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Ex vivo Evaluation in Porcine Nasal Mucosa Of PLGA Exaggerated Mucoadhesive Substances, $Al(OH)_3$ and Chitosan, as Nasal Vaccine Carrier

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Abstract
The mucosal immunization has been widely known to achieve its maximum advantage by the aid of delivery particles as well as mucoadhesive substances. However, an in dept analysis of particles transported mechanism by means of the full ex vivo evaluation in animal tissue model has been lacking even though this provides a vast advantage on the understanding of transported mechanism for the purpose of in vivo formulation screening. The aim of this present research was, therefore, to evaluate the full transported mechanisms which were; uptake, permeation, adhesion and cytotoxicity, of various sizes of poly (D,L lactic-co-glycolic acid) or PLGA particles, chitosan (CS) coated PLGA and $Al(OH)_3$ coated PLGA using porcine nasal mucosa as a model tissue. The uptake calculated by fluorescent intensities and observed by confocal laser scanning microscopy (CLSM) expressed that particulate uptake of particles up to approximate size of 1µm was rapidly taken up by and permeated through porcine nasal mucosa even though the adhesion on tissue surface was relatively lower compared to those of the larger size of 5 and 15µm. The tissue adhesion was improved after the exaggeration of 1µm particles with CS and $Al(OH)_3$ However, higher amount of particulate uptake and permeation of coated particles occurred only with $Al(OH)_3$. CS formulation, on the other hand, showed the converse result as the uptake content was quite low while the permeated content was relatively high. The transported mechanism of CS was mainly by paracellular path, consequence in the lower percentage of particulate uptake but the higher percentage of particulate permeation within the same period of time. Thus, the selected formulations for further study in experimental animal were the formulation of 1µm particles because of its appropriate size to be taken up, the coated formulation of 1µm particles with CS by its excellent properties of tissue adhesion and tissue permeation and the coated formulation of 1µm particles with $Al(OH)_3$ by its great characteristics of tissue adhesion, tissue uptake and tissue permeation. All chosen formulations were considerably non-toxic to porcine nasal tissue.
Key words : Ex vivo, PLGA, Chitosan, $Al(OH)_3$, Porcine nasal mucosa

INTRODUCTION

Most mucosal vaccine experiments, especially nasal vaccine, employ in vivo model rather than ex vivo or in vitro model in order to study the immunological response. Thus, the understanding of mucosal transported mechanisms which are tissue uptake, tissue permeability, tissue adhesion and toxicity on tissue of vaccine carriers has been lacking in which these mechanisms are important to sort the suitable formulation before applying in experimental animal. Few studies on mucosal transported mechanism have been reported. Most of those studies investigated only one or two transported mechanisms which were insufficient to predict the whole picture of mucosal transportation that would be really useful for the in vivo formulation screening. Some research studied the uptake mechanism of particles [1-4] while some research group investigated the permeation efficiency [5, 6]. The cytotoxicity had also been evaluated by many research groups [7-9] whereas several studied only depicted the morphology of cell or tissue involving transportation [1, 10-12]. The tissue adhesion had also been evaluated in some group [13, 14]. However, the understanding of overall mucosal transported mechanisms of any approaches has not yet been apparent. Therefore, in order to distinguish the vaccine formulation before trial in experimental animal and to understand more clearly of mucosal transported mechanism, the complete study of ex vivo evaluation which were the uptake, the adhesion, the permeation and the cytotoxicity of particulate formulation was conducted in this study.

Ex vivo such as nasal tissue derived from some animals have been shown to mimic the in vivo situation at the highest degree of possibility [15, 16]. This ex vivo has also been used as human tissue replacement by the reasons that the supply of human tissue has been continuously short [16] and the procedure to obtain human nasal tissue is relatively troubled regarding to the morphology and physiology of human nasal cavity [17-20]. Therefore, nasal tissue derived from animal could be an alternative approach beyond human nasal tissue.

Immunization of vaccine formulation via nasal route requires vaccine carriers to overcome nasal barriers, especially the mucociliary clearance [21-24]. Carriers that are deposited on nasal mucosa would either be taken up there or removed to the posterior part by mucociliary clearance. Thus, carriers with suitable size and mucoadhesive property to be taken up and to be adhered on tissue surface, respectively, would be more desired.

Studies in various animals have demonstrated the potential of copolymer of lactic and glycolic acid such as poly (D, L-lactic-co-glycolic acid) (PLGA) as vaccine carriers [25-27]. However, most studies of PLGA particle size suitable for mucosal delivery was widely variable in term of particle size range. One study discovered that PLGA particles of 1µm enhanced a great response via both nasal and oral route compared to smaller

particles of 0.2-1µm [28]. The other study from Tabata and coworker showed that the uptake of particles via mucosal route was increased when particles size increased from 0.6 up to 11µm, thereafter, decreased and become zero when diameters were 21µm or larger [29]. Another group discovered that the long lasting antibody titer could generally achieve by 2–8µm particles while particles of < 2µm and 10–70µm could elicit only the lower immune response [30, 31]. It can be seen that the size range of particles were vastly various. Thus, in this study, PLGA particles with a range of particles diameters between 1-20µm were prepared and applied into ex vivo nasal tissue in order to understand the effect of particles size on transportation efficiency before selecting the suitable formulation for in vivo study.

The use of mucoadhesive agents also offers a strategy for the facilitation of residence time as well as the vaccine efficacy [17]. Chitosan (CS) is a biodegradable polymer with mucoadhesive property widely used as transmucosal vaccine delivery by its well-known property of enhancing the permeation by opening the tight junction [32]. Indeed, CS-coated systems such as nanocapsules or nanoparticles showed an important capacity to enhance the mucosal absorption of many model substances such as peptide and protein [33]. Aluminium in the form of aluminium hydroxide, $Al(OH)_3$, is commonly used as an adjuvant in vaccine which could develop high and long lasting antibody titer after primary immunization [34, 35]. Thus, in this study, PLGA particles of selected size was exaggerated with mucoadhesive substances, $Al(OH)_3$ and CS for the purpose of improving the transportation efficiency of particles by rendering the clearance time of particles.

The aim of present study are to evaluate the mucosal transported mechanisms which are, the tissue uptake, the tissue permeability, the tissue adhesion, the tissue morphology involving transportation and the cytotoxicity of various sizes of PLGA, CS coated PLGA, (1C) and $Al(OH)_3$ coated PLGA (1A) particles using porcine nasal mucosa as ex vivo model to monitor and select the suitable formulation before applying in experimental animal.

