Development of Nanoadjuvants using Polymeric Carrier to Enhance Immune Response

A

Dissertation submitted

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by

Khayati Arora

Roll No.301507003

Under the Supervision of

Dr. Shekhar Agnihotri
Dr. Manoj Baranwal

School of Chemistry & Biochemistry

Thapar University

Patiala-147004

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CERTIFICATE

This is to certify that the work which is being presented in the dissertation entitled **"Development of Nanoadjuvants using Polymeric Carrier to Enhance Immune Response"** in partial fulfilment of the requirements for the award of degree of **Masters of Science** in **Biochemistry** submitted in School of Chemistry and Biochemistry, Thapar University, Patiala is an authentic record of candidate's (Ms. Khayati Arora) own work carried under the supervision of Dr. Shekhar Agnihotri (Supervisor) and Dr. Manoj Baranwal (Co-Supervisor) and refers other researcher's work which are duly listed in the reference section.

The matter embodied in this dissertation has not been submitted in part to any other university or institute for the award of any degree in India or abroad.

It is certified that the above statement made by the candidate is correct to the best of our knowledge and belief.

Dr. Shekhar Agnihotri
Assistant Professor
Department of Biotechnology
Thapar University, Patiala

Dr. Manoj Baranwal
Associate Professor
Department of Biotechnology
Thapar University, Patiala

DECLARATION

I hereby declare that the work being presented in the dissertation entitled **"Development of Nanoadjuvants using polymeric carrier to enhance immune response"** in the partial fulfillment of the requirements for the award of the degree of Master of Science in Biochemistry, submitted to School of Chemistry and Biochemistry, Thapar University, Patiala, is my own work under the supervision of **Dr. Shekhar Agnihotri (supervisor) and Dr. Manoj Baranwal (co-supervisor).** I have not submitted the contents embodied in this dissertation for the award of any other degree in India or abroad.

Khayati Arora

Patiala **Khayati Arora**

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Khayati Arora

Khayati Arora

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LIST OF SYMBOLS AND ABBREVIATIONS

Abbreviation	Name
%	Percentage
^{0}C	Degree Centigrade
µ	Micro
µL	Microlitre
$CaCl_2$	Calcium Chloride
CMI	Cell interceded invulnerability
DCs	Dendritic Cells
HCl	Hydrochloric Acid
IL	Interleukin
M	Molar
MHC	Major Histocompatability Complex
MTT	3(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)
mM	Milli-molar
NaOH	Sodium Hydroxide
Nm	Nanometer
O.D.	Optical density
PNPs	Polymeric Nanoparticles
Rpm	Revolutions per time
Th1	Type1 helper
TLR	Toll Like Receptor

ABSTRACT

Vaccines are the most successful scientific invention which is being utilized for the various diseases when any foreign antigen enters the body. An immune response is generated during the entry of an antigen which is mainly less immunogenic. Due to less immunogenic immune response by some vaccines, need of adjuvant has been raised. The most commonly used adjuvants are the aluminum adjuvants which are use for the human vaccination. But due to low efficiency, low stability, tolerability, and toxicity polymeric carrier for adjuvant is used. So there is need for the development of new adjuvants so that they can benefit the field of immunology. In the present research polymeric carrier using sodium alginate / Chitosan beads were formed in which the peptide was encapsulated. RAW bead interaction assay, MTT assay were performed in order to characterize the peptide loaded beads. Therefore, the effect of Chitosan/sodium alginate beads (with and without peptide) on peripheral blood mononuclear (PBMC) and RAW 264.7 cell lines were observed. Sodium alginate beads were found to have no effect on PBMC as the absorbance value was found similar to that for cells. While, Chitosan mixed sodium alginate beads was found to have little higher absorbance than cells only. When the effect of the beads was evaluated on Raw 264.7 cell line, it was observed that the absorbance is close to cells o1nly for both beads. Hence, these beads have no cytotoxic effect on peripheral blood mononuclear cells (PBMC) and RAW 264.7 cell line. In peptide encapsulated sodium alginate/ Chitosan beads PBMC interacted and proliferated more efficiently in the presence of peptide encapsulated in Chitosan/sodium alginate beads as the absorbance value was found to be more than cells only and peptide treated cells. Conclusively, Chitosan/sodium alginate beads can be utilized as a promising delivery vehicle after incorporating peptide based nanoadjuvants for enhancing immune response.

Keywords: Vaccines, immunogenic, adjuvant, polymeric carrier, immunology, cytotoxic.

CHAPTER 1

INTRODUCTION

1.1 Background and Motivation

There always exists a race between the modern health therapies and invasion of foreign contaminants to depress the immunological strength of individuals. Researchers have introduced an entity called vaccines, which are the biological soldiers solely work to improve the immunity of humans against a specific disease. The vaccine contains attenuated form of antigens which is formed from heat killed forms of the microbes to stimulate the immune response of an individual for development of the adaptive immunity towards the pathogen. The main problem affiliated with the formulation of the vaccines is their poor efficiency in terms of type of immune response they generate and their capacity to provide long term immunity with least no. of doses. For the correct evaluation of successful vaccines, it is suggested to have a prudent selection of the antigen based on known antigenic variations and strain diversity in a particular manner. It is essential to choose the right kind of delivery system to supply the antigen at the correct location based on duration and concentration (Gupta *et al*.,1995). Quality of vaccine production can be improved by the incorporation of adjuvants or immunomodulators with the use of modified vehicles like Liposomes, polymeric microspheres, Nanobeads etc (Sun *et al*., 2017). On the other hand, adjuvants are the compounds that improve or enhance the intensity of immune response against the antigen. In some cases antigen alone is less immunogenic. Iin such cases, adjuvants are employed to increase the magnitude of immune response (Mohan et al., 2013). For instance, the use of aluminium adjuvant was first demonstrated in 1926 for the treatment of diphtheria toxoid (Gaserod *et al*., 1998).

Nanotechnology has been a quickly creating and developing territory since the most recent decade for the twentieth century. To date, critical achievements have been made in the plan and control of materials at the nanoscale to impact their execution in biomedical applications (Aguilar *et al*., 2007). Many sorts of substances, including drugs, proteins and also immunizations can be conveyed by nanomaterial based conveyance frameworks to meet the criteria of high bioavailability, managed and controlled discharge profiles, focusing on, imaging et cetera (Elzatahry *et al*.,2009). Antigens conveyed by nanomaterials can be safeguarded from corruption and discharged in a supportable way,

and their take-up by antigen displaying cells (APCs) is more effective (Gregoriadis, 1990).

