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Preliminary characterization of *N*-trimethylchitosan as a nanocarrier for malaria vaccine

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ABSTRACT

Background & objectives: With the current snags from the use of Artemisinin-combination therapies (ACTs) in malaria treatment in addition to fear of cross-resistance to unrelated drugs, raising the immunocompetence of individuals in malaria endemic areas by vaccination is the best approach to malaria-free world.

Methods: Water-soluble cationic derivative, *N, N, N*-trimethylchitosan (TMC) was synthesized from chitosan. Nanoparticles of the TMC were prepared in various media [milliQ water, Na_2CO_3 (pH 10.92), Na_2HPO_4 (PBS, pH 9.01 and alhydrogel®] which were characterized as adjuvants for possible vaccine delivery. The nanoparticles were characterized for particle size, surface charge and morphology using microscopy (Phase contrast microscope and Confocal laser scanning microscope), and Malvern zetasizer Nano-ZS. Time-resolved particle size analysis was performed after one month storage of the TMC nanoparticles at 4°C.

Results: The result of the study showed that PBS was the best medium that produced cationic, monodispersed and stable TMC nanoparticles of <65 nm forming a compatibly homogeneous system even upon storage. Details of the polyelectrolyte-doped nanoparticles in PBS showed clear coatings due to Sodium poly (styrene sulfonate) [PSS, MW ~70 kDa] at the periphery of the particles and a fluorescent core with some tiny central hollow cavities implying that the nanoparticles can either entrap the vaccine candidate into the hollow cavities or adsorb them unto the surface of the peripheral polyelectrolyte coatings.

Interpretation & conclusion: This preliminary study established that TMC has the desired qualities for the intending antigen delivery. Further research regarding the biological activity of this TMC is indicated.

Key words Alhydrogel®; malaria; *N*-trimethylchitosan; PBS; nanocarrier; vaccine

INTRODUCTION

Malaria is a serious menace to countries in the tropic–Africa, Asia, etc. because it is geographically specific, affecting mostly children and pregnant women as well as having greater morbidity and mortality than any other infectious diseases of the world[1,2]. Currently, the only hope in chemotherapy of malaria lies with the artemisinin class of antimalarial drugs. The wide spread resistant malaria parasites (*Plasmodium* spp) to most common antimalarials, and cross-resistance to structurally unrelated drugs, emphasize the need for new therapeutic targets[3]. Raising the immunocompetence of individuals in malaria endemic areas by vaccination could significantly lower the death tolls due to clinically severe malaria. A viable malaria vaccine is regarded as the most cost-effective and best practical method of reducing the high human and economic toll of this devastating disease[4–6].

Plasmodium falciparum apical membrane antigen 1 (AMA1) is a surface protein expressed during the asexual blood stage of *P. falciparum,* and is a leading vaccine candidate, with several formulations being tested in malaria endemic areas in Africa[4,7]. Pre-clinical studies have shown that vaccination with AMA1 induces antibodies and protect against homologous parasite challenge in both rodent and monkey models of malaria infection[8]. Since single target antigens show loss of activity, the use of multiple allelic variants has improved this immune evasion and especially in co-formulation with an immunostimulant, CpG oligodeoxynucleotide (CpG ODN), a synthetic oligodeoxynucleotide[9–11].

Adjuvants are agents that augment immune responses to antigens (Ags) and generate immune responses to purified proteins and in their absence; Ags induce tolerance instead of immunity. To generate a more robust immunity, we report here a preliminary characterization of a

nanocarrier based on water-soluble chitosan, the protonation of the amine group which imparts a positive charge presumably responsible for boosting immunogenicity[12]. It is hoped that the result of this preliminary investigation will suggest the possible mechanism of antigen delivery by either entrapment in and/or adsorption unto the antigen (AMA1) in or onto the chitosan nanoparticles and subsequently decorating the nanoparticle-bearing antigen with other immune boosters such as uric acid (UA) and interleukin-12 (IL-12) for a longer-lasting immunity against the disease[13–15].