MATERIALS AND METHODS

Materials

Poly (D,L-lactic-co-glycolic acid), (PLGA) with lactide : glycolide 50 : 50 was purchased from Sigma-Aldrich (Saint Louise MO, USA). 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 use by membrane centrifuged tube (Nanosep®). Polyvinyl alcohol, PVA, (MW. 67,000) was obtained from Fluka chemical (Switzerland). BCA assay kit and Krebs-Ringer bicarbonate buffer (KBR) were purchased from Sigma-Aldrich (Saint Louise MO, USA). Fluorescein-5-isothiocyanate isomer I (FITC) was acquired from Invitrogen (Calsbad CA, USA). The other chemicals were of analytical grade and used as received.

Preparation of FITC-labeled JE

Japanese encephalitis (JE) vaccine was used as a model vaccine and 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.

Preparation and characterization of particles

The fluorescent particles were prepared from PLGA polymer by double emulsion solvent evaporation technique using FITC labeled JE as entrapped material. The optimized parameters used to prepare particles within the size range of 1-20µm are shown in Table 1. These sizes were chosen as a representative of small, medium and large size of particles within the range of 1-20µm. Briefly, the primary w/o emulsion was prepared by adding an appropriate amount of FITC-labeled JE into 8 ml of 5%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 was added to the primary emulsion and the mixture was then sonicated by either bath sonicator or probe sonicator to form particles of different sizes. The double emulsions were afterward diluted in 100 ml of 1% PVA and then stirred up to 3 hours at 500 rpm in order to eliminate the solvent. The resulting particles were collected by centrifugation at 10,000g for 5 minutes. The 1C particles were prepared by adding 0.2% CS [36] into 1% PVA of the final process of dilution of 1µm particles formulation. The 1A particles were prepared by adding a required amount of $Al(OH)_3$ into the sediment of the centrifuged particles of 1µm formulation by 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. $Al(OH)_3$ added into all formulations was calculated by the maximum limit of 0.85mg for one volume of single human

dose. The entrapped material in this study was FITC-labeled JE for uptake experiment and purified JE for the rest of the experiment.

Table 1 Optimized parameters to prepare the selected sizes of PLGA particles

Approximated size of particles (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 formation
[2] Volume ratio of water : oil in primary w/o emulsion formation
[3] Volume ratio of water/oil : water of secondary (w/o)/w emulsion formation
[4] %PVA of secondary emulsion

The size and size distribution of particles were observed under a particle size diffractometer (Mastersizer 2000, Malvern, UK) in order to confirm the desired size of particles. The morphology and fluorescence property of FITC-labeled JE loaded particles were determined by optical/fluorescence microscope (OM/FM microscope; E200, Nikon Eclipse, Japan) and confocal laser scanning microscope (CLSM, Olympus model FV 1000, Olympus, Germany) which was also exploited to observe the fluorescent intensity.

Porcine nasal mucosa preparation

Porcine nasal mucosa was prepared according to the method of Östh [3, 37] with slightly modification. In brief, nasal respiratory mucosa was removed from conchae within 5 minutes after slaughter at the certified slaughter house. Firstly, the snout was separated from the pig and was opened up to expose the conchae. After that, the posterior cavity tissues were carefully removed from conchae using forceps and scalpel, giving two pieces per snout. Then, tissues were immersed and swung in 250ml, 8°C 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 particles in porcine nasal mucosa Uptake study

Uptake study was evaluated by CLSM according to the method from Östh [3] and Smyth [2] with modification. Porcine nasal tissues of 1x1 cm were incubated with formulations of FITC-labeled JE loaded particles for 20 and 90 minutes. Control tissues were incubated with PBS. After incubation, tissues were washed by PBS to exclude the excess formulations and then fixed by 3% paraformaldehyde for 30 minutes. Subsequently, fixed 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 tissue sections of each formulation at each time point were monitored and photographed by the same objective lens with magnification of 20 in order to observe the fluorescent intensities of formulations taken up by tissues. The fluorescent intensities of formulations were also observed as references. Three photographs obtained from three tissue sections of three different animals were employed to calculate the average fluorescent 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 intensity of formulation itself. The percentage of uptake could be calculated using the following equation:

$$\text{Percentage of uptake} = \frac{AIt}{AIf} \times 100 \quad \ldots\ldots\ldots\ldots\ldots\ldots (1)$$

Where *AIf* represents the average fluorescent intensity of formulation

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

Tissue adhesion study

The mucoadhesive property of formulations on porcine nasal tissue was evaluated according to the method described by Harikarnpakdee [14] with modification. A freshly cut porcine nasal tissues were cleaned by rinsing with PBS. After that, the accurate amount of each formulation was applied onto a nasal tissue fixed on the petridish glass that slanted at an angle of 40° relatively to the horizontal plane. Then, PBS buffer was peristaltically flushed over nasal tissue surface at a rate of 6 ml/min. The amount of particles in washed solution at 0, 5, 10 and 30 minutes was determined by BCA assay with UV-VIS spectrophotometer (Jasco V-530, Schimidzu, Japan), at 560 nm to detect JE entrapped inside particles. The washed particles were demolished to obtain JE prior to UV determination. The tissues incubated PBS were served as blank in order to eliminate the interference of washed protein from tissues itself and the results shown has already been corrected by the blank.

Morphology of tissue surface involving transportation

Morphological evaluation of tissue surface involving transportation was determined by scanning electron microscopy (SEM, 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, following by distilled water and then, dehydrated three times by a gradient series of 30%, 50%, 70%, 90% and 100% alcohol, respectively. The critical point dryer was used to dry tissues. The tissues were then coated by a layer of gold and observed under scanning electron microscopy. The photographs of surface morphology were subsequently taken.

Cytotoxicity study

Cytotoxicity was determined by MTT assay as previously described by Wadell [19]. MTT was dissolved in PBS to the concentration of 2 mg/ml and stored in the dark 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 600 µg/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. 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 then extracted using DMSO. The absorbance was determined by a microplate reader (VICTOR^3, Perkin Elmer, USA) at 595 nm. The experiment was performed in triplicate.

Permeation study

Permeation study was performed by modified Franz diffusion cells. The receptor compartments were 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. One donor compartment was filled with PBS as reference. The withdrawn buffer was replaced with the same amount whenever it was sampled. The amount of permeation was determined by UV-VIS spectrophotometer at 560 nm. The amount of JE that was released from particles during the whole permeation time was also evaluated. The permeated particles were demolished by the solution of DMSO and SDS before the JE measurement by UV-VIS spectrophotometer using BCA assay kit as a color complex formation.

