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Phagocytic Uptake of Surface modified PLGA Microspheres Using Dendritic Cell

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ABSTRACT – The purpose of this study was to evaluate the phagocytic uptake of surface modified PLGA microspheres containing ovalbumin (OVA) into dendritic cell. In order to find the most suitable formulation for targeted delivery to antigen presenting cells (APC), OVA was encapsulated by a double emulsion solvent evaporation method with three PLGA microspheres (PLGA 50:50, PLGA 75:25 and PLGA 85:15) and two surface modified microspheres by chitosan and sodium dodecyl sulfate (SDS). Physicochemical properties were evaluated in terms of size, zeta potential, encapsulation efficiency, different scanning calorimeter (DSC), x-ray diffraction, morphology, and OVA release test from microspheres. Phagocytic activity was estimated using dendritic cells and analyzed by fluorescence activated cell sorter (FACS). The result showed that zeta potential of PLGA particles was changed to positive by the chitosan modification. The release profile of chitosan modified PLGA microspheres exhibited sustained release after initial burst. The chitosan modified microspheres had higher phagocytic uptake than the other microspheres. Such physicochemical properties and phagocytic uptake studies lead us to conclude that chitosan modified microspheres is more suitable formulation for the targeted delivery of antigens to APC compared with the other microspheres.

Key words – PLGA microsphere, Chitosan modified, Dendritic cell, Phagocytic uptake

Immune response occurs as antigen are phagocytosed by a antigen presenting cell (APC) such as macrophage, dendritic cell, B cell (Newman et al., 1998). APC express antigen through the major histocomporibility complex (MHC) molecules to the T cell and B cell antigens. Therefore, by controlling the extent of phagocytosis, the immune response can be adjusted.

Ovalbumin (OVA) peptide which induces the phagocytic function of the APC, can adjust immune response as an antigen (Ben Nasser et al., 2003). Generally, the form of particle is phagocytosed by APC easily than the solution, and it was reported that maximal phagocytosis of polystyrene microsphere took place when their size was in the range of 1.0~2.0 microns (Tabata et al., 1988).

Biodegradable poly (d,l lactic-co-glycolic acid (PLGA) microspheres has been studied for a variety of drugs and antigen delivery system (Okada, 1997; Wang et al., 1997; Barrow et al., 1998). PLGA is the most widely used biodegradable polymer and has been approved by FDA for certain clinical applications including drug delivery systems (Langer et. al., 1976; Yamaguchi et. al., 1993). These characteristics of PLGA is suitable for targeting APC through the encapsulation of OVA. Chitosan [$\alpha(1\rightarrow4)$2-amino 2-deoxy β-D-glucan] is a kind of linear polymamine with a high degree of deacetylation(i.e., a high percentage of glucosamine units compare to acetyl-glucosamine units). This amino group could make the chitosan polymer to coat nanoparticles by desolving cationic salt and crosslinking (Janes et al., 2001). Chitosan-modified microspheres have been reported as a pharmaceutical delivery vehicle and effective targetting delivery system to the cell(Kim et al., 2008) In this study, we have prepared several kinds of PLGA microspheres and surface modified PLGA microspheres containing OVA peptide by water-oil-water (w/o/w) emulsion technique. And we evaluated the physicochemical properties and the phagocytic uptake of our formulations into dendritic cell.

Materials and Methods

Materials

Ovalbumin, poly (d,l lactic-co-glycolic acid) (PLGA 50:50, 75:25, 85:15), chitosan and Fluorescein 5(6)-isothiocyanate (FITC) was purchased from Sigma-Aldrich (Sigma-Aldrich Inc., St. Louis, MO). We used BCA Kit from Thermo scientific. Dulbecco's modified eagle medium (DMEM), FBS, Penicillin/streptomycin solution for cell culture was purchased from HyClone Laboratories Inc. KCl, NaOH, KH_2PO_4, Poly-

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vinyl alcohol and Sodium Dodecyl Sulfate (SDS) were pharmaceutical grade and all other chemicals and solvents were of analytical grade.

