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Evaluation of ISCOM matrices clearance from rabbit nasal cavity by gamma scintigraphy

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ABSTRACT

Immune stimulating complexes and/or ISCOM matrices (adjuvant nanoparticles without antigen as a structural component) found potential applications as nasal vaccine adjuvant/delivery system owing to virus like particulate structure and saponin as potent Th1 adjuvant. One of important limiting factor for nasal vaccine delivery is the limited time available for absorption within the nasal cavity due to mucociliary clearance. In this report the clearance rate of ISCOM matrices from nasal cavity of rabbit was determined by gamma scintigraphy. ISCOM matrices were radiolabelled with ^{99m}Tc by direct labelling method using stannous chloride as a reducing agent. ^{99m}Tc labelled ISCOM matrices were administered into the nostril of female New Zealand rabbits and 1 min static views were repeated each 15 min until 4 h. Clearance rate of ISCOM matrices from nasal cavity was calculated after applying the physical decay corrections. The mean labelling efficiency for ISCOM matrices were calculated as ~58.4%. ISCOM matrices showed slower clearance rate compared to sodium pertechnetate control solution ($p < 0.005$) from nasal cavity that may be due to particulate and hydrophobic characters of ISCOM particles even though it was also cleared within 4 h from nasal cavity. Mucoadhesive ISCOM formulations that retain in nasal cavity for longer duration of time may reduce the dose/frequency of vaccine for nasal immunization.

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1. Introduction

Nasal delivery of vaccines is gaining prominence as a preferred mode of immunization due to improved safety, ease of use over needles and better patient compliance due to painless administration especially among the elderly and children. Further, with the use of appropriate adjuvant/delivery system, nasal vaccination elicits mucosal as well as systemic immunity, a feature that is difficult to obtain with needle-based vaccinations (Slütter et al., 2008; Csaba et al., 2009). In addition, potent immune responses in the respiratory and genital tracts could be induced by intranasal immunization as a consequence of the common mucosal immune system (Sharma et al., 2009).

However, nasal delivery of vaccine is impaired by the mucociliary clearance, which is movement of mucus from peripheral airways to larynx by ciliary activity of the underlying epithelium. It removes inhaled substances like dust, bacteria and viruses entrapped in mucus from the nasal cavity towards the throat preventing those potentially harmful substances to penetrate the nasal epithelium (Yang et al., 2008). This mucociliary transport system is essential for the protection of the conducting airway surfaces from ambient irritants and infectious agents and for maintenance of airway patency.

Among possible mucosal delivery systems, nanoparticles hold great promise because of their capacity to protect encapsulated antigens, to promote interaction with mucosae and to direct antigens towards lymphoid tissues as potential inductive sites (Csaba et al., 2009). One of potential mucosal adjuvant/delivery system is immune stimulating complexes (ISCOMs) (Sun et al., 2009). These 40 nm 'cage-like' particles are composed of phospholipid, cholesterol, saponin and incorporate antigen by virtue of hydrophobic interactions via membrane anchor sequences of viral envelope glycoproteins. An alternative form of this adjuvant system is ISCOMATRIX adjuvant, formed by the combination of saponin, phospholipid and cholesterol to give similar 'cage-like' structures without the inclusion of antigen in its structure. When this inherently more convenient ISCOMATRIX adjuvant is mixed with the appropriate antigen prior to immunization, the immunological outcomes are similar to those observed for ISCOM vaccines (Pearse and Drane, 2005). Antigens formulated as ISCOMs or physically mixed with ISCOM matrices induce augmented antigen-specific responses after either parenteral or mucosal immunization in animal models (Sun et al., 2009) or following parenteral administration to humans (Sanders et al., 2005). This adjuvant technology also found great

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potential as nasal delivery system for vaccines where humoral, cellular as well as mucosal responses are required especially in situations after bacterial and viral pathogens invade the host via the mucosal surface (Hu et al., 2001).