RESULTS AND DISCUSSIONS

Characterization of FITC-labeled JE loaded particles and purified JE loaded particles

The average particle size and uniformity of three selected FITC labeled JE loaded particles were 0.80(0.36), 7.96(6.47) and 17.18(0.77) µm while the particles size and uniformity of purified JE loaded particles were 0.92(0.27), 5.26(1.57) and 16.76(2.03) µm, respectively. These sizes represented the small, medium and large size of PLGA particles within the size ranges of 1-20µm. After coating the 1µm formulation with CS and $Al(OH)_3$, the effect of coating materials was obvious for $Al(OH)_3$ as particles were aggregated as a cluster as shown in Fig 1 while the characteristic of CS coated particles was relatively resemble to 1µm particles. The aggregation of 1A particles could probably occur by the adsorption property of $Al(OH)_3$ that formed a fine network around particles holding them together as a cluster [38]. The pictures taken by OM revealed the morphology of spherical particles without fluorescence property. FM expressed the same spherical shape of particles as shown in OM, but with the fluorescent properties. CLSM also revealed the particles of the same size as illustrated in OM and FM with the fluorescence properties.

Ex vivo evaluation of particles in porcine nasal mucosa Uptake study

The results of CLSM revealed the characteristic of particles inside tissues at 20 and 90 minutes, and the extent of particles taken up as shown in Fig 2-4. According to Fig 2, large amount of 1µm formulation (47.98%) was able to be taken up by nasal tissue at 20 minutes compared to those of 5 and 15µm formulations (5.02% and 14.43%). 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 5.02% to 5.51%, and declined from 14.43% to 9.34% for 15 µm particles, respectively as illustrated in Fig 2.

Thus, particles of approximate diameter 1µm were more prefer to be taken up by porcine nasal tissues compared to 5 and 15µm at both time points according to this study. It was relatively obvious that particles of 5µm or larger was less prefer to be taken up by nasal tissues compared to the smaller size in which these results were corresponded to the finding of Eldridge [39] who studied the effect of particles of less and more diameter than 5µm but using the tissue of gut as a model tissue. Eldridge found that the majority of particles in diameter of <5µm were transported to the efferent lymphatics more efficiently than the larger sizes of >5µm which corresponded to the result of size in this study.

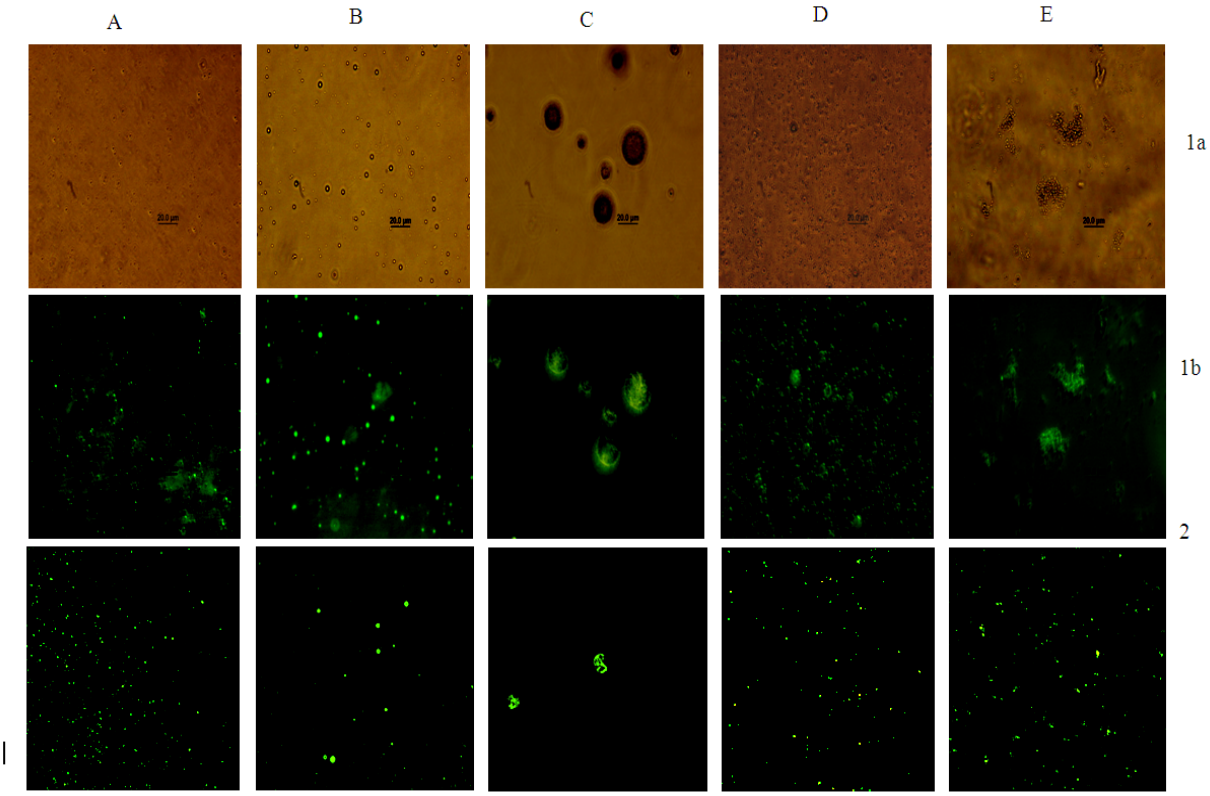

Figure 1 Morphology and fluorescence property of 1µm (A), 5µm (B), 15µm (C), 1C (D) and 1A (E) particles by optical/fluorescence microscope and CLSM (1a/1b and 2), respectively (objective x20).

