Pharmaceutical Research, Vol. 24, No. 12, December 2007 (© 2007)
DOI: 10.1007/s11095-007-9441-y

Research Paper

Microencapsulation of PEGylated Adenovirus within PLGA Microspheres for Enhanced Stability and Gene Transfection Efficiency

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Received April 3, 2007; accepted August 21, 2007; published online October 11, 2007

Purpose. Green fluorescent protein (GFP) encoding adenovirus (ADV) was surface modified with polyethylene glycol (PEG) for microencapsulation within poly(lactic-co-glycolic acid) (PLGA) microspheres with the aim of improving stability and gene transfection activity.
Methods. A series of PEGylated ADV (PEG-ADV) with different PEG seeding densities on the viral surface was prepared and the GFP expression efficiency of each PEG-ADV in the series determined. The physical stabilities of naked ADV and PEG-ADV were comparatively evaluated by exerting a high shear homogenization process or by exposure to low pH. Naked ADV or PEG-ADV was microencapsulated within PLGA microspheres using a water-in-oil-in-water (W/O/W) double emulsion and solvent evaporation method. *In vitro* cumulative ADV and PEG-ADV release profiles from PLGA microspheres were determined over a 10-day period. GFP transfection efficiencies into HeLa cells were quantified, and the relative extent of the immune response for ADV and PEG-ADV encapsulated within PLGA microspheres was analyzed using macrophage cells.
Results. The physical stability of PEGylated ADV was greatly enhanced relative to that of naked ADV under the simulated W/O/W formulation conditions, such as exposure to an aqueous/organic interface during high shear-stressed homogenization. PEG-ADV was also more stable than ADV at low pH. ADV and PEG-AD were both released from PLGA microspheres similarly in a sustained fashion. However, when the ADV and PEG-ADV encapsulated microspheres transfected into HeLa cells, PEG-ADV microspheres demonstrated a higher GFP gene transfection efficiency than ADV microspheres. The PEG-ADV microspheres also exhibited a reduced extent of innate immune response for macrophage cells.
Conclusions. PEGylated ADV could be more safely microencapsulated within PLGA microspheres than naked ADV due to their enhanced physical stability under the harsh formulation conditions and acidic microenvironmental conditions of the microsphere, thereby increasing gene transfection efficiency.

KEY WORDS: adenovirus; PEGylation; physical stability; PLGA microsphere.

INTRODUCTION

Viral vectors are known to have a high potential as gene carriers for treating various infectious diseases and cancer due to their high transduction efficiencies (1,2). Among these, the adenoviral vectors are an intrinsic part of approximately 40% of all gene therapy protocols in clinical trials (2). However, there are some inherent problems to be solved: (1) safety issues relating to innate immune response, (2) nonspecific cellular uptake, and (3) physical instability problems when the adenoviral vectors are microencapsulated within biodegradable polymer microspheres. It has been reported that polyethylene glycol conjugated (PEGylated) adenovirus (ADV) could reduce the innate immune response while retaining infectivity by preventing neutralizing antibodies from recognizing surface viral antigens both *in vitro* and *in vivo* (3–5). Furthermore, cell-specific cellular uptake and gene expression could be achieved by attaching a specific cell-recognizable targeting ligand onto the distal end of the PEG chain anchored on the viral surface (6–8).

Adenovirus has been recently microencapsulated within biodegradable poly(D,L-lactic-co-glycolic acid) (PLGA) microspheres with the aim of alleviating the safety problems and increasing gene transfection efficiency at a target tissue site (9–11). Poly(lactic-co-glycolic acid polymers are well-characterized, biodegradable, biocompatible, and Federal Drug Agency (FDA)-approved materials popularly used for implantable medical devices and as injectable carriers for tissue engineering and drug delivery. Various hydrophilic and macromolecular drugs, such as peptides, proteins, genes, and even viruses, have been encapsulated within PLGA microspheres by the double emulsion and solvent evaporation method (water-in-oil-in-water, W/O/W) (12–16). However, when highly labile protein drugs are microencapsulated within PLGA microspheres, they are prone to degrade and/or aggregate during the harsh formulation conditions, including the exposure to an aqueous/organic interface and the use of a

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high shear-stressing homogenization/sonication process for emulsification. Moreover, microencapsulated protein molecules were found to be chemically degraded and subsequently aggregated during the release period caused by acidic degradation products accumulated within the degrading microspheres, resulting in uncontrollable release profiles with a subsequent loss of activity after release (17). To address the protein instability problems, PEGylated proteins, including interferon, lysozyme, and epidermal growth factor, were microencapsulated within PLGA microspheres to minimize the extent of nonspecific adsorption or aggregation at the oil/water interface and onto the degrading PLGA surface (18–20). PEGylated proteins were found to leach out in better release profiles while maintaining their biological activity compared to unPEGylated ones, probably due to the presence of the protective stealth of PEG chains around the protein molecules.

