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Hybrid-modified poly(D,L-lactide-co-glycolide) nanospheres for a novel cellular drug delivery system

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ABSTRACT

We prepared surface-modified poly(D,L-lactide-co-glycolide) (PLGA) nanospheres (NS) for use as cellular drug and gene delivery systems using an emulsion solvent diffusion method. In this study, we screened for an appropriate surface modifier to improve NS cellular uptake. Poly-L-lysine (PLL)-modified PLGA NS were taken up by A549 cells in significantly higher amounts (17-fold) than unmodified NS. In order to provide a novel function, PLGA NS were hybrid-modified using both; a cationic polymer, PLL, and a nonionic surfactant, polysorbate 80, to improve cellular uptake in serum-containing medium (SCM). Hybrid modification abrogated the decreased PLGA NS cellular uptake in SCM as a result of better dispersion in serum compared to PLL-PLGA NS, which aggregated in SCM. Luciferase activity of Hybrid-NS/pCMV-Luc complexes in A549 cells in SCM was 122-fold higher than PLL-NS. Hybrid-PLGA NS were not cytotoxic for A549 cells. In conclusion, Hybrid-PLGA NS have great potential as effective cellular drug delivery carriers.

Biodegradable polymeric nanospheres (NS) have been developed as unique drug carrier systems due to their characteristic colloid-like behavior (Kawashima et al., 2000). Various research groups have encapsulated different types of therapeutic agents, ranging from low molecular weight to macromolecular drugs, including proteins and plasmid DNA (pDNA) (Panyam and Labhasetwar, 2003). Polymeric NS are used to deliver medicinal compounds because of their high stability, ease of cellular uptake via endocytosis, and their ability to target specific tissues or organs by adsorption or ligand-mediated binding (Lobenberg et al., 1997).

We successfully developed poly(D,L-lactide-co-glycolide) (PLGA) NS to improve peptide and gene delivery, both *in vitro* and *in vivo* (Kawashima et al., 1998, 2000; Yamamoto et al., 2005; Tahara et al., 2007, 2008). The physicochemical properties of PLGA NS are very important for the intracellular penetration of drug molecules during drug delivery. In addition, their surface properties have a significant influence on their stability and the intracellular fates of both drugs and carriers (Tahara et al., 2009).

In order to improve NS cellular uptake, we modified PLGA NS surfaces using chitosan (CS), a cationic polymer, and polysorbate 80 (P80), a nonionic surfactant (Tahara et al., 2010). This study aimed to determine the most suitable PLGA NS surface modifiers to improve NS cellular uptake and transfection efficiency and to find a novel function as a cellular drug delivery system for hybrid-modified NS, which were modified using two types of polymers. We evaluated hybrid-modified PLGA NS physicochemical properties and their potential as cellular drug carriers using cultured A549 human lung adenocarcinoma cells.

PLGA (lactide:glycolide = 75:25, MW = 20,000), poly(vinylalcohol) (PVA; MW = 25,000; hydrolyzation degree 88.0%), CS (MW = 20,000; deacetylation degree 84.2%), P80, poly-L-lysine hydrobromide (PLL; MW = 30,000–70,000), and branched polyethylenimine (PEI; MW = 25,000) were purchased from Wako (Osaka, Japan), Kuraray (Osaka, Japan), Katakurachikkarin (Tokyo, Japan), Kishida Chemical Co. Ltd. (Osaka, Japan), and Sigma (St. Louis, MO), respectively. Luciferase-encoding pDNA (pCMV-luciferase) was used.