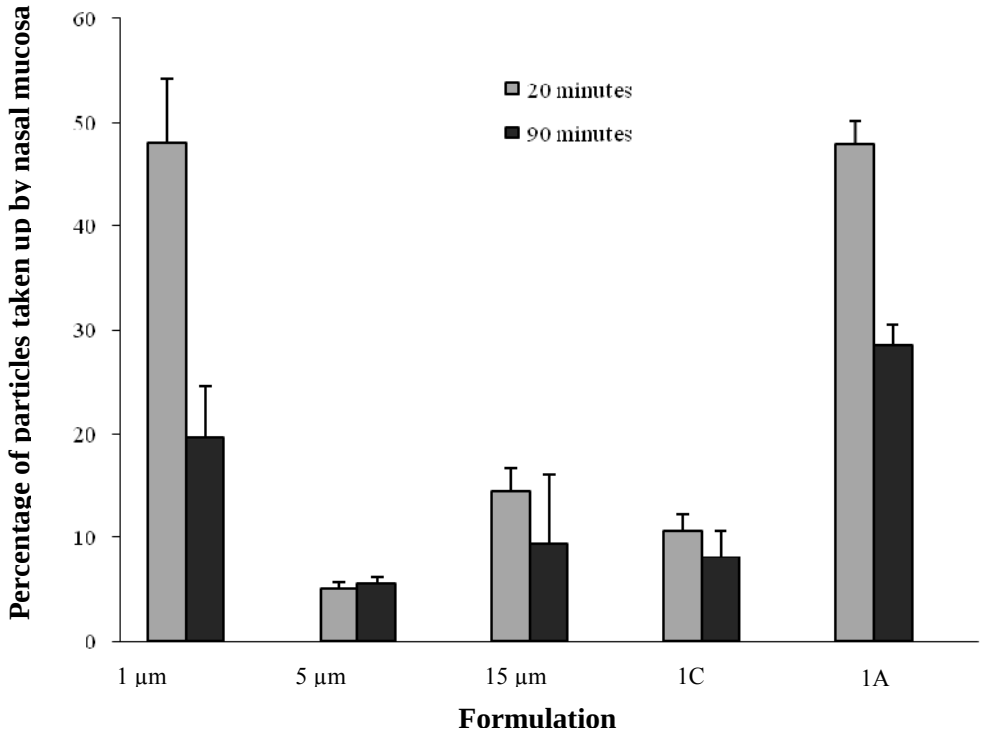

Fig 2 Percentage of particles taken up by porcine nasal mucosa of 1µm, 5µm, 15µm, 1C and1A particles at incubation time of 20 and 90 minutes. Data are represented as mean + S.D (error bar)

The transportation of particles depends on many factors such as particle size and transportation time. According to this present study, a large number of particles at all sizes, especially 1µm, were taken up by nasal tissues at 20 minutes and less number were taken up at 90 minutes which corresponded to the review of Smyth [2]. The review from Ermak [40] also showed the effect of time but with the oral route and found that by 30-45 minutes, particles administered orally were accumulated in the mucosal associated lymphoid tissue and after 90 minutes, large numbers of particles had already been traveled to the underlying region.

According to the characteristics of 1µm particles after taken up as shown by CLSM in Fig 3-4, it was found that 1µm particles taken up by tissues were grouped together in an irregular shape and scattered inside tissues at both 20 and 90 minutes. Generally, the uptake process of particles happens by phagocytosis which is the subtype of endocytosis. The endocytosis processes are described by the pinching of membrane vesicles from the plasma membrane following by the internalization of engulfed extracellular materials [41]. The internalized phagosome sometimes possibly contains several particles in which these particles could be observed as a particulate cluster. Then, the phagosome subsequently fuses with the basolateral membrane, emptying its contents into the intraepithelial pocket. After that, particles may then be engulfed by phagocytic cells in the pocket or pass through the basal lamina into the sub-epithelial region [40]. Thus, the particles appeared as the particulate cluster might be the results of the multiple particles taken up by phagosome.

The particles of 5 and 15µm appeared inside tissues at both 20 and 90 minutes as shown in Fig 3-4 were, nevertheless, not with an actual size of 5 and 15µm. This occurrence was due to some extends of small particles that included in 5 and 15µm formulations which could possibly be taken up by tissue beyond the precise diameter of 5 and 15µm and appeared as a small scattered group of particles. However, a particle of actual size of 5µm could be observed inside tissues at 90 minutes as shown in Fig 4.

Although size is an important parameter in particulate uptake, other characteristics such as surface property also influence the extent and the distribution of particles taken up by nasal tissues. As present in Fig 2, the amount of CS coated particles (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. These percentages were even lower than the percentage of 1µm particles taken up by nasal tissues at both time points. The characteristic of 1C inside tissues, on the other hand, was relatively similar to 1µm formulation at both 20 and 90 minutes as shown in Fig 3 and 4, respectively. This possibly happened by the following reasons.

Regarding to the transported mechanisms of particles, different pathways could be discerned which are paracellular and transcellular or endocytotic paths. Transcellular path is considered as the core pathway of mucosal transportation since paracellular spaces contribute only less than 1% of the total mucosal surface area [41]. Even though the attention of transportation is drawn to the transcellular process, paracellular process has its own advantage on which it could be enhanced by some polymers via several processes such as the charge mediated mechanism [41], the contraction of perijunctional actinomyosin ring, or the protein kinase/phosphatase-mediated changes in tight junction protein phosphorylation [42] ,that results in a structural reorganization of tight junction composition which improve the mucosal transportation. Thus, particles that could be transported via paracellular path such as hydrophilic particles may devour less time to reach a basement membrane compared to transcellular path as the forever process of taken up is less complicated. Chitosan (CS) can be considered as hydrophilic and non-antigenic polymer [43] that normally obtains an ability to enhance the paracellular transport by opening the tight junctions and reduce TEER values by means of charge mediated mechanism [18, 44, 45]. Obviously, in this study, the low amount of 1C taken up into nasal tissues at 20 and 90 minutes could possibly be attributable to the transported mechanism by paracellular path which was more favored for CS and was accordingly occurred before the time point of 20 minutes resulting in the low amount of particles retained inside tissues.

According to 1A, the percentage of particles taken up by nasal tissues expressed in Fig 2 was relatively equal to 1µm formulation at 20 minutes and higher than 1µm formulation at 90 minutes. The 1A particles characteristic at both time points was appeared as a group of particles scattered inside tissues with some extend adhered on tissues surface as could be seen in Fig 3-4. The characteristic of 1A inside tissues was quite alike to 1µm and 1C formulation, except that 1A could partly be adhered on tissue surface. The depth of penetration was not that deep since some extends of 1A remained adhere on tissue surface as shown in Fig 3 and 4.

