CHAPTER IV

Ex vivo evaluation of $Al(OH)_3$ and chitosan conjugated PLGA microparticles as nasal vaccine carriers in porcine nasal mucosa

Introduction

According to mucosal vaccine development, the ability of vaccine carrier and adjuvant on enhancing the systemic and mucosal immune response is predominantly necessary to evaluate. Up to date, three models has been employed to accomplish this approach which are ; in vitro, ex vivo and in vivo models. In vivo model limits by animal-to-animal and experiment-to-experiment to other models (Glorieux et al., 2007) while ex vivo and in vitro models offer the possibility of using pharmacological tools to get more precise information and are frequently used to determine the permeability of drugs across tissue, typically for ex vivo model (Florence, 1998). Additionally, ex vivo and in vitro models offer advantages over in vivo according to the ethical point of view (Wadell, Bjork and Camber, 1999). However, most of mucosal vaccine experiments, especially nasal vaccine, employ in vivo model rather than ex vivo or in vitro model. Thus, the understanding of mucosal transported mechanisms such as permeability, uptake and mucosal adhesion has been lacking. Many papers related to mucosal delivery have evaluated only one or two mechanisms of transportation in which the results could not be completely replied and correlated to the results of experimental animal such as the only uptake study from Torche et al. (2000), uptake and adhesion study from McClean et al. (1998), or the only permeability study of Jorgensen and Bechgaard (1994). In order to screen the vaccine formulation before trial in experimental animal and to understand the mucosal transported mechanisms with an intention to correlate to in vivo response, the in vitro and ex vivo evaluation should be investigated prior to the in vivo study.

Regarding to human nasal mucosa, the obvious difficulty in obtaining intact mucosa for in vitro studies is the key problem (Kissel and Werner, 1998). Porcine nasal mucosa could be an alternative approach as its morphology is comparable to human based on cell type present and physiological results. In the case of passive transport of drugs, a close correlation between human and porcine nasal mucosa could be observed (Wadell, Bjork and Camber, 2003; Wadell, Bjork and Camber, 1999).

Additionally, the anatomy, the histology and the biochemical aspect of porcine nasal mucosa are also identical to human (Pond and Houpt, 1987).

The nasal respiratory epithelium consists of four cell types; 1. non-ciliated columnar cell with microvilli, 2. goblet cell, 3. basal cell and 4. ciliated columnar cell, lying as a single layer covered by mucus. Below the respiratory epithelium is a thick lamina propria, composed of a loose mesh of fibroelastic connective tissue with many blood vessels, lymphatic vessel, nerve and gland (Merkus and Verhoef, 1997). Nasal associated lymphoid tissue (NALT) is a tissue consisting of plenty amount of lymphoid cells such as T-cell and B-cell aggregated together as follicles scattered and located among respiratory cell (Clark, Jepson and Hirst, 2001; Brayden and Baird, 2001; Jeong et al., 2000; Jung et al., 2000; Ermak and Giannasca, 1998 and McGhee et al., 1992). In order to stimulate the immune response through nasal route, the vaccine carriers need to be arrived at NALT by which anatomically are; lymphoid follicles, palatine, lingual and pharyngeal tonsils or Waldeyer's ring. Waldeyer's ring, a prominent component of NALT is located at the posterior part of nasal cavity (Chadwick, Kriegel and Amiji, 2010; Park et al., 2003; Tamura et al., 1998). The epithelial surfaces of NALT also contain specialized antigen–sampling cells known as M-cells. These cells represent themselves as a potential portal for nasal delivery due to their high endocytosis ability (Rieux et al., 2005; Brayden, Jepson and Baird, 2005; Foster and Hurst, 2005).

After entering of vaccine carriers, the released antigen are internalized and processed by antigen presenting cells such as dendritic cells and macrophages in the follicle and presented to T cells and B cells. Upon sensitization, B cells proliferate and switch to immunoglobulin committed cells (Ig cells). These immunoglobulin committed cells eventually leave the mucosal lymphoid follicle (inductive site) and migrate through the systemic circulation and to the various mucosal sites (Alpar et al., 2004; Lofthouse, 2002; Chen, 2000; Kamath and Park, 1997).

Nasal vaccination requires vaccine carriers to overcome nasal barriers. Carriers that are deposited on nasal mucosa will either be taken up there or be removed to the posterior part by mucociliary clearance. Thus, carrier with the suitable size and surface charge will be more desired to be taken up as they could be more adhered on nasal surface. However, if the carriers are removed by mucociliary

movement, they will probably be brought to Waldeyer's ring at the posterior part and possibly taken up there as well (Östh et al., 2003). Thus, nasal immunization need vaccine carrier with a suitable size to be taken up by M-cell and an appropriate surface charge to be adhered to mucosal surface. In our previous study, we optimized the formulation parameters to obtain PLGA particles with 1, 5 and 15μm in sizes and conjugated them with positively charged mucoadhesive substances, $Al(OH)_3$ and chitosan (CS) with an intention for the future goal of improving the transportation efficiency of particles and enhancing the mucosal immune response of entrapped vaccine inside particles (Tunsirikongkon and Ritthidej., in press).

The aim of present experiment is to study the mucosal transport mechanism which are; permeability, uptake, mucosal adhesion, cytotoxicity and tissue surface morphology involving transportation, of various size of PLGA, $Al(OH)_3$ conjugated PLGA, (1A) and CS conjugated PLGA (1C) particles using porcine nasal mucosa as ex vivo model to monitor the formulation before applying in experimental animal.

Materials and Methods

Materials

Krebs-Ringer bicarbonate buffer (KBR), Poly (D,L-lactic-co-glycolic acid), (PLGA) with lactide : glycolide 50 : 50 and BCA kit were purchased from Sigma-Aldrich (Saint Louise MO, USA). Fluorescein-5-isothiocyanate isomer I (FITC) was acquired from Invitrogen (Calsbad CA, USA). Polyvinyl alcohol, PVA, (MW. 67,000) was obtained from Fluka chemical (Switzerland). CS at molecular weight of 37kDA with 94% degree of deacetylation was procured from Seafresh® (Bangkok, Thailand). $Al(OH)_3$ was a gift from Bureau of Veterinary Biologic (Bangkok, Thailand). Japanese encephalitis vaccine, (JE) was kindly provided by Government Pharmaceutical Organization (Bangkok, Thailand). JE was purified and concentrated before using by membrane centrifuged tube. The other chemicals were of analytical grade and used as received.

Preparation of FITC-labeled JE

JE antigen was labeled by reacting with FITC solution at a ratio of 20:1 in 0.1M carbonate buffer. The reaction was processed for 2 hours before the elimination of excess FITC by dialysis against distilled water (Kusonwiriyawong et al., 2007).

Preparation and characterization of FITC-labeled JE loaded particles

The fluorescent particles were prepared from PLGA polymer by double emulsion solvent evaporation technique using FITC labeled JE as entrapped material. The CS and $Al(OH)_3$ conjugated PLGA microparticles,1C and 1A, respectively, were prepared as previously described (Tunsirikongkon and Ritthidej., in press) with slightly adaptation. The optimized parameters used for 1, 5 and 15μm PLGA particles formulation are shown in Table 4.1. Briefly, the primary w/o emulsion was prepared by adding appropriate amount of FITC-labeled JE into 8 ml of PLGA dissolved in dichloromethane (DCM). The mixture was immediately sonicated by 3-mm diameter standard probe sonicator at output control of 20 for 10 minutes to form primary emulsion. Then, the aqueous PVA solution of different concentrations was added to the primary emulsion and the resulting secondary emulsion was then sonicated by either bath sonicator or probe sonicator. The double emulsions were next diluted in 100 ml of 1% PVA and the solvent was eliminated by stirring up to 3 hours at 500 rpm. The resulting 1, 5 and 15μm particles were collected by centrifugation at 10,000 g for 5 minutes. The CS conjugated formulation was prepared by either adding 0.2% CS into the concave of centrifuged particles or blending 0.2% CS with 1%PVA at the final process of dilution of the selected preparation. The mixture of CS and particles were then vortexed for 15 minutes and shaken for 2 hours subsequently. After shaking, the mixture was left overnight and the excess of unconjugated CS was removed by centrifugation at 10,000g for 5 minutes. $Al(OH)_3$ conjugated formulation was obtained by adding required amount of $Al(OH)_3$ into the concave of the centrifuged particles following the regulation of US code of federal regulations (610.15(a)) that the amount of aluminium is limited to $\leq$ 0.85mg for a single human dose of vaccine. The entrapped material in this study was FITC-labeled JE for uptake experiment and JE for the rest of the experiment.

Table 4.1 Optimized preparation parameters for 1,5 and 15μm PLGA formulations

Size selected formulations (μm)	Input force[1]	w/o ratio [2]	(w/o)/w ratio [3]	% PVA [4] (w/v)
1	Probe sonicator	1:10	1:2	4%
5	Probe sonicator	1:2.5	1:4	4%
15	Bath sonicator	1:10	1:2	2%

[1] Input force of secondary emulsion

[2] Volume ratio of primary w/o emulsion

[3] Volume ratio of secondary (w/o)/w emulsion

[4] %PVA of secondary emulsion

The size and size distribution of samples were observed under a laser diffractometer (Mastersizer 2000, Malvern, UK) in order to confirm the desired size of particles. The morphology and fluorescence intensity of FITC-labeled JE loaded particles were determined by a confocal laser scanning electron microscope (CLSM, Olympus model FV 1000, Olympus, Germany) and were further confirmed by both optical microscope (E200, Nikon Eclipse, Japan) and fluorescence microscope. The photographs of all formulations were then taken.

Porcine nasal mucosa preparation

Porcine nasal mucosa was prepared according to the method of Östh et al. (2003) with slight modification. In brief, nasal respiratory mucosa was removed from conchae within 5 minutes after slaughter. First, the snout was separated from pig and opened up to expose the conchae. After that, the cavity tissue was carefully removed from conchae using forceps and scalpel, giving two pieces per snout. Then, tissues were immersed and swung in 250ml, 8^0C PBS for 3 seconds in order to clean the tissue surface. Finally, each piece was immediately immersed in 8°C KBR and placed on ice in order to transport to the lab.