Viruses have coat proteins on the surface that are very sensitive to denaturation against environmental pH, temperature, redox potential, and shear-stress (21–24). This has lead to the expectation that the microencapsulation of ADV within PLGA microspheres would result in a significant loss of viral infectivity due to the harsh physical conditions imposed by the double emulsion and solvent evaporation method. It has been reported that viruses dispersed in aqueous solution are physically damaged by high shear-stressing sonication or homogenization and subsequently form irreversible and insoluble aggregates (11,12). Adenovirus particles are also known to aggregate under acidic conditions, thereby reducing viral infectivity. To maintain viral physical stability, various additives, such as sucrose, mannitol, Pluronic, glycerol, and cations, have been used for storage and transduction (23).

In this study, PEG-ADV encoding the green fluorescent protein (GFP) gene was microencapsulated within PLGA microspheres in an attempt to maintain the physical stability of the ADV during the formulation of the microsphere and subsequently to attain improved gene transfection efficiency. The adenovirus was conjugated with PEG at different seeding densities on the surface, and the resultant PEG-ADV conjugates were subjected to a quantitative analysis of GFP gene expression levels. The physical stabilities of the ADV and PEG-ADV against a high shear-stressing condition and acidic pH environment were comparatively analyzed. The naked ADV and the PEG-ADV were microencapsulated within PLGA microspheres by the double emulsion and solvent evaporation method and their release profiles examined. After release from PLGA microspheres, GFP transfection efficiencies of HeLa cells were determined. The extent of the innate immune response was also assessed by determining the amount of interleukin-6 (IL-6) released from macrophage cells.

MATERIALS AND METHODS

Materials

Adenovirus containing the GFP gene was obtained from QBIOgene (AD5-CMV5-GFP) (Montreal, Canada). Methoxy-PEG-succinimidyl propionic acid (mPEG-SPA) (MW 2000) was a product of Nektar (Huntsville, AL). The dialysis membrane (MW cut-off 50,000) was purchased from Spectrum (Houston, TX). Poly(D,L-lactic-co-glycolic acid) with a lactic/glycolic molar ratio of 50:50 (PLGA 5010) was obtained from Wako Pure Chemical Industries (Tokyo, Japan). Polyvinyl alcohol (PVA, 88% hydrolyzed, MW 13,000~23,000) was obtained from Sigma (St. Louis, MO). Cell culture media and materials were the products of Gibco BRL (Grand Island, NY). HEK293 cells, HeLa cells and Raw264.1 macrophage cells were purchased from the Korea Cell Line Bank (Seoul, South Korea).

Methods

Adenovirus Preparation and PEGylation

Adenovirus expansion was performed according to an established procedure (6). HEK293 cells were plated in a 150-mm tissue culture dish. After reaching about 60% of confluency, 1×10^{10} of ADV particles (pts) were added and incubated at 37°C under a 5% CO_2 atmosphere. The cells were detached from the culture plate and collected for cell lysis. Adenovirus pts were separated by $CsCl_2$ gradient ultracentrifugation, and the number of ADV pts was determined by a spectrophotometric method using the equation: $A_{260} \times 1.1 \times 10^{12} = \text{pts/mL}$. The determination of viral particle number by a UV spectroscopic method has a quantification limitation of about 2.6×10^9 viral particles/ml (25,26). We used ADV pts at a concentration of 8×10^{11} in phosphate buffered saline (PBS) solution (pH 8.1) to produce PEGylated ADV. Various amounts of mPEG-SPA at different molar ratios of mPEG/ADV surface amine group (from 1 to 100) were added to the solution. The amount of primary amine groups on the surface of the ADV was determined by the fluorescamine assay, as described in our previous study (6). After reacting overnight at 4°C, PEGylated ADV was dialyzed against 3% (w/v) sucrose/PBS solution at 4°C and kept at −20°C until use. The degree of PEGylation on the surface of the ADV was determined by quantifying the amount of remnant amine groups following PEG conjugation.