MATERIAL & METHODS

Chitosan, low molecular weight (degree of acetylation >95%, molecular weight 400 kDa), Poly (sodium 4-styrenesulfonate) (PSS, Mw. 4.3 kDa), Rhodamine 123, aluminium hydroxide gel, acetic acid (purity 99%), formaldehyde, sodium borohydride, sodium hydroxide, *N*-methyl-2-pyrrolidinone, sodium iodide, methyl iodide (Sigma, USA), Sodium carbonate Sigma ultra and phosphate buffer solution (sodium phosphate dibasic Sigma ultra, 99%) were used as procured without further purification.

Derivatization of water soluble chitosan

Synthesis of N-methylchitosan

The method of Belalia *et al*[12], was adopted. Briefly, chitosan (4 g) was dissolved in 1% (v/v) aqueous acetic acid (400 ml). The solution was then filtered to eliminate the impurities and formaldehyde was added (3-fold excess to amine of chitosan). The solution was stirred at ambient temperature for 30 min. NaBH4 (0.33 g) was then added, and the solution was stirred at ambient temperature for 60 min. The pH was adjusted to 10 using 1 M NaOH. After filtration, the system was washed to reach pH 7. Finally, the excess of reagent was eliminated by extraction with a Soxhlet, using ethanol/diethyl ether (80:20 v/v). The product was dried at ambient temperature for 24 h.

Synthesis of N, N, N-trimethylchitosan (TMC)

N-methylchitosan was dispersed in 120 ml of *N*-methyl-2-pyrrolidinone with NaI (5 g) under vigorous agitation at 60 °C for 1 h. Fifteen percent NaOH (22 ml) and methyl iodide (10-fold excess to amine of chitosan) were then added. The mixture was stirred at 60 °C for 6 h. Finally, the quaternary ammonium salt of chitosan was precipitated using acetone (3-fold excess to the volume of *N*-methyl-2-pyrrolidinone)[16]. The product was dried at room temperature and subjected to ^{1}H NMR spectra on an Avance 300 NMR spectrometer using D_2O.

Formulation of chitosan nanoparticles in different adjuvant media

Three different media consisting of milliQ water, Na_2CO_3 (pH 10.92) and Na_2HPO_4 (pH 9.01) were employed to formulate nanoparticles of chitosan. Briefly, TMC (1 mg/ml) as well as the above salts (1 mg/ml) were separately dissolved in mQ water. Some 200 µl of the TMC nanoparticles was added dropwise to 200 µl of the buffer solutions. The particle size of the systems was analyzed with dynamic light scattering as well as zeta potential (Malvern Zetasizer Nano-ZS, UK). Room temperature storage stability analysis of the system was also done as well as storage at 4°C.

Preparation of polyelectrolyte doped-buffered-TMC nanoparticles

PSS and PSS-Rhodamine doped-buffered-TMC nanoparticles were prepared using layer-by-layer technique. Briefly, the solution of the TMC nanoparticle (360 µl) was added dropwisely into 40 µl polyelectrolyte solutions (10 mg/ml) upon vortexing. The solutions were aged for 5 min. The particles so formed were thoroughly washed with fresh mQ water and separated by centrifugation at 5000 g for 5 min. The particles were then characterized using techniques like microscopy, electrophoretic mobility and dynamic light scattering.

Preparation of PSS-Rhodamine doped buffered TMC nanoparticles

Buffered TMC solutions (360 µl) were added in drops to 40 µl of PSS-Rhodamine upon vortexing and aged for 10 min. The resultant particles were washed thrice with fresh mQ water upon centrifugation and later characterized in terms of particle size, morphology and zeta potential.

Preparation of PSS-Rhodamine doped alhydrogel® beads

Alhydrogel (360 µl) was added drop wise to a solution of the polyelectrolyte (40 µl) upon vortexing and the system incubated for 10 min. This was followed by thorough washing (3×) to remove the excess unadsorbed alhydrogel® beads. The particles were then characterized using confocal laser scanning microscopy.