An emulsion solvent diffusion method (Tahara et al., 2008) was used to prepare a PLGA NS-6-coumarin fluorescent label. PLGA (100 mg) and 6-coumarin (1 mg) were dissolved in 3 ml of an acetone/ethanol (2:1) mixture. The resulting organic solution was poured into a 2% PVA (25 ml) solution. The entire dispersed system was centrifuged at 43,400 × *g* for 10 min and the sediments were resuspended in distilled water. This process was repeated twice and the resulting PLGA NS suspension was freeze-dried. To prepare surface-modified PLGA NS, PVA (1%, w/v)-surface modifier with either a 0.1% CS, 1% P80, 1% PEI, or 1% PLL solution was used as the dispersing phase for the emulsion solvent diffusion process. For hybrid-modified NS, a mixture of 1% PVA, 1% P80, and 1% PLL was used.

To prepare PLL-PLGA NS or Hybrid-PLGA NS/pDNA complexes, 100 μl of Dulbecco's modified eagle medium was used to suspend

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Table 1
Surface-modified PLGA NS physicochemical properties and uptake by A549 cells. Significant differences in cellular uptake compared to unmodified (Non) PLGA NS are noted by asterisks ($P < 0.05$).

Modifier	Abbreviation	Particle size (nm)		Zeta potential (mV)	Polydispersity	NS cellular uptake in serum-free medium
		Before freeze-drying	After freeze-drying			(% of unmodified, $n = 3$)
Unmodified	Non	251.2	254.5	−29.6	0.098	100.0 ± 14.5
Polysorbate 80	P80	247.4	239.0	−31.1	0.084	385.9 ± 99.7*
Chitosan	CS	298.5	309.1	6.2	0.190	250.7 ± 15.8*
Polyethyleneimine	PEI	322.2	342.1	45.5	0.298	1588.9 ± 481.1*
Poly-L-lysine	PLL	332.1	395.0	22.4	0.137	1704.8 ± 71.9*
Poly-L-lysine and P80	Hybrid	310.0	331.2	6.8	0.307	1058.9 ± 147.3*

1 mg PLGA NS. This suspension was rapidly added to an equal volume of Tris–EDTA buffer containing pDNA. Particle sizes and zeta potentials were determined using a Zetasizer Nano ZS90 (Malvern Instruments Ltd., Malvern, UK). NS cytotoxicity was evaluated by a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay using a CellTiter 96® Aqueous One solution assay (Promega, Madison, WI, USA). This assay is based on the ability of viable cells to reduce a water-soluble yellow dye, MTS, to a purple colored formazan product by the actions of the mitochondrial enzyme succinate dehydrogenase. NS cellular uptake into A549 cells was determined using a previously described method (Tahara et al., 2009). Luciferase activity was analyzed using a Luciferase Assay system (Promega).

Table 1 shows the physicochemical properties and relative cellular uptake percentages into A549 cells of modified NS compared to unmodified NS. In this study, four types of surface modifiers were used: CS, P80, PEI, and PLL. Cationic polymers, such as CS, PEI, and PLL have been used as modifiers for nonviral gene vectors and as drug absorption enhancers (Amiji, 2005). P80, a nonionic surfactant, has been reported to modify polymeric nanoparticles, which could enhance drug delivery into the brain via brain microvessel endothelial cells (Kreuter, 2001). Therefore, we considered that these cationic polymers and P80 would be potential surface modifiers for hybrid-modified NS.

PLGA NS sizes ranged from 200 to 300 nm, depending on the type of surface modifier used (Table 1). The sizes of modified cationic polymers and Hybrid-PLGA NS increased because polymer molecular layers formed on their surfaces. The zeta potential of unmodified PLGA NS (Non-PLGA NS) carried a negative charge after dissociation of the PLGA carboxyl group in distilled water. CS, PEI, PLL, and Hybrid-PLGA NS had high positive charges in distilled water due to protonation of the amino group.