To show the transduction efficiency of PEG-ADV relative to naked ADV, ADV and PEG-ADV were transfected into HeLa cells. HeLa cells were maintained in DMEM media supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2. The cells were regularly passaged at sub-confluence and plated on a 6-well plate (5×10^6 cells/well) for 24 h, following which the cells were incubated with medium containing 1.6×10^{10} pts of ADV or PEG-ADV [multiplicity of infection (MOI)=32] for transduction. After a 2-day incubation, the cells were harvested by treatment with a cell lysis buffer solution (1% triton X-100 in PBS) and centrifugation. The level of GFP fluorescence in the supernatant was determined using a spectrofluorophotometer (model SLM-AMINCO 8100; SLM Instruments, Rochester, NY) with an excitation and emission wavelength of 488 and 507 nm, respectively. All values were normalized by amounts of cellular protein, as determined by the Micro-BCA protein assay (Pierce, Rockford, IL). All experiments were performed in triplicate, and each data point was expressed as mean ± standard deviation.

Physical Stability upon Exposure to the Water/Oil Interface During Homogenization and Under Acidic Conditions

Native ADV or PEG-ADV at a concentration of 1.4×10^{11} pts in 100 μl of PBS solution was added to 900 μl of

dichloromethane in an Eppendorf tube. A water-in-oil emulsion was produced by homogenization at 3500 rpm using PowerGen 700 homogenizer (Fisher Scientific, Germany). The samples were taken as a function of homogenization time. Following the complete removal of dichloromethane, 500 μl of PBS solution was added and the solution centrifuged at 14,000 rpm for 20 min. The amount of ADV in the supernatant was determined using a UV spectrophotometer as described above. The fractional amount of intact and partially aggregated ADV remaining in the supernatant was regarded as the soluble ADV fraction in this study.

To determine the physical stability of the ADV or PEG-ADV pts (1.4×10^{11} pts in 100 μl PBS solution) was diluted in buffer solutions with different pH values. The total changes in the associative states of ADV and PEG-ADV with decreasing pH were measured as optical density at 350 nm, as reported previously (21). All experiments were performed in triplicate, and each data point was expressed as the mean ± standard deviation.

Preparation and Characterization of PLGA Microspheres

For encapsulating ADV and PEG-ADV within PLGA microspheres by the W/O/W double emulsion and solvent evaporation method, ADV or PEG-ADV at a concentration of 8×10^{11} pts was first dispersed in 100 μl of PBS solution; 1 ml of this solution was then added to dichloromethane containing 50 mg PLGA (5010, Wako). The primary W/O emulsion solution was briefly homogenized for 15 s at 3500 rpm and then added into 50 ml of dichloromethane pre-saturated aqueous solution containing 1% (w/v) polyvinyl (PVA) alcohol. The secondary W/O/W emulsion was homogenized for 60 s at 1500 rpm and agitated with a magnetic stirring rod for 4 h to evaporate off the dichloromethane. The hardened PLGA microspheres were centrifuged at 1500 rpm for 5 min, washed three times with deionized water and stored at −20°C. The loading amount of ADV within the PLGA microspheres was determined by digesting PLGA polymer with 1 *N* NaOH for 24 h to extract the ADV particles into the aqueous solution. The number of ADV pts was then determined by UV spectroscopy. The loading efficiency (in percentage) was then calculated by dividing the amount of encapsulated viral particles by the initial number of viral particles used. All experiments were carried out in triplicate.

Poly(lactic-co-glycolic) acid microspheres were observed by scanning electron microscopy (SEM, Philips 535M). The dried microspheres were mounted on a brass stub using a double-sided adhesive tape and vacuum coated with a thin layer of gold particles. The average size of the microspheres was determined by measuring the diameter of at least 50 microspheres.

Release Profile of Adenovirus from PLGA Microspheres

In vitro release profiles of ADV and PEG-ADV from PLGA microspheres were determined in PBS solution (pH 7.4). PLGA microspheres (10 mg) were suspended in 1 ml of PBS solution and incubated at 37°C. At pre-determined times, samples were centrifuged at 1500 rpm for 5 min and the supernatant used to quantify the amount of released ADV by determining absorbance at 260 nm. PLGA microspheres encapsulating PBS were used as a blank.

