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Uptake of antigen encapsulated in polyethylcyanoacrylate nanoparticles by D1-dendritic cells

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Polyethylcyanoacrylate (PECA) nanoparticles were prepared by interfacial polymerization of a water-in-oil microemulsion. Nanoparticles were isolated from the polymerization template by sequential ethanol washing and centrifugation. A nanocapsule preparation yielding the original particle size and distribution following redispersion in an aqueous solution was achieved by freeze-drying the isolated nanoparticles in a solution of 5% w/v sugar. The cytotoxicity and uptake of nanocapsules by dendritic cells was investigated using a murine-derived cell line (D1). PECA nanoparticles were found to adversely effect cell viability at concentrations greater than 10 μg/ml of polymer in the culture medium. In comparison to antigen in solution, cell uptake of antigen encapsulated within nanoparticles was significantly higher at both 4 and 37 °C. Following a 24 h incubation period, the percentage of cells taking-up antigen was also increased when antigen was encapsulated in nanoparticles as compared to antigen in solution. The uptake of nanoparticles and the effect of antigen formulation on morphological cell changes indicative of cell maturation were also investigated by scanning electron microscopy (SEM). SEM clearly demonstrated the adherence of nanoparticles to the cell surface. Incubation of D1 dendritic cells with nanoparticles containing antigen also resulted in morphological changes indicative of cell maturation similar to that observed when the cells were incubated with lipopolysaccharide. In contrast, cells incubated with antigen solution did not demonstrate such morphological changes and appeared similar to immature cells that had not been exposed to antigen.

1. Introduction

A prerequisite for the induction of an immune response to an antigen or pathogen is that the antigen/pathogen is first taken up (endocytosed) and processed by specialised antigen presenting cells (APCs). Following processing, the antigens are displayed as peptide fragments on cell surface molecules (MHC molecules), together with other co-stimulatory molecules, to naive T cells (Banchereau and Steinman 1998). During the process of antigen-uptake and processing, APCs become activated and undergo morphological changes from cells able to efficiently sample and take up antigen to cells that are efficient at presenting antigenic epitopes to activate T cells.

APCs include macrophages, B cells and dendritic cells (DCs). Over the last decade or so, the importance of DCs in the initiation of an immune response has become increasingly recognised. DCs now have the status of being the most potent APCs, essential for the initiation of the immune response with an extraordinary capacity for inducing differentiation of naive CD4+ or CD8+ cells into helper and cytotoxic T cells respectively in both primary and secondary T cell responses (Banchereau and Steinman 1998; Hart 1997; Regnault et al. 1999).

DCs are present in extravascular tissues in an "immature" state. In this state, DCs are extremely efficient at antigen uptake and processing but display insufficient co-stimulatory molecules for T-cell activation (e.g. CD80 and CD86). Exogenous (for example, microbial components) or endogenous (inflammatory cytokines) molecules provide "danger signals" necessary to induce DC maturation and migration out of the inflammatory site into the lymphatics. Mature DCs are no longer able to capture antigen but have an increased capacity for antigen presentation (Banchereau and Steinman 1998; Bell et al. 1999; Bhardwaj 2001; Cella et al. 1997).

The uptake of particulate antigen delivery systems by APCs has been investigated both *in vivo* (Alpar et al. 1989; Jani et al. 1990; LeFevre et al. 1989) and *ex vivo* using cell cultures or cell lines. Using cell cultures/cell lines of APCs often allows better and more ready assessment of the interaction of particles with these cells as it removes the complexities of the biological environment. However, to ensure that data of *ex vivo* cell cultures/cell lines can be extrapolated back to the *in vivo* situation, the selection of the cell culture model for investigating particulate uptake must be appropriate.

Most studies investigating the uptake of particulate antigen delivery systems by APCs have used macrophages (Ahsan

et al. 2002; Chavany et al. 1994; Denis-Mize et al. 2000; Pinto-Alphandary et al. 1994; Roser et al. 1998; Schafer et al. 1992; Thiele et al. 2001; Torche et al. 1999; Whyte et al. 2000; Zhang et al. 1998). However, despite having a role in inducing and regulating the immune response, macrophages, unlike DCs, are incapable of stimulating naive T cells (Inaba et al. 1990; Thiele et al. 2001).

Owing to the increasingly recognised importance of dendritic cells in the induction of the immune response, the *ex vivo* uptake of particulate antigen delivery systems by these cells has recently also received some attention (Copland et al. 2003; Denis-Mize et al. 2000; Reece et al. 2001; Scheicher et al. 1995; Thiele et al. 2001; Thiele et al. 1999).

DC cultures, suitable for assessing particle uptake, can be obtained by culturing and differentiating cells isolated from humans (Copland et al. 2003; Reece et al. 2001; Thiele et al. 2001; Thiele et al. 1999) or animals (Denis-Mize et al. 2000; Scheicher et al. 1995). Such cultures are termed primary cultures and generally comprise a heterogeneous population of cells, which are at various stages of maturation and differentiation (Pizzoferrato et al. 1994). Thus, in a primary culture, the heterogeneity of the cell population may not give a true reflection of cell uptake of a co-incubated antigen formulation, as it will contain cells at various stages of differentiation.

A murine-derived DC cell line was successfully developed by Winzler et al. (1997). The immortal cell line comprises a homogeneous population of DCs in their immature state. The immature state of this cell line renders it useful for investigations involving uptake of antigen or delivery systems. An aim of this paper was therefore to use this DC cell line to investigate the uptake and cytotoxicity of polyalkylcyanoacrylate (PACA) nanoparticles. Further, the effectiveness of these nanoparticles in delivering antigen to these cells would also be investigated by encapsulating the model antigen, ovalbumin within the nanoparticles.

We have previously reported on a procedure for the preparation of PACA nanocapsules involving the interfacial polymerization of water-in-oil microemulsions (Watnasirichaikul et al. 2000). The procedure enables the efficient entrapment of macromolecules within the nanocapsules and also the release rate of the encapsulated bioactive can be controlled by manipulation of the wall-thickness which can readily be accomplished using this procedure (Watnasirichaikul et al. 2002b). The procedure results in the nanocapsules being dispersed in the microemulsion template which may have beneficial effects in terms of their application in drug delivery (Watnasirichaikul et al. 2002a). However, in order to investigate the uptake of these PACA nanoparticles by antigen-presenting cells, the nanocapsules have to be firstly isolated from the microemulsion template. Hence, another aim of this study was to develop a process for the isolation of PACA nanoparticles from the microemulsion template, which overcomes any resulting aggregation problems to yield a stable formulation that can readily be redispersed in an aqueous solution.