Ex vivo evaluation of formulations in porcine nasal mucosa

Uptake study

Uptake study was evaluated by a CLSM according to the method from Osth et al. (2003) and Smyth et al. (2007) with slight modification. Porcine nasal tissues of 1x1 cm. were incubated with FITC-labeled JE loaded formulations for 20 and 90 minutes, respectively. Control tissues were also incubated with PBS. After incubation, tissues were washed by PBS to clean the excess formulations on tissue surface and then fixed with 3% paraformaldehyde for 30 minutes. Subsequently, tissues were soaked in Tissue Tex®, and then frozen by liquid nitrogen and immediately sliced by Cryostat section into 5µm thickness. Sections were mounted by glycerol and fixed on glass slide to observe under confocal microscope. At least three sections of each formulation at each time point were monitored and

photographed by the same objective (x20) in order to observe the fluorescence intensities of tissues. The fluorescence intensities of formulations themselves were also observed. Three photographs obtained from three tissue sections were employed to calculate the average fluorescence intensity of each formulation at each time point.

The percentage of uptake was analyzed by comparing the average intensity of tissue incubated formulation at each time point with the average beginning intensity of formulations themselves. The percentage of uptake could be calculated using the following equation:

$$\text{Percentage of uptake} = \frac{AIt \times 100}{AIf} \quad \text{.....(2)}$$

Where AIf represents the average fluorescence intensity of formulation

AIt represents the average fluorescence intensity of tissue incubated formulation at each time point

Tissue adhesion study

The mucoadhesive property of formulations on porcine nasal surface was evaluated by an adapted method described by Harikarnpakdee (Harikarnpakdee et al., 2006). A freshly cut porcine nasal tissues were cleaned by rinsing with PBS. An accurate amount of each formulation was applied onto nasal surfaces attached on petridish glass that fixed in an angle of 40° relative to the horizontal plane. Then, PBS buffer was peristaltically flushed over nasal surface at a rate of 6 ml/min. The washed were collected at 0, 5, 10 and 30 minutes, respectively and the amount of washed particles was determined by BCA kit and measured by UV-VIS spectrophotometer at 560 nm. The washed particles were demolished prior to OD determination.

Morphology of tissue surface involving transportation

Morphological evaluation of tissue surface involving transportation was determined by scanning electron microscopy (SEM, JEOL, JSM-5410LV, Jeol, Japan). Porcine nasal tissues were incubated with formulations for 10 minutes and directly fixed with 2% glutaraldehyde for 30 minutes. Tissues were rinsed for 5 minutes by PBS pH7.4, following by distilled water and then, dehydrated three times with a gradient series of 30%, 50%, 70%, 90% and 100% alcohol, respectively. The critical point dryer was used to dry tissues. The tissues were coated by a layer of gold and observed under scanning electron microscopy. The photographs of surface morphology were then taken.

Cytotoxicity study

Cytotoxicity was determined by MTT assay as previously described by Wadell et al. (1999). MTT was dissolved in PBS buffer to the concentration of 2mg/ml, then kept in the dark and stored at 4°C. Porcine nasal tissues from cavity mucosa were cut into 1x1cm and incubated with three concentrations of each formulation which were 100, 300 and 600µg/ml for 7 hours. Tissues incubated with PBS were served as reference. After incubation, formulations were removed and tissues were then rinsed three times with PBS, pH7.4. MTT solution was subsequently added and incubated with tissues for 4 hours. After that, MTT solution was removed and tissues were gently flushed with PBS and extracted using DMSO. The absorbance was determined by a microplate reader (VICTOR^3, Perkin Elmer, USA) at 595 nm.

Permeation study

Permeation study was performed by modified Franz diffusion cell. The receptor compartment was filled with PBS and the system was maintained at 37°C for the whole process. Porcine nasal tissues were used within 1 hour after slaughter as permeated membrane. Each formulation was added to the donor compartment and 2 ml of PBS in receptor compartment was withdrawn at 0.5, 1, 2 and 4 hours, respectively. The withdrawn buffer was replaced with the same amount whenever it

was sampling. The amount of permeated was determined by UV-VIS spectrophotometer at 560 nm. The permeated JE was separated from permeated particles by centrifugation. The permeated particles were demolished by the solution of DMSO and SDS before the OD measurement by UV-VIS spectrophotometer (Jasco V-530, Schimidzu, Japan), using BCA kit as a color complex formation.

Results and discussions

Characterization of FITC-labeled JE loaded particles

The average particles sizes of FITC labeled JE loaded particles were 0.80, 7.96 and 17.18 μm which represented 1, 5 and 15μm particles, respectively. The uniformity values of 1, 5 and 15μm were 0.36, 6.47 and 0.77, accordingly. Theoretically, the particle size of less than 10 μm usually deposit in the upper respiratory tract, whereas particles size of less than 0.5 μm is normally exhaled by breathing (Arora, Sharma and Garg, 2002; Rice-Ficht et al., 2010). This occurrence is applied for the droplet and powder particles. Even though, the particles administered in solution form probably provide a different result, the theory of air born particles deposition could be applied as a guideline to select the suitable size of particles for nasal administration. Thus, the sizes of particles selected in this study were 1, 5 and 15μm, respectively. The particles sizes and uniformity values of 1, 5 and 15μm particles in this study were relatively satisfied. The particles sizes (uniformity) of 1C and 1A were 0.65(6.81) and 0.81(1.35) μm, respectively which represented the effect of 0.2% CS and 0.75% $Al(OH)_3$ on uniformity of 1μm size particles. The uniformity value of 1C and 1A were relatively elevated when compared to 1μm particles which could probably be a result of an adsorption property of CS and $Al(OH)_3$ that adsorbed the nearby particles. Additionally, the high probability of particulate impaction of 1μm particles themselves compared to the same volume of 5 and 15μm preparations which resulted in the particulate aggregation could as well be correlated to the high uniformity value (Mitsumata, Hachiya and Nitta, 2008).

Optical microscope demonstrated spherical particles of all sizes including the characteristics of conjugated particles. The particles of 1, 5 and 15μm were relatively

independent from each other as demonstrated in Fig 4.1,A1-C1, respectively. The effect of conjugated materials on morphology of particles was obvious for $Al(OH)_3$ as particles were aggregated as shown in Fig 4.1,E1 while the morphology of CS conjugated particles was relatively resemble to 1μm particles as displayed in Fig 4.1, D1 which corresponded to the results of particles size and uniformity value. However, the fluorescence property and morphology of fluorescence particles were also needed to be evaluated. Fluorescence and confocal laser scanning microscope were utilized and revealed that the particles of all sizes and all conjugated particles provided the fluorescent property as shown in Fig 4.1,A2-E2 and Fig 4.1,A3-E3, respectively.

Ex vivo evaluation of formulations in porcine nasal mucosa

Uptake study

The results from CLSM revealed the extent of particles taken up and the characteristic of particles at the underlying tissues at 20 and 90 minutes as shown in Table 4.2 and Fig 4.2-4.3, respectively. From the results, large amount of 1μm formulation (47.98%) was able to be taken up by nasal tissue at 20 minutes compared to those from 5 and 15μm formulations (5.02 and 14.43%), respectively. Moreover, none of any particle at precise size of 5 and 15μm could be observed at the underlying tissues by 20 minutes. At 90 minutes, the percentage of 1μm particles taken up by nasal tissues was dropped off from 47.98% to 19.59% while 5μm particles seemed to have the stable uptake of about 5.02% to5.51%, and 14.43% to 9.34% for 15 μm particles, respectively.

Thus, particles of the approximate diameter of 1μm were more prefer to be taken up by nasal tissue compared to 5 and 15μm at both time points according to this study which corresponded to the review from Donovan and Huang (1997) that particles were taken up into nasal tissue with the size of up to approximately 1μm as well as the study from Östh et al. (2003) that the particles with the size of around 2μm could indisputably be taken up by porcine nasal tissue and the study from Mcclean et al. (1998) also showed that particles of up to 1μm size undergo the uptake pathway by intracellular trafficking regarding to intestinal epithelia.

Table 4.2 Percentage of particles taken up by porcine nasal mucosa

Formulations	% Particles uptake (SD)	
	20 minutes	90 minutes
PLGA 1μm	47.98(6.25)	19.59(4.93)
PLGA 5μm	5.02(0.64)	5.51(0.67)
PLGA 15μm	14.43(2.18)	9.34(6.68)
1C	10.68(1.49)	8.03(2.62)
1A	47.85(2.29)	28.46(2.08)

The time points were chosen from the review of Smyth et al. (2008) that the early time point of less than 30 minutes allowed the higher observation of the particulate taken up using the small intestine as a model which could probably be applied to the nasal model. In accordance with this study, more number of 1μm particles at the underlying tissue could be observed at 20 minutes and less amounts of particles was noted at 90 minutes which corresponded to the results from Smyth. In addition, the article of Ermak and Giannasca (1998) underlined the parameters influenced the tissue uptakes were particle size and time. This article stated that PLGA microparticles of particle size around 1–4 μm were taken up by mucosal cells in rabbit peyer's patch within 30–60 minutes and translocated to the basolateral pocket region containing lymphocyte afterward. All these studies were corresponded to the size and time points for intranasal administration of particles in this study.

The particles of 5 and 15μm formulations disclosed at the underlying tissues at both 20 and 90 minutes were, nevertheless, not with an actual size of 5 and 15μm. This occurrence was due to the size distribution of 5 and 15 μm formulations themselves that refer to the variation of the diameter within the same formulation. Thus, the smaller particles included in 5 and 15 μm formulations could be taken up by tissues beyond the precisely size of 5 and 15μm and the uptaken amount was relatively stable at both 20 and 90 minutes.