Transduction Efficiency of Adenovirus

HeLa cells were maintained in DMEM media supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2. The cells were regularly passaged at sub-confluence and plated on a 6-well plate (5×10^6 cells/well) 24 h before transduction. The following day, cells were incubated with different amounts of PLGA microspheres encapsulating the same number of ADV or PEG-ADV pts (3×10^{10} pts: MOI=60) in DMEM media with 2% FBS. Green fluorescent protein expression within HeLa cells was observed by fluorescence microscopy. To quantify the amount of GFP gene expression, HeLa cells incubated with PLGA microspheres for 3 days were harvested by treating with a cell lysis buffer solution and the amount of GFP expression quantified using a spectrofluorophotometer. Relative GFP expression level was then calculated as relative percentage of GFP gene expression compared to those transfected with the same amount of naked ADV. All experiments were performed in triplicate, and each data point was expressed as the mean ± standard deviation.

Assay of Immune Response

Raw 264.1 macrophage cells were seeded on a 12-well plate at a density of 5×10^5 cells/well containing RPMI1640 supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. After 1 day, the culture media was replaced with 1 ml of fresh media containing PLGA microspheres containing 1×10^{10} pts of ADV or PEG-ADV. Blank PLGA microspheres were used as a control. After 24 h, the level of IL-6 in the culture media was determined by an enzyme-linked immunosorbent assay (ELISA) (Quantikine, Torrance, CA). All experiments were performed in triplicate.

RESULTS AND DISCUSSION

PEGylated ADV was prepared by conjugating an amine reactive mPEG-SPA derivative (MW 2000) to primary amine groups on the surface coat proteins of ADV, as shown in Fig. 1a. After conjugating mPEG onto the surface of ADV at various molar ratios of viral amine/mPEG, we analyzed the degree of PEG conjugation by calculating the remnant amount of surface amine groups, using the fluorescamine assay as described in (6). The GFP expression level of the HeLa cells was then determined as a function of PEGylation degree (Fig. 1b). Fig. 1b shows that the relative GFP expression efficiency of PEG-ADV decreased with increasing PEGylation degree. In particular, the extent of GFP gene expression decreased sharply at a viral amine:mPEG feed molar ratio above 1:1. Although the degree of PEGylation increased slightly when the molar feed ratio was increased from 1:1 to 1:10, the relative GFP expression percentage decreased drastically – from 93.8±2.6% to 51.6±2.6% – probably because excessive PEGylation above a critical level was able to extensively damage the surface protein structures and destroy the inherent binding efficiency of the fiber on the

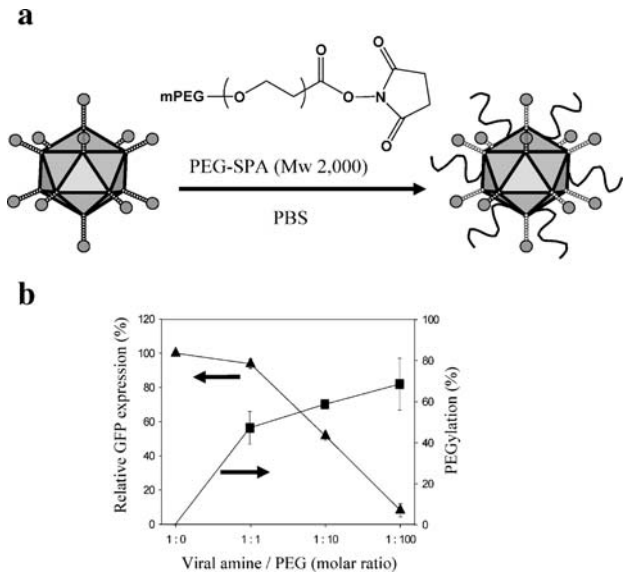

Fig. 1. **a** A scheme of PEGylation of adenovirus (ADV), **b** PEGylation degree and relative green fluorescent protein (GFP) expression under various reaction conditions.