We then screened for the best cationic polymer to improve NS cellular uptake (Table 1). PLGA NS cellular uptake in serum-free medium (SFM) increased after surface modification using cationic polymers in the following order: CS < PEI < PLL. The mechanism for cationic polymer-modified PLGA NS cellular uptake was thought to be an electrostatic interaction between NS and the cell membrane. However, these uptake results were not correlated with the PLGA NS zeta potentials, which were in the following order: CS (+6.2 mV) < PLL (+22.4 mV) < PEI (+45.5 mV). This suggested that not only the zeta potential, but also the affinity of a surface modifier for a cell was an important factor for cellular uptake. P80-PLGA NS showed a higher cellular uptake than Non- and CS-PLGA NS. A number of possibilities might account for the mechanisms of P80-PLGA NS cellular uptake; one of them being P80 on the PLGA NS surfaces which may increase cell membrane fluidity.

We attempted PLGA NS hybrid modifications using two types of materials with different physical properties, i.e., PLL, the best cationic modifier to improve cellular uptake, and P80, the characteristics of which are different from cationic polymers. The zeta potential of Hybrid-modified PLGA NS (+6.8 mV) was lower than PLL-PLGA NS (+22.4 mV) due to P80 coadsorption on the NS surfaces

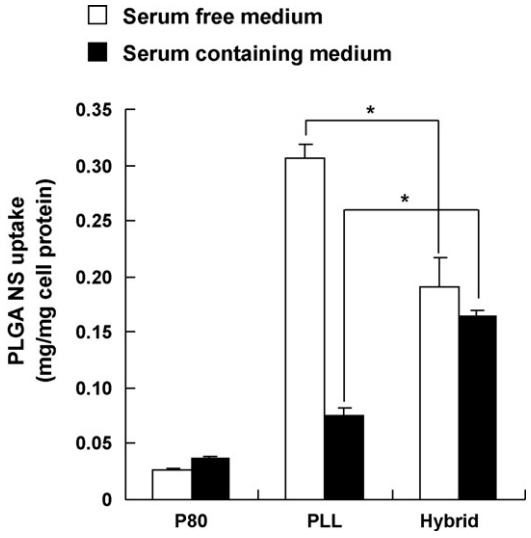

Fig. 1. Effects of serum-containing medium (SCM) on surface-modified PLGA NS cellular uptake. Results are mean ± SD ($n = 3$). Significant differences in cellular uptake compared to PLL-PLGA NS are noted by asterisks ($P < 0.05$).

with a cationic polymer (Table 1). Hybrid-PLGA NS cellular uptake in SFM was lower than PLL-PLGA NS. However, in serum-containing medium (SCM), Hybrid-PLGA NS cellular uptake was much higher than that of PLL-PLGA NS (Fig. 1). This provided for a novel function of PLGA NS due to PLL–P80 hybrid modification. PLL-PLGA NS were partially aggregated in SCM (Fig. 2); therefore, PLL-PLGA NS cellular uptake in SCM was lower than that in SFM. Furthermore, the luciferase activity of A549 cells containing Hybrid-PLGA

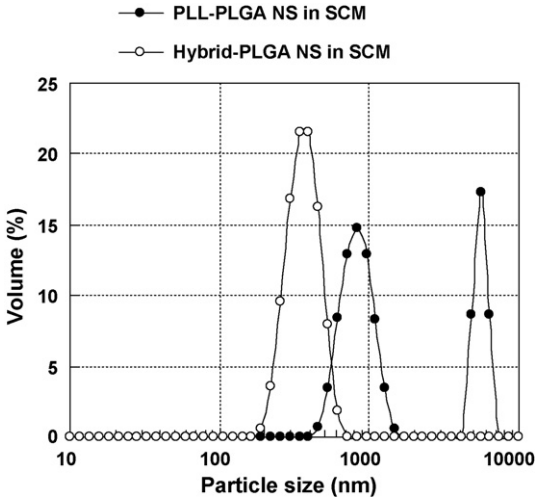

Fig. 2. PLL and Hybrid-PLGA NS size distributions in SCM.