Preparation of PLGA Microspheres

The Preparation condition of PLGA microspheres and CS-coated PLGA microspheres are discribed elsewhere (Tobio et. al., 1998). PLGA microspheres were obtained by the W/O/W emulsion solvent evaporation methods. 200 μL of aqueous solution of OVA 100 mg/mL) was added to 1 mL of ethyl acetate containing 100 mg PLGA and vortex for 30 sec (Vortex, G-560, Scientific industries INC). The mixture was homogenated (Homogenizer, T 25-S1, JANKE&KUNER GMBH&CO KG) in ice bath. After 3 mins of emulsification, 4 mL of aqueous solution of PVA (5%, w/w) was added to the w/o emulsion to form a w/o/w double emulsion and homogenated further for 5 min. To solidify the particles, the double emulsion poured into 100 mL of aqueous solution of 0.1% (w/w) PVA and stired for 2 hrs. The latter was evaporated for complete removal of ethyl acetate for 2 hrs (Evaporater, CCA-1110, EYELA Co, Ltd). The resulting dispersion was centrifuged at 3,000 rpm for 15 min, washed two times with phosphate-buffered saline (PBS, pH 7.4), removed supernatant and was freeze dried (Centrifuge, FD-5N, EYELA Co, Ltd). Three kinds of PLGA microspheres were made respectively, PLGA 50:50, PLGA 75:25, PLGA 85:15.

In case of chitosan modified PLGA 50:50 microspheres, the double emulsion pour into chitosan acetic acid solution (2.5 mg/mL) included PVA (1%, w/w) under agitation for 3hrs. For SDS-modified PLGA 50:50 microsphere, the double emulsion poured into aqueous solution of 1% (w/w) SDS for 2 hrs.

Size & zeta potential measurement

Size of microsphere was determined by the light scattering method (ELS-Z, Otsuka Electrocics, Osaka, Japan) with suspension in PBS solution. The zeta potential of microsphere was measured by using an electrophoretic light-scattering spectro-photometer (ELS-Z, Otsuka Electrocics, Osaka, Japan) after suspention of microspheres in PBS solution or acetic acid solution. All measurement was conducted at 25°C.

OVA encapsulation efficiency

Microspheres were digested in 950 μL of 0.1 N NaOH containing 0.1% (w/v) sodium laulyl sulfate (SLS) on a 37°C incubator for 24 hrs until the complete dissolution of the microspheres. The sample was centrifuged at 15000 rpm for 10 min and bichinchonic acid (BCA) protein microassay was used to determine the OVA concentration in the supernatant. Absorbance was measured by Fluorescence Spectrophotometer (Spectra Max M2e, innotech) at 575 nm. Each sample was assayed in triplicate.

$$\text{Encapsulation efficiency}(\%) = \frac{\text{amount of OVA recovered from microsphere}}{\text{amount of OVA used in preparation of microspheres}} \times 100$$

Differential scanning calorimeter(DSC)

To confirm the encapsulation of OVA into microsphere, Thermal analyzer(DSC2910, TA Insteruments) were used. In aluminum pan, the temperature was increased with 10°C/min from room temperature to 150°C to get the differential scanning calorimetry thermograms.

X-ray diffraction

X-ray diffractometry (X-ray Diffractometer-II, D8 Discover with GADDS, Bruker AXS) was conducted to examine the existing status of the OVA in the microsphere and crystrallization of microsphere. The diffraction angle was recorded from 5° to 70°.

Table I. *Size, zeta potential and encapsulation efficiency of PLGA microspheres having different composition, chitosan-modified PLGA 50:50 microsphere, SDS-modified PLGA 50:50 microsphere in PBS solution[a)] and in aceticacid solution[b)]*

Shell	Size	PI*	Zeta potential	Entrapment efficiency ±S.D.(%)
PLGA 50:50	981.2 nm	0.089±0.013	-4.68 mv[a)] -4.35 mv[b)]	19.80±5.39%
PLGA 75:25	1150.8 nm	0.203±0.050	-4.89 mv	30.33±4.36%
PLGA 85:15	1202.4 nm	0.306±0.075	-7.93 mv	24.44±10.16%
Chitosan-modified PLGA	1221.4 nm	0.072±0.020	-0.03 mv[a)] 13.22 mv[b)]	13.57±1.18%
SDS-modified PLGA	1440.2 nm	0.099±0.025	-14.14 mv	14.51±0.28%

*PI(Polydispersity Index): The polydispersity index is a measure of the distribution of molecular mass in a given polymer sample. The PI calculated is the weight average molecular weight divided by the number average molecular weight.

Scanning electron microscopy(SEM)

The appearance and shape of microsphere surfaces were examined by scanning electron microscopy using a Hitachi S-2500C scanning electron microscopy.