surface of ADV to the coxsackievirus-adenovirus receptor (CAR) or integrin receptor present on the cells, respectively (27). Viral transduction into cells starts with the binding of ADV to CAR by a knob portion of the fiber protein. As the surface structural proteins of ADV consist of 88.2% hexon, 7.4% penton base, and 4.4% fiber, the conjugation of PEG onto ADV was most likely to occur through binding onto the hexon protein (5). When PEG conjugated mainly to the hexon protein, viral transduction efficiency would not be affected to any large extent. However, at a higher PEGylation degree, the fiber protein could be PEGylated while significantly damaging the ADV/CAR interaction, resulting a decreased viral transduction efficiency (6,28). For PEG-ADV prepared at a feed molar ratio of 1:1, the extent of PEG conjugation was 47.8±7.9% and GFP expression efficiency was 93.8±2.6% relative to that of naked ADV. Based on these GFP transfection results, we prepared PEG-ADV conjugates from a molar ratio of 1:1 for subsequently stability and encapsulation experiments.

A double emulsion and solvent evaporation process has been the most popular method to date for microencapsulating viruses within PLGA microspheres. This method involves a series of critical detrimental steps for the stability of viruses. Viral particles can be denatured and aggregated upon exposure to the interface between water and oil phases during the double emulsion process. Additionally, high shear stress should be provided to generate fine emulsion droplets; however, this may cause irreversible viral inactivation via physical aggregation. We previously reported that PEGylated proteins can show a higher survival rate than their unPEGylated counterparts under such harsh conditions, probably due to the protective shielding effect of PEG chains on the protein surface (18): the extent of aggregation of the PEGylated proteins was noticeably lower than that of the native proteins during the simulated double emulsion formulation process. We postulated that PEGylated ADV would exhibit a better physical stability than naked ADV during the formulation process of PLGA microspheres. Fig. 2a shows the physical stabilities of ADV and PEG-ADV as a function of homogenization time. An aqueous solution containing ADV or PEG-ADV pts was emulsified in dichloromethane by homogenization for 120 s in 90% dichloromethane/10% PBS solution; the recovered soluble fractions of ADV and PEG-ADV were 18.0±0.2% and 55.7±2.7%, respectively. There were approximately three-fold more PEG-ADV pts in the soluble fraction than ADV pts. PEGylated ADV was much more resistant to aggregation than naked ADV upon exposure to the water/oil interface during the homogenization, most likely due to the presence of PEG chains on the surface of the ADV. Thus, it is highly probable that the PEG chains conjugated onto the surface of ADV physically protected the viral surface coat proteins from being denatured and aggregated upon exposure to the water/oil interface under high shear stress conditions. The amount of soluble ADV fraction did not continuously decrease with increasing homogenization time, suggesting that ADV was mainly aggregated and precipitated by exposure to the interface between the water and oil phases during the homogenization process (29). Thus, the extent of viral aggregation may be saturated even after prolonged homoge-