The uses of nanomaterial as immunization adjuvant have been progressively examined for resistant assurance and immunotherapy for irresistible sicknesses and harmful malignancies, and these materials have indicated implicational advantages (Klabunde, 2001).Yet at the same time the part of adjuvant in human antibodies has involved solid open deliberation in light of the fact that for around 80 years, aluminium salts were the main adjuvant which got endorsed for investigate reason by the Food and medication organization(Silva *et al*., 2004).

Indeed, even today, alum based adjuvant or in mix with extra safe activators are the main adjuvants which have been endorsed for human utilization (Eisenbarth *et al*., 2008). To beat the issues of adjuvant, like low efficiency, low dependability and high poisonous quality, polymeric nanoparticles are being utilized (Lewis *et al*., 1995). Polymeric nanoparticles (PNPs) are the particulate scatterings or the strong particles which are 10-1000 nm in the size range. They are being utilized as bearers for little and substantial atoms in particulate conveyance frameworks (Bomford *et al*., 1992). Pharmacokinetic and pharmacodynamic properties of different sorts of medication atoms has been enhance and adjust by the utilization of particulate frameworks like nanoparticles (Owens *et al*., 2006). In pharmaceutical and restorative fields polymeric nanoparticles are as a rule broadly utilized on account of their controlled and supported discharge properties, sub cellular estimate, biocompatibility with tissue and cells (Soppimath *et al*., 2001). A few strategies have been created to plan polymeric nanoparticles and these methods are classified by whether the molecule arrangement includes a polymerization response or nanoparticles form straightforwardly from a macromolecule or preformed polymer (Nagavarma *et al*., 2012). In this study natural polymeric particles like chitosan and sodium alginate were used for the formation of beads. After the formation of chitosan/ sodium alginate beads peptide was encapsulated in them which showed enhanced proliferation of PBMC. Therefore this study sheds new light on developing polymeric particle which can enhance the intensity of the immune response and act as good adjuvant.

CHAPTER 2

LITERATURE REVIEW

2.1 Adjuvants

Adjuvant has been generally used to build the greatness of a resistant reaction to an immunization, in view of counter acting agent titre or capacity to anticipate contamination, yet second part for adjuvant has turned out to be progressively critical i.e. controlling the sort of versatile reaction to create the best types of invulnerability for every particular pathogen (Sylvester *et al*., 2000). An adjuvant is important for consolidation into antibody for the accompanying reasons: (1) dosage saving, which implies the adjuvant advances adequate insusceptible reactions with less antigen or less quantities of inoculation, (2) empowering a wide counter acting agent reaction against pathogen with antigenic float or varieties, (3) the capacity to shape the safe reaction toward a practically proper sort to give subjective and solid insurance against diseases, and (4) advancement of a more quick invulnerable reaction and to accomplish subjective change of the resistant reaction(Ayanian *et al*.,2003). For antibodies right now being worked on, adjuvant are progressively used to advance sorts of resistance not viably produced by the non-adjuvant antigens. For instance, adjuvant have been utilized as a part of pre-clinical and clinical investigations to (1) give practically suitable sorts of insusceptible reaction, (2) increment the era of memory, particularly T-cell memory, (3) increment the speed of beginning reaction, which might be basic in a pandemic flare-up of disease and (4) change the specificity or liking of the reaction (Ferraro *et al*., 2011).

2.2Adjuvant Selection

A portion of the components required in adjuvant determination are the antigen, the species to be inoculated, the course of organization and the probability of reactions. preferably, adjuvant ought to be steady with long retire –life, biodegradable, modest to deliver , not initiate invulnerable reactions against themselves and advance a proper safe reaction that might be cell or humoral insusceptibility relying upon the prerequisites for assurance. Critical characteristics of a perfect adjuvant are as per the following; (Pashine *et al*., 2005)

1. It must be protected, including opportunity from quick and long haul symptoms.

2. Adjuvants should be biodegradable or expelled from the body with ease.
3. It should exhibit a more vigorous defensive response to evoke even when the same antigen attacks an individual again.
4. It must be characterized artificially and organically, so that there is no parcel to-part variety in the made item, in this manner guaranteeing steady reactions in immunizations amongst studies and extra time.
5. Adequacy must be accomplished utilizing less dosages or lower groupings of the antigen.
6. It ought to be steady on the rack to be economically and clinically helpful.
7. The adjuvant should be cost effective and economically viable.

2.3 Delivery Systems of Adjuvant

An adjuvant is a compound that intensifies or amplifies the insusceptible reaction against an immunization antigen. "Adjuvant" originates from the latin word "adjuvare", signifies "help" or to "upgrade", can be characterized as any item or relationship of parts that increments or adjusts the humoral or cell immune reaction against an antigen (Nochi *et al*., 2010). By and large, the antigen itself is feebly immunogenic; thusly an adjuvant is expected to escalate the resistant reaction. Adjuvant can likewise be incorporated into antibody to manage the kind of insusceptible reaction produced (Jiang *et al*., 2008). This might be particularly imperative when creating antibody for tumour, human immunodeficiency (HIV). Interestingly, a more immunogenic antigen may profit by a particular conveyance vehicle. This segment may encourage focusing on or controlled arrival of the antigen to dendrite cells (DCs). Late investigations, using Toll like receptor (TLR) ligands, have demonstrated that antigens related with their ligands can deliver astoundingly high counter acting agent and fast insusceptible reactions(Singh *et al*.,2001). Adjuvants have likewise been appeared to shield antigens from corruption, despite the fact that this for the most part relies upon the idea of adjuvant (Baudner *et al*., 2003). For instance, chitosan –adjuvant nanoparticles were found to balance out ovalbumin while on the opposite side, the model protein antigens are really destabilized by the customary aluminium salt adjuvant. The adjuvants can be ordered in light of their five potential methods of activity: (1) immunomodulation, (2) Presentation (3) cytotoxic

T-lymphocytes (CTL) induction, (4) Targeting specific cells, and (5) Depot generation (Marciani *et al*., 2003).

2.3.1 Immunomodulation

This alludes to the capacity of numerous adjuvants to change the cytokine organize. By and large, just immuno-modulatory adjuvant will apply an adjuvant impact when exhibited at a different time or site to the immunogen. Immuno-modulation may bring about a general up-regulation of the whole resistant frame-work; however most regularly brings about up-regulation of specific cytokines and an attending down control of others (Kazatchkine *et al*., 2001). Two noteworthy subsets of CD4+ T cells, by means of Th1 and Th2 have been all around depicted for mouse and man and their reality is populated for other creature species (Schepetkin *et al*., 2006). Th1 reactions commonly incite supplement settling counter acting agent and solid postponed sort extreme touchiness (DTH) responses and are related with gamma interferon , IL2 and IL12 while Th2 reactions result in high circling and secretory immune response levels, often IgE and IL6, IL5, IL10 and IL4 cytokines(Erickson *et al*.,2000). Th1 and Th2 reactions mainly show inhibition. Determination of the suitable immuno-modulatory adjuvant won't just prompt an improved invulnerable reaction yet will likewise decide the IgG isotope in which different immuno-globulins and the amount CD4+ coordinated, (CMI) cell interceded invulnerability is created. Resistant reactions never swing absolutely one way or other. The most striking swings are delivered by aluminium salts greater than 90% Th2 and end toxins of bacteria and subordinates which instigate a predominately Th1 sort reaction (Perros *et al*.,2016).