RESULTS & DISCUSSION

Mechanisms of chitosan quaternization

TMC, otherwise called quaternized chitosan was

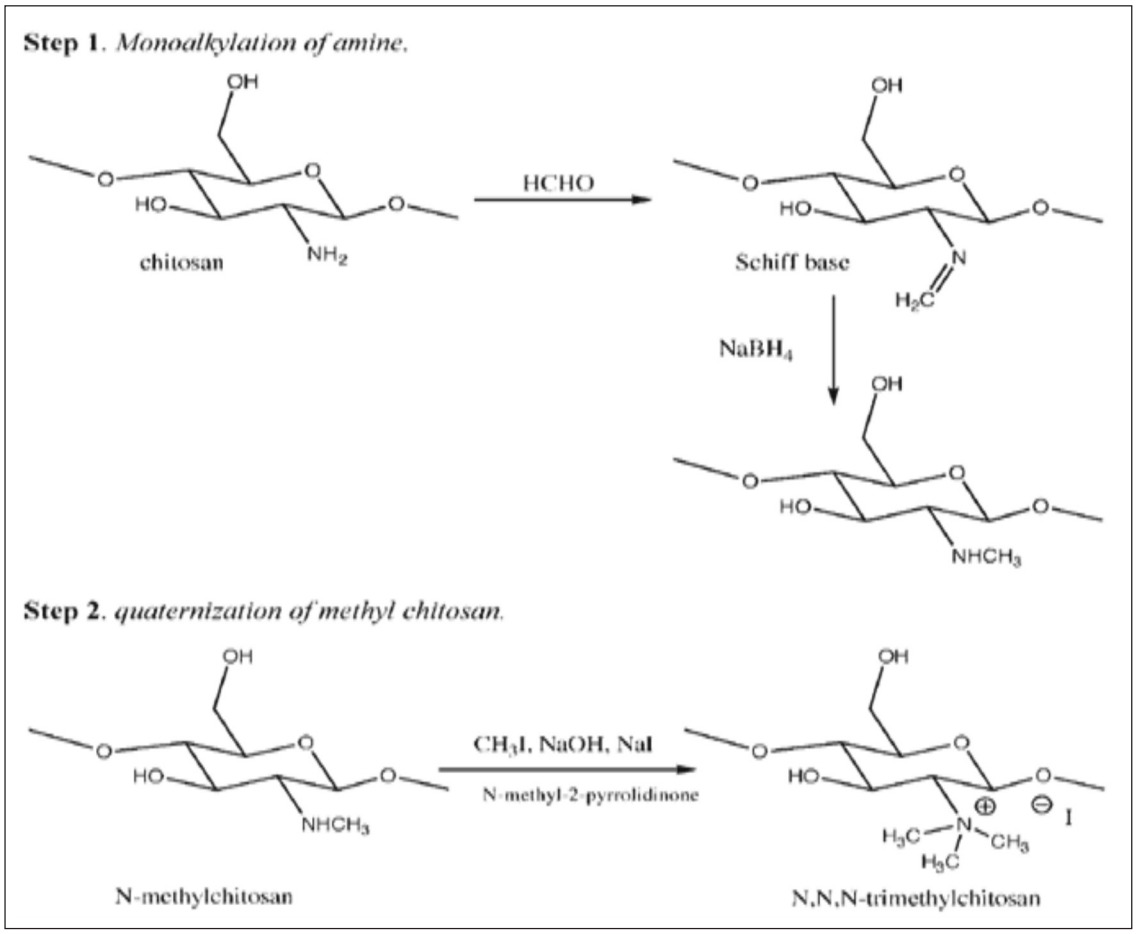

Fig. 1: Synthesis of N,N,N-trimethylchitosan[12].

derivatized to improve the solubility of chitosan in water or other solvents with the ultimate aim of generating non-pH-dependent positive charges which has been shown to boost its antimicrobial activity[12]. The quaternization process was done in two steps (Fig. 1); Step 1: monoalkylation of the amine group; and Step 2: quaternization of the alkyl chitosan. Aldehydes and ketones form hemiaminals with amine groups. The hemiaminals resulting from primary amines of anhydroglucosamine units of chitosan, easily lose water, inducing a double carbon-nitrogen bond. The reduction of the double bond allows the formation of *N*-alkylchitosan. The quaternization of *N*-methylchitosan was further performed using methyl iodide with sodium hydroxide at 60°C for 6 h under vigorous agitation.

