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Rapid communication

PEGylated J591 mAb loaded in PLGA-PEG-PLGA tri-block copolymer for targeted delivery: In vitro evaluation in human prostate cancer cells

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Abstract

J591 monoclonal antibody (mAb) has high affinity for prostate specific membrane antigen (PSMA) on prostate cancer (PCA) cells. We coupled polyethylene glycol-J591 (PEGylated J591) to a salicyl hydroxamic acid (SHA)–derivatized polyethylenimine (PEI)/DNA-βgal vector to investigate the specificity and efficiency of targeting PSMA in PCA cells through encapsulation. Coupling was facilitated via the high affinity interaction between phenyl(di)boronic acid (PDBA) and SHA molecules yielding J591/PEG/PEI/DNA-βgal polyplex. After encapsulation with poly(D,L-lactic-*co*-glycolic acid)-*b*-polyethylene glycol-*b*-poly(D,L-lactic-*co*-glycolic acid) (PLGA-PEG-PLGA) tri-block copolymer, 8–10-fold increment of gene transfection levels were attained at the optimum concentration of 0.25% (w/v) using Pluronic F68 tri-block copolymer as a control. The enhanced transfection efficiency was attributed to increased internalization and uptake of the radiolabeled plasmid in the presence of PLGA-PEG-PLGA tri-block copolymer. The release of plasmid DNA (pDNA) from microparticles containing SHA–PEI-complexed pDNA showed little initial burst release followed by a 5% release over 48 h. The release accelerated thereafter and approximately 60% was released after 28 days. Deconvolution confocal microscopy showed polyplex/microparticle formulation localized in the cell nucleus as opposed to the polyplex without PLGA-PEG-PLGA indicating that an optimal concentration of PLGA-PEG-PLGA tri-block copolymer can be utilized to enhance endocytic process of J591-mediated targeting of PCA cells.

Keywords: Polyethyleneimine (PEI); Phenyl(di)boronic acid (PDBA)–salicyl hydroxamic acid (SHA); PLGA-PEG-PLGA; J591mAb; Prostate specific membrane antigen (PSMA); Targeted gene delivery

One of the greatest limiting factors to effective gene therapy is the choice of delivery vector (Verma and Somia, 1997; Luo, 2004). Although producing high levels of transduction, viral vectors as a whole have many disadvantages for use in gene therapy, such as difficulty in construction, toxicity, immunogenicity, and inability to target (Brown et al., 2001). Consequently, the generation of safe and effective gene delivery vectors has resulted in numerous reports about targeting tumor cells without eliciting adverse effects (Cristiano et al., 1993; Ziady et al., 1999; Jenkins et al., 2001; Moffatt et al., 2005). An increasing focus has also been placed on the use of biodegradable non-viral vectors to enhance, as well as control gene expression of non-viral vectors (Men et al., 1997; Hedley et al., 1998).

Prostate specific membrane antigen (PSMA) is a potential prostate target that is over expressed in prostate tumors (Horoszewicz et al., 1987; O'keefe et al., 2000). In order to target PSMA, anti-PSMA mAb, J591, has already been generated and shown cellular internalization (Liu et al., 1998). Therefore, we envisaged that an efficient delivery system of J591-mediated polyplex could be harnessed in targeting PSMA in the prostate tumor. We recently reported the interaction between phenyl(di)boronic acid (PDBA) and salicyl hydroxamic acid (SHA) molecules to couple the disulphide bridged tumor-specific Cys-Asn-Gly-Arg-Cys (CNGRC) peptide (Moffatt et al., 2005) and also J591 mAb to the polyethyleneimine (PEI)/DNA-βgal vector (Moffatt et

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al., 2006) for targeting purposes. Several advantages of this complex including stability under a wide variety of conditions, reversible interaction and a lack of immune response (Stolowitz et al., 2001) make this system very attractive.