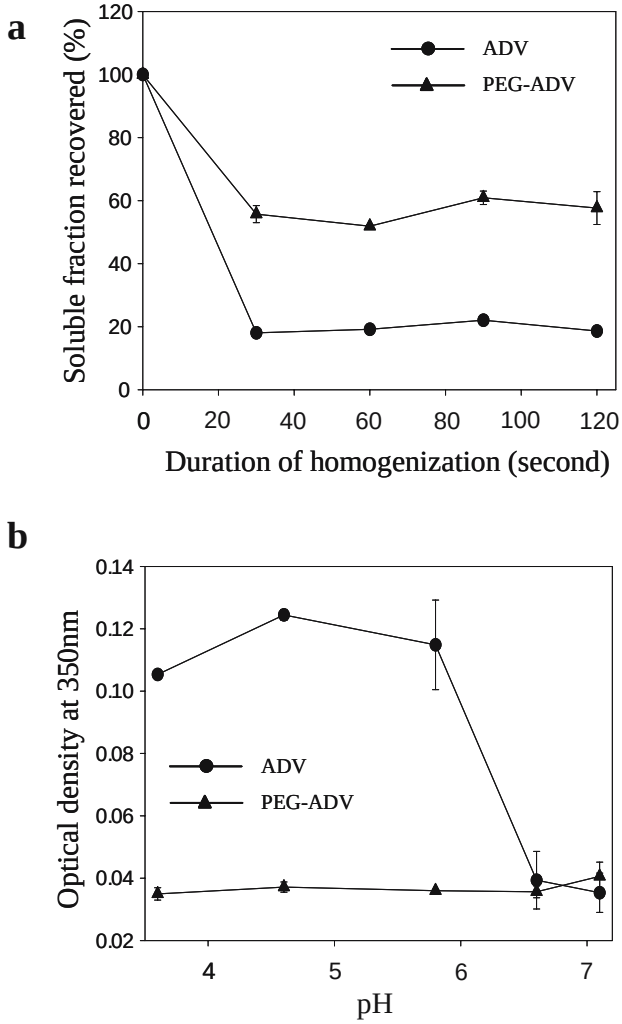

Fig. 2. **a** Physical aggregation of naked ADV (1:0) and PEGylated ADV (PEG-ADV; 1:1) induced by the homogenization of solution dispersed in dichloromethane, **b** aggregation property of naked ADV (1:0) and PEG-ADV (1:1) under various pH conditions.

nization due to the limited interfacial area in the water/oil emulsion solution.

Viral particles are also known to be sensitive to environmental pH, tending to aggregate and precipitate under acidic conditions (21). Since the interior microenvironment of the PLGA microspheres is acidic due to polymer degradation, the physical stability of viral particles under acidic pH conditions would be an important factor for ADV-loaded PLGA microspheres. To assess any physical change in the associative state of ADV and PEG-ADV under acidic conditions, we measured the optical density value at different pH values (Fig. 2b) and found that the absorbance value at 350 nm was well correlated with the physical status of viral particles (21). The PEG-ADV exhibited no change in turbidity down to pH 3.6, while ADV showed a significant increase in scattering (turbidity) below pH 5.8, which was probably due to the aggregation of viral particles under acidic conditions. This suggests that PEGylated ADV may be physically more stable than naked ADV in the acidic microenvironment that was generated within the degrading PLGA microspheres. Characterization of the PLGA microspheres encapsulating ADV and PEG-ADV (Fig. 3a) revealed that PLGA microspheres encapsulated with ADV and PEG-ADV were 9.4±2.1 and 9.3±2.1 μm in diameter, respectively. There was no apparent difference in diameter and surface morphology between the two groups. The loading efficiencies of ADV and PEG-ADV within PLGA microspheres were 74.2±8.6 and 100.3±15.5%, respectively. When the molecular weight of a viral particle (1.76×10^8 g/mol virus) was taken into consideration, the loading amounts (w/w percentage) of ADV and PEG-ADV within the PLGA microspheres were 0.34±0.04 and 0.46±0.07%, respectively

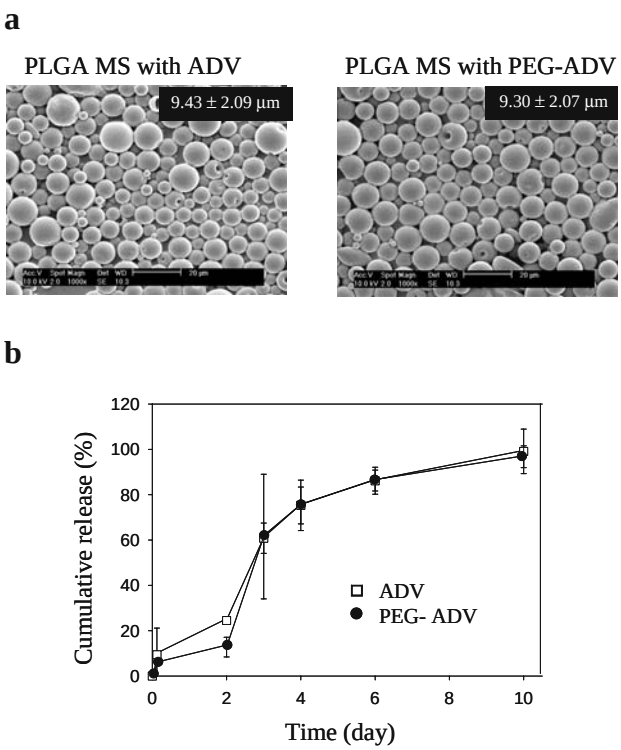

Fig. 3. Scanning electron microscopic (SEM) image **a** and sustained release profiles **b** of PLGA microspheres encapsulating naked ADV and PEG-ADV (1:1).