The TMC was characterized by ^{1}H NMR and the spectrum showed an intense signal at 3.16 ppm corresponding to the trimethylammonium group in addition to other functional groups (Fig. 2). A quaternization degree of 95% was achieved. The TMC was freely soluble in water at any pH.

In order to ascertain the most stable media for nanoparticle formulation, TMC nanoparticles were formulated in mQ water, carbonate and phosphate buffers and analyzed for surface charge and zeta potential both immediately after preparation (Fig. 3) and after storage for one month (Fig. 4). Figure 3 showed that the nanoparticles of TMC were most stable in PBS as well as had the smallest particle size of 186 nm where as upon storage for one month at 4°C, the particles grew smaller

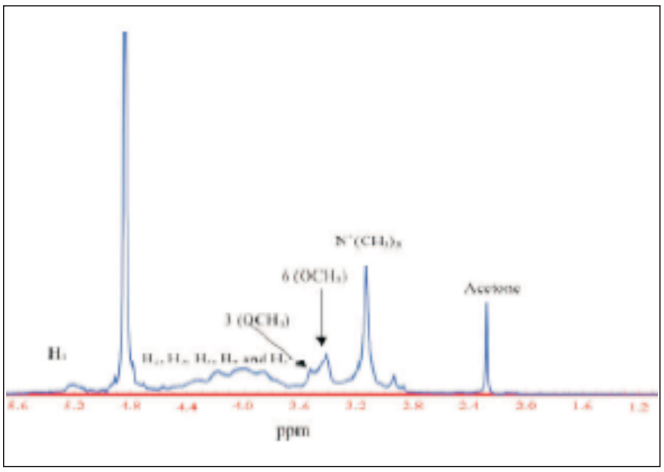

Fig. 2: ^{1}H NMR spectrum of TMC.