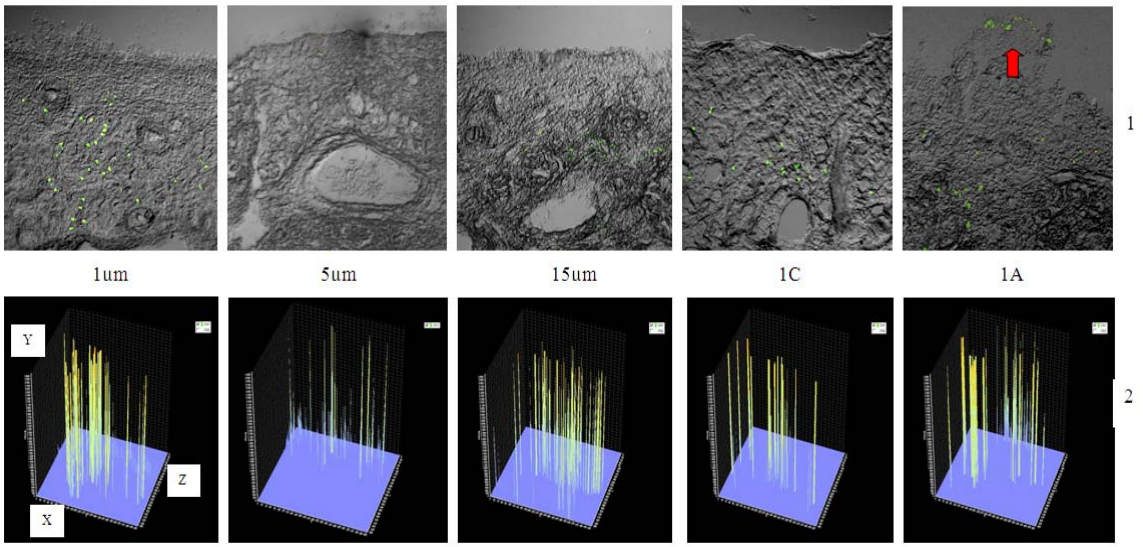

Figure 3 Representative confocal laser scanning micrographs of particulate characteristics (1) and fluorescence intensities (2) after taken up by porcine nasal mucosa of 1µm, 5µm, 15µm, 1C and 1A particles; incubation time 20 minutes (objective x20); red arrow indicate the tissue surface adhesion of 1A particles; y-axis of three dimensional graphs represent the fluorescence intensities while x and z axis represent the area of tissue

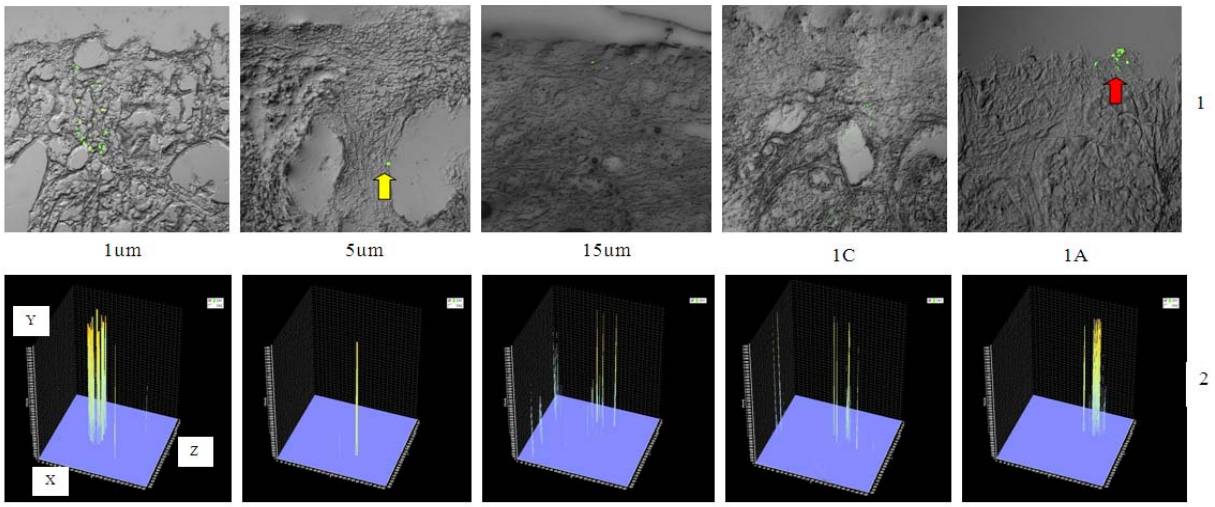

Figure 4 Representative confocal laser scanning micrographs of particulate characteristics (1) and fluorescence intensities (2) after taken up by porcine nasal mucosa of 1µm, 5µm, 15µm, 1C and 1A particles; incubation time 90 minutes (objective x20); red and yellow arrows indicate the tissue surface adhesion and tissue uptake characteristic of 1A and 5µm particles, respectively; y-axis of three dimensional graphs represent the fluorescence intensities while x and z axis represent the area of tissue

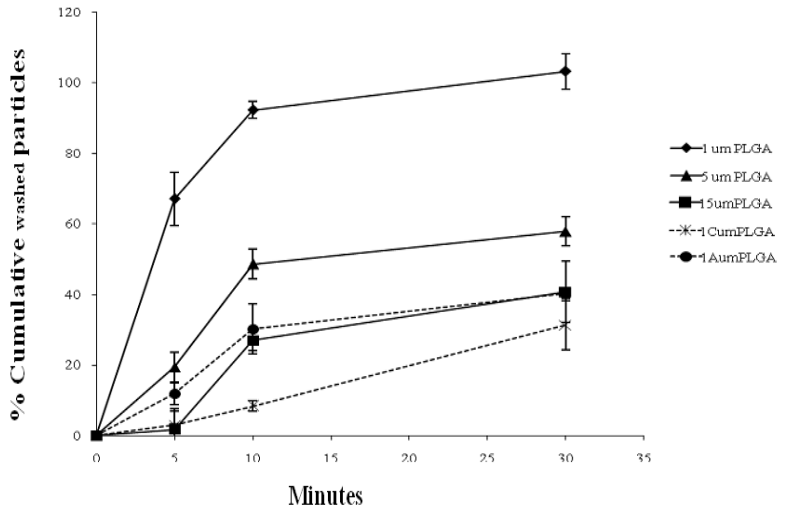

Figure 5 The percentage of washed particles represents the adhesion property of particles on porcine nasal mucosa of 1µm, 5µm, 15µm, 1C and 1A particles. Data are represented as mean ± S.D. (error bar)

It was relatively obvious that the amount of 1A on tissues surface was due to the effect of mucoadhesive property of $Al(OH)_3$. The interaction between $Al(OH)_3$ and tissue surface could either be by electrostatic interaction or ligand exchange between $Al(OH)_3$ and mucus protein [46, 47]. At pH values of less than an isoelectric point of $Al(OH)_3$, $Al(OH)_3$ presents positive charge such as in the pH 6.8 of nasal secretion, which assists the adhesion of $Al(OH)_3$ on tissues surface. Beside the electrostatic interaction, adsorption could also be occurred by ligand exchange between accessible phosphate groups of mucus protein and surface hydroxyl groups of $Al(OH)_3$. Moreover, $Al(OH)_3$ could promote the attachment on tissues surface by generating a fine network around the delivery particles holding them together as a depot on tissue surface [38, 48]. Thus, these are the reasons for the good tissue adhesion property of 1A. The uptake mechanism of 1A into tissues was probably happened by both transcellular and paracellular pathway. The study from Aspenstrom-Fagerlund [49] advocated that aluminium (Al) absorption could occur mainly by paracellular pathway. Moreover, several mechanisms also play an important role in aluminium uptake such as the energy-dependent transport involving Ca-ATPase [50] and some other-essential-metals channels [51, 52]. These possible pathways have gained $Al(OH)_3$ formulation more channel to be taken up beyond the only pathway of particulate endocytosis or paracellular process. The unique characteristic of $Al(OH)_3$ could prolong the residence time of particles on tissue surface in order to obtain a better opportunity to be taken up by tissue.