2.3.2 Presentation

It eludes the capacity of an adjuvant in order to protect the conformational respectability of the antigen to display this to the fitting insusceptible effectors cells. It takes place when an adjuvant is ready to associate with conformational epitomes' which are antigens, more successfully. The fundamental advantages are enhanced in vivo movement and an expanded time span of usability. Therefore antigen introduction gives three noteworthy advantages, right off the bat it will amplify the measure of conformation ally pertinent immunizer, furthermore it will impact the partiality of the counter acting agent lastly it can impact and span of the insusceptible reaction. Proficient antigen introduction by MHC on APCs is imperative for the

enlistment of versatile insusceptible reaction. It has been suspected that numerous adjuvant including oil-based emulsions, alum and micro particles act by "focusing on" antigens to APCs bringing about upgraded antigen introduction by the MHC (Guéry et al., 1996; Schijns and Lavelle, 2011). Alum was appeared to expand antigen take up by DCs and change the extent and term of antigen introduction. Similarly the adsorption of antigen on alum, leads to the expansion in the disguise of an antigen. Late examinations by Flach et al. (2011) demonstrated that the alum does not enter into the DCs straightforwardly yet it conveys an antigen through fruitless phagocytises. These occasions in the long run prompt take-up of antigen that is adsorbed on alum, DC enactment, expression of MHC-II (Didierlaurent *et al*., 2014).

2.3.3 Induction of CD8+ Cytotoxic T-lymphocyte (CTL) Responses

Enlistment of CTL reactions by an antigen handled inside the cytosol of the cell where peptides by and large 9-mers, wind up plainly fused inside the shut end depression of the MHC-I atom and then these communicated on the surface of cell. Recent proof, recommends that the turnover of cell proteins, happen in 26 S, multi-chemical complexes. Proteolytic part of this is the proteasome complex, profoundly saved 20S structure including of 24S and 28S subunits. The dominant part of the proteins goes through the perplexing way out, the peptides, additionally handled by exopeptides to the amino acids. Be that as it may, a little extent are specifically transported to endoplasmic reticulum by (TAP1 and TAP2) transporter proteins, they are joined in furrow of the framing of MHC-I and means of the golgi passed to the phone surface. There is an expanding proof that the low sub-atomic mass protein is a proteasome where two of the subunits are encoded by MHC (Liu *et al*., 2015). The nearness of these subunits encoded by MHC, inside the proteasome may change the cleavage of proteolyte towards peptides which are MHC good. LMP creation, unregulated by gamma-IFN and enticing it to guess an instrument whereby the extent of peptide fit for inclusion into the MHC-I, expanded in the light of the cytokine cautioning signal. For an adjuvant to be valuable for the CTL enlistment, must encourage consolidation or industriousness of suitable peptide in the MHC-1. The best approach to accomplish this for adjuvant to the cooperate somehow with cell layers so antigen related with an adjuvant is kept inside the cytosol in a frame appropriate ordinary handling in proteasome. This may happen by combination with the outside layer or by endocytosis taken after by endosome film combination or

burst. Fuse of an immunomodulator inside this an adjuvant detailing, particularly one which instigates gamma IFN creation, could be relied upon to an increment of pertinent MHC-I peptide expression. Albeit MHC-I expressed by most of the cells, for CTL acceptance the best target cell, is an APC and most presumably a DC (Siewe *et al*., 2014).

2.3.4 Targeting

It Characterizes capacity of the adjuvant, to convey an immunogen to the resistant effector cells, for the most of the part by means of APCs. Albeit little information exists, likely that most of the immunization conveyed is lost either by first pass evacuation in liver or by corruption of serum protease. This type of adjuvant action may not adjust the sort of insusceptible reaction but instead will influence measure of immunogen required to accomplish a given impact i.e. effectiveness resistant reaction era. Nonetheless, if focusing on can be particular for macrophages as opposed to DC, or the opposite, the sort of safe reaction might be considerably altered where consumption of macrophages prompted a solid Th2 move in response(Marques *et al*.,2014). There are a few routes in which an adjuvant can accomplish this impact. The most widely recognized is to interface with antigen so as to frame multimolecular totals. These totals will empower take-up by macrophages, DC and an immunomodulatory adjuvant is incorporated, will guarantee that antigen and immunomodulator are conveyed to a similar APC. Adjuvants that have this property are named "particulate adjuvants". In particular applications, particles of 1-10 um can be conveyed orally to improve take-up by Peyer's patches.

2.3.5 Generation of Depot

This can be accomplished as here and now or long haul warehouse, the last giving either continuous or the beat discharge. Here and now warehouses are exemplified by aluminium salts and without emulsions, where antigen is caught at the infusion site and consequently can't be lost by liver leeway. Extraction of the infusion site 8-10 days in the wake of dosing has close to nothing if any impact on extent or term of reaction proposing antigen has either been evacuated or walled off by that stage. Long haul terminals are best accomplished utilizing engineered polymers, for example, Poly-lactide co-glycolide (PLG) to create microspheres which debase to yield beat conveyance. The microspheres are ideally of a size >10 um with the goal

that they should stay at the infusion site until the point that biodegradation grants evacuation of their substance by APC. Discharge times from 1 to 6 months can be accomplished with sensible exactness. The development of a warehouse at the infusion site is maybe the most established and most generally perceived instrument of activity of adjuvant. As of not long ago, terminal impact was viewed as a great instrument of activity of numerous adjuvant. Glenny et al., 1926 were the first to research the significance of station development in an adjuvant action of alum. Antigens are basically adsorbed onto the alum however the coupling is proposed to be because of solid electro-static cooperation amongst antigen and alum, which improved antigen take-up and introduction by APC. Different adjuvants, for example, water-in-oil emulsions [Complete Freunds Adjuvant (CFA)] and biodegradable miniaturized scale and nano-particles were appeared to act by stop impact to produce drawn out and managed high immune response titers (Brito *et al*., 2014). AS04, an adjuvant mix comprising of monophosphoryl lipid A (MPL) and alum was appeared to instigate ideal invulnerable reactions just when co-restricted with antigen. The nearness of alum in AS04 is critical in balancing out the MPL and antigen inside the immunization, alongside giving a terminal impact. The cationic adjuvant definition (CAF) 01, a blend of dimethyldioctadeclammonium/trehalose-6, 6-dibehenate (DDA/TDB), which is as of now in stage I clinical trial, is additionally thought to prompt dependable terminal impact.