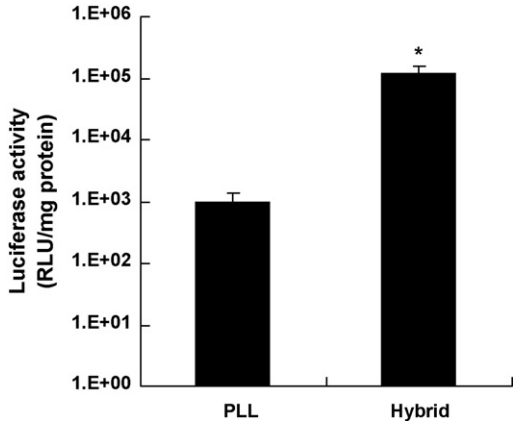

Fig. 3. Effects of pDNA/surface-modified PLGA NS surface properties in SCM on luciferase activity. Doses are amounts of pDNA equivalents in 5 μg/well. Results are mean ± SD ($n = 3$). Significant difference in cellular uptake compared to PLL-PLGA NS is noted by an asterisk ($P < 0.05$).

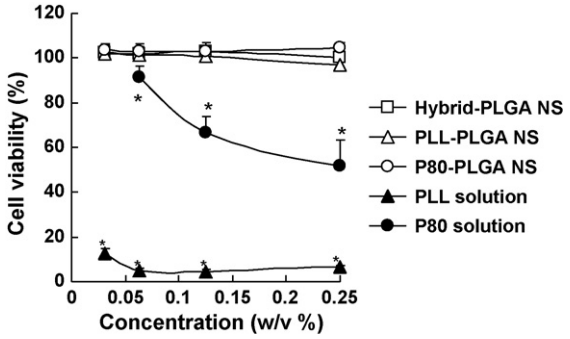

Fig. 4. Cytotoxicities for A549 cells by surface-modified PLGA NS formulations and surface modifier solutions. Treated cells' viabilities were determined by MTS assay. Cell viability (%) relative to the control wells containing cell-culture medium without NS was determined by: $[A]_{test}/[A]_{control} \times 100$, where $[A]_{test}$ is the absorbance of the test sample and $[A]_{control}$ is the absorbance of the control. Symbols represent mean ± SD ($n = 6$). Significant differences in cellular uptake compared to control are noted by asterisks ($P < 0.05$).

NS/pDNA complexes was much higher than those with PLL-PLGA NS complexes in SCM (Fig. 3).

Several authors have found that cationic polymers and lipids used as intracellular drug carriers, such as a DNA nonviral vector, can also interact with serum compounds. These form aggregates that are subject to phagocytic capture *in vivo* and these complexes accumulate in the fine capillary beds (Moret et al., 2001). In general, preventing aggregate formation by the adsorption of serum proteins can be achieved using surface modifications with polyethylene glycol (PEG). However, once taken up by a target tissue, PEG inhibits the interactions between drug carriers and cells which results in a significant loss of pharmacological effects. Although PEG is useful for controlling pharmacokinetics, it is undesirable for cellular associations of drug carriers in tissues. Thus, using PEG presents a dilemma (Hatakeyama et al., 2007) which may be resolved by hybrid modification by preventing adsorption of serum proteins while simultaneously maintaining PLGA NS cellular associations, as Hybrid-PLGA NS cellular uptake did not decrease in the experimental conditions involving the serum.

The use of PLL and P80 to improve PLGA NS delivery may be potentially cytotoxic (Fig. 4). PLL and P80 solutions were shown to be toxic for cells, depending on their concentrations. Surface-modified PLGA NS were not cytotoxic at concentrations used in our study. This was possibly due to low concentrations of free PLL or P80 in the formulations, because any excess surface modifier not adsorbed to the NS surface was removed during the centrifugation step of PLGA NS preparation.

We conclude that Hybrid-PLGA NS can be highly recommended as carriers for cellular drug delivery because of their lack of cytotoxic effects and increased interactions with cells in the presence of serum.

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