Tissue adhesion study

The adhesion property of each formulation was illustrated by the percentage of washed particles from tissue surface over time. The high percentage of washed particles represents 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 5. The percentage of washed particles was dramatically high for 1µm formulation compared to all other formulations at all points of time and reached 100% by 30 minutes. In contrast, the percentages of washed particles of 5 and 15µm at the first five minutes were less than 20% for both formulations and gradually increased to only 57.82% and 40.68%, within 30 minutes, respectively. According to these results, the adhesion property of particles could be ranked 15µm > 5µm > 1µm, respectively. Even though the small size of 1µm did not adhere very well as 5 and 15µm, the results of tissue uptake was relatively satisfied since the size of 1µm was the most preferable size to be taken up by tissue according to the uptake study.

Mucus is an adhesive gel that covers and protects mucosal surfaces. The foreign particles are normally trapped inside mucus layers by either steric obstruction or mucosal adhesion. Trapped particles are typically removed from the mucosal tissue by mucosal clearance, thereby strongly limiting the duration of drug delivery locally [53]. The transport of particles via mucus is relatively complex and controlled by mucus layer as well as particles size, charge and surface wet-ability of particles. The mucus adhesion of particles could be counted as size dependent phenomena [54]. The adhesion between particles and mucus is probably recognized by analyzing the hydrodynamic drag forces (*f*) that required for moving particles through a continuous liquid according to Stokes derived equation as shown in equation 2.

$$f = 6\pi\mu r \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)$$

where f represented hydrodynamic drag force coefficient, r is hydrodynamic radius of the diffusing molecule or particles and µ represents a medium viscosity [55].

Regarding to the drag force coefficient, it was variable directly to the particle size. As a consequence, large size of particles appeared to be adhered on mucosal surface for a longer period of time compared to small particles.

Upon coating with CS, the adhesion property of 1µm particles was not only increased but also superior than 15µm particles as shown in Fig 5. The percentage of washed particles of 1C at the first 5 minutes was only 2.93% and rose slightly to 31.28% at 30 minutes which represented the excellent adhesion efficiency of chitosan formulation on tissues surface. After coating with $Al(OH)_3$, the adhesion property of 1µm particles was also elevated until it was comparable to adhesion property of 15µm particles by 10 minutes. The washed particles of $Al(OH)_3$ reached 40.15% at 30 minutes which relatively equal to the percentage of 15µm formulation. Thus, coating particles with CS and $Al(OH)_3$ obviously improved the adhesion property of particles and the effect of CS was more stated than $Al(OH)_3$ according to the tissue adhesion characteristic.

Most of vaccine delivery vehicles are generally prepared by either hydrophobic materials such as PLGA [28] or cationic materials such as polyethylenimine [53], chitosan [32] 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 the advantage of each material. For the formulation of 1C and 1A, both had the characteristics of hydrophobic from PLGA and electrostatic from cationic coating materials of chitosan and $Al(OH)_3$ which could be very well adhered on tissue surface by polyvalent adhesive interaction. Polyvalent adhesive interaction is considered as the primary mechanism of mucus adsorption in that the mucus could efficiently trapped the particles [53, 54]. As a result, the adhesion of 1C and 1A were more pronounced than 1µm formulation. However, the amount of washed 1C particles was less than 1A as a consequence of the higher permeation of 1C that moved most particles through tissue at any short specific time compared to 1A particles.

Tissue surface involving transportation

According to the tissues transportation of different size and surface property of particles, it was observed that there was none of any 1µm particles found on tissue surface after the transportation as shown in Fig 6 since the uptake property of 1µm was excellent but the adhesive property of 1µm particles was very deprived as aforementioned in the above section. Thus, the particles of 1µm size could not be seen on tissues surface. Regarding to 5 and 15µm transportation, a great number of 15µm particles were observed on tissue surface while 5µm formulations was hardly noticed. These results corresponded to the results of uptake and adhesion study that the particles of 5µm or larger, such as 15µm, were non-favorable to be taken up but more prefer to be adhered on nasal surface. Thus, they could easily be observed by SEM.

Even though, all SEM pictures in this study did not clearly express the depiction of M-cells or the non-ciliated cells with membrane protrusion [56, 57], these SEM pictures obviously showed the different types of cell which were long-ciliated and short-ciliated microvillus cells in all tissues transported formulations which was corresponded to the fact that total of 15–20% of respiratory cells were covered by long cilia of size 2–4 mm [20]. The particles of specific size were shown to adhere on tissues surface which could be concluded that porcine nasal tissue composed of various cell types in which the particular size of larger than 5µm preferably adhere on tissue surface.

The coating material, CS, influenced the morphology of tissues surface involving transportation as shown in Fig 6. 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 incubation time as with 1µm particles. This was because 1C had advantage over 1µm particles by an excellent adhesion property which could render the clearance time and enhanced the particles transportation. In the case of 1A, the characteristic of cilia was shown in Fig 6. 1A formulation was detected as white materials that coated on the cilia of tissues. They could be seen easily on tissues surface. These results corresponded to the results of adhesion study of 1A that 1A had an excellent adhesion characteristics on tissue surface.