OVA release test from microspheres

Release pattern of OVA from microsphere was measured at 4ºC and 37ºC. Microspheres were diluted with 5 mL of PBS in a capped centrifuge tube. 100 μL of supernatant was taken and replace same amount of PBS at specified time points (0, 1, 3, 7, 14, 21, 28 Day). Absorbance was measured from each sample by BCA kit and Fluorescence Spectrophotometer (Spectra Max M2e, innotech) at 575 nm.

Phagocytic activity

To measure the phagocytic activity, Fluorescein 5(6)-isothiocyanate (FITC) is encapsulated into microspheres. We made the microspheres in same way mentioned above except one step; Instead of adding 1 mL of ethyl acetate containing 100 mg of PLGA, we added to 1 mL of ethyl acetate containing 100 mg of PLGA and 0.02 mg of FITC mixture. Dendritic cell line were employed to study the phagocytic uptake of PLGA, chitosan PLGA and SDS-PLGA microspheres. Dendritic 2.4 cells were incubated in DMEM medium with 10% FBS (fetal bovine serum) and antibiotics (100 U/mL penicillin G sodium, 100 mg/mL streptomycin sulfate) at 37ºC, CO_2 5% (Incubator, MCO-17AC, SANYO Electric Biomedical Co., Ltd). Dendritic cells were prepared at 2×10^6 cells/6well in 3 mL of complete DMEM-10 and added with 150 μL of the microspheres preparing in the way mentioned above. Dendritic cells were incubated at 37ºC, 5% CO_2 for 20 minutes and aspirate supernatant from each well. Dendritic cells were washed with 5 mL of pre-warmed PBS twice. Then, Dendritic cells were incubated for 20 minutes with ice. That cells were harvested by pipetting, and washed with 10 mL of cold PBS twice to remove the extra microspheres. After final washing, cells were fixed in 1% paraformaldehyde in PBS and flow cytometric analysis was performed on a FACS Canto II (Becton-Dickinson). Dead cells and microspheres were gated out by their low intensity.

Results and Discussion

Size, zeta potential and drug encapsulation efficiency

The size and zeta potential of microsphere are summarized in Table I. It was found that the maximal phagocytosis of polystyrene microspheres took place when their size was in the range 1000~2000 nm (Tabata et.al., 1988). So we made microspheres with size about 1000 nm. The size and their PI (polydispersity index) increased as ratio of lactic acid increased. The zeta potential of PLGA microsphere was negative and absolute value of zeta potential increased with ratio of lactic acid increased. SDS modified microsphere was more negative than PLGA microspheres.

After chitosan modification, the zeta potential changed to positive. Chitosan is poly cationic in acidic media and the zeta potential was increased as the medium becomes more acidic (Khunawattanakul etal., 2008). Because of the presence of free amines on chitosan, chitosan is ionized in acidic environment. Therefore, the zeta potential of chitosan modified microsphere was significantly influenced by pH.

OVA encapsulation efficiency of PLGA microspheres ranged from about 20% to 30% (PLGA 50:50, 19.80±5.39%; PLGA 75:25, 30.33±4.36%; PLGA 85:15, 24.44±10.16%). Encapsulation efficiencies of chitosan-modified microspheres and SDS modified microspheres were 13.57±1.18% and 14.51±0.28% each.

Differential scanning calorimetry

Figure 1 shows thermograms of PLGA, PLGA microspheres having different compositions, chitosan-PLGA microsphere, and SDS-PLGA microsphere. Peak of PLGA appeared 46.87ºC, peak of microspheres also appeared about 50ºC clearly, and no other peaks appeared. Therefore, the result suggested that OVA encapsulate into PLGA microspheres.

X-ray diffraction

Figure 2 shows the X-ray diffraction pattern of OVA, PLGA 50:50, PLGA micropheres. In case of OVA X-ray diffraction pattern, there is a peak at 8.91º but there is no peak at that point