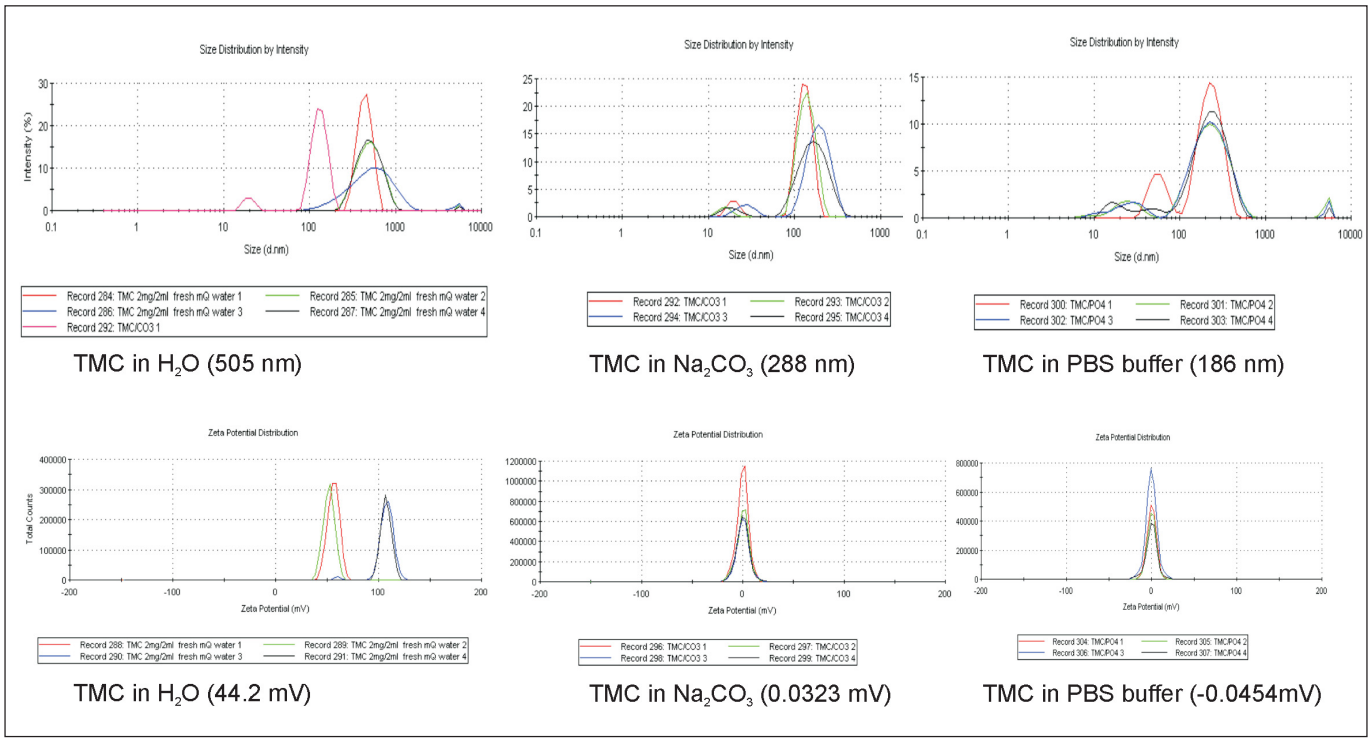

Fig. 3: Characterization of TMC nanoparticles in adjuvant buffers immediately after preparation.

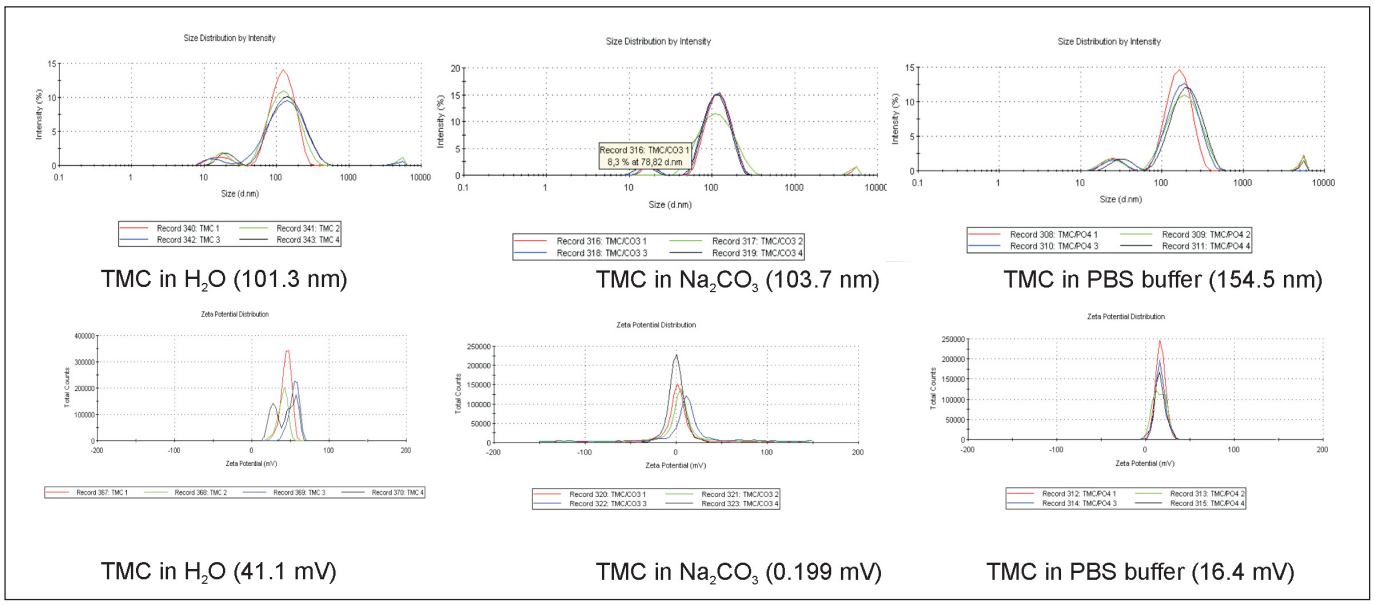

Fig. 4: Time-resolved particle size analysis of the TMC nanoparticles after storage for one month at 4 °C.

in size (154.5 nm) with a large positive charge of 16.4 mV (Fig. 4), showing that the nanoparticles were permanently cationic. This property has been shown to be responsible for the improved performance of quaternary chitosan against the parent chitosan (water insoluble) in terms of the antimicrobial activities against Gram negative and Gram positive bacteria, lower but sustained permeation through epithelial cells as well as improved opening of the tight junction.