Gamma scintigraphy imaging technique provides clearance data from the entire airway and being non-invasive includes the capability to restudy animal subjects when time course and/or prolonged treatment(s) are being considered. This technique relies on the use of radioactive tracers included into the medicament and selected so as to enable an optimum detection by a gamma camera. It has proved valuable and versatile in the assessment transit times of nasal sprays and drops (Bryant et al., 1999; Di Giuda et al., 2000), deposition patterns of nasal sprays (Harris et al., 1988; Suman et al., 1999; Eyles et al., 2001) and bioadhesive behaviour (Illum et al., 1987; Soane et al., 1999).

The aim of the work presented here was to study the nasal deposition and clearance characteristics of ISCOM matrices from rabbit nasal mucosa using external gamma scintigraphy to monitor the deposition pattern and clearance rate.

2. Materials and methods

2.1. Materials

Quil A (purified fraction of saponin mixture from Chilean tree *Quillaja saponaria* Molina) was kindly provided by Branntag Biosector, Frederikssund, Denmark. Cholesterol, L-α-phosphatidylcholine (PC) from egg yolk, MEGA-10, stannous chloride dehydrate ($SnCl_2{\cdot}2H_2O$) and sephadex G-25 were purchased from Sigma–Aldrich Private Ltd. (St. Louis, MO). All other chemicals and solvents were of analytical reagent grade and were used without further purification. Distilled deionized water (18 Ω A Milli-Q™ Water system, Millipore Corporation, Massachusetts, USA) was used throughout the study.

2.2. Experimental methods

2.2.1. Preparation of ISCOM matrices

ISCOM matrices were prepared by lipid hydration method (Demana et al., 2004). Briefly, 8 mg PC and 4 mg cholesterol were dissolved in 1 ml chloroform. The solution was evaporated to dryness at 45 °C for 1 h using rotary evaporator (Rotavapor 210 R, Büchi, Switzerland). 4 mg of Quil A dissolved in 3 ml of PBS, pH 7.0 (phosphate buffered saline contained 1.7 mM KH_2PO_4, 7.9 mM Na_2HPO_4, 2.7 mM KCl, 250 mM NaCl, pH 7.0) was then added to the dried lipid films. It was stirred for 3 h at 4 °C to ensure complete hydration. Formulations were then freeze-dried (Heto Drywinner, Germany) overnight followed by rehydration with 3 ml PBS, pH 7.0. Particles formed were purified by sucrose density gradient (10–60%) ultracentrifugation at 200 000 × *g* for 6 h (L-7 Ultracentrifuge, Beckman Coulter, USA) followed by dialysis for 48 h at 4 °C against PBS, pH 7.0 to separate the sucrose. It was concentrated to 2 ml by ultrafiltration using a 10,000-molecular weight cut-off membrane (Millipore, USA) and a 10 ml filtration cell (Amicon, Beverly, Massachusetts, USA) pressurized to 200 kPa.

2.2.2. Characterization of ISCOM matrices

The morphology of ISCOM matrices was determined by transmission electron microscopy (Philips EM268D, The Netherlands). One drop of aqueous dispersion was placed over a 400-mesh carbon-coated copper grid followed by negative staining with phosphotungstic acid (3%, w/v, adjusted to pH 4.7 with KOH) and placed at the accelerating voltage of 80 kV. The particle size and size distribution of the ISCOM matrices was determined by the laser diffraction method (Zeta Nano ZS 90, Malvern Instruments Inc., Worcestershire, UK). Nanoparticle suspension (1.0 ml) was dispersed in 4.0 ml ultrapure deionized water. The mean particle size and size distribution were determined at 25 ± 1 °C by scattering the light at 90°. The zeta potential of the ISCOM matrices was determined by laser doppler anemometry using a Zetasizer (Malvern Instruments, UK) following 1:300 dilution in PBS pH 7.0 An electric field of 150 mV was applied to measure the electrophoretic velocity of the particles. All the measurements were made in triplicate.