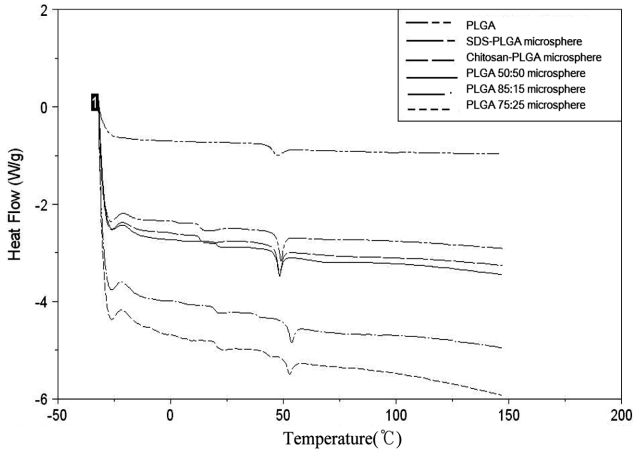

Figure 1. DSC thermograms of microspheres - PLGA, PLGA50:50 microsphere, PLGA75:25 microsphere, PLGA85:15 microsphere, Chitosan-modified microsphere, SDS-modified microsphere.

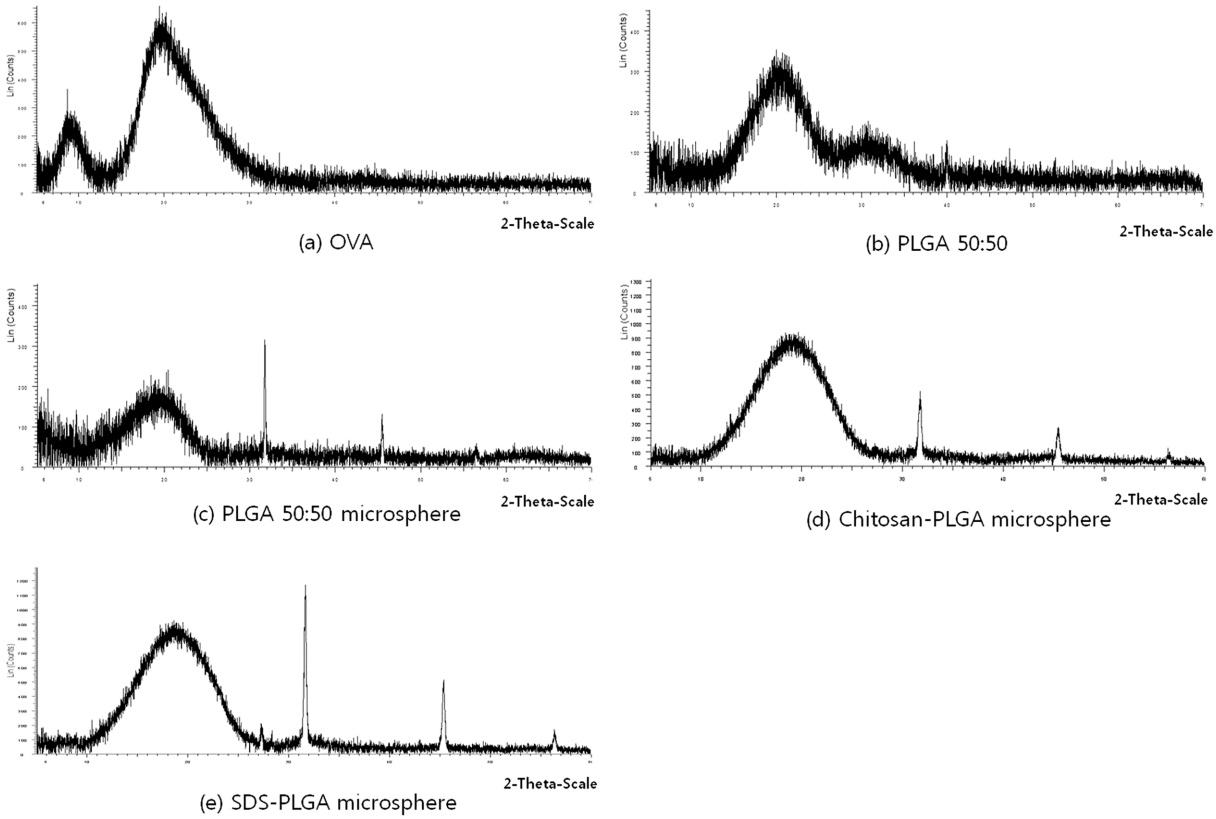

Figure 2. X-ray diffraction pattern of (a) OVA, (b) PLGA 50:50, (c) PLGA 50:50 microsphere, (d) Chitosan-PLGA microsphere, (e) SDS-PLGA microsphere.