An adjuvant can act in more than one route, adding to inspire a gainful safe reaction against an antigen. Over the most recent couple of years, the adjuvant properties of immunomodulation have been ascribed to a few macromolecular segments of microorganisms which are perceived by pathogen-related atomic examples (PAMPs), display on cells of intrinsic safe framework. These parts are called sub-atomic examples in light of the fact that these are structures much of the time experienced in microorganisms that encourage the inborn insusceptible reaction against them. Cases of insusceptible tweak by these segments incorporate authoritative of mixes like lipopolysaccharides (LPS), lipopeptides and CpG themes to unmistakable individuals from TLR family, prompting macrophage and DCs initiation and the official of glycoproteins or glycolipids to mannose receptor on phagocytes. Albeit numerous parts of this class have been decontaminated and tried with various immunization plans focusing to evoke an appropriate resistant reaction

against a particular antigen, yet to play out the adjuvant ought to be as one at a similar site since the antigen-introducing cells (APCs) which prepare the antigen ought to likewise be actuated for a back initiation of a gullible T-cell. To take care of these issues, a few plans and bearer frameworks have been created, for example, emulsion, liposome, microspheres, insusceptible invigorating edifices (ISCOMs) and nanospheres. These bearers share a portion of the accompanying properties: insurance of antigen from debasement following its organization by various courses including mucosal, capacity to support the antigen discharge over a broadened timeframe, intracellular conveyance of antigen adding to cytotoxic T-cell incitement and focusing at APCs (Perrie *et al*., 2016**).** Subsequently, with the point of evoking wide insusceptible reaction particularly with solid cell intensifies, the pattern has been to join adjuvant or to define these to accomplish warehouse arrangement, enlistment and initiation of APCs within the sight of the coveted antigen.

2.4. Adjuvants in Vaccine Research

The advancement of adjuvants which new for immunization of human turned into an extending research field over most recent thirty one years, in order to create more grounded antibodies fit for instigating defensive and durable insusceptibility in people. The achievement of adjuvant in improving the insusceptible reaction for antigen recombination has driven may look into re-center their antibody improvement programs. Improvement of effective antibody requires knowing the adjuvant which are utilized and knowing the plan of the adjuvant and antigen to accomplish steady, sheltered and vaccines which are immunogen. Adjuvant are atoms, mixes or macromolecular edifices that lift the strength and life span of particular insusceptible reaction to antigens, yet cause negligible danger or dependable safe consequences for their own. Expansion of adjuvant to upgrades of antibodies coordinates and supports the immunogenicity of antigens, viably regulating suitable resistant reactions, decreasing the measure of antigen or number of inoculations required and enhancing the proficiency of immunizations in babies, or in immunocompromised people. Adjuvants have low proficiency unless legitimately planned, along these lines both adjuvant segments and definition are critical for upgrading immunization potency.

Conventional live antibodies in light of lessened pathogens normally don't require the expansion of adjuvants. Similarly, immunizations in light of inactivated

viruses or microorganisms are frequently adequately immunogenic without included adjuvants, albeit some of these for instance split influenza infection or entire cell pertusis can be defined with adjuvants to further enhance the insusceptible responses. By differentiate , protein based antibodies , in spite of the fact that offering significant focal points over customary immunizations as far as security and cost of generation, much of the time have restricted immunogenicity and require the expansion of adjuvants to incite a defensive and enduring insusceptible response. Late advances have started to reveal insight into the cell and sub-atomic nature of inborn insusceptibility and adjuvant action. The insusceptible framework perceives pathogen-related sub-atomic examples (PAMPs) by methods for pathogen – acknowledgment receptors (PRRs) , incorporates the TLRs, C-sort receptors like lectin, oligomerization of cytosolic nucleotide area like receptors and retinoic corrosive given quality – based –I-like receptors . These receptors tie microbial ligands including cell divider segments, lipoproteins, lipopolysaccharides, proteins, RNA and DNA of microorganisms, infections, protozoa and growths in order to increase the distinctive sorts of resistant reactions. The PAMPs, particularly those coupling the TLRs, are the premise of numerous adjuvants. Moreover, cytokines, glycolipids and bacterial poisons that change antigen preparing are being utilized as a part of adjuvants and adjuvant plans use different mixes and systems to accomplish the coveted immunological enhancement (Reed *et al*., 2013). These components incorporate the era of enduring antigen terminals, expanded immunological introduction of immunization dendritic cell antigens (DC) initiated through the engagement of PRR or harm – related atomic example (DAMP) receptors and acceptance of CD8+, cyto-toxic T-lymphocyte (CTL) reactions or CD4+ and T-partner lymphocyte reactions (TH1or TH2).

According to sources component adjuvant to be classified into the physiochemical properties or action mechanisms. Adjuvant two classes are commonly found in modern vaccines includes:

Immunostimulants-They specifically follow the insusceptible framework to expand reactions to antigens. Cases are cytokines, TLR ligands, bacterial exotoxins and saponins that invigorate safe responses.

Vehicles-In the present antibody antigens is the safe framework in ideal way, includes a controlled discharge, warehouse conveyance frameworks in order to expand the particular insusceptible reaction to the antigen. The vehicle can likewise serve to convey the Immunostimulants (Baert *et al*., 2016). Illustrations are minerals salts, emulsions, liposome, virosomes, and biodegradable polymer microspheres.

2.5 Categories of Adjuvants

2.5.1 Mineral Salts

From the period of Glenny et al., salts of aluminium, essentially hydroxide or phosphate of aluminium, generally utilized adjuvant in people. Sadly, salts of the alum are moderately poor adjuvant much of the time, especially at initiating cell insusceptible reactions. The component whereby aluminium salts work stays obscure albeit one proposal is that they work by the development of an antigen stop at the vaccination site. Other conceivable system whereby aluminium salts work stays obscure albeit one recommendation is that they work by the development of an antigen terminal at the immunization site. Other conceivable components of activity may include supplement actuation, or eosinophils or macrophage initiation. Normal granulomas only when the alum is managed through the subcutaneous or intradermal as opposed to intramuscular course. Opposite symptoms of alum are expanded IgE generation, allergenicity and potential neurotoxicity. Typically, aluminium is discharged by the kidneys, albeit under specific conditions, for example, diminished renal capacity aluminium is gathered in the body and can wind up plainly lethal. Predominate level of aluminium levels in body increases the cerebrum and bone tissues causing lethal neurological disorder and dialysis related dementia. Aluminium inebriation is likewise possibly connected to amyotrophic parallel sclerosis and Alzheimer's illness. Then again, iron the salts of calcium, and zirconium has likewise been utilized to adsorb antigens. Specifically, calcium phosphate has been utilized for diphtheria-lockjaw pertusis antibodies.