2. Investigations, results and discussion

2.1. Determination of the type and concentration of cryoprotectant required for the formulation of freeze-dried nanocapsules

Nanocapsules separated form the microemulsion template and freeze-dried in water were found to form aggregates upon reconstitution and could not be fully redispersed in aqueous medium with sonication. Thus, various types of sugars (glucose, maltose and sucrose at 1%, 3% and 5% (w/v) in water) were evaluated for their ability to aid redispersibility of freeze-dried nanocapsules. In the presence of sucrose and maltose, "fluffy" cakes were produced whereas in the presence of glucose, a shrunken, sponge-like matrix was formed.

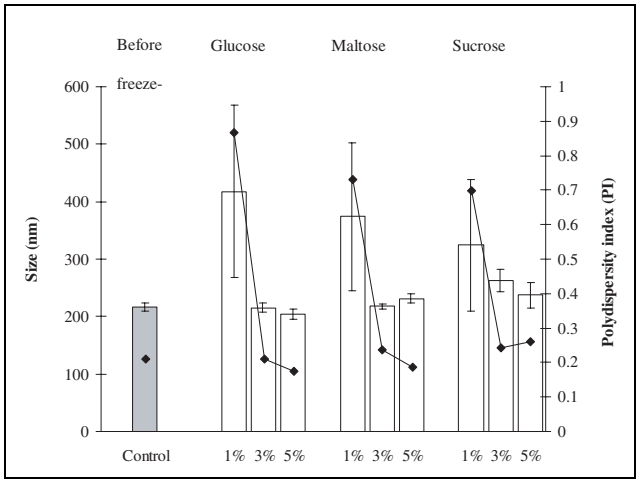


Fig. 1: Size and polydispersity index of redispersed nanocapsules prepared using 1% (w/w) of ethyl 2-cyanoacrylate monomer and freeze-dried in the presence of different types and concentrations of sugar solutions. ☐ size, —◆— PI

The effect of type and concentration of the various sugar solutions on particle size and polydispersity index (PI) of empty polyethylcyanoacrylate (PECA) nanocapsules is shown in Fig. 1. Redispersibility (determined from the least change in mean particle size and PI following reconstitution) of the freeze-dried nanocapsules improved with increasing the concentration of sugar in solution. Complete redispersion of the nanocapsules, i.e. redispersion that lead to the same mean particle size of the nanocapsules after freeze-drying as before, was achieved when sugars were added at a concentration of 5% (w/v). The lowest PI was also obtained at this concentration. The PI of nanocapsules freeze-dried in the presence of 5% (w/v) sugar solutions was about 0.2, indicating that uniform dispersion of nanocapsules following reconstitution had been achieved. All sugars at a concentration of 5% (w/v) exhibited a comparable ability in retaining mean particle size and PI of the nanocapsules.

As the freeze-dried nanocapsules were to be used to investigate uptake using cell cultures, glucose was selected to prepare freeze-dried PECA nanocapsules because it is commonly used as a supplement in cell culture media.

2.2. Physico-chemical characterisation of antigen-loaded nanocapsules

The Table shows particle size, PI and zeta potential for empty and ovalbumin (OVA) loaded nanocapsules before and after freeze-drying and redispersion. Whilst it can be stated that both empty and OVA-loaded nanocapsules remained practically unchanged following freeze-drying and redispersion, particle size and PI of the nanocapsules increased when OVA was incorporated.

It has been suggested in several studies that the size of the droplets in the polymerisation medium influences or determines the size of the resulting nanoparticles (Chouinard et al. 1991; Vranckx et al. 1996; Wohlgemuth et al. 2000). Further, it has been reported that addition of proteins (ly-

Table: Particle size, polydispersity index (PI), zeta potential and loading content of empty and OVA-loaded nanocapsules dispersed in the polymerisation template (microemuslion) and after freeze drying

Sample	Particle size (nm)	PI	Zeta potential (mV)	Loading content (mg OVA/mg polymer)
Empty PECA-NC (in microemulsion)	217 ± 7	0.20 ± 0.07	-24.0 ± 2.8	–
Empty PECA-NC (redispersed after freeze drying)	210 ± 11	0.19 ± 0.12	-23.5 ± 3.3	
OVA-loaded PECA-NC (in microemulsion)	405 ± 39	0.61 ± 0.18	-24.5 ± 1.4	0.32 ± 0.02
OVA-loaded PECA-NC (redispersed after freeze drying)	425 ± 76	0.55 ± 0.20	-24.5 ± 1.6	0.40 ± 0.04

(values represent mean ± SD of 4 batches)

sozyme and chymotrypsin) to micellar systems results in an increase in the size of the micelles (Luisi and Magid 1986). The enlargement of the micelle size may be due to the rearrangement of the surfactants in order to accommodate the protein (Luisi et al. 1988). We have shown in an earlier study, that the size of nanocapsules obtained by interfacial polymerisation of microemulsion is not equivalent to the size of the microemulsion "droplets", which suggests that structural collapse of the microemulsion occurs during polymerisation (Watnasirichaikul et al. 2000). However, an increase in size of the nanocapsules upon addition of protein to the microemulsion will still be correlated to changes in the "droplet" size of the polymerisation template.