Upon further particle analysis of the TMC doped-polyelectrolytes of PSSRh in carbonate and phosphate buffers, the nanoparticles contained in the PBS showed consistently 62.66 nm size with a more positive surface charge of 4 mV, superior to that of carbonate buffer (Fig. 5). The morphology of the nanoparticles showed monodispersed small sized particles both after preparation and storage

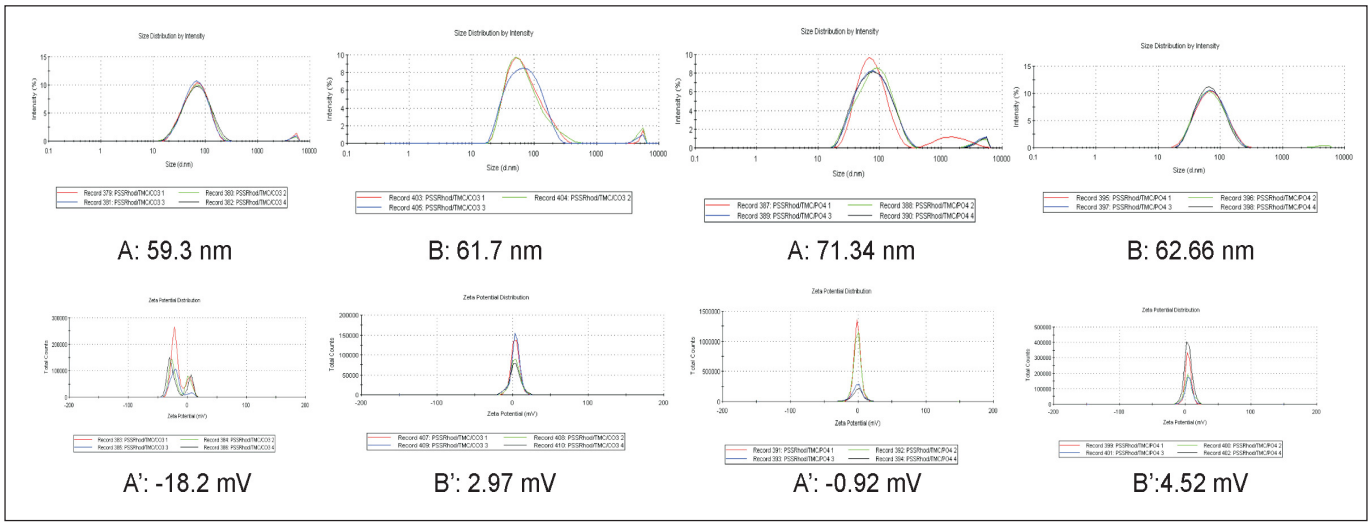

Fig. 5: Time-resolved particle size analysis of LBL polyelectrolyte doped- TMC nanoparticles immediately after preparation [A and A´] and after one month storage at 4 °C [B and B´].

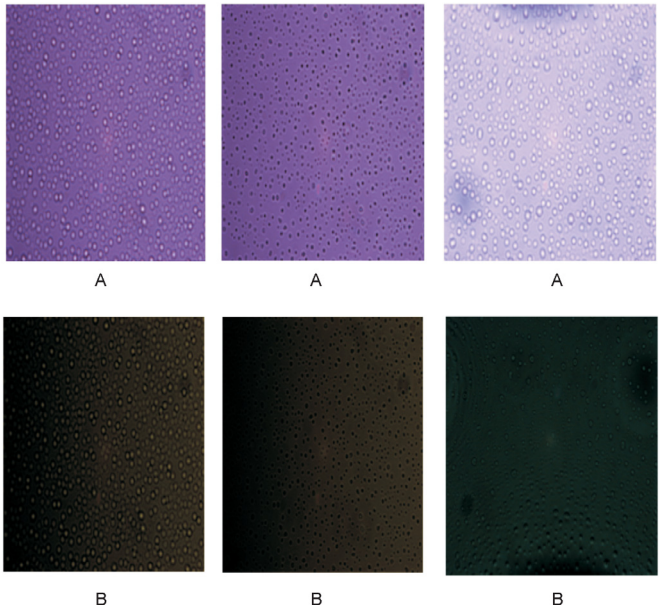

Fig. 6: Morphology of PSSRhod/TMC in PBS buffer immediately after preparation (A) and after one month of storage at 4°C (B)