Aluminium Salts- These are an insoluble, gel like accelerate of aluminium hydroxide, aluminium phosphate or alum with a molecule estimate from 100 to 1000 nm. Immunogen is bound by electrostatic collaboration to pre-shaped gel or amid gel arrangement *in situ*.

They have been generally utilized as a part of human and veterinary immunizations since 1930 and have a superb wellbeing record. They instigate solid Th2 reactions, great focusing on, result in a direct warehouse impact however actuate negligible CTL or CMI enlistment. Solid IgE reactions are much of the time announced. Aluminium salts are modest, safe, and easy to figure.

2.5.2 Tensoactive Compounds

Quil A will be a saponin, gotten as a watery concentrate from the Quillaaja saponaria bark. Parts cleaned from the concentrate by turn around stage chromatography, for example, QS-21; can initiate solid cell reactions against pathogen –derived and HIV-1 antigens. The Quil A will be characteristic item made out of more than 23 unique saponins and is excessively dangerous for human utilize. the Quil A-determined saponin QS-21, while less dangerous than Quil A, has a considerable lot of similar issues and is comparably unsatisfactory for most use of human other tumour antibodies where higher poisonous quality might be acknowledged or at generally low dosages.

2.5.3. Microorganism Derived Adjuvant

Have strong immuno-stimulatory limit, contagious substances form a profitable wellspring of valuable adjuvant. Bacterial cell divide peptide-glycan or LPS upgrades resistant reaction whiles not being themselves profoundly immunogenic. The adjuvant action is interceded by the initiation of Toll-like receptors (TLRs) that intervene the peril signals enacting the host resistant protection framework. Distinctive types of microscopic organisms utilized as a wellspring of adjuvant incorporate Mycobacterium spp., C. parvum, C. granulosam, B. pertusis and N. meningitides. As entire executed microorganisms, these are very harmful to be in any way utilized as human adjuvant. Be that as it may, it shows up the real adjuvant action of these microscopic organisms is intervened by N-acetyl muramyl-L-alanyl-D-isoglutamine, additionally called muramyl dipeptide (MDP). In saline, MDP for the most part improves humoral invulnerability, while when fused into liposome or blended with glycerol it incites solid cell resistance. Mixes with adjuvant movement gotten from MDP incorporate treonyl-MDP.

2.5.4. Emulsions

This class include water in oil or oil in water emulsions, for example, montanide, (FIA) Freund's deficient adjuvant, an adjuvant 65 or lipovant. System of an adjuvant emulsion activity involves development of the terminal at site of infusion, empowering the antigen moderate arrival and the incitement of plasma cells which counter acting agent creating:

Water in Emulsions- These are micro-droplets of water, settled by surfactant in a continous oil stage. Freunds deficient adjuvant (FTA) has been utilized for human and veterinary immunizations yet is presently to a great extent ruined because of a low frequency of site reactivity. Emulsions in view of metabolizable oils have a prevalent security profile. They are ineffectively immunomodulatory, give great here and now stops are modest, moderately easy to plan and incite great counter acting agent reactions particularly for hydrophilic immunogens. W/o emulsions give a great plan into which dissolvable immune-modulators can be consolidated. Emulsions can be unsteady.

Oil in Water Emulsion- These are micro-droplets of water, settled by surfactant in a continous oil stage. Freunds deficient adjuvant (FTA) has been utilized for human and veterinary immunizations yet is presently to a great extent ruined because of a low frequency of site reactivity. Emulsions in view of metabolizable oils have a prevalent security profile. They are ineffectively immunomodulatory, give great here and now stops, are modest, moderately easy to plan and incite great counter acting agent reactions particularly for hydrophilic immunogens. W/o emulsions give a great plan into which dissolvable immunomodulators can be consolidated. Emulsions can be unsteady (Hawkes *et al*., 2015).

2.6 Delivery System of Antigen

In addition to some stop affect, it is particulate in nature that on a very basic level picks whether the antigen-movement structure will be productive in inciting a safe response. If this essential is completed, the blend course of action of vaccination picks which kind of resistant response make, like B cells antibodies isotope will convey, and which T cell cytokines release , and by combining the antigen with immuno-modulatory can be controlled or also with co-stimulatory particles. A couple

of by far most of the analyzed adjuvant is of consolidated into class "particulate antigen transport structures": Polymeric, liposome, microspheres, immunostimulating complexes (ISCOMSs), nano-globules, disease like particles (VLPs), among the most fundamental antigen movement systems. This type of adjuvant is broadly used as bearers for protein subunit and DNA antibodies. There is an understanding, the expansive focus, on their natural affiliations and frameworks of action related to size and substance (Slütter *et al*., 2016).

2.6.1 Liposomes

The Liposomes are engineered circles involved by bilayers of lipid that epitomize antigens and go about both as an immunization conveyance an adjuvant or a vehicle. The power of liposome relies upon the quantity layers of lipid, charge, structure and technique for readiness. Late outcomes have recommended that, picking lipid segments for liposome, antigens which are surface coupled liposome may relevant for the improvement of tumour immunizations to introduce tumour antigens to antigen-showing cells (APC) and incite anti-tumour reactions. Capacity in order to initiate the cross introduction of an liposome coupled Ag was higher in those comprising of greasy acid which is unsaturated (Alving *et al*., 2016). The similar impact in case of liposomal co-entangled DNA and protein has been appeared to surpass the outstanding adjuvant impacts of plasmid liposome and DNA (Yazdi et al., 2010). This new way to deal with inoculation named "co delivery" and might get from the concurrent introduction of antigen through MHC-1 i.e. DNA and MHC-II pathways to CD8+ and 4+ cells at a similar antigen showing cell-a mode of introduction that would normally happen with live popular pathogens, opening uses for this new innovation. Be that as it may, steadiness, assembling and quality affirmation issues appear to main considerations increasing the utilization of liposome as adjuvant in humans.

2.6.2 Polymeric Nanoparticles

Among the particulate and polymer frameworks, poly micro-spheres broadly considered. These are bio-compatible and also bio-degradable microspheres of nano-meter to micro-meter estimate ready to consolidate diverse antigens. One of their points of interest includes ability which control debasement energy through changing

the relative convergence of their parts, in this way controlling the time of antigen discharge.