A proposed mechanism for the finding that the particle size and PI of the nanocapsules in the current study increased when OVA was incorporated, may be based on the fact that microemulsion droplets are exchanging their contents with that of other droplets in a dynamic fashion (Fletcher and Horsup 1992). The inter-droplet exchange involves continuous coalescence of the droplets to form short-lived fused droplets (transient fusion) followed by their separation. Although the droplet size of the microemulsions remains apparently constant because the exchange process between the droplets is very fast (Fletcher and Horsup 1992), this inter-droplet exchange has been reported to lead to an increase in size of resulting nanoparticles (Kim et al. 1999; Munshi et al. 1997). It has also been reported that the inter-droplet interaction can be controlled by means of increasing the interfacial rigidity of the droplets (Munshi et al. 1997). As discussed above, in the presence of OVA, the size of aqueous droplets of the microemulsions is likely to have increased. Assuming the number of water droplets in the OVA-loaded microemulsion, at the initial stage, is constant in comparison to the antigen-free microemulsions, one could expect that with the larger droplet size, the amount of monomer per unit surface for the OVA-loaded microemulsion will be lower. This may lead to droplets that are less rigid and more prone to transient fusion, which may result in a polymerisation template that yields larger particles. When monomer is added to the microemulsion, polymerisation is highly likely to be initiated at the "water-oil-interface". The use of a low concentration of monomer (1% w/w of the microemulsion) may result in the initial formation of thinner and weaker polymer films (due to less monomer being available per unit surface area), which are not so resistant to transient fusion. As a result, transient fusion of microemulsion "droplets" could occur more readily with the OVA-loaded microemulsion resulting in templates yielding nanoparticles having a larger size.

2.3. Cytotoxicity of empty PECA nanocapsules towards D1 cells

The limited number of studies that have investigated the uptake of nanoparticles by dendritic cells have used particles made from either non-degradable polystyrene (Reece et al. 2001; Scheicher et al. 1995; Thiele et al. 2001; Thiele et al. 1999) or slowly degradable polymers, such as polylactide-co-glycolide (Denis-Mize et al. 2000). In contrast, polyalkylcyanoacrylates (PACAs) degrade much faster, releasing antigen and products of polymer decomposition over a period of days to weeks. The relatively fast rate of degradation of PACA nanocapsules may render them more suitable as delivery systems for vaccine antigens compared to other more slowly degradable polymers since it has recently been proposed that the optimal period of antigen presentation which is critical for driving T cell expansion and differentiation is only the first few days. Antigen presentation for a longer period than this can lead to T cell death and tolerance (Jelley-Gibbs et al. 2000). However, when using polymers that degrade relatively quickly, the toxicity of the degradation products towards the cells needs to be considered.

Toxicity of the degradation products is a particularly important consideration when using cell cultures to investigate interaction between delivery systems and the cells. In such circumstances, the toxicity may not be indicative of the biocompatibility of the delivery system in a "whole" biological system. In cell cultures/cell lines, the local con-

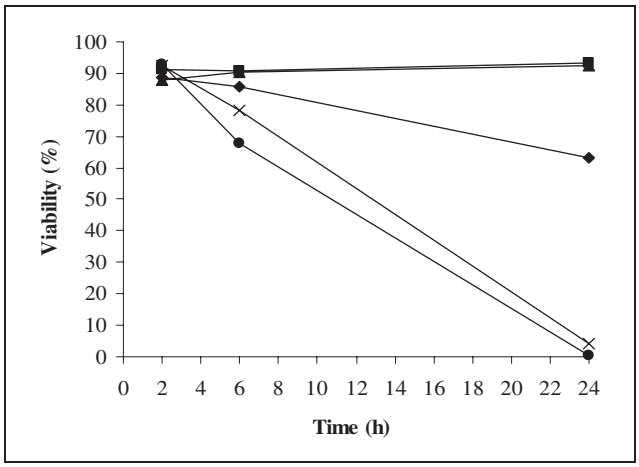


Fig. 2: Percentage viability of D1 cells treated with nanocapsules prepared using 1% ethylcyanoacrylate at polymer concentrations of 0 (■), 10 (▲), 20 (◆), 30 (×) and 40 (●) μg/ml for various times (values represent mean of duplicate cultures)