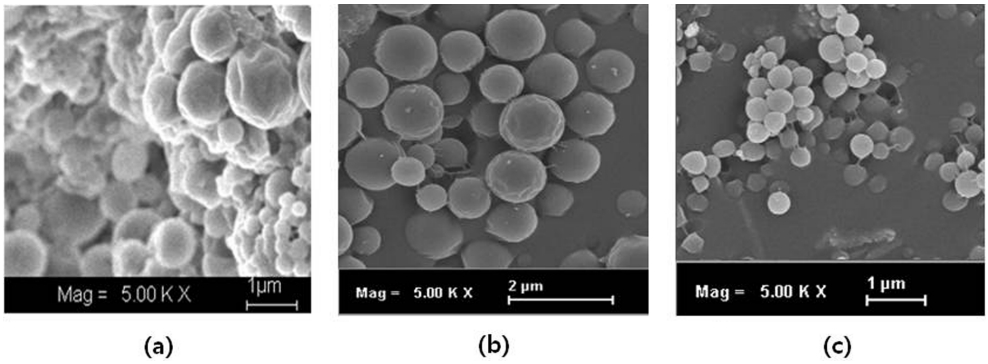

Figure 3. Morphology of microspheres. (a) PLGA 50:50 microsphere, (b) chitosan-PLGA microsphere, (c) SDS-PLGA microsphere.

in PLGA microspheres, so we could assume that OVA is encapsulated in microspheres successfully.

There are two peaks at 31.7° and 45.5° in X-ray diffraction pattern of micropheres and we could know that crystality of PLGA was changed during the preparation of microspheres.

Scanning electron microscopy

Figure 3 shows the appearance and shape of microsphere surface. They were analyzed by SEM (Scanning electron microscopy, S-2500C, Hitachi). We could confirm the morphology of PLGA 50:50 and chitosan-modified microspheres. The microspheres appeared to be spherical in shape and their surfaces were smooth.

Release test

We evaluated the amount of OVA released from the PLGA microspheres, chitosan-modified and SDS-modified microspheres for 28 days at 37°C (Figure 4) and 4°C (data is not shown here). The release profiles of the microspheres shows a similar biphasic configuration. But the amount of OVA

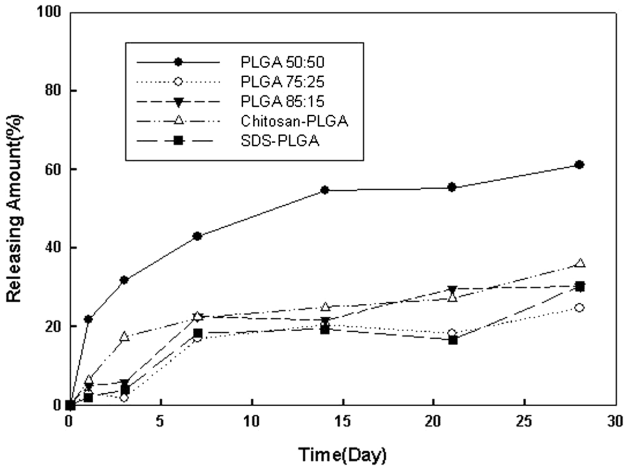

Figure 4. OVA releasing profiles from microspheres at 37°C.- PLGA 50:50 microspheres, PLGA 75:25 microspheres, PLGA 85:15 microspheres chitosan-modified PLGA microsphere, SDS-modified PLGA microsphere.

released from the PLGA 50:50 was much more than other microspheres.

A remarkable sustained release profile of OVA from chitosan modified PLGA microspheres was found in PBS. After the initial release burst, drug release was sustained until 29 days. The sustained release at later stage was caused by the diffusion of OVA through PLGA matrix as well as the erosion of polymer.

Phagocytosis of microspheres

The phagocytic uptake of microspheres by dendritic cell is shown in Figure 5 and Figure 6. In Figure 5, three kinds of PLGA microspheres make the peak movement to the right comparing the pure cell and this signified that microspheres were phagocytized by dendritic cells. And the point of peaks are similar, so the phagocytic uptake is also similar among the three kinds of PLGA micropheres. In Figure 6, we compared the phagocytic uptake of chitosan-PLGA and SDS-PLGA microspheres with PLGA microspheres by dendritic cells. In this case, the pattern of chitosan-PLGA microsphere is moved further right to PLGA microsphere. It means that the phagocytic uptake of chitosan-PLGA microsphere is higher than that of PLGA microsphere. The modified surface properties like as less negatively charged by chitosan might influence the phagocytic uptake into dendritic cells.