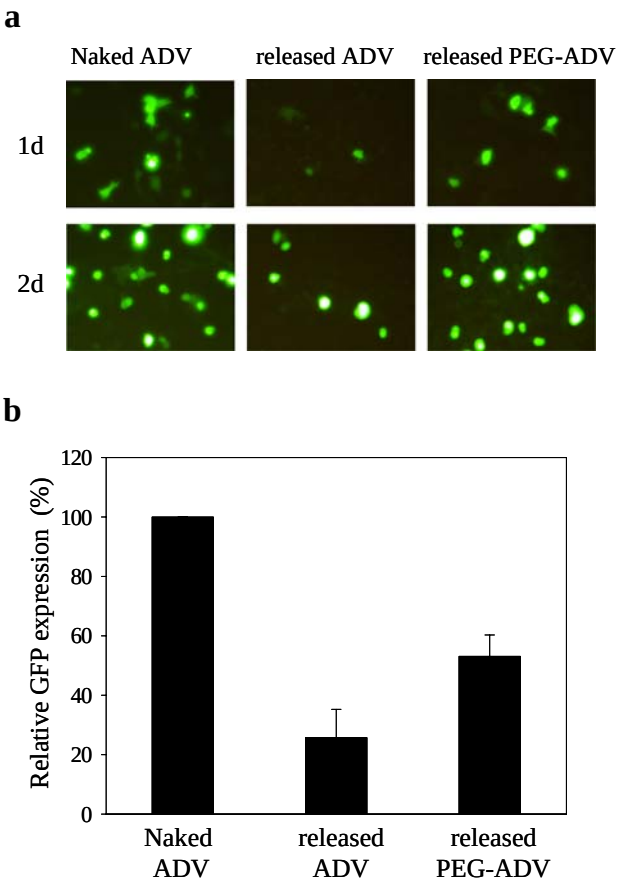

Fig. 4. Fluorescence image **a** and quantitative fluorescence intensity **b** of reporter gene (GFP) expression in HeLa cells following an incubation with poly(lactic-co-glycolic acid) (PLGA) microspheres encapsulating naked ADV or PEG-ADV(1:1).

(30). It is of interest to note that PEG-ADV was encapsulated within the microspheres at almost a 100% loading efficiency. This may be due to PEG-ADV being larger in terms of hydrodynamic size than naked ADV. The PEGylated viral

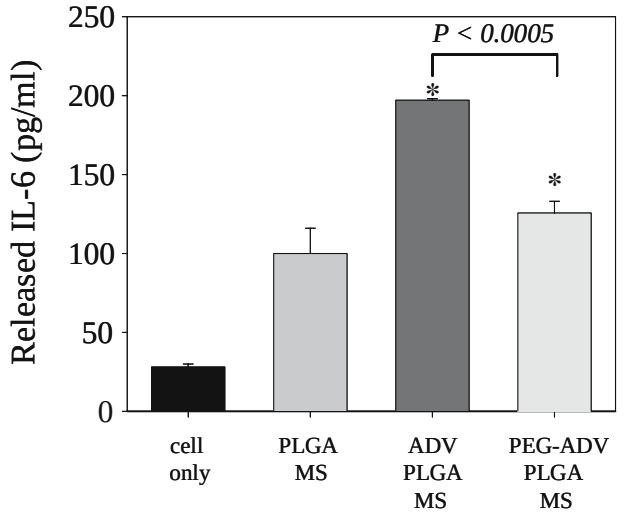

Fig. 5. The amount of IL-6 released from macrophage cells (Raw264.1) after incubation with naked ADV or PEG-ADV encapsulated PLGA microspheres for 24 h.