2.2.3. Gamma scintigraphy

2.2.3.1. Radiolabelling of ISCOM matrices. The prepared ISCOM matrices were labelled with ^{99m}Tc using the stannous reduction method as described previously (Garg et al., 2008). Briefly, 1 ml of the ISCOMATRIX dispersion (diluted to 5 mg/ml with PBS, pH 7.0) was mixed with stannous chloride dihydrate solution (100 μg in 100 μl of 0.10 N HCl). Before dissolving stannous chloride, sterile, pyrogen-free water was bubbled for 30 min with nitrogen in order to expel most of the oxygen to exclude the possibility of the oxidation of tin to the unreactive stannic form. The pH was adjusted to 7.00 ± 0.20 using 50 mM sodium bicarbonate solution. 1 ml of technetium pertechnetate (75–400 MBq) in sterile saline was then added; the mixture was shaken vigorously for 1 min by vortexing at 1200 rpm (Vortex mixer, Fischer Scientific, India) and left to react at room temperature for 30 min with continuous nitrogen purging. The final volume was made up to 2.5 ml using 0.9% (w/v) sodium chloride solution. The effects of incubation time, pH, and stannous chloride concentration on labelling were studied to achieve optimum reaction conditions.

Labelling efficiency of the purified radiolabelled formulations was determined by ascending instant thin layer chromatography (ITLC) using silica gel (SG)-coated fiber sheets of approximately 10 cm in length (Gelman Science Inc., Ann Arber, MI, USA) at room temperature (25 ± 1 °C). The ITLC was performed using 100% acetone as the mobile phase. A tiny drop (2–3 μl) of the radiolabelled formulation was applied at a point of 1 cm from one end of an ITLC-SG strip. The strip was developed in acetone and the solvent front was allowed to reach approximately 8 cm from the origin. The strip was cut into two equal halves and the radioactivity in each segment was determined in a well-type gamma-ray counter (gamma-ray scintillation counter, Type CRS 23C, Electronics Corporation of India Ltd., Mumbai, India). The free $^{99m}TcO4^-$ moved with the solvent (Rf = 0.9) while the radiolabelled formulation remained at the point of application. Percent labelling efficiency was calculated from the formulae:

$$\text{Labelling efficiency}(\%) = \frac{T \times 100}{T + B}$$

where *T* is the counts at top and *B* is the counts at bottom.

In vitro stability of the labelled formulations was determined by incubating 100 μl of the labelled formulation with 2.0 ml of simulated nasal fluid, pH 6.4 (Lorin et al., 1972) at room temperature and change in labelling efficiency was monitored over a period of 6 h by ITLC as described above.

2.2.3.2. Scintigraphic evaluation. In order to study the clearance characteristics, ISCOM matrices were administered to healthy female New Zealand rabbits (*n* = 4 per preparation, weight 3.40 ± 0.20 kg age 90–140 days). Animals were housed at animal house facility of the Institute of Nuclear Medicine and Allied Sciences (INMAS), New Delhi, India. Experiments were performed under strict supervision of associated technical specialist of the same laboratory. All animal studies were carried out under the guidelines compiled by CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Culture, Government of India) and all the study protocols were approved by institutional animal ethics committee.

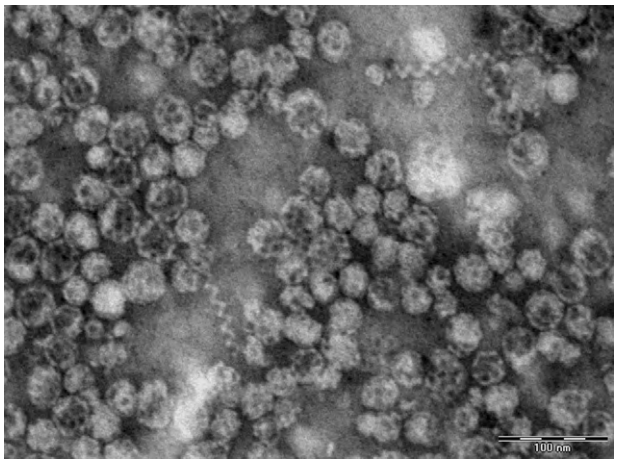

Fig. 1. Transmission electron photomicrograph of ISCOM matrices prepared by lipid hydration method (40,000×).