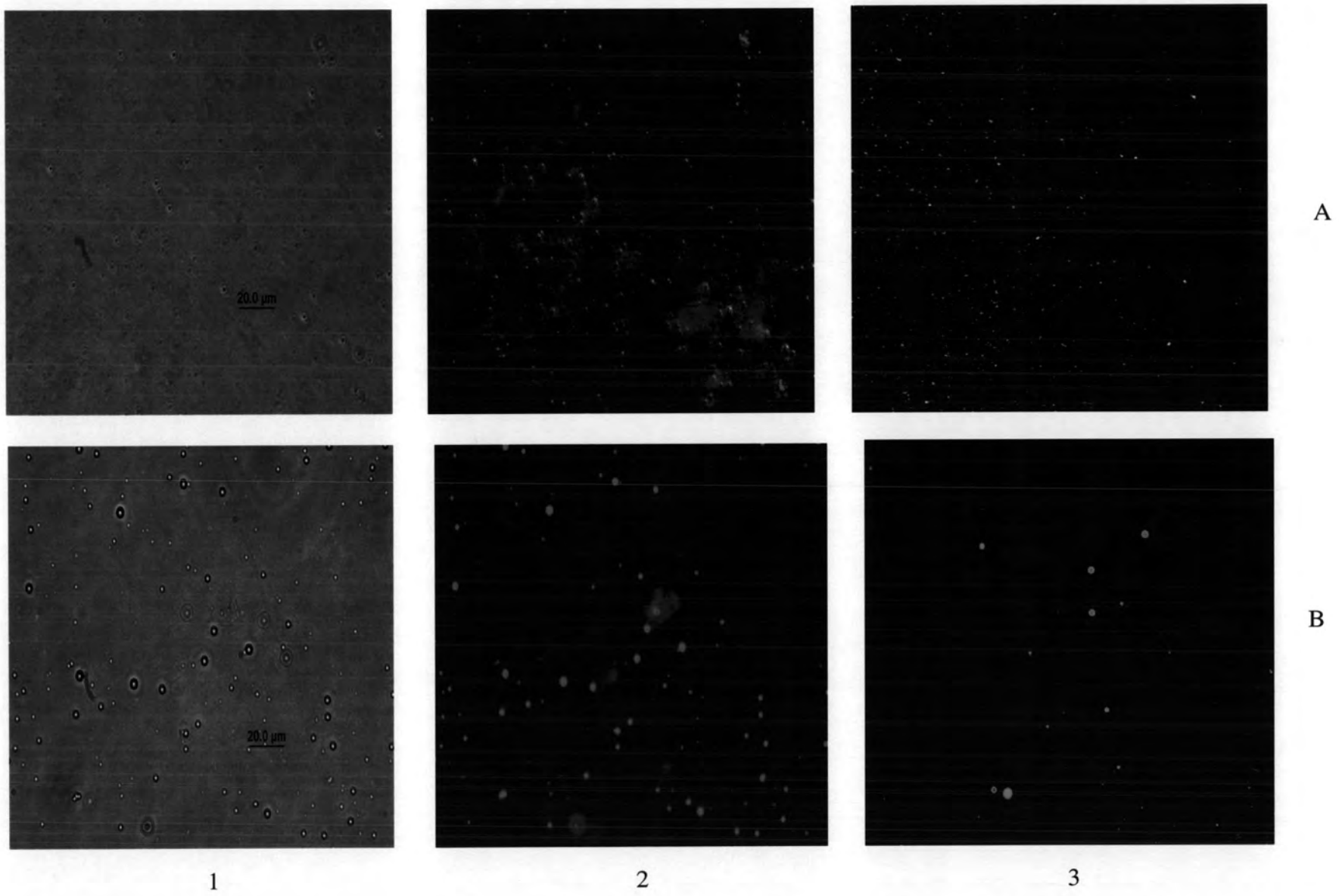


Figure 4.1 Morphology and fluorescence property of 1 µm (A), 5 µm (B), 15 µm (C), 1C (D) and 1A (E) particles by optical, fluorescence and confocal laser scanning microscope (1→3), respectively.

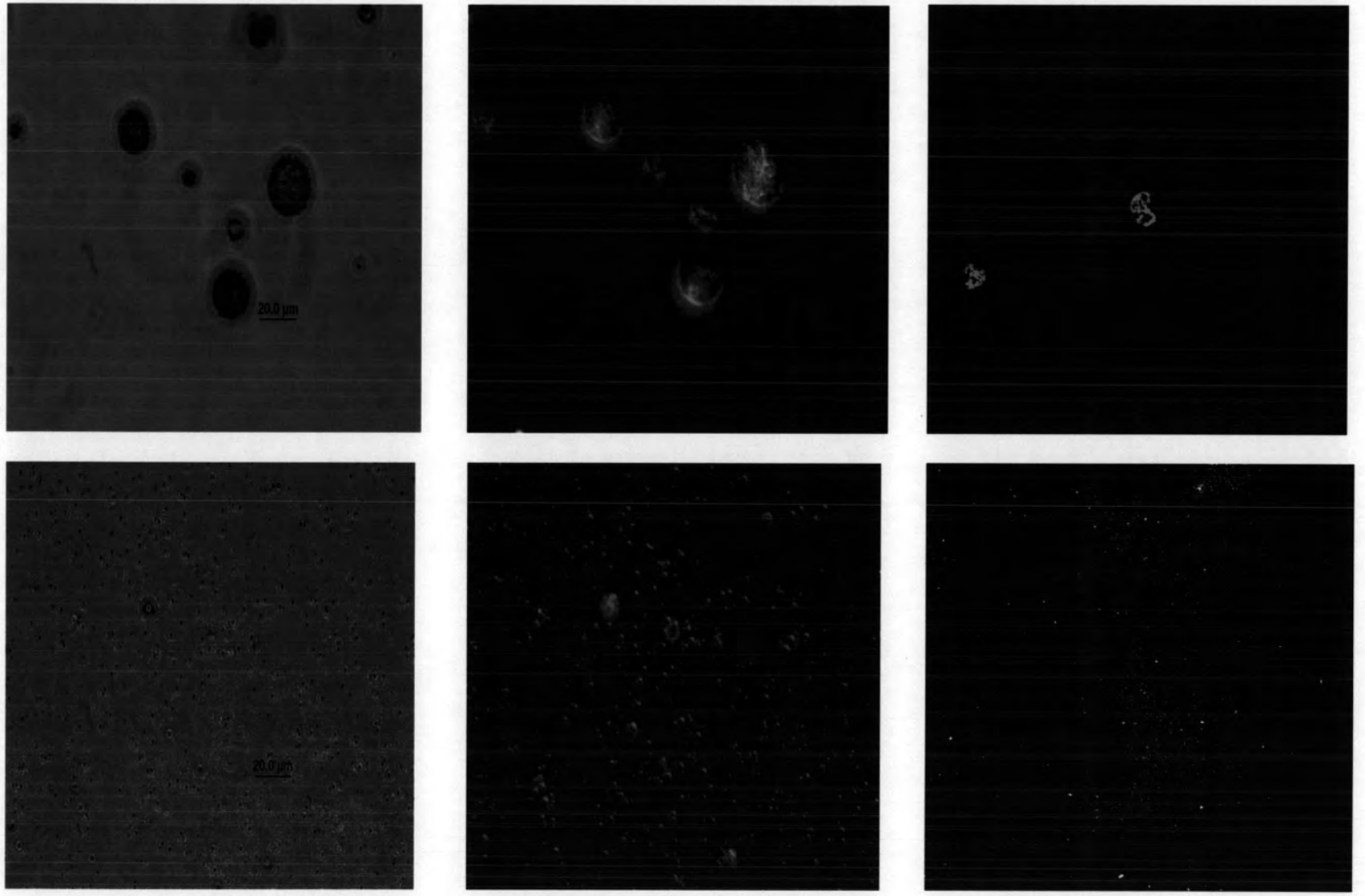


Figure 4.1 (cont.) Morphology and fluorescence property of 1 µm (A), 5 µm (B), 15 µm (C), 1C (D) and 1A (E) particles by optical, fluorescence and confocal laser scanning microscope (1→3), respectively.

E

1 2 3

Figure 4.1 (cont.)Morphology and fluorescence property of 1 µm (A), 5 µm (B), 15 µm (C), 1C (D) and 1A (E) particles by optical, fluorescence and confocal laser scanning microscope (1→3), respectively.

Since the fraction of small particles of both 5 and 15μm formulations could not be exactly identified, the accurate amount of small particles taken up was not able to determine precisely as well. Though, a single particle of actual size of 5μm could remain be observed inside tissues at 90 minutes as shown in Fig 4.3B. The transportation of particles depended on many factors in which particles size and transported time were deemed as significant factors. It was relatively obvious that particles of larger size than 5μm was less prefer to be taken up by mucosal cells in this study in which the results were corresponded to the finding of Eldridge et al (1991), that particles of greater size than 5μm after orally administered remained within the patches whereas particles of less than 5μm diameter remained within Peyer's patches and were transported to mesenteric lymph nodes and peripheral lymphoid organs, respectively. According to the influence of transported time, the study from Pappo and Ermak (1989), illustrated that by 30 to 45 minutes, particles administered orally were accumulated in the follicle associated lymphoid tissue in a pattern that correlated with the distribution of M-cells and after 90 min, large numbers of particles were translocated into the subepithelial region of gastrointestinal tract which could be concluded that by 90 minutes, most particles were mainly traveled to the underlying region. Thus, formulation of 5μm obtained more opportunity to be taken up and required less time to be transported by tissues compared to 15μm particles. These might be the reason why the uptake amount of 5μm particles was initially less and slower declined according to time compared to 15μm particles. However, the amount of small articles in each 5 and 15μm formulations was also an essential feature. According to the characteristics of 1μm particles after taken up, it was found that 1μm particles taken up by tissues were grouped in an irregular shape and scattered inside tissues at both 20 and 90 minutes as shown in Fig 4.2A and 4.3A, respectively. These particles were penetrated into the underlying tissues. Particles uptake initially involves contact with the microvillus projections on cell surfaces followed by rapid phagocytosis through apical membrane. Phagocytosis is a subtype of endocytosis by mean of particles or virus-liked particulate uptake. The internalized phagosome sometimes probably contains several particles in which the particles could be observed as a particulate cluster. Then, the phagocytic vesicle subsequently fuses with the basolateral membrane, emptying its contents into the intraepithelial pocket.