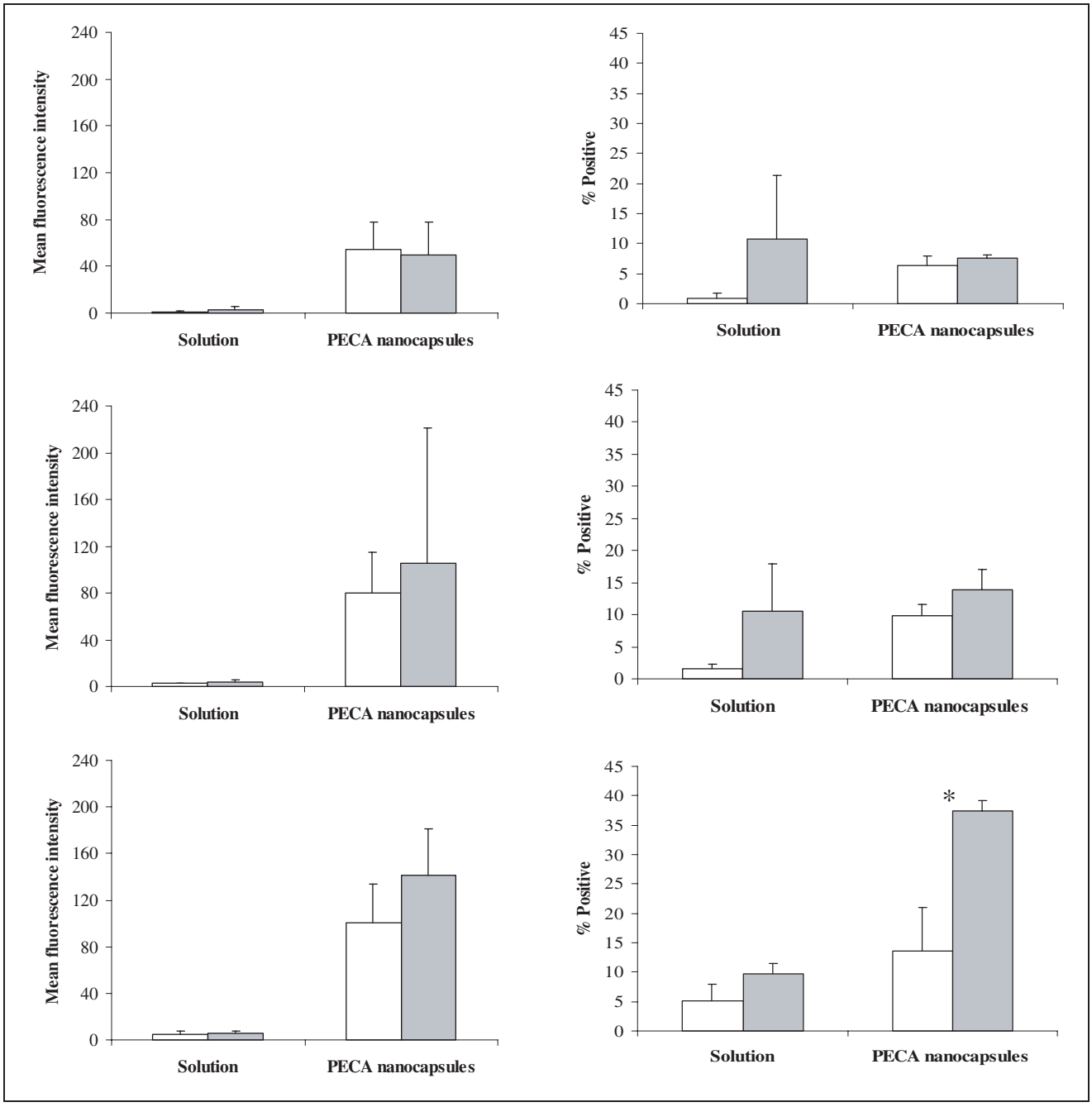


Fig. 3: Mean fluorescence intensity (FITC-OVA) of D1 dendritic cells (left column) and percentage of dendritic cells having associated FITC-OVA (% positive) (right column), following incubation with FITC-OVA solution and FITC-OVA entrapped in PECA nanocapsules prepared using 1% monomer for 3 h (top row), 6 h (middle row) and 24 h (bottom row) at 4 °C (white bars) or 37 °C (gray bars) (values represent mean ± SD of triplicate cultures)

centration of degradation products can become quite high in the absence of mechanisms to eliminate these. The degradation products of PACA are alcohol and formaldehyde, both of which can be toxic if the local concentration in the cell is high (Lherm et al. 1992). Thus, to study the interaction of PACA with APCs it is important to ensure that the polymer concentration used does not result in the build up of cytotoxic degradation products to levels that may be harmful to the cells.

Percentage viability of murine-derived cell line (D1) cells following incubation with nanocapsules was observed as a function of time and polymer concentration. The results are illustrated in Fig. 2. A decrease in percentage viability with increasing amount of polymer added to the culture was noted. At polymer concentration of greater than 10 μg/ml, percentage viability of D1 cells decreased with increasing incubation time. No cytotoxicity up to a 24 h period was observed at a polymer concentration of 10 μg/ml. As a result, a concentration of 10 μg/ml of PECA nanocapsules in the cell culture was selected for further investigations.

2.4. Uptake of PECA nanocapsules by D1 cells

2.4.1. Investigation by fluorescence activated flow cytometry

Investigation of the uptake of FITC labeled OVA (FITC-OVA) loaded PECA nanocapsules by D1 cells was carried out at the apparently non-toxic concentration of 10 μg

polymer/ml of culture. As the loading content of FITC-OVA in freeze-dried nanocapsules was about 0.40 mg OVA/mg polymer the amount of FITC-OVA per 220 µl of culture medium (per tube) was approximately 0.88 µg. To compare the extent of uptake of antigen in soluble and particulate forms, a FITC-OVA solution having a concentration of 4.5 µg/ml was prepared such that each tube would contain 1 µg of FITC-OVA.

Uptake of FITC-OVA from solution and nanocapsules is expressed as the mean fluorescence intensity (MFI) and percentage positive cells. The results are illustrated in Fig. 3. For the nanocapsule formulation, MFI increased with increasing time of incubation at both 4 and 37 °C. For FITC-OVA in solution, MFI was much lower compared to MFI of cells incubated with nanocapsules and did not appear to increase with increasing incubation time. At 24 h of incubation at 37 °C, the MFI measured for D1 cells pulsed with FITC-OVA entrapped in PECA nanocapsules was significantly greater than from those pulsed with FITC-OVA solution ($p < 0.05$). MFI at 37 °C was greater than that at 4 °C (although this difference was not statistically significant).

Percentage positive cells (representing the percentage of the total population of D1 cells taking up FITC-OVA) increased as a function of incubation time (Fig. 3). At the earliest incubation time point of 3 h and at 37 °C, % positive cells appeared highest following incubation of cells with FITC-OVA solution. However, with increasing incubation time, % positive cells increased only following incubation with nanocapsule formulations such that at 24 h, % positive cells was greatest following incubation of the cells with nanocapsules and was significantly higher than that obtained following incubation of the cells with FITC-OVA solution ($p < 0.05$). As with MFI, % positive cells was higher when cells were incubated with formulation at 37 °C compared to 4 °C.

These findings are consistent with those reported by Scheicher et al. (1995) who demonstrated that the uptake of conalbumin by DCs was greater when it was adsorbed onto particles than when it was delivered in solution. Zhang et al. (1998) also demonstrated that the uptake of gentamicin by mouse peritoneal macrophages was significantly increased by binding gentamicin to polybutylcyanoacrylate nanoparticles.

Both MFI and percentage positive cells was slightly lower at 4 °C compared to 37 °C. The reduction in uptake at 4 °C can be explained by the fact that both phagocytosis and pinocytosis are metabolic, energy-dependent processes (Silverstein et al. 1977). Therefore, at 4 °C both of these processes are suppressed resulting in lower uptake of antigen from solution or encapsulated within particles. The association of antigen with the cells at 4 °C is likely to be due to the adsorption of the antigen/particles to the surface in the absence of internalisation (Silverstein et al. 1977). Although D1 cells were washed a number of times to remove the excess formulation, MFI obtained upon incubation of D1 cells with FITC-OVA loaded nanocapsules at 4 °C was not significantly different ($p > 0.05$) from that obtained at 37 °C. This may indicate that, to some extent, the nanocapsules are tightly bound to the D1 cells. In general, the membrane surface of the cells possesses a negative charge, which should repel negatively charged particles such as PECA nanocapsules. However, the effect of surface charge of particles on the uptake by APCs is not clear. A high uptake of negatively charged particles such as benzyl cellulose microspheres by macrophages has been reported by Tabata and Ikada (1988 and 1990). Thiele et al. (2001) have suggested that not only surface charges but also the type of material of the particles may have an effect on phagocytic activity. PECA polymers are hydrophobic and widely used as biological adhesives. Thus, it is possible that these properties led to the firm adherence of PECA nanocapsules to the surface of D1 cells.