2.6.3 Nanobeads

Strong latent dots with an antigen which are adsorbed on surface have already been utilized to empower T CD8+cell reactions, with ideal dab distance across size of 1um and <0.5 um revealed as mediocre in focusing on antigens for MHC class1 limited introduction to T cells. As of late, the utilization of strong inactive globules of nano-metric estimate (0.04-0.05um) was accounted for as an exceptionally encouraging system to accomplish productive antigen conveyance to APC, creating powerful and joined humoral and CD8+ T cell insusceptibility.

2.6.4 Virus- Like Particle

These include particles which are dormant, discharge capsids of infections, excluding the genetic material from the infection itself. They may hold the structure of an infection and are delineated in such a way so that the antigens are connected. Particles which are comparable in shape as well as in size acquired through hereditary designing which includes antigen by non- viral or either viral sources are additionally viewed as VLPs.

2.6.5 Cytokines

When in doubt, cytokines are incorporated into the cutting edge arrangement of adjuvants. For instance, granulocyte-macrophage state fortifying element (GM-CSF) improves the essential invulnerable reaction by actuating and selecting APC. Cytokines are for the most part glycoproteins of atomic weight around 20 kDa which are proposed as human and veterinary immunization added substances. They have different activities, e.g. IL-1, gamma-IFN, and GM-CSF. IL-12 has as of late been appeared to initiate solid Th 1 moves and may have potential as an adjuvant in human immunizations. Cytokines are costly, species-particular, and also there are concerns with respect to soundness, harmfulness and potential autoimmunity. On the off chance that these issues can be defeated, they may end up noticeably critical parts of some prophylactic and most helpful immunizations (Matyas *et al*., 2013).

2.7 Adjuvant Formulations

New adjuvant definitions have been come about because of the blend of atleast two adjuvants with various activity systems. The point of this system is to additionally upgrade or balance the safe reaction against a given antigen contrasted with the adjuvant alone and now and again, to consolidate conveyance change and adjustment.

A surprising adjuvant definition involving MPL and alum has been as of late incorporated into the endorsed antibody plan Fendrix , utilized this vaccination in the patients which are suffering from renal diseases which are averse with respect to hepatitis B. It is the immunization that builds up a high, quick, extraordinary and enduring invulnerable reaction contrasted and the control antibody in these high hazard gatherings, demonstrating wellbeing and clinically adequate nearby responses like other authorized hepatitis B immunizations. The ASO4 adjuvant detailing has been tried additionally as a piece of promising HPV antibody advancement (Agger *et al*., 2016).

2.8 Problems in the Development of Adjuvant

New adjuvant definitions have been come to fruition due to the mix of no less than two adjuvants which are different in their action of framework. It involves the response against a particular antigen appeared differently in relation to the adjuvant alone and from time to time, to unite transport change and alteration. . A adjuvant development main problem is the development of vaccines for human routine use, is that adverse reactions which are rare, like in thousand several doses, until late may not be detected in development programs. Additionally, during adjuvant development there are some other problems have been encountered for vaccines for human use which are discussed as follows:

2.8.1 Limited Adjuvanticity

With particular antigens, few adjuvant act and are not viable with different types of antigens. For example, aluminium mixes did not show an adjuvant impact when utilized with typhoid antibody, flu haem-agglutinin antigen and haemophilic flu sort b (Hib) polysaccharide capsular conjugated to lockjaw toxoid. Antigens which are standard like ovalbumin and flu haem-agglutinin have been proposed to ponder adjuvant city of details which are new. The model of antigen might be helpful for

beginning preparatory screening, yet it is suggested that the greater part of the advancement work should be possible with the antigen for which adjuvant is being created. Being less immunogenic, ovalbumin might be a reasonable antigen for assessment in adjuvant yet there a few issues with utilization as a standard or model antigen:

1. It does not have clinical criticalness about ova albumin.
2. Research in creatures utilizing ova albumin have utilized dosages of protein which are clinically not adequate and might maximal measurements which would not able to separate little contrasts among adjuvant definitions.
3. There is no measure for utilization of immune response because ovalbumin does not have an organic action. Preparatory assessment of adjuvant, utilization of antibody and antigens, for example, lockjaw toxoid at dosages which are not for this creature model or the edge measurements suggested. Diphtheria toxoid discovered especially valuable, as it is immunogenic which is poor.

2.8.2 Sub-optimal use of Aluminium Adjuvant

The aluminium mixes, the main adjuvant utilized for the human immunizations, these turned into the reference or benchmark arrangements for assessing new adjuvant formulations for human antibodies. Along these lines, it is imperative that aluminium adjuvants be utilized ideally to get the right relative assessment of a trial adjuvant. Aluminium mixes utilized as immunization adjuvants incorporate aluminium hydroxide and alum encouraged vaccines. Alum was utilized initially to incompletely refine protein antigens, principally lockjaw and diphtheria toxoid, by accelerating them within sight of anions including particles of bicarbonate and phosphate bringing about a blend of mixes, for the most part aluminium phosphate and aluminium hydroxide. Aluminium hydroxide indicated higher adsorption of lockjaw toxoid and diphtheria toxoid than aluminium phosphate at RT overnight at 6.0pH. Sparck discovered 10-20 times more egg whites human serum adsorption on aluminium hydroxide in comparison to phosphate of aluminium. Hydroxide of aluminium has been observed to be a better intense adjuvant than phosphate of aluminium. This might be because of higher adsorption limit and better adsorption of specific antigens at impartial pH by aluminium hydroxide than aluminium phosphate. Aluminium hydroxide adjuvant antigens demonstrated

comparative or unrivalled counter acting agent reactions than the antigens given with FCA (Freund's total adjuvant). We watched that diphtheria toxoid adsorbed onto phosphate of aluminium under proper conditions demonstrated counter acting agent levels in rabbits like those evoked by diphtheria toxoid given with FCA. Aluminium hydroxide is good adjuvant for frail antigens in mice however saponin and FCA are more intense adjuvant than aluminium hydroxide for solid antigens. To limit the varieties and to keep away from non reproducibility because of utilization of various arrangements of aluminium aggravates, a particular planning gel, aluminium hydroxide, from superfros was picked as a logical standard in assessment of adjuvant definitions which are new.

Adjuvant of aluminium has been portrayed as hard to make in a physio-chemically reproducible manner. Antigen adsorption on adjuvant of aluminium relies on the nonattendance of abundance phosphate particles in the response blend. Adsorption of lockjaw and diphtheria toxoid onto hydroxide gel of aluminium was not touchy to the states of pH and abundance phosphate particles (Moyer *et al*., 2016). However, finish adsorptions of every antigen under examination on adjuvant of aluminium ought to be checked and states of adsorbed improved, if essential. Because of these confinements of adjuvant polymeric nanoparticles have as of late been appeared to have huge potential as medication conveyance frameworks.