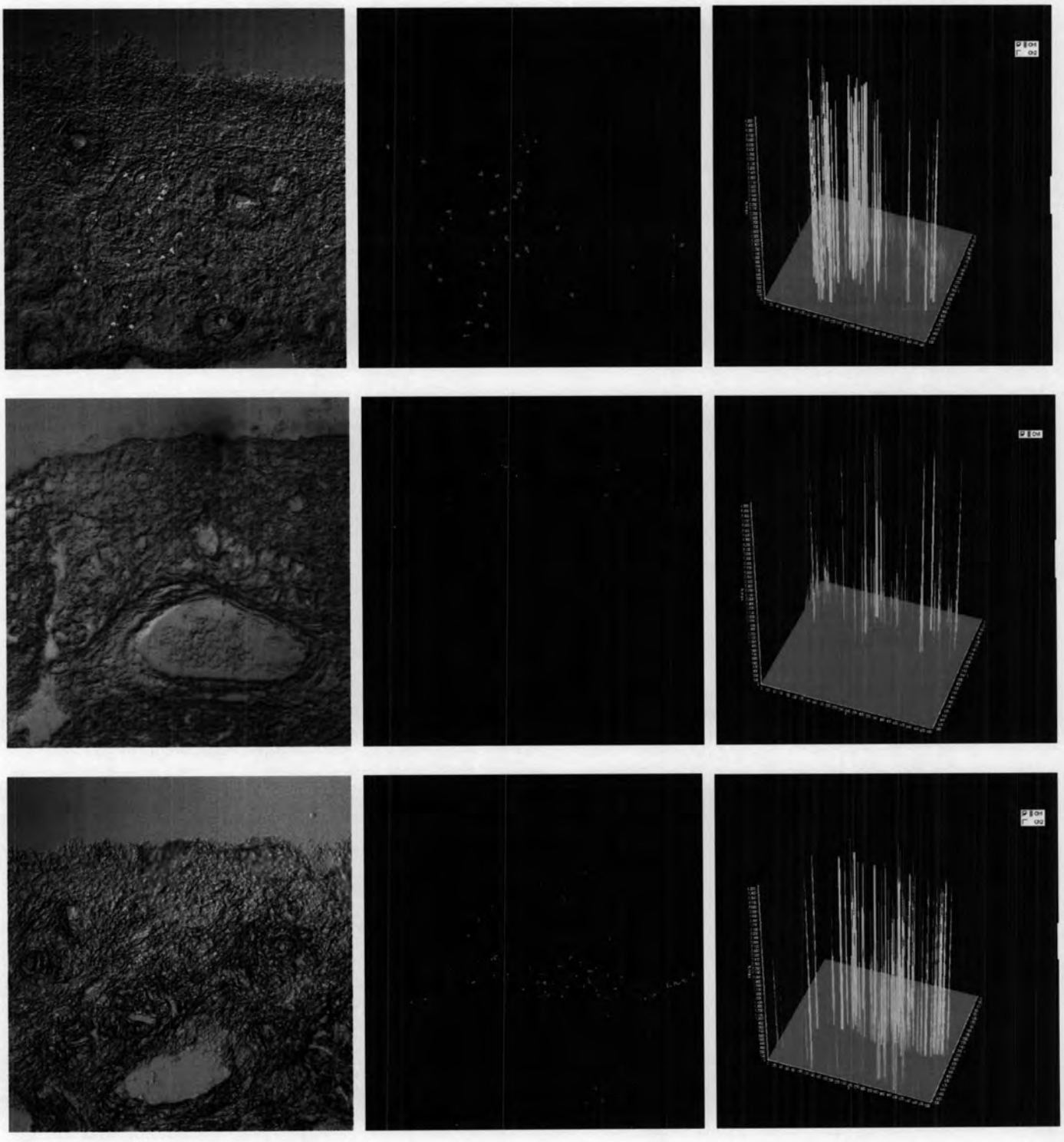


Figure 4.2 Characteristic of particle taken up into porcine nasal mucosa of 1μm (A), 5μm (B), 15μm (C), 1C (D) 1A (E) particles and PBS (F) by CLSM; incubation time 20 minutes

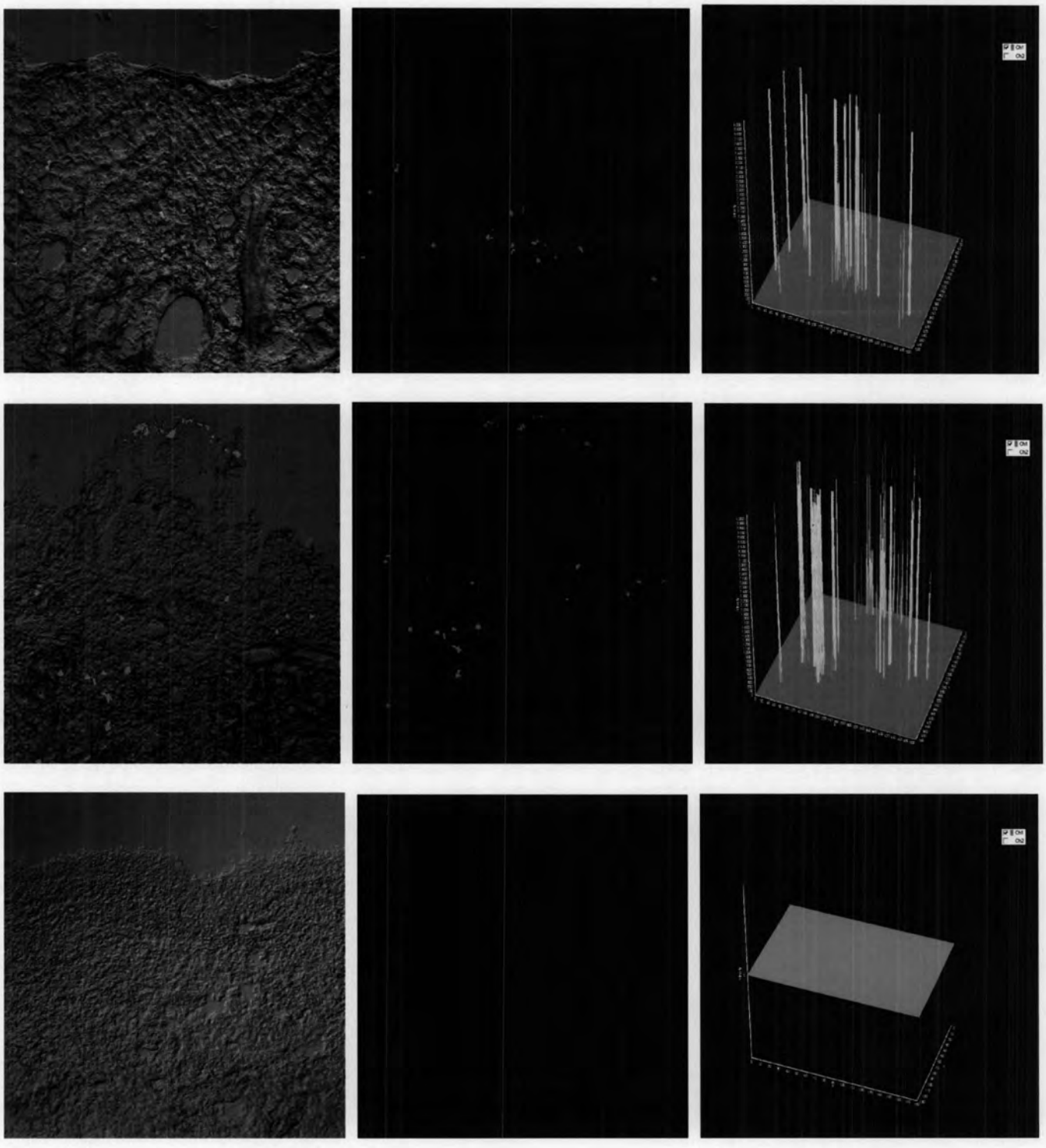


Figure 4.2 (cont.) Characteristic of particle taken up into porcine nasal mucosa of 1μm (A), 5μm (B), 15μm (C), 1C (D) 1A (E) particles and PBS (F) by CLSM; incubation time 20 minutes

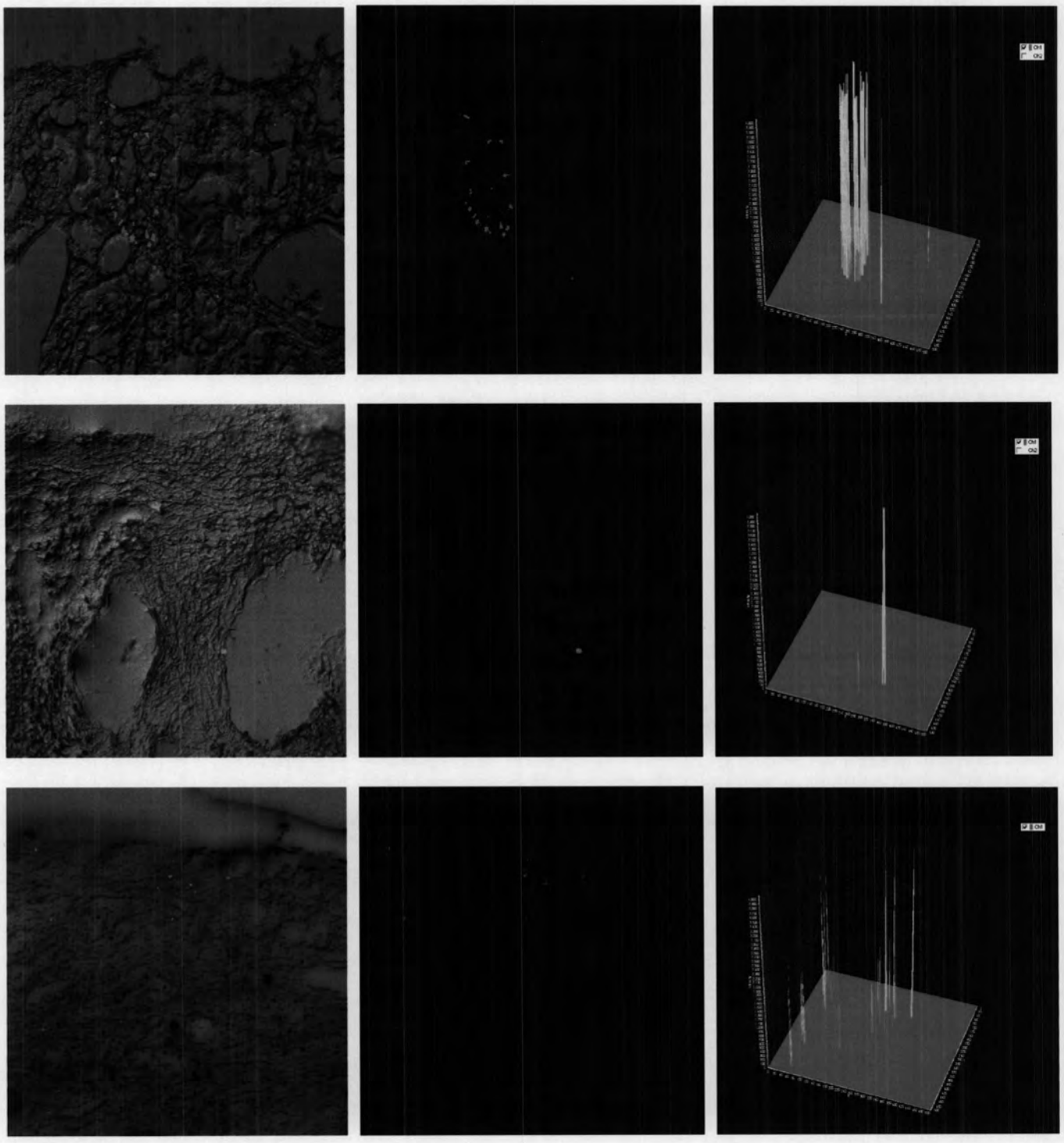


Figure 4.3 Characteristic of particle taken up into porcine nasal mucosa of 1μm (A), 5μm (B), 15μm (C), 1C (D) 1A (E) particles and PBS (F) by CLSM; incubation time 90 minutes