Labelled ISCOM matrices were administered in a dose of 5 μl per nostril (total of 20 μl; radioactivity of 2 MBq) at an interval of 5 min with the help of a micropipette (Rankem, India). Rabbits were held in supine position both during and post-administration. Sodium pertechnetate solution (20 μl, radioactivity of 2 MBq) was taken as control and administered similarly as above.

Immediately after installation, anterior and posterior views of whole manually restrained animal were measured in a supine position by a gamma camera (Model SYS00000L, Elgems Millenium VG Wilconsin, USA) equipped with a low-energy (140 keV) high resolution parallel collimator. In order to evaluate the retention of the inhaled ISCOMs, scans were repeated at intervals of 15 min up to 4 h after nasal installation. Regions of interest (ROI) were manually drawn around nasal cavity zone. Each image was manually aligned and count rate from region of interest (ROI) corrected for radioactive decay and background. Immediately after dosing, highest count rate at ROI, i.e. nasal cavity was assigned 100% value, which was then used to calculate the percentage remaining for the other time points. An estimate of the half-time of nasal clearance (T_{50}) was determined by interpolation of a plot of percentage nasal radioactivity versus time for each individual animal. Counts from the anterior and posterior views were combined by taking geometric mean values. Geometric mean counts were corrected for the room's background measured separately from each image and for radioactive decay.

2.3. Statistical analysis

Statistical analysis including student *t* tests was performed using GraphPad Prism version 5.03 for Windows, GraphPad Software, San Diego California USA. $p < 0.05$ was considered as statistically significant difference.

3. Results

3.1. Preparation and characterization of ISCOM matrices

Lipid hydration method used for preparation of ISCOM matrices yield in nanometric formulations with cage-like morphology as confirmed by TEM (Fig. 1). The size of the particles obtained was in the range of 42–67 nm as measured by photon correlation spectroscopy with polydispersity index of 0.216. These results are in agreements with other reports (Demana et al., 2004; Lendemans et al., 2005) demonstrating that lipid hydration method produces more heterogeneous formulation as compare to dialysis method.

Table 1
Stability of ^{99m}Tc labelled ISCOM matrices formulations. For *in vitro* stability 100 μl of the labelled formulation was mixed with 2.0 ml of simulated nasal fluid (pH 6.4) and incubated at room temperature and labelling efficiency was monitored over a period of 6 h. All values are expressed as mean ± SEM ($n = 6$).

Time (h)	*In vitro* stability (% radiolabelled)
0	58.3 ± 3.4
0.25	58.1 ± 2.1
0.5	58.1 ± 3.2
1	57.9 ± 3.6
2	57.4 ± 4.1
6	57.2 ± 2.9

Surface charge of these colloidal particles was found to be approximately −18.6 mV in PBS pH 7.0. Negative surface charge of ISCOM matrices might be explained by the presence of glucoronic acid in Quil A.

3.2. In vivo clearance studies by gamma scintigraphy

ISCOM matrices were labelled with the gamma-ray emitting radionucleide ^{99m}Tc, which has ideal radiation energy (140 keV) for use with a gamma camera. The short half-life of ^{99m}Tc (6 h) coupled with a very 'clean' radiation emission profile which contains few beta-particles, results in very low radiation doses so that satisfactory scintigraphic data can be obtained using only a fraction of the radiation dose (Newman and Wilding, 1998).

Technetium pertechnetate ($^{99m}TcO_4^-$) was used to directly label preformed ISCOM matrices using stannous chloride as a reducing agent. It is known that $^{99m}TcO_4^-$ is a non-reactive species and does not label any compound by direct addition thus prior reduction of $^{99m}TcO_4$ from 7^+ state to a lower oxidation state (4^+) is required. The technetium used for the study was reduced to its lower valency state (4^+) using stannous chloride dehydrate and then pH was adjusted to neutral; other variables like amount of pertechnetate, pH, incubation temperature and time was kept constant before mixing it with ISCOM matrices.

Radiolabelled formulations were optimized for maximum labelling efficiency and stability. The pH range of 6.0–6.5 and 100 μg (in 100 μl) of stannous chloride with incubation time of 30 min were selected as conditions for the optimum radiolabelling.