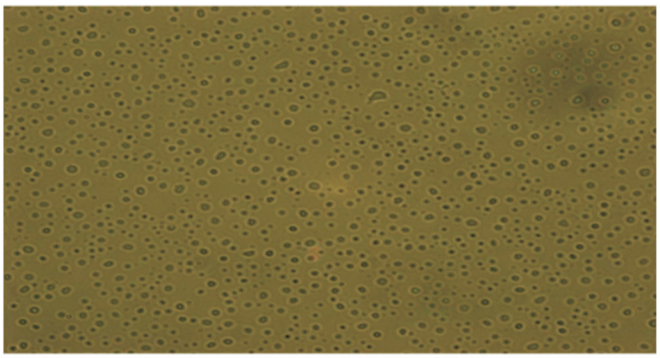

Fig. 7: PSSRhod-doped TMC nanoparticles in PBS showing fluorescent hollow cores (cavities) and peripheral coatings due to PSS.

(Fig. 6). The morphology of the TMC nanoparticle-doped polyelectrolytes showed fluorescent hollow cavities in the core of the nanoparticles with peripheral coatings due to PSS (Fig. 7). This suggests two possible mechanisms by which the system would pick the antigen: firstly by entrapment into the hollow core of the nanoparticle or secondly, by adsorption unto the peripheral PSS layer which can be tuned to multilayered assembly system using LBL polyelectrolyte approach to decorate the resulting system with other immune boosters (UA and IL-12) for a longer-lasting immune recognition (Fig. 8).

The conventional vaccine carrier (Alhydrogel®) was subjected to polyelectrolyte coating of PSSRhodamine 123 to check the particle size and morphology of the system as a comparison to the TMC nanoparticles. The confocal image showed polydispersed particles of 1.6 µm size, with the fluorescent dye covering the entire surface of the particles (Fig. 9). Even though the alhydrogel beads were

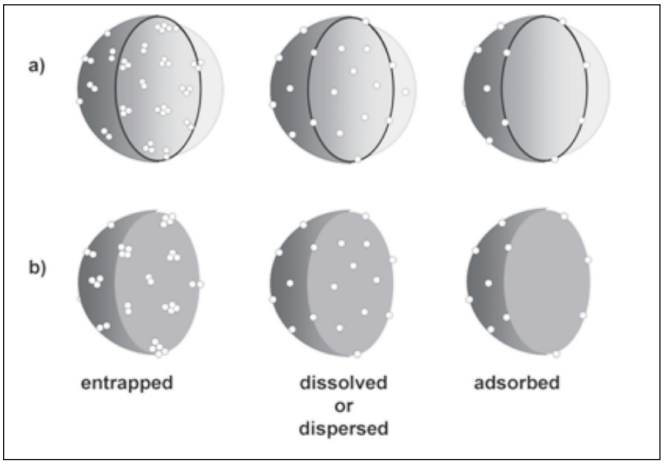

Fig. 8: Overall mechanism of TMC adjuvancity.