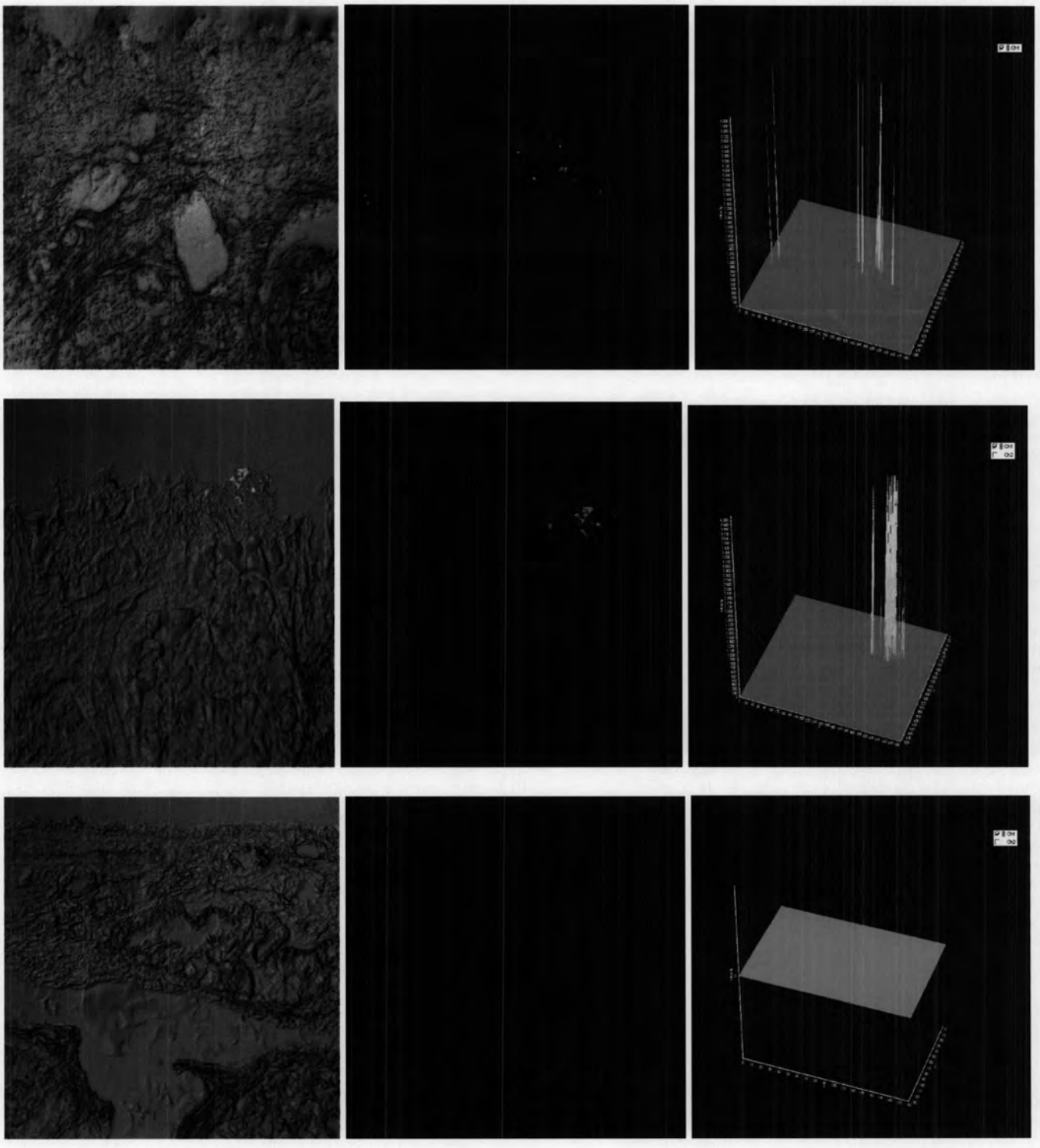


Figure 4.3 (cont.) Characteristic of particle taken up into porcine nasal mucosa of 1µm (A), 5µm (B), 15µm (C), 1C (D) 1A (E) particles and PBS (F) by CLSM; incubation time 20 minutes

After that, particles may then be engulfed by phagocytic cells in the pocket or pass through the basal lamina into the sub-epithelial region (Ermak and Giannasca., 1998). Thus, the particles appeared as the particulate cluster might be the results of the multiple particulate taken up into the phagosome. Additionally, the smaller particles in 5 and 15 μm formulations taken up by nasal tissues could also be merely observed as a small group of particles with the same reason as 1 μm particle as illustrated in Fig. 4.2B-C and 4.3B-C. Although size was an important parameter in particulate uptake, other characteristic such as surface property also influenced the extent and the distribution of particles taken up by nasal tissues. As seen in Table 4.2, the amount of 1C taken up by nasal tissues at 20 minutes was surprisingly low, only at 10.68% and slightly declined to 8.03% at 90 minutes. This percentage was much lower than the percentage of 1μm particles taken up by nasal tissues at both time points. However, the morphology of 1C inside tissues was relatively similar to 1μm formulation at both 20 and 90 minutes as shown in Fig 4.2D and 4.3D, respectively.

Regarding to the transport of particles, different pathways could be discerned which refer paracellular and transcellular or endocytotic paths. Transcellular path is possibly considered as the core pathway of transported mechanism according to the more majority of the cell's surface membrane against the small length channels of paracellular path. The exacting attention of transportation is the transcytotic process at mucosal surfaces by which macromolecules or particles internalized at the apical plasma membrane of epithelial cells and transported to the contralateral plasma membrane and released to the basolateral compartment (Jung et al., 2000; Okamoto., 1998).

The paracellular path, on the other hand, is less considerable compared to transcellular path as it is normally processed via pore channels covering by tight junction that contributes only 1% of the whole mucosal surface area. However, paracellular path could be enhanced by some polymers with mainly process by charge mediated mechanism that results in a structural reorganization of tight junction composition (Jung et al., 2000). Thus, particulate transport via paracellular path devoured less time to reach a basement membrane compared to transcellular path as the forever process of taken up is less complicated. Chitosan can be considered as

one of the cationic polymer which normally obtained the ability to enhance the paracellular permeability by the dilation of paracellular space and reduce TEER values with their charge excluding the damage of the cell membrane (Agrawal, Strijkers and Nicolay, 2010; Harikarnpakdee, et al., 2006; Yu et al., 2004; Hejazi and Amiji., 2003; Illum, 2003; Karlsson et al., 1999; Kotze et al., 1997) and could also be considered as a biodegradable natural polymer with a great potential for pharmaceutical applications due to its biocompatibility, high charge density, non-toxicity and mucoadhesive properties (Sinha et al., 2003; Senel et al., 2000). Obviously, in this study, the slight low amount of 1C taken up into nasal tissues at both 20 and 90 minutes could possibly be attributable to the fast transported mechanism of paracellular path by mean of chitosan which was more preferred than transcellular path and accordingly occurred before the time point of 20 minutes.

According to 1A, the percentage of particles taken up by nasal tissues expressed in Table 4.2 was relatively equal to 1μm formulation at 20 minutes and decreased from 47.85% to 28.46% at 90 minute. However, the amount of 1A inside tissues was relatively higher than 1μm particles at time point of 90 minute. The particles of both time points appeared to group together inside tissues and also adhered on tissues surface. The characteristic of 1A inside tissue was quite alike to 1μm and 1C formulation except 1A which adhered on tissue surface. The depth of penetration was not that deep as there were a number of 1A remained attach on cells surface as shown in Fig 4.2E and 4.3E.

It was relatively obvious that the amount of 1A remained more on tissues surface compared to 1μm particles due to the effect of positively charge and the unique mucoadhesive property of $Al(OH)_3$. The interaction between $Al(OH)_3$ and tissues surface could be counted either on electrostatic interaction or ligand exchange with devoid of any changes of the conformational mucus protein (Hansen et al., 2009; Dong et al., 2006). At pH values of less than an isoelectric point of $Al(OH)_3$, $Al(OH)_3$ presented positive charge, such as at pH 6.8 of nasal, which assisted the adhesion of $Al(OH)_3$ on tissues surface. Beside the electrostatic interaction, $Al(OH)_3$ promoted the attachment on cell or tissues surface by mucoadhesive property known as "depot forming" since the presence of alum or $Al(OH)_3$ generated a fine network

around the delivery particles holding them together (Kanchan, Katare and Panda., 2009; Du et al., 2009) and would slowly released the embraced substance such as the particles for a period of time (Sokolovska et al., 2007). The great transport of 1A at both time points happened by the suitable size of particles that attached very well on tissues surface by the help of $Al(OH)_3$.in which the mechanism of transportation could be both transcellular and paracellular pathway. The study from Aspenstrom-Fagerlund et al. (2009) advocated that aluminium (Al) absorption could occur mainly by the paracellular pathway. However, many studies proposed other mechanism of transportation for aluminium. The energy-dependent transport played an important role in aluminium uptake, possibly involving Ca-ATPase which replied to the important of calcium channels as an additional entry site for aluminium (Cochran et al., 1990; Van der Voet et al., 1989). Aluminium absorption and uptake might be also well occurred along the pathway for other-essential-metals such as iron (Van der Voet et al., 1987). These possible pathways have gained $Al(OH)_3$ formulation more channel to be taken up beyond the only pathway of particulate endocytosis compared to other formulations. However, the amount of 1A taken into the inside tissues was less articulated than 1μm formulation according to CLSM pictures as several fraction of 1A formulations adhered on tissues surface. Nevertheless, the unique characteristic of $Al(OH)_3$ could prolong the residence time of particles adhering on tissue surface in order to obtain a better opportunity to be taken up by tissue for a period of time.