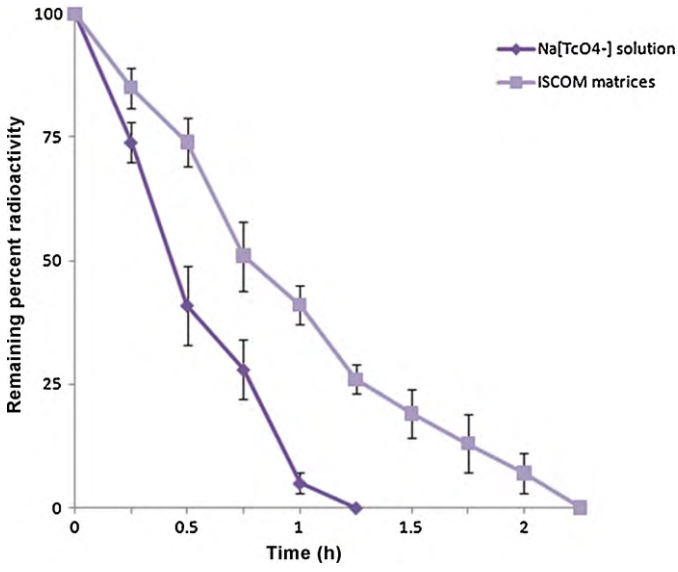

Fig. 2. The percent mean radio activity remaining in the rabbit nasal cavity, at different time points after nasal administration of radiolabelled ISCOM matrices and control solution.