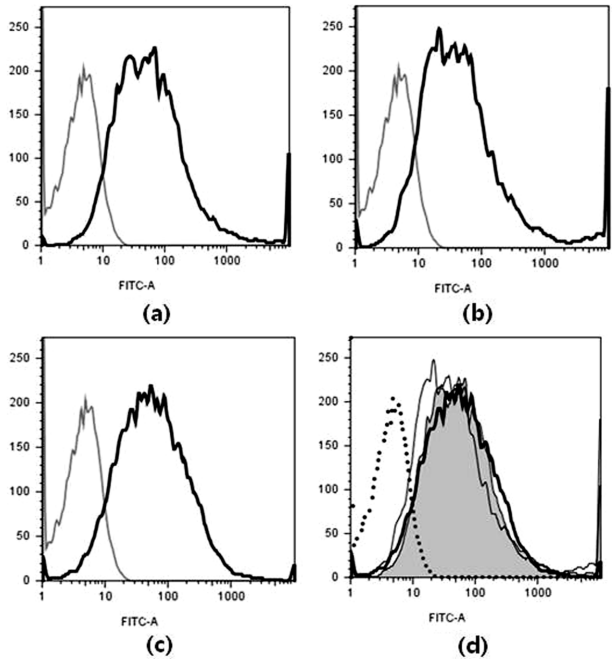

Figure 5. Phagocytic activity of PLGA microspheres - (a) PLGA 50:50, (b) PLGA 75:25, (c) PLGA 85:15 microsphere, (d) comparison of phagocytic activities - dot; none, shade; PLGA 50:50, line; PLGA 75:25, bold; PLGA 85:15.

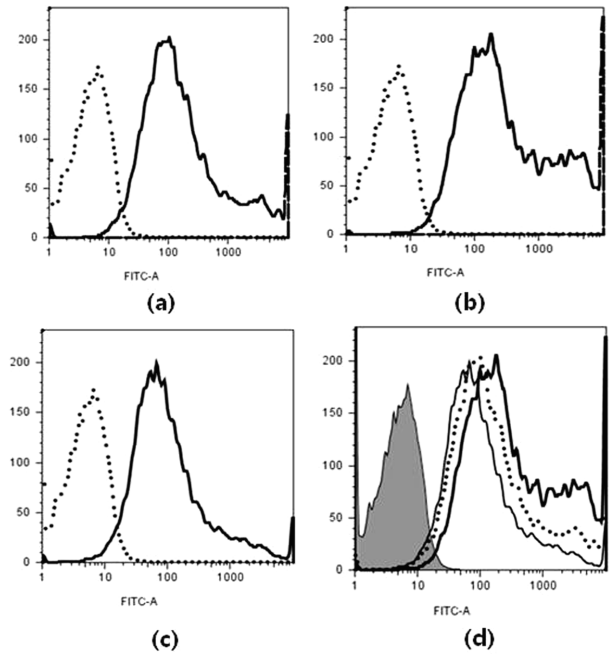

Figure 6. Phagocytic activity of modified PLGA microspheres - (a) PLGA 50:50, (b) Chitosan-PLGA, (c) SDS-PLGA, (d) comparison of phagocytic activities - shade; none, dot; PLGA 50:50, line; SDS-PLGA, bold; Chitosan-PLGA.

Conclusion

In this study, we characterized the various PLGA microspheres and surface modified PLGA microspheres and compared their phagocytic uptake by dendritic cell. The size and PI of microspheres increased with ratio of lactic acid increased. The size of modified-PLGA microspheres increased slightly, but the encapsulation efficiency decreased. It was confirmed

that microspheres are spherical and have smooth surfaces through differential scanning calorimetery and scanning electron microscopy. The result of X-ray diffraction shows that crystallinity has been changed during the preparation of microspheres.

Comparing the OVA release rate from PLGA microspheres and surface modified-PLGA microspheres, the release rate of modified-PLGA microspheres is slower and more sustained than that of PLGA microspheres.

Phagocytic uptake was not significantly different among the different portion of PLGA and SDS-PLGA microspheres. But, phagocytic uptake of chitosan-PLGA microspheres is higher than others. The modified surface properties by chitosan might influence the phagocytic uptake into dendritic cells.

These studies suggest that chitosan-modified PLGA microspheres could be one of the optimized model for targetting antigen presenting cells.

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