Tissue adhesion study

The adhesion property of formulations was illustrated by the percentage of washed particles from tissue surface over time. The high percentage of washed particles represented the low efficiency of particles to be adhered on tissue surface. The results of adhesion property examined by the percentage of washed particles at each time point revealed that the large particles of 15μm could adhere on tissue surface for a longer period of time compared to the small particles of 1 and 5μm as shown in Fig 4.4. The percentage of 1μm washed particles was dramatically high compared to all other formulations at all points of time and reached 100% by 30 minutes. In contrast with 5 and 15μm particles, the percentages of washed particles at the first five minutes were less than 20% and gradually increased to 57.82% and

40.68%, respectively, within 30 minutes for 5 and 15μm formulations. According to the results, the adhesion property of particles could be ranked; 15μm > 5μm > 1μm, respectively. Even though the small size of 1μm was not as good on tissue adhesion as 5 and 15μm as a consequence of a rapid and easy wash by nasal clearance, the results of tissue uptake as stated in the above results was considerably higher for the reason that the size of 1μm was more preferred to be taken up. Regarding to this study, particles size was more stated to influence the tissue taken up than tissue adhesion property.

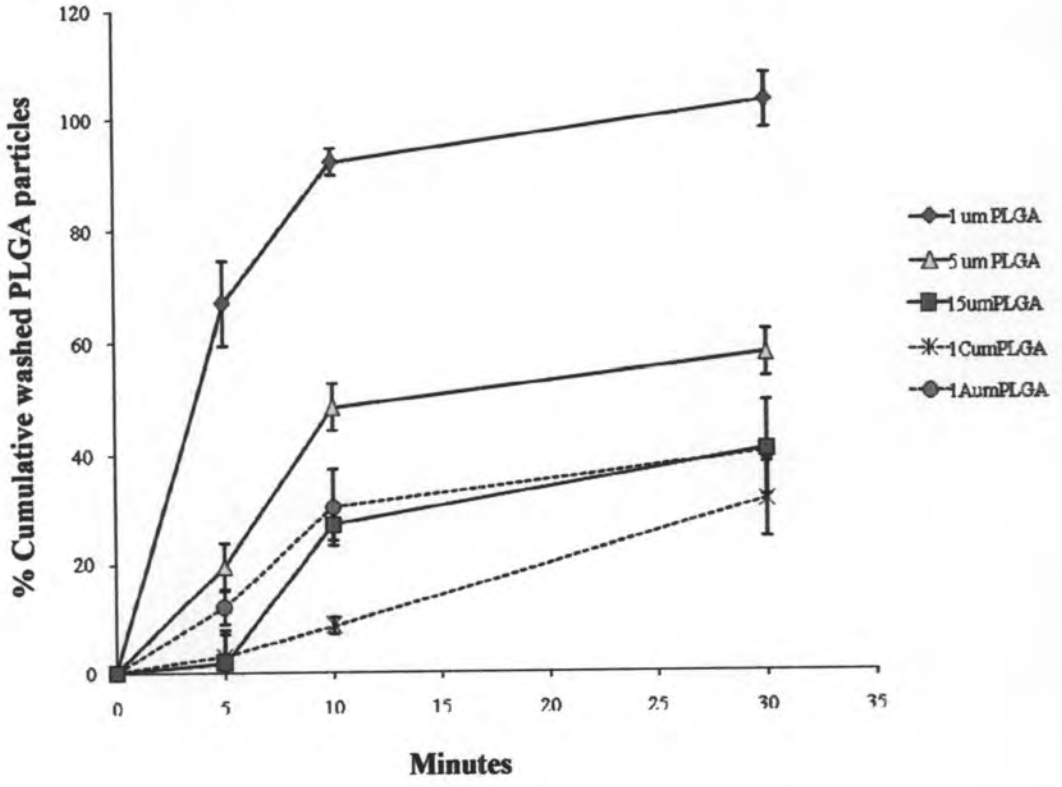


Figure 4.4 The percentage of washed particles representing the adhesion property on porcine nasal tissue of 1μm, 5μm, 15μm, 1C and 1A particles.

Mucus is an adhesive gel that covers and protects mucosal surfaces which foreign particulates are normally efficient trapped inside mucus layers by steric obstruction and/or adhesion. Trapped particles are typically removed from the mucosal tissue by mucosal clearance, thereby strongly limiting the duration of sustained drug delivery locally (Lai, Wang and Hanes, 2009; Cu and Saltzman, 2009).

The transport of particles via mucus is relatively complex and controlled by mucus layer as well as size, charge and surface wettability of particles in which the mucus adhesion of particles could be counted as size dependent phenomena (Cu and Saltzman, 2009; Ugwoke et al., 2005). The adhesion between particles and mucus is probably recognized by hydrodynamic drag force (f), produced by interactions of particles and mucus pertaining from the Stokes–Einstein equation, that provides a means to estimate the diffusion coefficient (D) from its physical size,(r):

$$D = \frac{k_B T}{f}, \textit{ where } f \textit{ represented:} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (3)$$

$$f = 6\pi\mu r \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (4)$$

which D represented diffusion coefficient, k_B stood for Boltzmann's constant, T symbolized absolute temperature, f represented drag force coefficient, r meant hydrodynamic radius of the diffusing molecule or particles and μ as a medium viscosity (Cu and Saltzman, 2009).

Regarding to the drag force coefficient which represented the adhesion force between mucus and particles, it was variable directly to the particle size according to equation 4. As a consequence, large size of particles required more force in order to eliminate particles from mucosal surface. Thus, large size of particles appeared to be adhered on mucosal surface for a longer period of time. However, large size rendered the lower uptake efficiency in regards to the diffusion coefficient of the Stokes–Einstein equation that increasing the particles size resulted in the diffusion coefficient dropping which could be implied from the equation that the larger size of particles had less efficiency to diffuse to the mucus layer resulting in a low uptake efficacy.

After conjugation by $Al(OH)_3$, the adhesion property of 1μm particles on tissues surface was significantly elevated and comparable to adhesion property of 15 μm particles within 10 minutes as shown in Fig 4.4. The percentage of washed 1A at the first 5 minutes was only less than 12% which represented the good adhesion efficiency. The washed particles of $Al(OH)_3$ reached 40.15% at 30 minutes which relatively equal to the percentage of 15μm formulation. Upon conjugation by CS, the

adhesion property of 1μm particles was not only increased but also superior than 15 μm particles. The percentage of washed particles of 1C at the first 5 minutes was only 2.93% and slightly rose to only 31.28% at 30 minutes which represented their excellent adhesion efficiency on tissues surface. Thus, conjugation of particles by CS and $Al(OH)_3$ obviously improved the adhesion property of particles and the effect of CS was evidently better than $Al(OH)_3$ according to the tissue adhesion characteristic.

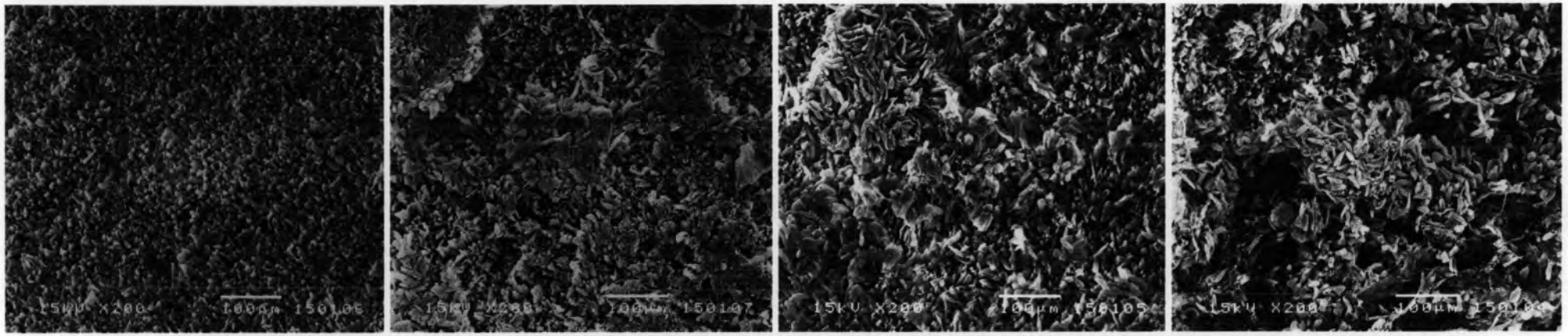
The adhesion could probably be the result of adsorption in which the adsorption theory of particle and tissue could be considered from: (1) primary chemical bonds that were relatively permanent and (2) secondary chemical bonds such as van der waals, hydrogen, hydrophobic and electrostatic forces which were a temporary bond. The electrostatic theory proposed the existence of an electrical charge double layer at the interface between the adhesive particles and the tissues surface from the difference of their electronic structure (Ugwoke et al., 2005). Polyvalent adhesive interactions with mucus can be achieved via electrostatic interactions since the glycosylated group of mucin that densely coated with negative charges could bind to the positive charge particles with high avidity. It might also interact via hydrophobic force which represents a particularly challenging problem for polymeric particles designed to deliver drugs and genes, since many commonly used biomaterials are either hydrophobic, such as poly (lactide–co-glycolide) and polyanhydrides or cationic, such as polyethylenimine, chitosan and polylysine (Lai, Wang and Hanes., 2009) which could propose only hydrophobic or electrostatic interaction, respectively. Thus, combination of hydrophobic and cationic material would be an ideal design to gain the most benefit by each advantage of both materials. For the formulations of 1C and 1A, both obtained positive surface charge which could very well adhere to the negative surface charge of biological tissue. As a result, the surface adhesion of 1C and 1A were more pronounced than 1μm formulation. However, the amount of washed 1C particles was less than 1A which could probably be the consequence of the high tissue uptake efficiency of 1C that uptake most particles through tissue at any short specific time compared to 1A particles.

Tissue surface involving transportation

The porcine nasal tissues composed of short-ciliated and sparser group of long-ciliated microvillous cells organized on tissue surface as shown in Fig 4.5. According to 1μm transportation in this study, there was none of any 1μm particles observed on tissue surface after the transportation since the adhesive property of 1μm particles was very deprived as obviously aforementioned in adhesion study. Thus, the particles of this size could not be seen. Regarding to 5 and 15μm transportation, a great numbers of 15μm particles were observed on tissue surface while 5μm formulations was hardly noted. These results corresponded to the results of uptake and adhesion study that the particles of 5μm or larger were non-favorably taken up but more adhered on nasal surface. Thus, they could easily be observed by SEM.