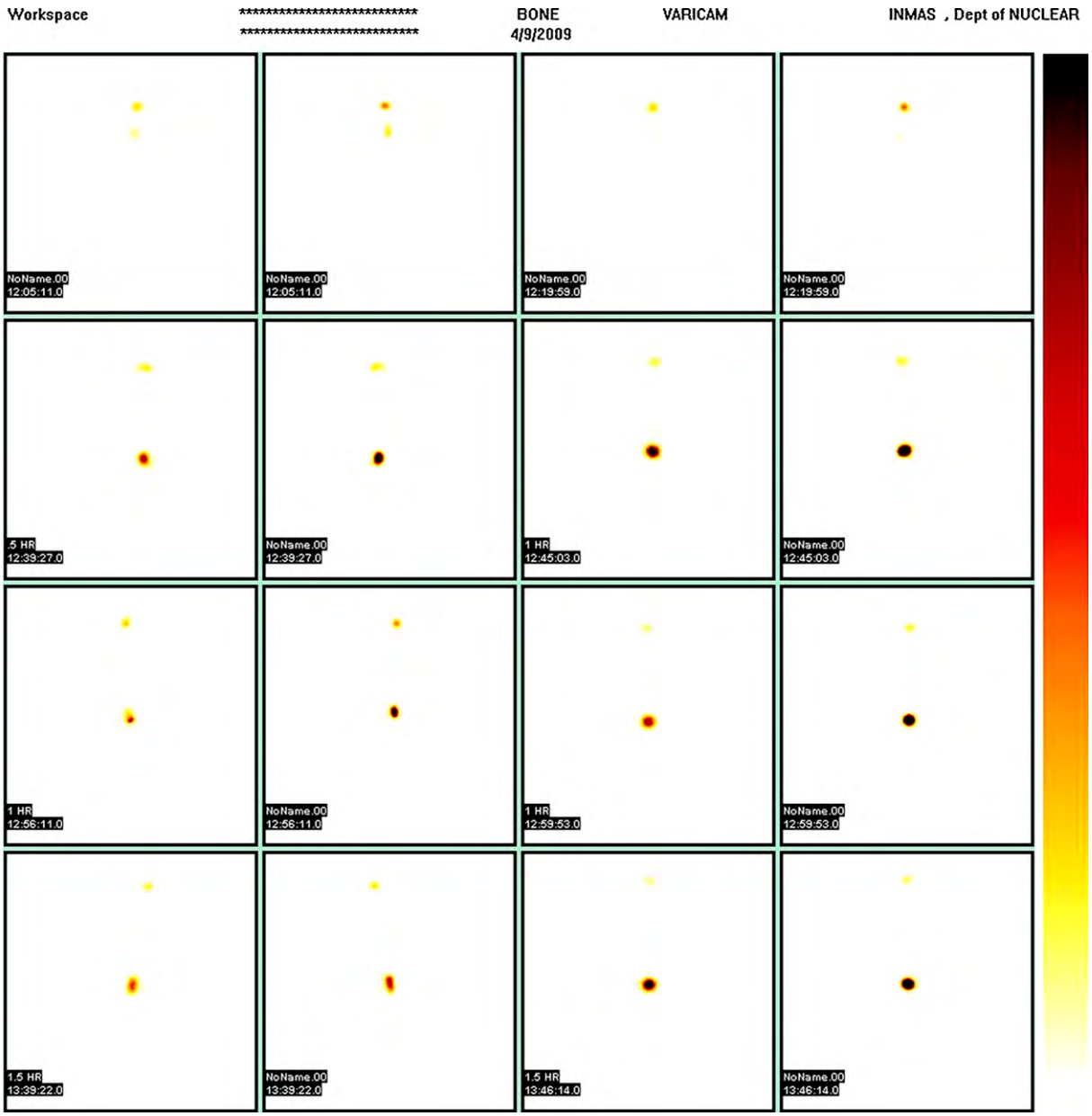

Fig. 3. Scintigraphic images of rabbit after administration of ^{99m}Tc labeled ISCOM matrices at different times post-nasal administration.

To remove the possible radiocolloids (reduced/hydrolyzed ^{99m}Tc) contaminant formed during labelling process; labelled formulations were purified using gel permeation chromatography by passing them through a column of Sephadex G-25 (Lazorová et al., 1996). *In vitro* stability of the labelled formulations was evaluated in simulated nasal fluid (pH 6.4). Both formulations exhibited excellent *in vitro* stability. Less than 2.0% of the radioactivity was lost from ISCOM matrices in 6 h (Table 1).

Labelling efficiency of ISCOM matrices was found to be 58.4 ± 3.4% (mean ± SEM). The labelling efficiencies reported by other groups with polymer particles were greater than 90% by using the same method of labelling (Illum et al., 1987; Soane et al., 1999). Observed low labelling efficiency might be due to the open cage-like structure of ISCOM matrices that do not provide entrapment of radioactive material. Further, few functional groups were available for radioactive binding due to strong bond formation between the saponin and lipid; specifically cholesterol. It is presumed that ^{99m}Tc was attached to the outer surface of ISCOM matrices bonded with the hydroxyl groups of phospholipids (Soane et al., 1999). Presently, role of Quil A and cholesterol in binding with ^{99m}Tc is not known and required to be confirmed further by other advanced analytical techniques.

The percent of the formulations cleared from the nasal cavity in the time course of study (4 h) is shown in Fig. 2. Clearance of ISCOM-bound ^{99m}Tc was comparatively slower than radioactive control solution. A mean of 41% (4.3) of the total nasal dose was detected in the nasal cavity after ISCOM nasal installation. After 2 h, the ISCOM retention on average was 7% (2.1). The planar 15-min images showed formulation dependent progressive migration of radioactivity with time from the nasal cavity to the stomach and intestine. Fig. 3 shows gamma camera images for time dependent clearance of formulations from nasal cavity of ISCOM particles.

In addition, by using the averaged clearance data, time taken for 50% of the formulation cleared from the nasal cavity ROI was also calculated. This averaged data shows that the control Na[TcO_4^-] solution was cleared rapidly, with a half-life of 24.50 ± 2.2 min (mean ± SD), whereas ISCOMs had longer half-life, i.e. 74.03 ± 6.2 min (mean ± SD). Data generated from nasal clear-

ance of control solution are in agreement with previous studies reported that normal half-life of clearance for solution is about 15–20 min (Illum, 2004).