2.4.2. Investigation by scanning electron microscopy

Scanning electron micrographs of D1 cells incubated with OVA-loaded PECA nanocapsules for 24 h are shown in Fig. 4. From the micrographs, especially at a higher magnification, it can clearly be seen that the nanocapsules are adhered on the membrane surface of D1 cells (shown by arrow). Adherence of a particle to the membrane surface of a cell is known to be a prerequisite step leading to

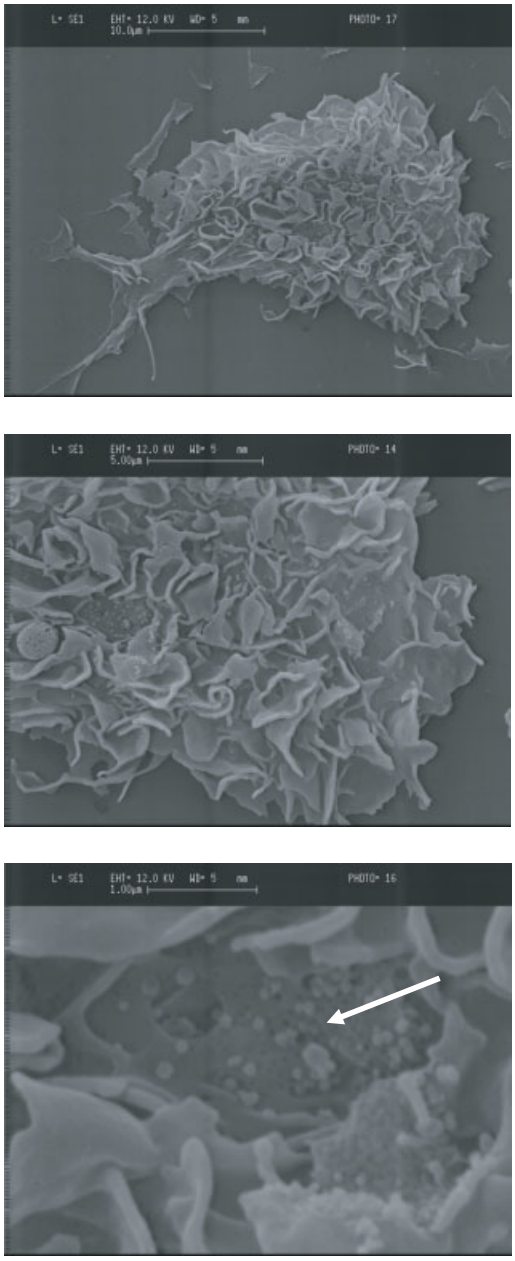


Fig. 4: Scanning electron micrographs of D1 cells pulsed with PECA nanocapsules containing ovalbumin at 37 °C in 5% CO_2 for 24 h. The micrographs were taken at magnification × 2.76 K (a), × 5.49 K (b) and × 25.9 K (c). Arrow indicates nanocapsules adhered on the surface of the D1 cells

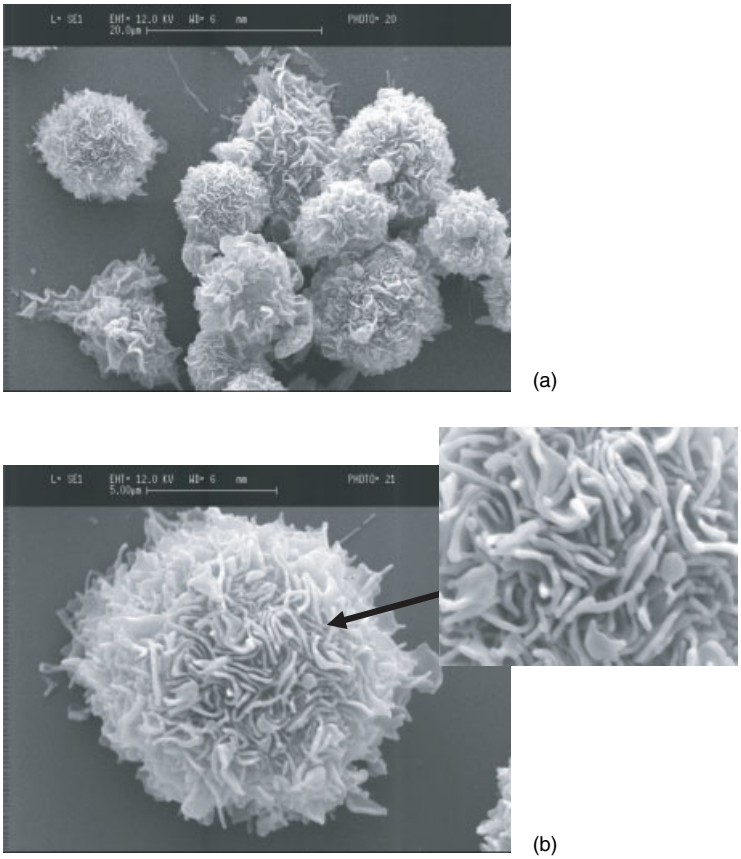


Fig. 5:
Scanning electron micrographs showing the morphology of immature (untreated) dendritic cells. The micrographs were taken at magnification $\times$ 2.07 K (a) and $\times$ 6.14 K (b). Enlarged insert shows short and dense sheet-like veils

internalisation (Silverstein et al. 1977; Tabata and Ikada 1990). Therefore, PECA nanoparticles that are tightly bound to the surface of the cells may be suitable for the antigen uptake.

It is known that following antigen uptake, DCs mature and change their morphology. Mature DCs become irregular in shape with long veils and/or dendrites. These structures help to increase the cell surface area to facilitate antigen presentation to T cells (Bell et al. 1999).