particles, with their lower diffusion coefficient, would have very little tendency to migrate from the inner aqueous W_1 emulsion droplet phase into the outer aqueous W_2 continuous phase during the double emulsion and solvent evaporation process. Fig. 3b shows the release profiles of ADV and PEG-ADV from PLGA microspheres. Both exhibited similar tri-phasic release behaviors with the same extent of a small initial burst. Almost all viral particles were released out of the PLGA microspheres within 10 days. The current formulation prepared by homogenization may enable viral particles to be encapsulated homogeneously within PLGA microspheres, which in turn may assist the entrapped viral particles to be released completely. The result is in contrast to previous results of ADV-encapsulating PLGA microspheres, which showed incomplete release profiles over a 10-day incubation period (9,31). This difference can be attributed to the fact that different polymers, morphologies, sizes, formulation recipes, and processing conditions were used to fabricate ADV-encapsulated PLGA microspheres. Hence, the ADV release profiles observed in this study can not be directly compared with other ones reported earlier. The tri-phasic release profiles suggest that ADV and PEG-ADV were released primarily by a polymer erosion/diffusion mechanism, as reported previously (15,32), with those particles located in the vicinity of the surface being preferentially leached out at an early incubation stage as an initial burst. After a short lag period, they were released out in an accelerated fashion. The later rapid release phase can be attributed to the polymer erosion-induced diffusion of ADV and PEG-ADV. Because the ADV and PEG-ADV had average sizes of around 100 nm in diameter, they would show very low diffusivity in the intact PLGA polymer matrices prior to degradation. Polymer degradation may generate porous channels and defects, through which entrapped viral particles would be released out. This is the most plausible release mechanism for viral particles entrapped within biodegradable polymer microspheres. It is of particular interest to note that there was no significant difference in the release pattern between ADV and PEG-ADV, which is in contrast to the results obtained from PEGylated proteins released from PLGA microspheres (18,20). As shown in the aforementioned stability studies, PEG-ADV was superior to ADV in terms of physical aggregation stability under homogenization and acidic conditions. It is not clearly understood why ADV and PEG-ADV having different physical states and sizes were released from the microspheres in a similar kinetic pattern, but it is likely that the increased hydrodynamic radius of PEG-ADV was delicately counterbalanced with the improved physical stability provided by PEGylation, resulting in similar release rates for PEG-ADV and ADV.

To visualize reporter gene (GFP) expression by the released ADV and PEG-ADV from PLGA microspheres, PLGA microspheres encapsulating ADV and PEG-ADV were incubated with HeLa cells for 1 or 2 days. Fig. 4a shows the fluorescence microscopic image of HeLa cells expressing GFP, clearing revealing that naked ADV being released from PLGA microspheres had a lower GFP expression efficiency than PEG-ADV. It is conceivable that the enhanced physicochemical stability of PEGylated ADV resulted in a higher gene transfection efficiency. To quantitatively determine the extent of GFP expression efficiency, fluorescence intensity after cell lysis (Fig. 4b). The extent of ADV and PEG-ADV release from PLGA microspheres in terms of GFP expression percentage were 25.7±9.6 and 53.1±7.2%, respectively, relative to that of unencapsulated ADV (100%). The GFP expression levels were roughly correlated to the results of the recovered soluble fraction for ADV and PEG-ADV (18.0±0.2 and 55.7±2.7%, respectively; Fig. 2a.

To determine the extent of nonspecific immune response, ADV- and PEG-ADV-encapsulated PLGA microspheres were incubated with macrophage cells (Raw264.1). The nonspecific immune response could be elicited when viruses were used as gene carriers (33,34). Interleukin-6 is one of the pro-inflammatory cytokines released from macrophage cells, and the released amount of IL-6 is directly related to the innate immune response caused by ADV (3,6,33,34). The amount of IL-6 released from macrophage cells was quantified by ELISA (Fig. 5): the amount of IL-6 released from untreated macrophage cells was 28.1±1.8 pg/ml and that by exposure to naked ADV was 118.1±3.4 pg/ml. The amounts of IL-6 released from cells treated with blank PLGA microspheres, ADV/PLGA microspheres, and PEG-ADV/PLGA microspheres were 99.9±15.9, 197.0±0.9, and 125.6±7.4 pg/ml, respectively. The amount of released IL-6 induced by naked ADV was much lower than that treated by ADV/PLGA microspheres, but comparable to that treated by PEG-ADV/PLGA microspheres, most likely due to a greater cellular uptake of PLGA microspheres than naked ADV particles within macrophage cells via a phagocytic pathway. However, PEG-ADV-encapsulated PLGA microspheres showed significantly reduced cytokine release than ADV-encapsulated PLGA microspheres (p value=0.0002).

In conclusion, PEGylated ADV exhibited enhanced physical stability against harsh formulation conditions, such as high shear stress and low pH, during encapsulation within PLGA micropsheres. PLGA microspheres encapsulating PEG-ADV showed enhanced gene transfection activity and reduced immune response *in vitro*. PEGylation of other viral vectors could be potentially applied for local and sustained gene therapy.

ACKNOWLEDGEMENTS

This study was supported by the grant of the National Cancer Center, Republic of Korea (0620240-1).

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