A total of 15–20% of respiratory cells was covered by long cilia of size 2–4 mm which increase the surface area of cell substances and water transportation between cells (Glorieux et al., 2007; Illum, 2003). These cilia moved in a coordinated way to propel mucus across the epithelial surface towards the pharynx. In relation to the previous finding, the non-ciliated microvillus cells could be the homologues of M-cell previously described in NALT, GALT and BALT of various species of animals. NALT played in important role in mucosal immunity against foreign antigen in the upper respiratory tract (Jeong et al., 2000) and could be rearranged as membrane protusion after contacting specific environment (Brayden, Jepson and Baird, 2005). The flat circular cilia happened in 1μm transportation might be the result of a cilio-inhibiting that affected the cilia movement and cilia beat frequency by 1μm formulation.

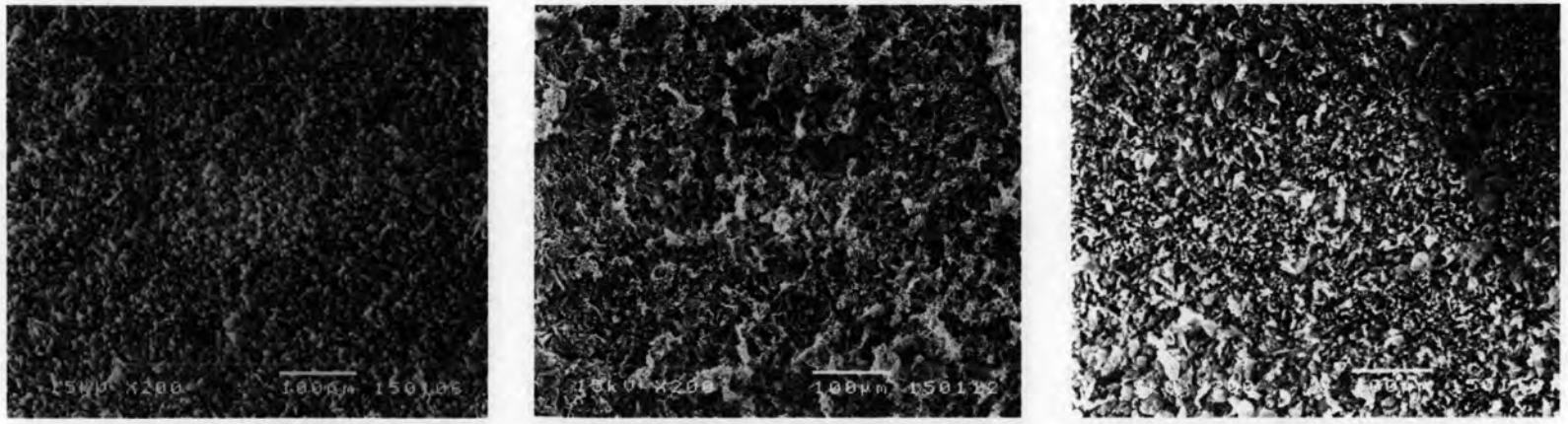
Even though, all SEM pictures did not express clearly the depiction of M-cells, the pictures obviously showed the different types of cell which were long-ciliated and short-ciliated microvillus cells of all tissue transported formulations. Additionally, the particles of specific size were adhered on tissues surface which could be concluded that porcine nasal tissue composed of various cells type including M-cell in which the particles of particular size could be adhered on its surface.



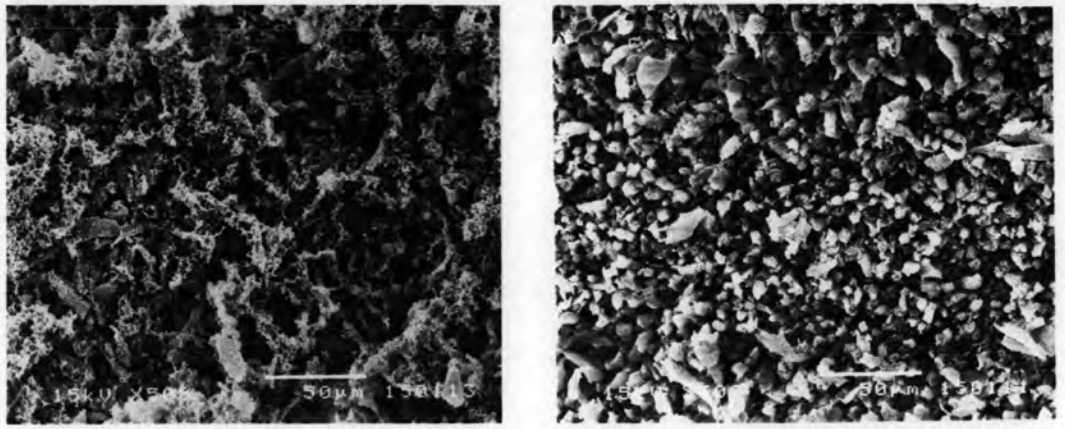
Blank(x200) 1 μm (x200) 5 μm (x200) 15 μm (x200)

1 μm (x500) 5 μm (x500) 15 μm (x500)

Figure 4.5 Morphology of porcine nasal mucosa involving transportation of 1 μm, 5 μm,15 μm, 1C and 1A particles by SEM technique



Blank(x200) 1Cμm (x200) 1Aμm (x200)



1Cμm (x500) 1Aμm (x500)

Figure 4.5 (cont.) Morphology of porcine nasal mucosa involving transportation of 1 μm, 5 μm,15 μm, 1C and 1A particles by SEM technique.

The conjugated material; CS, was observed to influence the morphology of tissues involving transportation as shown in Fig 4.5. The cilia of tissues incubated with 1C were covered by the mesh of 1C formulation which could be observed as a network of particles even though it was examined at the same 10 minutes period of incubation time as with 1μm particles. This was because 1C had advantages over 1μm particles by the excellent adhesion property and relatively fast and good uptake efficiency, presumably by paracellular process. These results were corresponding to the uptake and adhesion study that 1C could be rapidly transported through nasal tissue by means of the great adhesion property. In the case of 1A, the characteristic of cilia was also shown in Fig 4.5. The 1A was investigated as white materials coated on the cilia of tissues. They could be seen easily on tissues surface. These results were corresponding to the uptake and adhesion study that the 1A was moderately taken up by nasal tissue via various processes, mainly by both transcellular and paracellular path and the adhesion property of 1A was considerably excellent.

Cytotoxicity study

The results of cell viability represented the cytotoxicity to cells of each formulation as shown in Fig 4.6. The high percentage of cell viability indicated the low toxicity of formulations to cells. The results revealed that the cell viability of tissues incubated formulations could be ranked as follows, 15μm < 5μm < 1μm, respectively, in regard with the same dose as shown in Fig 4.6. However, the higher of the dose tended to cause the lower of the viability. Cytotoxicity or cells viability could be considered as a parameter that gained an increase attention in studies where freshly excised membranes are attained to mimic in vivo conditions. The technique normally used to investigate the cytotoxicity is a MTT assay which can successfully discriminate between various experimental parameters (time and temperature) and shows a good inter-animal reproducibility (Nafee et al., 2009; Manca et al., 2008). Cells viability could normally be affected by many factors such as organic solvent and some toxic chemicals. According to the results of organic solvent residue in previous study (Ritthidej, Tunsirikongkon., in press), it was observed that the amount of dichloromethane residue could be ranked as 15μm >5μm >1μm, respectively. Thus,

the large size of particles that obtained a higher amount of organic solvent appeared to be more toxic than the smaller size particles. The conjugated CS and $Al(OH)_3$ were associated with the decrease in percentage of cells viability, especially CS. The percentage of cells viability were ranked; 1C< 1A< 1μm, respectively, according to an equal dose of formulations. This tendency could be determined at all doses of formulations. The high percentage of cytotoxicity might be due to the strong interaction between cationic molecules and cell membranes of the cilia (Amidi et al., 2006) which affected the ciliary beat frequency that caused the cytotoxicity of cell or tissue. Thus, the viability of both cationic particles in this study was less than that of 1μm particles. Additionally, cytotoxicity was associated with an irreversible decrease in TEER value due to the destruction of tight junctions of the epithelial cells which might be considerably caused by the acidic effect of CS formulation. As a result, the percentage of cells viability could theoretically be ranked as 1C< 1A< 1μm, respectively which corresponded to the result in this study.

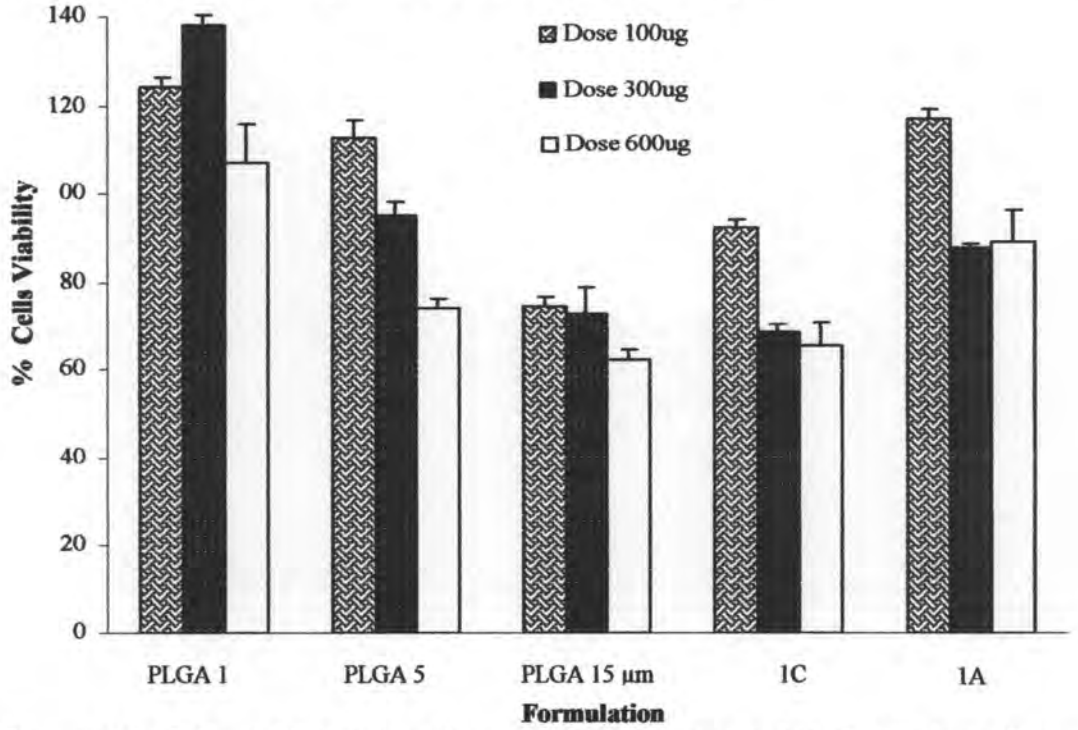


Figure 4.6 Cells viability of porcine nasal mucosa, co-incubated with 1μm, 5μm, 15 μm, 1C and 1A particles

The dose, particles sizes and conjugated materials were considerably affected the percentage of cells viability. The small size of particles comprised less toxicity

compared to the larger one and the conjugated materials; CS and $Al(OH)_3$ could reduce the cells viability of tissue, especially CS. Moreover, the high dose led to the lower percentage of cells viability.