Electron micrographs of untreated (immature) D1 cells (Fig. 5) showed the cells to be round or oval in shape having dense cell membrane processes (or veils), which were either fine or sheet-like. The size of the D1 cells was approximately 10 μm. Upon incubation with LPS (10 μg/ml for 24 h at 37 °C in 5% CO_2), morphological changes of the D1 cells were observed. They became irregular in shape with longer veils (Fig. 6) indicative of maturation. Following incubation with nanocapsules containing OVA, the D1 cells had a similar appearance to those that have been incubated with lipopolysaccharides (LPS), i.e. morphology of mature cells (Fig. 7) whereas those incubated with OVA solution had a similar appearance to immature cells (Fig. 8).

These observations imply that the internalisation of the antigen-loaded nanocapsules by D1 cells has indeed been taking place leading to morphological changes required for the efficient presentation of antigenic peptide epitopes.

3. Experimental

3.1. Materials for the preparation of the nanocapsules

Caprylic/capric triglycerides (Crodamol GTCC®), polysorbate 80 (Crillet 4®) and sorbitan mono-oleate (Crill 4®) were obtained from BTB Chemicals Ltd. (Auckland, NZ). Caprylic/capric mono-/diglycerides (Capmul MCM®) was obtained from Abitec Corp. (Columbus, OH, USA). Ethyl 2-cyanoacrylate (ECA), sucrose, ovalbumin (grade V), fluorescein 5-isothiocyanate (isomer I) and sucrose (AR grade) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). FITC-OVA was prepared as described previously Pitaksuteepong et al. (2002). Glucose and maltose (AR grade) were obtained from BDH Chemicals Ltd (Poole, UK).

3.2. Materials for cell culture and uptake studies

Dulbecco's phosphate buffered saline (DPBS), ethylenediaminetetraacetic acid (EDTA), L-glutamine, 2-mercaptoethanol, trypan blue solution (0.4%) and poly-L-lysine solution (0.1% w/v in water) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Iscove's modified Dulbecco's media (IMDM), foetal calf serum 2-mercaptoethanol (5.5×10^{-2} M in DPBS) and a solution of 10,000 IU/ml penicillin G sodium and 10000 μg/ml streptomycin sulfate in 0.85% saline were purchased from Gibco BRL (Life Technologies, Melbourne, Australia). Phycoerythrin conjugated antibodies to the mouse cell surface marker MHCII was obtained from PharMingen (Becton Dickinson, CA, USA). Sodium azide was obtained from BDH Chemicals Ltd. (Poole, UK). DAKO® fluorescent mounting medium (DAKO Corporation, CA, USA) was obtained through Med-Bio Limited (Christchurch, NZ). Glutaraldehyde solution (25%, EM grade) and osmium tetroxide solution (4%, EM grade) were obtained from ProSciTech (Queensland, Australia). Paraformaldehyde (AR grade) was obtained from Agar Scientific (Stansted, UK).

3.3. Preparation and isolation of polyethylcyanoacrylate nanocapsules

Polyethylcyanoacrylate nanocapsules were prepared as previously described (Watnasirichaikul et al. 2000) using a microemulsion containing 7.6 g of an oil mixture (Crodamol GTCC : Capmul MCM 3 : 1 w/w), 1.4 g of a surfactant mixture (Crillet4 : Crill4 3 : 2 w/w) and 1.0 g of 0.001 M phosphate buffer (pH 7.4). Ethylcyanoacrylate monomer was dissolved in chloroform (ratio 1 : 3 w/w) and slowly added to the microemulsion under mechanical stirring at 4 °C. Following stirring overnight at 4 °C for polymerisation, the empty nanocapsules were separated from the polymerisation medium by centrifugation at 51,500 $\times$ g for 60 min at 25 °C. They were then washed twice in pure ethanol in order to remove oil and surfactant residues. After each wash, nanocapsules were separated immediately from ethanol by centrifugation at 18,500 $\times$ g for 10 min at 25 °C. Following the final washing, nanocapsules were redispersed in an aqueous sugar solution (glucose, maltose or sucrose at concentration of 1, 3 or 5% (w/v)), to give a final polymer concentration of 5 mg/ml. The redispersion of nanocapsules in each step was carried out using a sonicating water bath (Bandelin, Berlin, Germany). Four ml of the resulting suspension was then placed in 20 ml glass vials. The suspension was frozen at −84 °C for 1 h and then freeze-dried for 2 days at −84 °C and a vacuum of 5.5×10^{-2} mbar

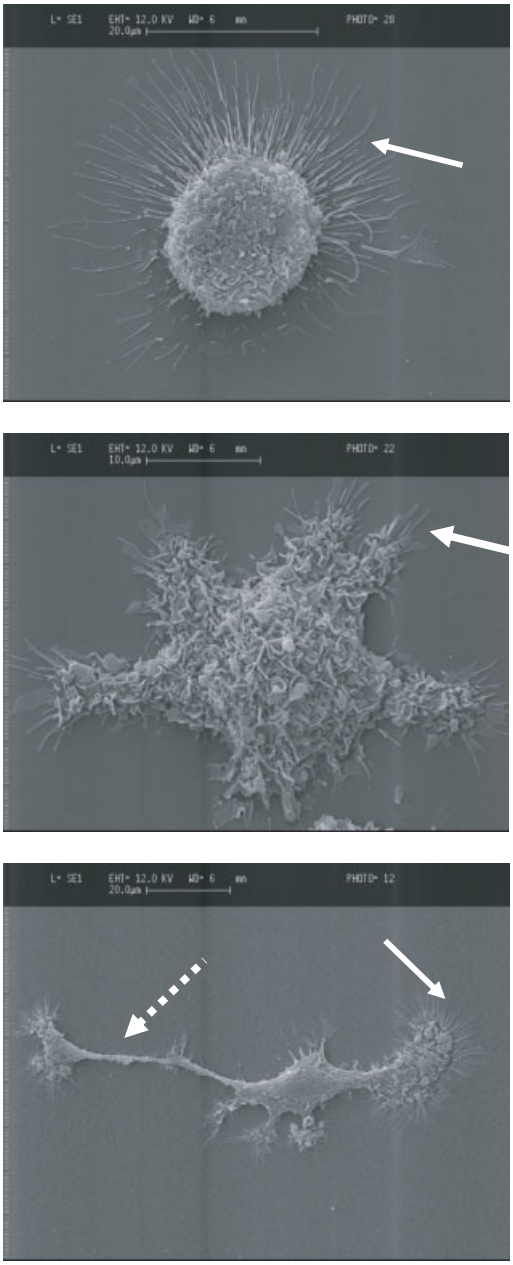


Fig. 6: Scanning electron micrographs showing various morphologies of dendritic cells following incubation with lipopolysaccharides (10 µg/ml) at 37 °C in 5% CO_2 for 24 h. The micrographs were taken at magnification × 2.04 K (a), × 2.71 K (b) and × 0.995 K (c). Arrow indicates long thin veils, dashed arrow indicates dendrites