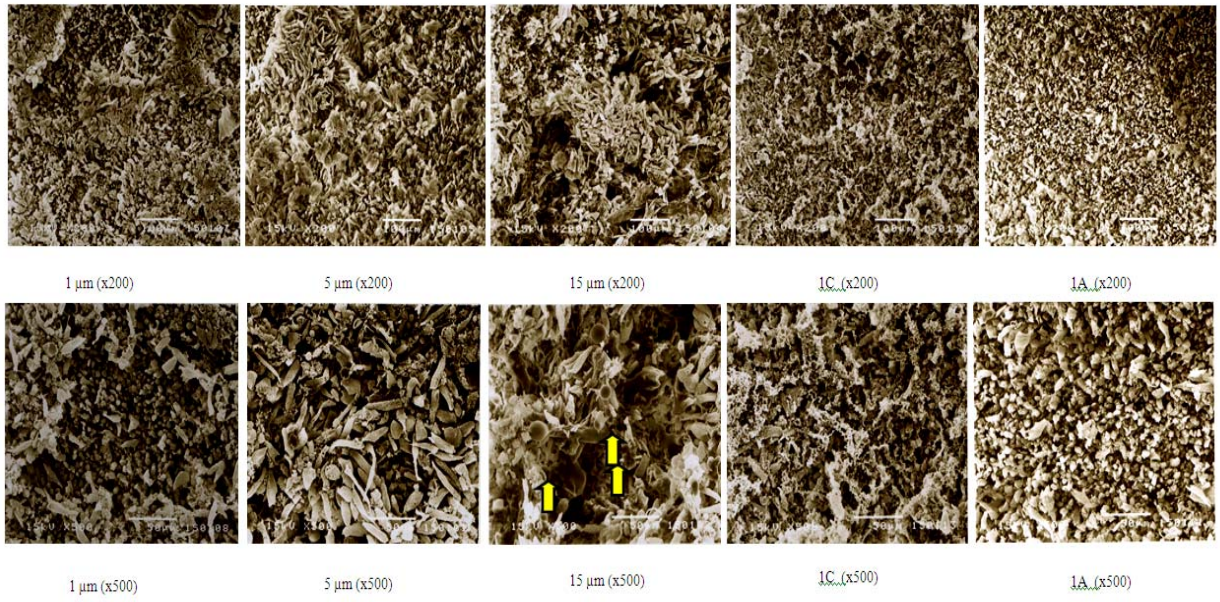

Figure 6 Morphology of porcine nasal mucosa involving transportation of 1 µm, 5 µm, 15 µm, 1C and 1A particles by SEM technique (Magnification x200 and x500); yellow arrows show the particles of 15µm in diameter

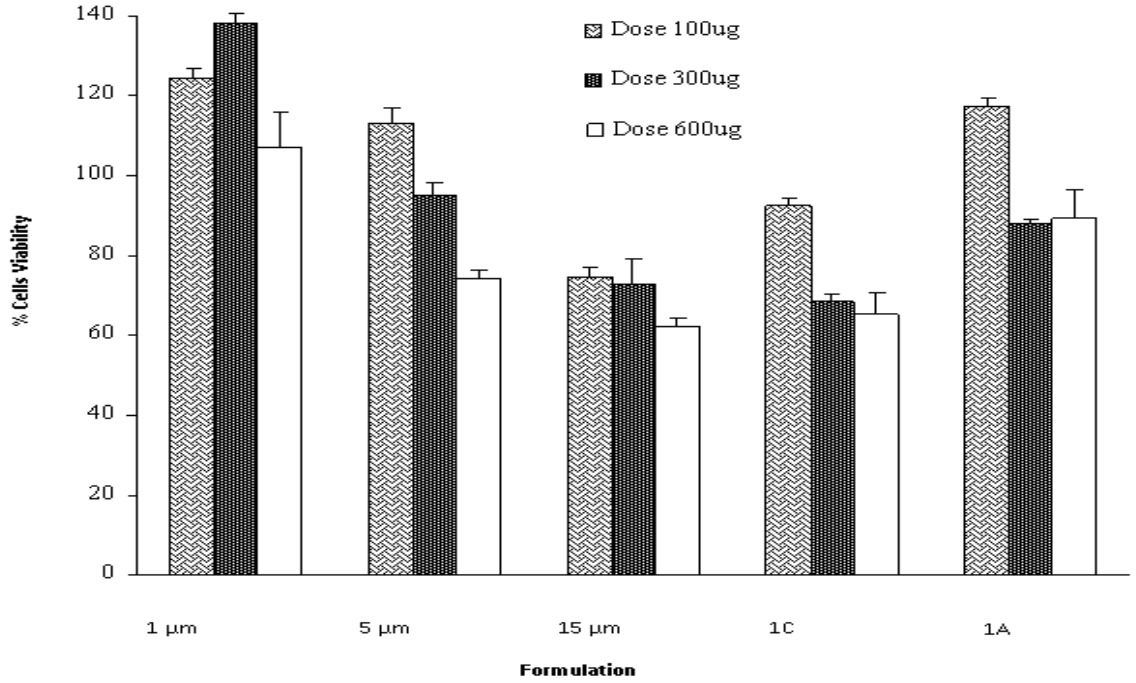

Figure 7 Cells viability of porcine nasal mucosa incubated with 1µm, 5µm, 15 µm, 1C and 1A particles. Data are represented as mean + S.D. (error bar)

Cytotoxicity study

The results of cell viability that represent the cytotoxicity of each formulation on viability of cells was shown in Fig 7. The high percentage of cell viability indicates the low toxicity of formulation to cells. The result revealed that the cell viability of tissues incubated by each formulation could be ranked 15µm < 5µm < 1µm, respectively, in regard with the same dose. According to different dose, higher dose tended to result in lower viability as illustrated in Fig 7. Cell viability is normally affected by many factors such as organic solvent or some of other toxic chemicals. According to organic solvent residue left in particles prepared by solvent evaporation method, the amount of organic solvent residue in large particles was higher than small particles due to the slower evaporation rate of solvent of large particles that contained less surface area. Thus, the organic solvent residue could be ranked 15µm >5µm >1µm, respectively. As a result, the large size of particles appeared to be more toxic by the more solvent residue compared to small size particles.

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 equal dose of formulations. However, the toxicity of 1A was not that obvious as 1C formulation. This tendency could be determined at all doses of formulations. The results of dose corresponded to the study of Prego [33] who identified the toxicity of CS coated nanosystem and found that the cell viability was ranged from 60%-80% according to the dose from 606-152µg/cm^2, respectively. The low percentage of cell viability was possibly due to the interaction between cationic molecules of CS and $Al(OH)_3$ on cell membranes and on cell membranes of cilia [58] which affected the ciliary beat frequency that caused the cytotoxicity of cell or tissue. Additionally, cytotoxicity was associated with an irreversible destruction of tight junctions of the epithelial cells which might be considerably caused by the acidic effect of CS formulation [59].

Permeation study

For the particulate permeation, about 10%-15% of 1, 5 and 15µm particles were permeated through porcine nasal mucosa at 0.5 hours and were increased gradually as shown in Fig 8A. The rate of particulate permeation could be ranked 5µm > 15µm > 1µm, respectively. The rates of 5µm and 15µm formulations were relatively comparable while the permeation of 1µm formulation was slightly slower. For particles of approximately 1µm in size, most particles were taken up by endocytosis pathway into the inside tissue [16, 60] and merely a quantity of particles were permeated through the tissue. Thus, the results of permeation corresponded to the results of uptake in that 1µm was retained inside the tissue and merely permeated while 5 and 15µm permeated more. However, the permeation of 5 and 15µm was

possibly by the great tissue adhesive property and by the uptake of specific cell type that preferred to uptake large microparticles rather than small particles such as M-cell [61, 62]. Since the basolateral surface of M-cells invaginated and formed the intraepithelial pocket that could effectively shortens the transcellular pathway of particles across the M-cell cytoplasm [40, 63], this shorten pathway was a possible results of the fast permeation of large particles

The permeation of 1C and 1A were superior than 1µm at all points of time, especially for 1C. The permeation of 1C reached 70.86% within 4 hours while 1A achieved 48.26% at 4 hours. For CS, the pathway of transportation could either be the paracellular pathway or the transcellular pathway, but preferably for the paracellular pathway. CS exerts an effect on opening tight junctions to increase the permeability of cells similar to that shown for the intestinal epithelium [17] and nasal cells [14] resulting in a high permeability of particles. This could be the reason for the low uptake amount but the high permeation of CS formulation.

