolecular Delivery Vehicle for Protein Based Vaccines: Acid Degradable Protein Loaded Microgels. *Proc. Natl. Acad. Sci. 100*, 4995−5000.

(39) Murthy, N., Thng, Y. X., Schuck, S., Xu, M. C., and Fréchet, J. M. J. (2002) A Novel Strategy for Encapsulation and

Release of Proteins: Hydrogels and Microgels With Acid-Labile Acetal Cross-Linkers. *J. Am. Chem. Soc. 124*, 12398−12399.
(40) Freshney, R. I. (1994) *Culture of Animal Cells: A Manual of Basic Technique*, 3rd ed., Wiley-Liss, New York.
(41) Higgins, D. E., Shastri, N., and Portney, D. A. (1999) Delivery of Protein to the Cytosol of Macrophage Using Escherichia Coli K-12. *Mol. Microbiol. 31*, 1631−1641.
(42) Barton, J. (1996) Free-Radical Polymerization in Inverse Microemulsions. *Prog. Polym. Sci. 21*, 399−348.
(43) Candau, F., Leong, Y. S., and Fitch, R. M. (1985) Kinetic Study of the Polymerization of Acrylamide in Inverse Microemulsion. *J. Polym. Sci. A: Polym. Chem. 23*, 193−214.
(44) Harding, C. V., and Song, R. (1994) Phagocytic Processing of Exogenous Particulate Antigens by Macrophages for Presentation by Class I MHC Molecules. *J. Immunol. 153*, 4925−4933.
(45) Ando, S., Putnam, D., Pack, D. W., and Langer, R. (1999) PLGA Microspheres Containing Plasmid DNA: Preservation of Supercoiled DNA Via Cryopreparation and Carbohydrate Stabilization. *J. Pharm. Sci. 88*, 126−130.
(46) Wang, D., Robinson, D. R., Kwon, G. S., and Samuel, J. (1999) *J. Controlled Release 57*, 9−18.
(47) Piña, B., Haché, R. J. G., Arnemann, J., Chalepakis, G., Slater, E. P., and Beato, M. (1990) Hormonal Induction of Tranfected Genes Depends on DNA Topology. *Mol. Cell. Biol. 10*, 625−633.
(48) Harland, R. M., Weintraub, H., and McKnight, S. L. (1983) Transcription of DNA Injected in Xenopus Oocytes is Influenced by Template Topology. *Nature 302*, 38−43.
(49) Kawabata, K., Takakura, Y., and Hashida, M. (1995) The Fate of Plasmid DNA After Intravenous Injection in Mice: Involvement of Scavenger Receptors in Its Hepatic Uptake. *Pharm. Res. 12*, 825−830.
(50) Caulfield, M. J., Qiao, G. G., and Solomon, D. H. (2002) Some Aspects of the Properties and Degradation of Polyacrylamides. *Chem. Rev. 102*, 3067−3083.
(51) Hemmi, H.; Takeuchi, O.; Kawai, T.; Kaisho, T.; Sato, S.; Sanjo, H.; Matsumoto, M.; Hoshino, K.; Wagner, H.; Takeda, K.; Akira, S. (2000) A Toll-like Receptor Recognizes Bacterial DNA. *Nature 408*, 740−745.
(52) Wagner, H. (2002) Interactions Between Bacterial CpG-DNA and TLR9 Bridge Innate and Adaptive Immunity. *Curr. Top. Microbiol. Immunol. 5*, 62−69.
(53) Ahmad-Nejad, P., Häcker, H., Rutz, M., Bauer, S., Vabulas, R. M., and Wagner, H. (2002) Bacterial CpG-DNA and LPS Activate Toll-Like Receptors at Distinct Cellular Compartments. *Eur. J. Immunol. 32*, 1958−1968.

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