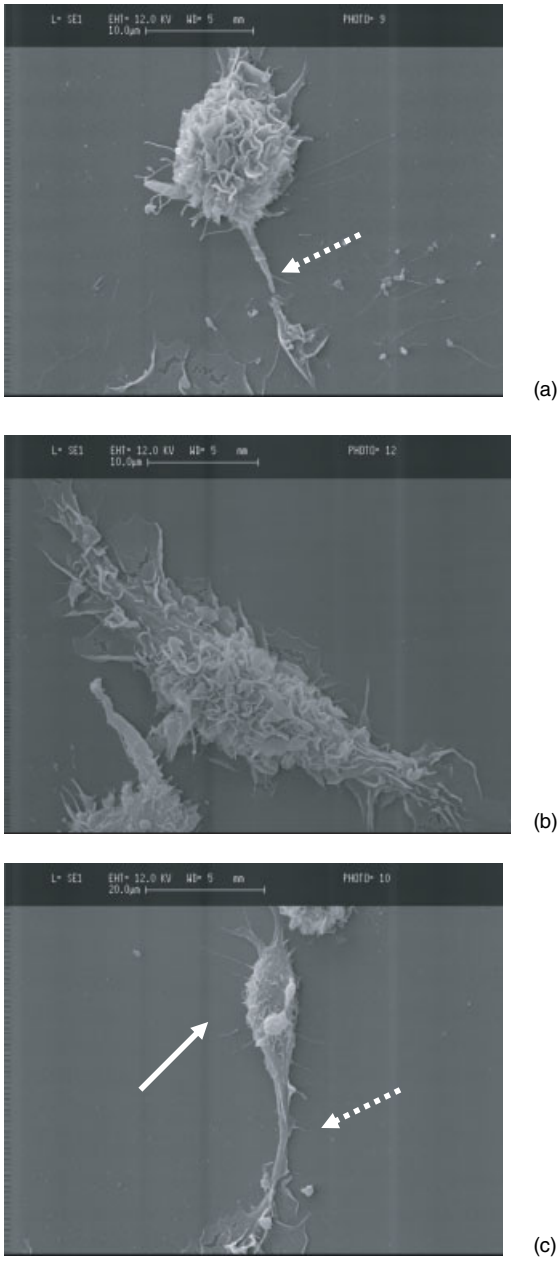


Fig. 7: Scanning electron micrographs showing various morphologies of dendritic cells following incubation with freeze-dried polyethylcyanoacrylate nanocapsules prepared using 1% monomer containing ovalbumin at 37 °C in 5% CO_2 for 24 h. The micrographs were taken at the magnification × 2.61 K (a), × 2.62 K (b) and × 1.44 K (c). Arrow indicates long thin veils, dashed arrow indicates dendrites

using a FreeZone Plus 6 (Labconco, USA). The freeze-dried nanocapsules were subsequently kept in a dessicator (over silica gel) at 4 °C until required.

To assess the most suitable type and concentration of sugar solution for use as cryoprotectant, particle size and PI of nanocapsules before and after freeze-drying were measured by photon correlation spectroscopy (Zetasizer 3000, Malvern Instruments Ltd., UK). The type and concentration of sugar solution that gave the least change in nanocapsule size and polydispersity was selected for further studies.

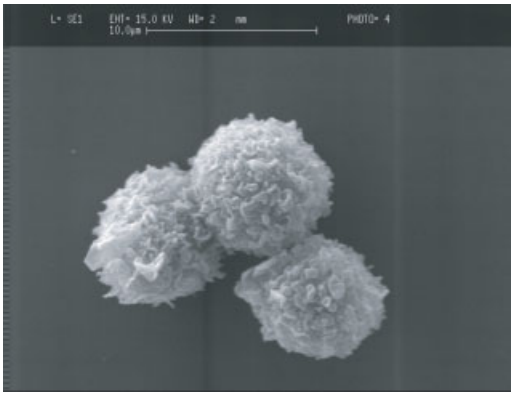
After a suitable type and concentration of sugar solution had been established, freeze-dried PECA nanocapsules containing OVA were prepared by dissolving 50 mg of OVA or FITC-OVA in the aqueous fraction of microemulsion (0.001 M phosphate buffer pH 7.4) prior to mixing with the oil/surfactant mixture. The resulting microemulsions were then polymerized by adding monomer at 1% (w/w) of the microemulsion and subsequently treated as described above.

3.4. Physico-chemical characterisation of nanocapsules

Particle size, polydispersity and zeta potential of empty and antigen-loaded nanocapsules were determined using a Zetasizer 3000 (Malvern Instruments Ltd., UK). The FITC-OVA content of freeze-dried nanocapsules was determined by dissolving the nanocapsules (12.5 mg) in 25 ml of 0.5 N sodium hydroxide. Samples were stirred for 6 h at 37 °C. The resulting solutions were centrifuged at 12,000 g for 12 min at room temperature. The amount of FITC-OVA in the supernatant was determined by spectrofluorimetry (excitation 500 nm; emission 518 nm) following appropriate dilution. The loading content was expressed as the amount of FITC-OVA (mg) associated with 1 mg of nanocapsules.