Permeation study

For the particulate permeation, about 10% of 1, 5 and 15μm particles were permeated at 30 minutes and gradually increased as shown in Fig 4.7A. The rate of particulate permeation could be ranked; 5μm ≈ 15μm > 1μm, respectively with slightly different in percentage. The rates of 5μm and 15μm formulations were relatively comparable while the permeation of 1μm formulations was slightly slower.

Particulate uptake was certainly occurred in nasal mucosa with the particle size of approximately 1 μm that had been shown to rapidly enter the bloodstream following intranasal administration (Donovan, Huang, 1998., Almeida et al., 1993). Additionally, the endocytosis could be considered as a basically typical feature of the nasal uptake mechanism of most particles (Schmidt et al., 1998) and all particles used in vaccine formulations normally had a comparable size to the pathogens recognized sizes during the evolution by an immune system which usually was less than 5 μm (Xiang et al., 2006). Thus, for 1μm particles, most particles were taken up by endocytosis pathway into the underlying tissue and merely a quantity of particles were permeated through tissue at an equal point of time compared to 5 and 15μm particles. The 5 and 15 μm particles, however, obtained a high permeation through the tissue and not retaining inside the tissue as well as they attained higher cytotoxicity compared to 1 μm particles. Accordingly, nasal tissue was considered as a very leaky tissue that the leaky effect was coordinated with the cytotoxicity resulted in enlarging the paracellular channel. However, the cytotoxicity on tissues of large particles in this study did not yield the tissue viability of less than 70% according to the above results. Surprisingly, the permeation of 1C and 1A were superior than 1μm at all points of time, especially CS. The permeation 1C reached 70.86% within 4 hours while 1A only achieved 48.26%. Regarding to the permeation, conjugated substances: CS and $Al(OH)_3$ could enhance the tissue permeation of particles. The ionic attraction

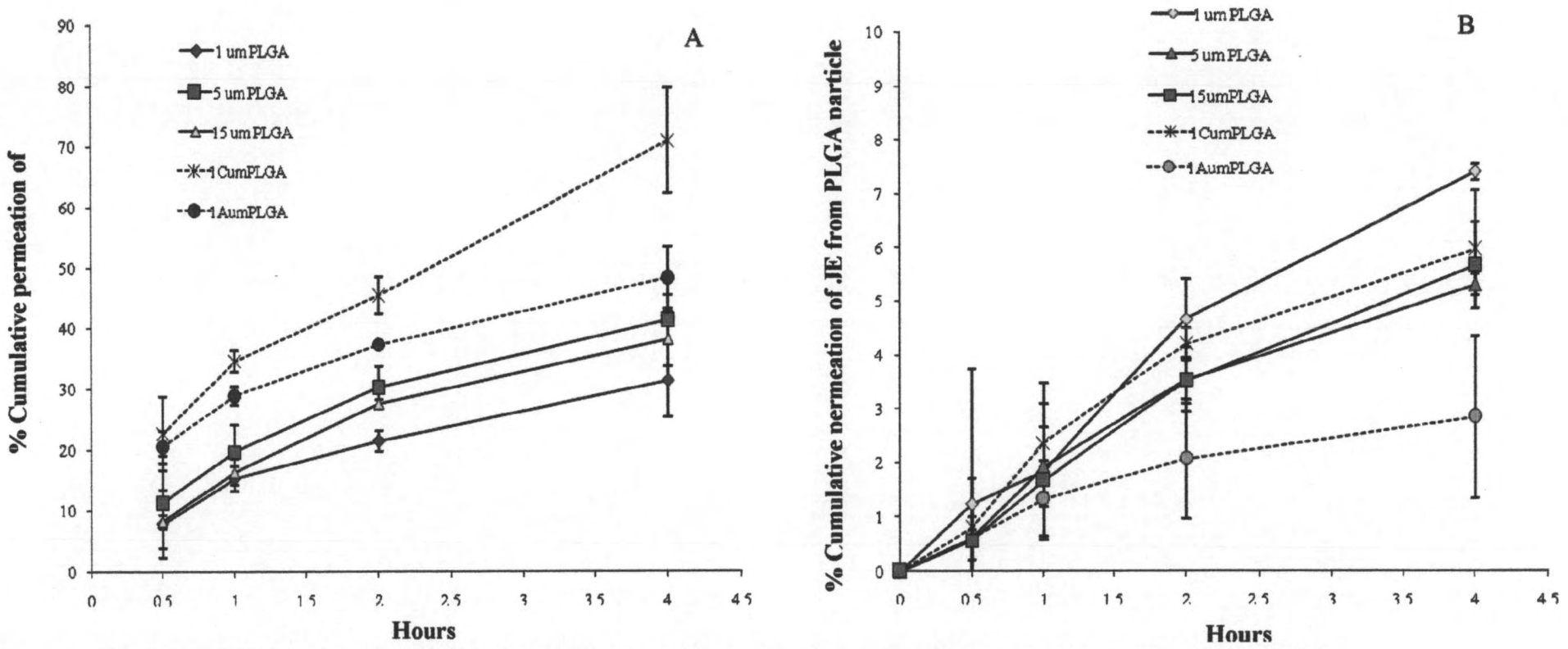


Figure 4.7 The permeation profile of particles (A) and JE released from particles (B) of 1μm, 5μm, 15μm, 1C and 1A particles

between the positive charge particles and the negative charge tissue surface was likely to represent a strong stimulus that initiated binding and subsequent internalization (Xiang et al., 2006) which most likely resulted in an enhancing of permeation. For CS, the main pathway of transportation was presumably paracellular path in which transcellular path was less considerable but not totally distinguished. CS may exert an effect on tight junctions similar to that shown for the intestinal epithelium (Alpar et al., 2005) and nasal cells (Harikarnpakdee et al., 2002). However, the combination of the cytotoxicity effect of CS which widened the paracellular channel and the small particles size that obtained a better opportunity to depart through the paracellular channel, caused the highest amount of particulate permeation of CS formulation. According to $Al(OH)_3$, the gel forming-liked structure of $Al(OH)_3$ embrace a relatively high viscosity. In fact, there are many factors affecting the nasal permeability, viscosity of formulation could be considered as one of the factor as higher viscosity of the formulation increases contact time between the substances and the nasal mucosa thereby decreasing the nasal clearance. At the same time, highly viscous formulations interfered the normal functions like ciliary beating or mucociliary clearance and thus enhanced the permeability of drugs by decreasing the clearance (Arora, Sharma and Garg, 2002). Additionally, the characteristic of most adjuvant particularly $Al(OH)_3$ was the induction of local tissue necrosis that consequently induce a large number of dendritic cells and also enhanced dendritic cell activation (Hansen et al., 2007; Brewer, 2006; Morefield et al., 2004; Rimaniol et al., 2004; HogenEsch, 2002) in which this process was normally found inside the specialized antigen sampling cells of mucosal surface know as M-cells (Clark, Jepson and Hirst, 2004). The basolateral surface of M-cells was invaginated forming an intraepithelial pocket that could effectively shortens the transcellular pathway of particles across the M-cell cytoplasm to be process by dendritic cell (Ermak and Giannasca, 1998) and set off further to the lymphatic circulation located beneath. Thus, particles acquired a shorten time to reach the lymphatic circulation compared to the other process. Moreover, particles were able to be accessed to M-cells without any M-cells targeting (Brayden and Baird, 2001). Thus, 1A formulation likely obtained a fast permeation by these processes. Apart from M-cell process, it has been suggested that aluminium absorption could occur as well by the paracellular route

(Aspenstrom-Fagerlund et al., 2009) which might also be the possible rapid tract of transportation for 1A formulation to the serosal side. As a result, permeation of 1A formulation was ranked the second in all tested formulations. According to the free JE released from permeated particles, it was found that the slight amounts of less than 7.5% of JE were observed from all formulations as shown in Fig 4.7B. Regarding to JE released from conjugated particles, it was evident that the release of 1A was the slowest among all as the released JE which obtained the negative charge could encounter with positive charge of $Al(OH)_3$ resulting in the lowest amount of release. However, the amounts of JE released from permeated particles were only a little and less concerned compared to the percentage of particulate permeation.

Conclusions

The mucosal transported mechanisms were studied by various methods in order to understand the mechanism of particulate transportation via tissues or cells more clearly. The investigation carried out in porcine nasal tissue using particles and fluorescence particles indicated that small size of 1μm particles was more preferred to be taken up and permeated through porcine nasal tissue compared to the larger particles of 5 and 15μm even though the small size was less adhered on tissue surface. The main mechanism of small size transportation was likely by transcellular route. The conjugation of small particles with CS and $Al(OH)_3$ conferred the positively surfaced charge that obviously assisted the adhesion of particles on tissue surface as well as the tissue permeation of both formulations. However, the uptake of CS conjugated particles was surprisingly lower than PLGA particles and $Al(OH)_3$ conjugated particles as CS had been known to aid the transportation by opening the tight junction recognizing as paracellular path which was the more rapid process compared to transcellular path. The result of $Al(OH)_3$ conjugated particles suggested that $Al(OH)_3$ had a major influence on increasing the tissue attachment resulting in the more quantity of particulate uptake mainly by transcellular path compared to PLGA and CS conjugated particles. All formulations showed the viability on porcine nasal tissue of more than 70% approximately according to the applied dose. In vivo study of chosen formulations was underway of examination to evaluate the effectiveness of selected formulations as mucosal vaccine carrier.