2.9 Polymeric Nanoparticles

Polymeric nanoparticles are the strong particles which are set up from biodegradable and biocompatible polymers, between 10-1000nm measure where the medications is disintegrated, ensnared, exemplified or joined to a nanoparticle lattice. In the field of polymers, nanoparticles is rapidly growing and assuming an imperative part in a wide range of territories running from gadgets, photonics, leading materials, prescription, sensors, biotechnology, contamination control and ecological innovation. Polymeric nanoparticles are promising vehicles for medicate conveyance by simple control to get ready transporters with the goal of conveying the medications to particular target; such leverage enhances the medication security. Polymer based nanoparticles viably convey medications, proteins, and DNA to target organs and cells. Their nanometer measure advances successful saturation through cell films and security in the circulatory system. Polymers are exceptionally advantageous materials

for the fabrication of incalculable and changed sub-atomic plans that can be coordinated into one of a kind nanoparticle develops with numerous potential medicinal applications (Nagvarma *et al*., 2012). Advantages of polymeric nanoparticles are as follows:

1. They expand the dependability of any unstable pharmaceutical operators, effectively and inexpensively manufactured in substantial amounts by a huge number of techniques.
2. Polymeric nanoparticles offer a huge change over customary oral and intravenous techniques for organization as far as productivity and adequacy.
3. They convey a high convergence of pharmaceutical specialist to a coveted area.
4. The decision of polymer and the capacity to change medicate discharge from polymeric nanoparticles have made them perfect contender for growth treatment, conveyance of immunizations, preventative and conveyance of focused antibodies.
5. PNPs can be effectively joined into different exercises identified with medicate conveyance, for example, tissue designing.

2.9.1 Polymers used in Preparation of Nanoparticles

The body compatible polymers in terms of their adaptability and should be biodegradable and bio-compatible in nature. The natural polymers which are common in use under the polymeric nanoparticles are:

Chitosan- It is a cationic polysaccharide, comparative in structure to cellulose. Both are made by straight beta (1-4) - connected monosaccharide in any case, a critical distinction to cellulose is that chitosan is made out of 2-amino-2deoxy-beta-D-glucan consolidated with glycosidic linkages. The essential amine bunches render exceptional properties that make CS extremely helpful in pharmaceutical applications. Contrasted with numerous other regular polymers, chitosan has a positive charge and is mucoadhesive. It is gotten from the deacetylation of chitin, an actually happening and bounteously accessible biocompatible polysaccharide. In the course of recent years, chitosan NP planning procedure has been produced in light of chitosan micro particles innovation.

Sodium Alginate- Alginate is a normally anionic polymer ordinarily acquired from dark colored ocean growth. It can be characterized as an anionic copolymer including

mannuronic corrosive and glucoronic corrosive units orchestrated in an unpredictable piece astute example of differing extents of GG, MG, and MM squares. Alginate has been broadly explored and utilized for some biomedical applications, because of its biocompatibility, low danger, generally minimal effort, and mellow gelation by expansion of divalent cations, for example, calcium particles or sodium particles. Sodium alginate is broadly utilized for nourishment, drink, pharmaceutical and bioengineering enterprises. It has number of free hydroxyl and carboxyl gatherings disseminated along the spine. It can all around broken up in water because of adversely charged carboxyl gathering (Cheng *et al*., 2015).

3.0 Objectives of the Work

Adjuvants are important in the present scenario but there is need to enhance their efficacy and stability for proper utilization. Therefore, considering the importance of present study following objectives have been designed:

- Synthesis and characterization of chitosan/ alginate beads.
- Encapsulation of peptide based adjuvant in chitosan/ alginate beads.
- Evaluation of cytotoxic effects of these polymeric beads on RAW 264.7 and Peripheral Blood Mononuclear cell lines.

CHAPTER 3

METHODOLOGY

3.1 Chemicals & Reagents

Chitosan (purity 99%; deacetylation degree 85%; pH 5-6; MW 100,000–300,000 Da) was purchased from Nano Wings Pvt. Ltd., Telangana. Sodium alginate was obtained from Sigma Aldrich Ltd. For PBMC isolation, blood was obtained from rajendra hospital, Patiala. Calcium chloride, PBS buffer, DMEM media (Dulbecco's modified eagle's medium), RPMI media (Rosewell park memorial institute), H1 peptide were procured from research lab-3, department of biotechnology, Thapar University. For all experiments distilled or deionized water was used.

3.2 Sterilization Procedure

Glasswares were first washed with detergent and then kept in oven at 40-50 °C till dried. After drying, glass wares were treated with Aqua regia for 15-30 minutes to dissolve metals and salts, followed by washing with water. Finally, they were kept in distilled water for 15 minutes and oven dried. All the tips and forceps were autoclaved at 121°C for 15 minutes.

3.3 Reagent Preparation

- **Chitosan 0.5% w/v-** 0.125g of chitosan dissolved in 24.5ml of distilled water with 500µL acetic acid.
- **3% w/v $NaC_6H_7O_6$-** 0.75g of sodium alginate dissolved in 25ml of warm distilled water.
- **Aqua regia**- HCl : HNO_3 of 3:1.
- **0.2M $CaCl_2$-** 0.73g of $CaCl_2$ dissolved in 50 ml of distilled water.

3.4 Preparation of Chitosan/ Sodium Alginate Beads

The chitosan /sodium alginate beads were prepared as per following procedure; Sodium alginate was dissolved in warm distilled water at room temperature under stirring conditions until complete dissolution of the resulting mixture was obtained. The

suspension was then added dropwise into chilled $CaCl_2$ solution through syringe under stirring conditions at room temperature at 300 rpm for about 4 hrs. At the end of incubation white colored sodium alginate beads were formed. The formed beads were chitosan solution under stirring conditions and left for about 3 hrs for the coating process. The coated beads were then separated and washed with distilled water for further use.

3.5 Peptide Encapsulation of Chitosan/ Alginate Beads

The preparation of chitosan/alginate beads for peptide encapsulation was performed as follows. In warm distilled water, sodium alginate beads were dissolved under stirring conditions at room temperature. 25 µg of H1 peptide was added to alginate solution under stirring conditions to obtain homogenous suspension of alginate and H1 peptide. The obtained suspension was then added dropwise into chilled $CaCl_2$ solution through syringe under stirring conditions at room temperature at 300 rpm for about 4 hrs. At the end of incubation, peptide encapsulated sodium alginate beads were formed. In 0.5% chitosan solution, sodium alginate peptide encapsulated beads were transferred under stirring conditions and kept for about 3 hrs for the coating process. Hence peptide encapsulated sodium alginate/chitosan beads were formed which were then separated and washed with distilled water for further use.