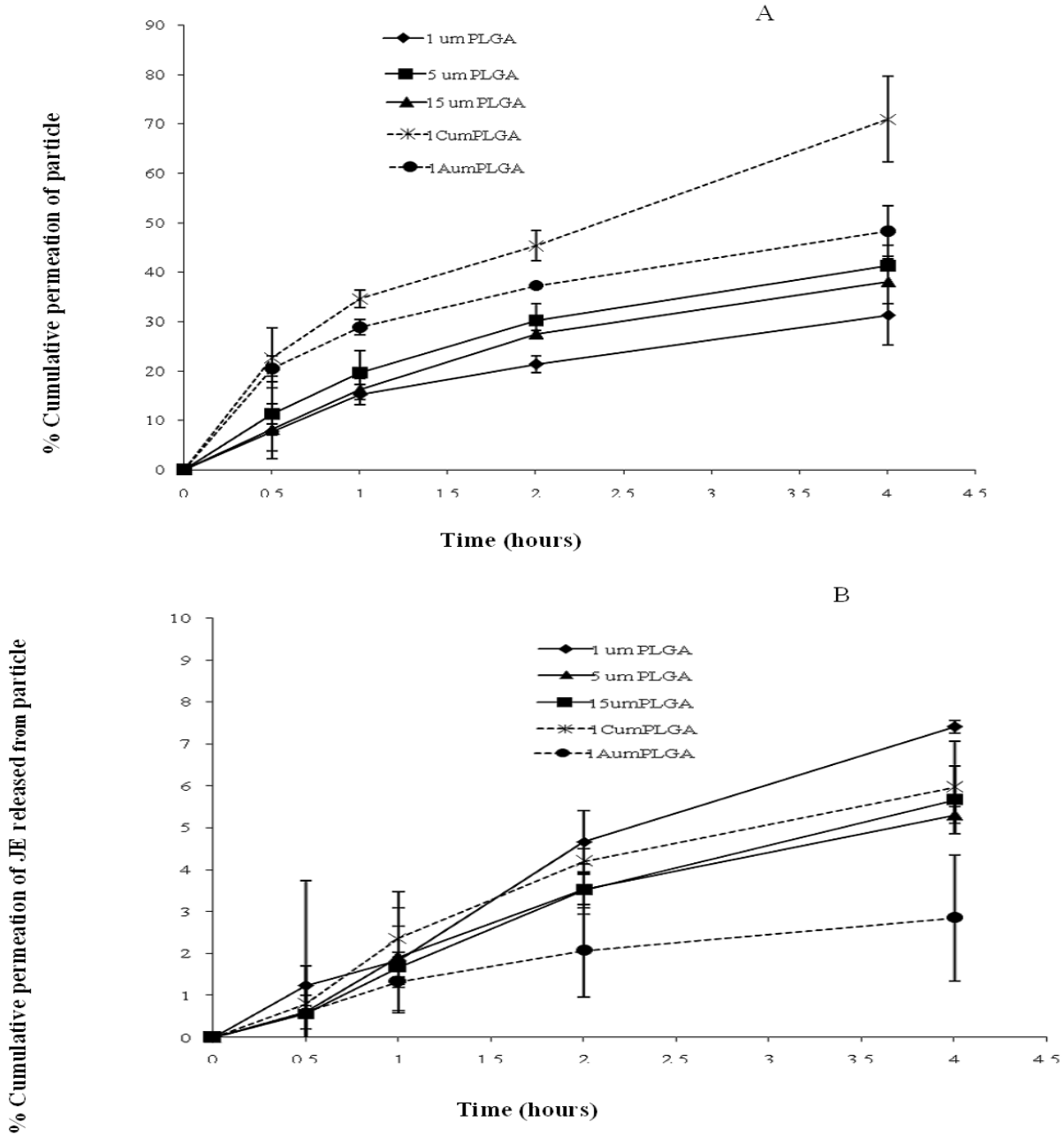

Figure 8 The permeation profile of particles (A) and JE released from particles (B) of 1µm, 5µm, 15µm, 1C and 1A particles using porcine nasal mucosa as permeated membrane at any specific points of time. Data are represented as mean ± S.D. (error bar)

According to $Al(OH)_3$, the gel forming-liked structure of $Al(OH)_3$ embraces a high viscosity that increases the contact time between particles and the nasal mucosa thereby increasing the time for permeation. At the same time, highly viscous formulation interferes the normal function such as mucociliary beating and thus enhances the permeability of drugs by decreasing the mucociliary clearance [64]. Additionally, it has been suggested that aluminium absorption could occur as well by the paracellular route [49]. As a result, permeation of 1A formulation was ranked as the second of all tested formulations.

According to the free JE released from particles during the permeation experiment, it was found that the slight amounts of less than 7.5% of JE were observed from all formulations at 4 hours as shown in Fig. 8B. Regarding to JE released from coated particles, it was evident that the release of 1A was the slowest among all. The JE released from particles would encountered the positive charge of $Al(OH)_3$ resulting in the incomplete release of negatively charged JE. However, the amounts of JE released from permeated particles of other formulations were only in a small amount and less concern compared to the percentage of particulate permeation.

CONCLUSIONS

All four evaluations of uptake, adhesion, permeation and cytotoxicity are essential to be evaluated in order to understand the transported mechanisms more clearly. In the context of biological product or particles that the main transported mechanism is the transcellar, the uptake and adhesion studies are highly recommended and should be together evaluated. In this study, the results of uptake and adhesion were correspondent in which the particles that appeared more inside tissue was less observed on tissue surface. In the context of soluble drug, hydrophilic substances, toxic substances or any other substances apparently transported via paracellular path, the evaluation of permeability as well as cytotoxicity should be together investigated since the cytotoxicity has a huge impact on tissue structure leading to the permeation of substances via paracellular path. The results of permeability and cytotoxicity of all formulations in this study were relatively relational in that the high toxic formulation tended to have the higher permeation efficiency.

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