While encapsulation of DNA in biodegradable poly(D,L-lactic-*co*-glycolic acid) (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 microparticles for targeted gene delivery to PCA cells. Previous studies have shown that drug release from PLGA microspheres is very low due to the hydrophobicity of both drug and PLGA. In a recent study, only 50% of paclitaxel could be released within 3 months (Feng et al., 2004; Mu and Feng, 2001) when conjugated to PLGA which is insufficient for therapy from a controlled delivery device. It is therefore conceivable that incorporation of a hydrophilic segment such as PEG into the hydrophobic PLGA chain forming the tri-block copolymer poly(D,L-lactic-*co*-glycolic acid)-*b*-polyethylene glycol-*b*-poly(D,L-lactic-*co*-glycolic acid) (PLGA-PEG-PLGA) would greatly facilitate the drug release. Indeed, studies have demonstrated that this tri-block copolymer increases the release rate of hydrophilic proteins (Deng et al., 1990; Li et al., 2000). 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 J591-containing polyplex to yield a targeting gene delivery vehicle for evaluation of transfection efficiency in PCA cells. The primary objective of this communication was to investigate enhancement of targeted delivery as well as sustained release in PCA cells through encapsulation with PLGA-PEG-PLGA copolymer, with the ultimate aim of evaluating the targeted polyplex in a biodegradable formulation for gene therapy purposes. We examined the transfection efficiency of J591/PEG/PEI/DNA-βgal targeting polyplex loaded within different concentrations of biodegradable PLGA-PEG-PLGA tri-block copolymer in a human PSMA-positive PCA cell line, PC3-MM2, and compared it to the unencapsulated polyplex or similar concentrations of the commonly used Pluronic F68 tri-block copolymer.

The addition of PEG (Sigma, $M_r = 3000$) as a spacer between PDBA and J591 and the final coupling of the mAb was done in a stepwise manner as described previously (Moffatt et al., 2006). Molar ratios of PDBA:J591 as well as J591:vector was estimated quantitatively using the formula by Prolinx Inc., (Seattle, WA, USA). The formation of the SHA–derivatized PEI (SHA–PEI) in which 5% of the primary amine groups of PEI (Aldrich Chemical Company, Inc., Milwaukee, WI, USA) was replaced with SHA, was done by Prolinx. The generation of the working amine to phosphate (N/P) ratio of 2.7/1, as well as the addition of DNA and PDBA-PEG-J591 to yield J591/PEG/PEI/DNA-βgal polyplex has adequately been described (Moffatt et al., 2005). The hydrodynamic radii were measured by dynamic light scattering.

PC3-MM2 cells was cultured in RPMI with 10% FBS supplemented with 1% antibiotic–antimycotic (penicillin–streptomycin–amphotericin, GibcoBRL) and 1% L-glutamine in a 37 °C incubator with 5% CO_2. Cells were plated at a density of 1×10^4 cells/ml in 12-well plates 24 h before transfection. The plasmid pCMVβgal contains the *Escherichia coli* βgal gene under the control of the cytomegalovirus enhancer and promoter. Polyplex containing 6 μg DNA and four different concentrations of either PLGA-PEG-PLGA or Pluronic F68 copolymer dissolved in PBS (0%, 0.25%, 0.5% and 1% w/v) in a total volume of 40 μl was incubated for 15 min at room temperature before adding to cells at 37 °C. Expression of βgal was determined from cells using a Galacto-light chemiluminescent reporter assay (Tropix) after 24 h of culture and measurements normalized for protein concentration using a BCA protein assay kit (Pierce, Rockford, IL, USA). Statistical analyses were processed and analyzed by Sigma-Plot 8.0 software (SPSS, IL) and *P*-value less than 0.05 was considered statistically significant. Whereas no significant difference ($P < 0.01$) in transfection efficiency was observed between PEI/DNA and SHA–PEI/DNA polyplexes, there was approximately 8–10-fold increase over that of the unencapsulated formulation or the control Pluronic F68 polymer at the optimum concentration of 0.25% in the J591-targeted formulation. These results indicate that an optimal PLGA-PEG-PLGA concentration can be used as a more effective polymer for enhancing transfection efficiency over our previously reported unencapsulated J591-targeted polyplex in PCA cells. Microparticles did not cause any significant cell death in PC3-MM2 cells after overnight incubation, as assessed by spectrophotometry.