3.5. Cell culture

The murine dendritic cell line, D1, as reported by Winzler et al. (1997) was kindly supplied by Professor Paola Ricciardi-Castagnoli from the Department of Biotechnology and Bioscience, University of Milano Bicocca, Milan, Italy. The D1 cell line was grown in a culture medium at $2–2.5 \times 10^5$ cells/ml at 37 °C in 5% CO_2. The cells were used on day 3 (where day 0 is taken as the day of cell-splitting). The medium used for the culture of D1 cells comprises 30% (v/v) of R1 medium in complete Iscove's modified Dulbecco's media (cIMDM). R1 medium was generated from mouse fibroblasts (NIH/3T3) transfected with granulocyte-macro-



(a)

(b)

Fig. 8: Scanning electron micrographs showing various morphologies of dendritic cells following incubation with ovalbumin solution (4.5 μg/ml) at 37 °C in 5% CO_2 for 24 h. The micrographs were taken at magnification × 3.50 K (a) and × 5.60 K (b)

phage colony-stimulating factor (supplied by Professor Paola Ricciardi-Castagnoli and prepared according to a specified protocol). The cIMDM was prepared from IMDM by addition of 10% heat inactivated FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 50 μM 2-mercaptoethanol.

3.6. Cytotoxicity of PECA nanocapsules

Freeze-dried empty PECA nanocapsules dispersed in glucose were reconstituted in sterile milli-Q water by shaking and subsequent sonication. The nanocapsule suspension was diluted with culture medium (30% (v/v) R1 media in cIMDM) to yield polymer concentrations of 110, 220, 330 and 440 μg/ml before adding to the cell culture.

Prior to incubation with nanocapsules, D1 cells were diluted with the culture medium to 1×10^6 cells/ml. Two hundred μl of this suspension and 20 μl of the various nanocapsule suspensions having different polymer concentrations as indicated above were incubated together in wells of a 96-well culture plate, yielding final polymer concentrations of 10, 20, 30 and 40 μg/ml of the culture. For control, 20 μl of the culture medium was used instead of the nanocapsule suspension. After 2, 6 and 24 h of culture at 37 °C and 5% CO_2, the medium was removed and transferred to a 0.5-ml Eppendorf tube. One hundred μl of warm 2 mM EDTA in DPBS (EDTA/DPBS) was added to each well of the culture plate and left for 5 min to dislodge the adherent cells. The EDTA/DPBS solution containing the cells was thoroughly mixed with the medium in the Eppendorf tube. The viability of the cells was determined using the trypan blue exclusion test, where D1 cells were incubated with trypan blue solution. Blue stained cells resulting from the diffusion of trypan blue into the cells and non-stained cells were observed under a microscope as non-viable and viable cells, respectively. The viable and non-viable cells were counted using a haemocytometer and the percentage of viable cells was calculated (% viability = (viable cells × 100)/(viable cells + non-viable cells)).

3.7. Uptake of PECA nanocapsules

The uptake of freeze-dried PECA nanocapsules containing FITC-OVA by D1 cells was investigated by FACS, and scanning electron microscopy (SEM).

3.7.1. Fluorescence activated flow cytometry

Aliquots of 200 μl of D1 cells (at a concentration of 1×10^6 cells/ml in culture medium) were dispensed into FACS tubes (polystyrene tubes, size 75 × 12 mm) (Biolab, NZ) and the cells pre-incubated for 30 min at either 4 °C or 37 °C. The cells were then pulsed with 20 μl of FITC-OVA solution (4.5 μg/ml) or 20 μl FITC-OVA loaded PECA nanocapsule (10 μg/ml polymer containing 4 μg/ml FITC-OVA). At 3, 6 and 24 h, the supernatants (medium and excess formulation) were removed and the uptake stopped by adding 2 ml of ice-cold 5% FCS in DPBS. Cells were then centrifuged at 250 g at 6 °C for 7 min. The cell pellet was washed twice with 2 ml of ice-cold 5% FCS in DPBS and 0.02% (w/v) sodium azide solution (azide/FCS/DPBS) to remove any remaining non-associated protein. After each wash, the cells were separated from the DPBS solution by centrifugation at 250 g at 6 °C for 7 min. The cells were finally suspended in 0.5 ml of azide/FCS/DPBS and kept on ice until analysed by FACS (FACScalibur® and CellQuest® software, Becton-Dickinson, USA). A parallel protocol was carried out using empty freeze-dried nanocapsules as a negative control.

The uptake of FITC-OVA either from solution or encapsulated in nanocapsules was expressed as mean fluorescence intensity (MFI) of FITC associated with the cells and also as percentage of dendritic cells having associated FITC fluorescence as compared to total cells analysed (percentage positive). MFI and percentage positive for a population of 10,000 cells were recorded in each analysis. The autofluorescence and percentage positive cells following the incubation of D1 cells with empty nanocapsules (negative control) were subtracted from the values measured in each analysis.

3.7.2. Scanning electron microscopy

D1 cells (1×10^6 cells/ml; 1.5 ml) were added into each well of a 6-well tissue culture plate, each well containing a 13 mm round coverslip, and incubated with 150 μl of a OVA-loaded freeze-dried PECA nanocapsule suspension in culture medium, to yield a final polymer concentration of 10 μg/ml in the culture. Cultures were maintained at 37 °C and 5% CO_2. At 24 h, the cells adhered to the coverslip were washed several times with 0.1 M phosphate buffer pH 7.4 to remove the excess or unassociated nanocapsules. Cells were then fixed with 2.5% (v/v) glutaraldehyde for a minimum of 2 h followed by 2 washes with 0.1 M phosphate buffer pH 7.4. Subsequently, cells were postfixed in 1% (v/v) osmium tetroxide for at least 20 min and then washed 2–3 times with the buffer. The samples were dehydrated using a graded series of ethanol solutions before critical point drying for 3 h (CPD030, Balzers, Liechtenstein). The coverslips were mounted on aluminum stubs and sputter coated with gold. Cells were examined using a Stereoscan 360 SEM (Cambridge, UK).

The morphology of D1 cells pulsed with nanocapsule formulations was observed in comparison to that of D1 cells pulsed with OVA solution at a concentration of 4.5 μg/ml. Untreated D1 cells and cells treated with lipopolysaccharides (LPS) at 10 μg/ml were used as negative (immature cells) and positive controls (mature cells), respectively.

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