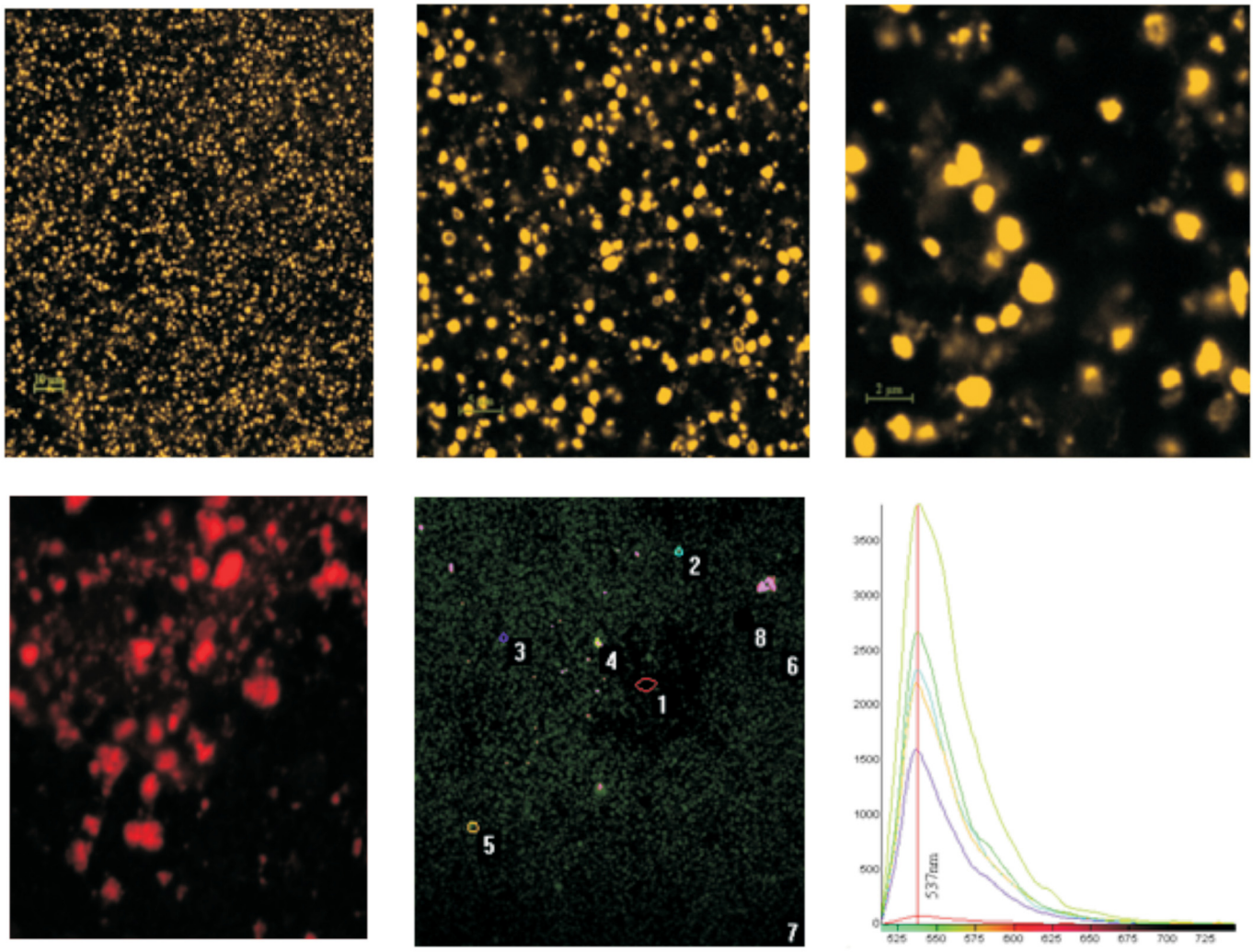

Fig. 9: CLSM of alhydrogel®-coated PSS-labeled Rhodamine 123.

stable, the TMC nanoparticles contained in PBS would make better carrier for the intended antigen delivery due to their smaller particle sizes and stability.

CONCLUSION

The investigation however identified phosphate buffer (PBS) as a compatibly better carrier for the TMC nanoparticles than carbonate buffer, even upon storage. The particles obtained were cationic, monodispersed and stably less than 65 nm in size upon one month storage at 4°C unlike the large particles obtained using conventional alhydrogel beads. The mechanism of the antigen delivery based on this result would therefore be by entrapment into the hollow cavities of the nanoparticles or adsorption unto the surface of the peripheral polyelectrolyte coatings. Having preliminarily established that our carrier (TMC) has the desired qualities for the intending antigen delivery, further research regarding the biological activity will therefore be pursued in the next phase of the work.

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