Equally important was to investigate if the PLGA-PEG-PLGA aided the intracellular uptake of polymer/DNA complexes in comparison to the pluronic F68 controls. Plasmid pCMVβgal was labeled with [α-^{32}P]dATP (400 Ci/mmol, 10 mCi/ml) (Amersham, Piscataway, NJ, USA). About 1×10^5 cells was cultured for 24 h. Labeled DNA formulated with SHA–PEI and PLGA-PEG-PLGA or Pluronic F68 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 or unencapsulated complexes. The level of plasmid DNA uptake increased by five-fold in the presence of 0.25% PLGA-PEG-PLGA over the unencapsulated polyplex at 37 °C with no significant DNA uptake at 4 °C, and approximately three-fold over the Pluronic F68 controls, further confirming that the cellular endocytosis process is suppressed at this temperature, with PLGA-PEG-PLGA as a more superior tri-block copolymer for encapsulation. Relative DNA uptake was calculated by relating the radioactivity of the polyplex with PLGA-PEG-PLGA formulation to that of the group without the formulation. These results may also provide evidence that encapsulation protects the DNA integrity thereby increasing the overall DNA delivered to the cells as opposed to the unencapsulated polyplex.

The in vitro release profile of plasmid DNA from microparticles was carried out in 30 mM PBS (pH 7.4) at 37 °C. After

centrifugation, the concentration of pDNA in the supernatant was determined by fluorescence spectrophotometry using the PicoGreen dye assay (Molecular Probes, Eugene, OR, USA). Two standards (i.e., free and complexed pDNA [1:0.6 wt./wt. complexation]) were used to construct a standard curve to determine in vitro release of free and complex-containing microparticles, respectively. The release of pDNA from microparticles containing SHA–PEI-complexed pDNA showed little initial burst release. Approximately 5% released in a linear fashion over 48 h. The release accelerated thereafter and approximately 60% was released after 28 days. The sustained-release profile observed for microparticles containing SHA–PEI-complexed pDNA is presumed to be either a result of the lower solubility of complexed pDNA in the release medium or the increased association of the more hydrophobic complex with the hydrophobic polymer matrix.

PC3-MM2 cells were transfected with 40 μl J591/PEG/PEI/DNA-βgal polyplexes alone or encapsulated within either PLGA-PEG-PLGA or Pluronic F68 microparticles (0.25%, w/v), 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, cells were exposed to rhodamine-albumin-labeled (Sigma–Aldrich, St. Louis, MO) particles for 6 h (5 μg/ml), fixed with 1% formaldehyde, and permeabilized with Triton X-100 (0.1%). Cells were labeled with a fluorescent phalloidin to delineate the actin cytoskeleton, washed thoroughly to remove non-adherent or extracellular particles before staining with Alexa Fluor 488 phalloidin (Molecular Probes), according to manufacturer's instructions. Fluorescence microscopy images were acquired using a Zeiss (Oberkochen, Germany) Axiovert microscope, and deconvolution analysis was performed with Openlab Deconvolution Software (Improvision, Lexington, MA, USA). The cell suspensions were adhered to a polylysine-coated slide and subjected to confocal microscopy. After incubation at 37 °C, PC3-MM2 cells were observed to be closely associated with PLGA-PEG-PLGA microparticles unlike cells with the pluronic control polymer or the unencapsulated polyplex. However, if cells were incubated at 4 °C, no particles were visible in association with the cells, suggesting that the uptake of particles was an energy-dependent process. These data indicated that J591-coupled SHA–PEI/DNA polyplexes loaded within PLA-PEG-PLGA microparticles are preferentially and avidly endoocytosed by PSMA-positive cells. Furthermore, deconvolution analysis of acquired images confirmed that the particles were localized in the cell nucleus, thus excluding the possibility that the intracellular localization of encapsulated polyplex was due to cell surface-adherent microparticles creating high local concentrations at the cell membrane. Our data support the view that these microparticles, having diameters of less than 10 μm, were taken up by receptor-mediated endocytosis. While detailed cellular localization was not analyzed in our previous report using the unencapsulated J591 targeted polyplex, its conceivable that encapsulation with PLGA-PEG-PLGA copolymer in this communication aid in the nuclear localization of the targeted microparticles in PC3-MM2 cells. The morphologies of selected particles were assessed by scanning electron microscopy (SEM) using an AMR-1000 at 10 kV with a gold–palladium conductive coating. The SEM showed that the microparticles have a smooth spherical surface with a size of less than 5 μm and there was no significant difference between shapes and sizes of PLGA-PEG-PLGA- and Pluronic F68-encapsulated microparticles.

To our knowledge, this is the first biodegradable microparticle formulation of J591 polyplex targeting PSMA. These elegant results warrant the focus of future studies on the incorporation of a therapeutic drug in the PLGA-PEG-PLGA-encapsulated J591/PEG/PEI microparticles to assess the effect on tumors in rodent models.

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