4. Discussion

ISCOM and ISCOM matrices have been appreciated as potential adjuvant/delivery systems for a variety of antigens by nasal route of immunization. Recently, we have reported that nasal administration of recombinant hepatitis B surface antigen incorporating ISCOMs were able to induce strong humoral, mucosal and cellular immune response in BALB/c mice although high dose and multiple administrations were required to boost the potent immune response (Pandey and Dixit, 2010).

The mucociliary clearance mechanism in the respiratory tract provides competent defence against inhaled particles including bacteria and irritants. Particulates trapped in the viscous mucus covering the nasal epithelium are transported from nasal cavity to the pharynx and oesophagus; ultimately reach to the stomach (Proctor, 1985).

The clearance of inhaled materials from the nasal cavity of man and other animals has been shown to follow a biphasic pattern (Hardy et al., 1985; Suman et al., 1999). This biphasic pattern is the result of an initial fast rate of clearance of material from the ciliated regions of the nose, followed by a comparatively slow second phase of clearance associated with material deposited on the anterior region of the nose (Soane et al., 2001). ISCOM matrices showed biphasic pattern of clearance and control solution showed monophasic pattern which may be due to initial deposition of formulations into the nasal cavity. ISCOM matrices were predominantly deposited on the anterior region of nose. Previously, it was reported that rate limiting step for the nasal clearance of nasally administered particulate systems is their dislocation from the initial site of deposition and interaction of particles with mucus layer. The rest of nasal passage does not significantly affect the clearance time (Tafaghodi et al., 2004).

In present study, low volume spaced installation of formulations minimized overflow of liquids into the gastrointestinal tract during installation and that certainly had some impact in clearance time. It is already reported that low volume (10 μl) solution into the nostril resulted in the deposition of the majority of the poorly absorbed distribution marker ovalbumin in the nasal cavity (Minne et al., 2007).

It has been shown that negatively charged polymers/liposomes have some mucoadhesion potential (Ahuja et al., 1997) and also the negative charged particles could be taken up by Peyer's patches (Borges et al., 2006). Therefore it seems that negatively charged ISCOM matrices could possibly penetrate within the glycoprotein network of mucin and remain there because of the negative charge repulsion of mucin (Amin et al., 2009).

Particle size and hydrophobicity also played important role in particle uptake by nasal mucosa and ultimately the clearance from nasal cavity. It is generally accepted that nasal associate lymphoid tissue (NALT) and M cells (Microfold cells characterised by a basolateral cytoplasmic invagination possess a high capacity to transport a wide range of materials by transcellular vesicular transport to underlying intraepithelial cells) can rapidly uptake nanoparticles (Csaba et al., 2009). This is supported by the enhanced uptake by mucosa and immunogenic response induced by other delivery systems of size range similar to viruses, for example virosomes and Supra molecular biovector (Gluck, 1999; von Hoegen, 2001). Similar correlation between hydrophobicity of particles and uptake by nasal mucosa after intranasal administration of particles has also been reported by Alpar and Almeida (1994).

Overall, difference in nasal clearance observed in this study might be due to the binding to the mucin and biological membranes as well as uptake of the nano-complexes by mucosal epithelium (Pandey and Dixit, 2010).

However, factors such as type of formulation (solution vs. powder), administration device and aerodynamic properties of the liquid droplets or powders can affect insufflations and deposition patterns, and ultimately mucociliary clearance (Ugwoke et al., 2000). The site of drug deposition in the nose is also highly dependent on the dosage form. Nasal sprays deposit drugs more anteriorly, resulting in a slower clearance of sprays than drops (Bryant et al., 1999; Hardy et al., 1985).

In conclusion, ISCOM matrices showed some degree of mucoadhesion and slower clearance from nasal cavity of rabbit as compared to the radioactive control solution. Even though rapid clearance of ISCOM matrices as compared to other mucosal carrier systems such as alginate, PLG, chitosan and dextran micro/nanoparticles etc. warrants formulation of mucoadhesive ISCOM formulations those retain in nasal cavity for longer duration of time and facilitate in reducing the dose/frequency of vaccine for nasal immunization.

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