3.6. Cell Culture Studies

3.6.1 Maintenance of RAW 264.7 Cell Line

The RAW 264.7 is a macrophage like cell line derived from tumours induced in male BALB/c mice by the Abelson murine leukemia virus. The cell line was maintained in DMEM (Dulbecco's modified eagle's medium) medium supplemented with 100U of amphotericin, pencillin, streptomycin and 10% FBS (complete media) in T -25 cell culture flask. Cells were trypsinized when 70-80% of confluency was reached. Warm trypsinization method was carried out in which 3ml of trypsin was added to the T- 25 flask and incubated at 37C for 5min in CO_2 incubator. Equal amount of DMEM complete media was added in the T-25 flask followed by the centrifugation at 2000rpm for 10min. the cell plate was resuspended in 1ml DMEM complete media.

3.6.2. RAW-Bead Interaction Assay

The wells were seeded at the density of 10^4 cells / well. 24 h of the incubation was provided for the surface attachment. Required samples were added and the culture was incubated for 48h at 37C in CO_2 incubation with 5 % CO_2. After the incubation of 48h, 20µl of MTT (5µg / ml in PBS) was added to each well which was further incubated for 4h. it was followed by the removal of 170µl of of media and addition of 100µl of DMSO. The absorbance was observed at 570nm keeping 620nm as reference.

3.6.3 PBMC Isolation

Isolation of peripheral blood mononuclear cells (PBMCs) from blood was done by density gradient centrifugation. Blood was drawn from healthy donor by experienced technicians of Rajendra Hospital, Patiala. In a sterile 15-mL falcon tube, 5mL of whole blood was layered over 5mL of histopaque^R-1077 and centrifuged at 400xg for 30 minutes at room temperature in swinging bucket rotor. This density based centrifugation technique fractionates blood into plasma, red blood cells (RBC) and peripheral blood mononuclear cells (PBMC). After centrifugation, upper plasma layer was carefully disposed of and the layer containing PBMCs was transferred in a sterile 15mL conical centrifuge tube. Resuspended the cells in 10mL of PBS (1X) and centrifuged at 250xg for 10 minutes. This washing step was done twice. Then the supernatant was discarded and cell pellet was resuspended in 1mL of complete RPMI media supplemented with 10% FBS (Foetal Bovine Serum). Cells were counted with the help of haemocytometer and were assessed for viability by tryphan blue dye exclusion method.

$$Cell\ count = \text{No. of cells (in 1mL)} = Average\ number\ of\ cells * 10^4 * D.F.$$

3.6.4 MTT Assay

MTT assay is a colorimetric assay for assessing cell metabolic activity. Two assays were carried out. In the first assay, PBMC were seeded at the density of 2×10^5 cells / well in 96 well microtitre plates. 2 beads were added per well as sample. One was sodium alginate coated chitosan beads and second was only sodium alginate beads. The culture was incubated for 48h at 37C in CO_2 incubation with 5% CO_2. After the incubation of 48h, 20µl of MTT was added to each well which was further incubated for 4h. It was followed by the removal of 170µl of media and addition of 100µl of DMSO. The absorbance was observed at 570nm keeping 620nm as reference.

In the second assay, samples (peptide, control peptide and peptide beads)) were added to wells with PBMC containing RPMI media in triplicates. $2*10^5$ cells were added per well and the concentration of H1 peptide was kept at 25ug/ml. Volume in each well was made up to 200ul with the help of RPMI media. Blank and media including cells as well as peptide beads were used as control. The culture was incubated for 48h at 37C in CO_2 incubation with 5% CO_2. After the incubation of 48h, 20µl of MTT was added to each well which was further incubated for 4h. It was followed by the removal of 170µl of media and addition of 100µl of DMSO. The absorbance was observed at 570nm keeping 620nm as reference.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Formation of Chitosan/ Sodium Alginate Beads

Beads were formed according to the protocol discussed in the materials and methods section 3.4. White colored and oval shaped chitosan/sodium alginate beads were developed (Fig 1).

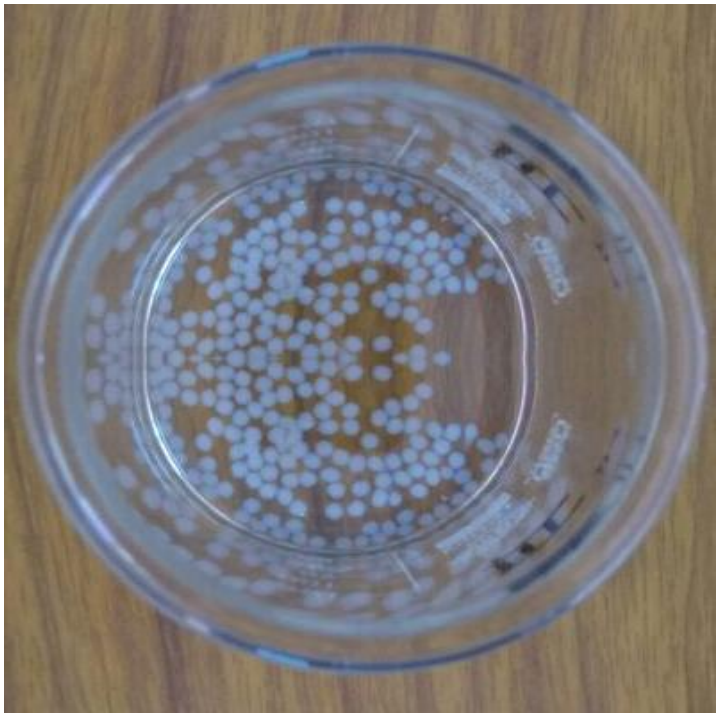

Fig 4.1 Formation of Chitosan/sodium alginate beads.

4.2 Effect of beads on peripheral blood Mononuclear Cells (PBMC) and RAW 264.7 Cell Line

MTT assay was carried out to observe the effect of prepared beads on PBMC and Raw 264.7 cell line. Sodium alginate beads were found to have no effect on PBMC as the absorbance value was found similar to that for cells (Figure 4.2 (A)). While, chitosan mixed sodium alginate beads was found to have little higher absorbance than cells only (Figure 4.2). When the effect of the beads was evaluated on Raw 264.7 cell line, it was observed that the absorbance is close to cells only for both beads (Figure 4.2 (B)). Hence,

these beads have no cytotoxic effect on peripheral blood mononuclear cells (PBMC) and RAW 264.7 cell line.

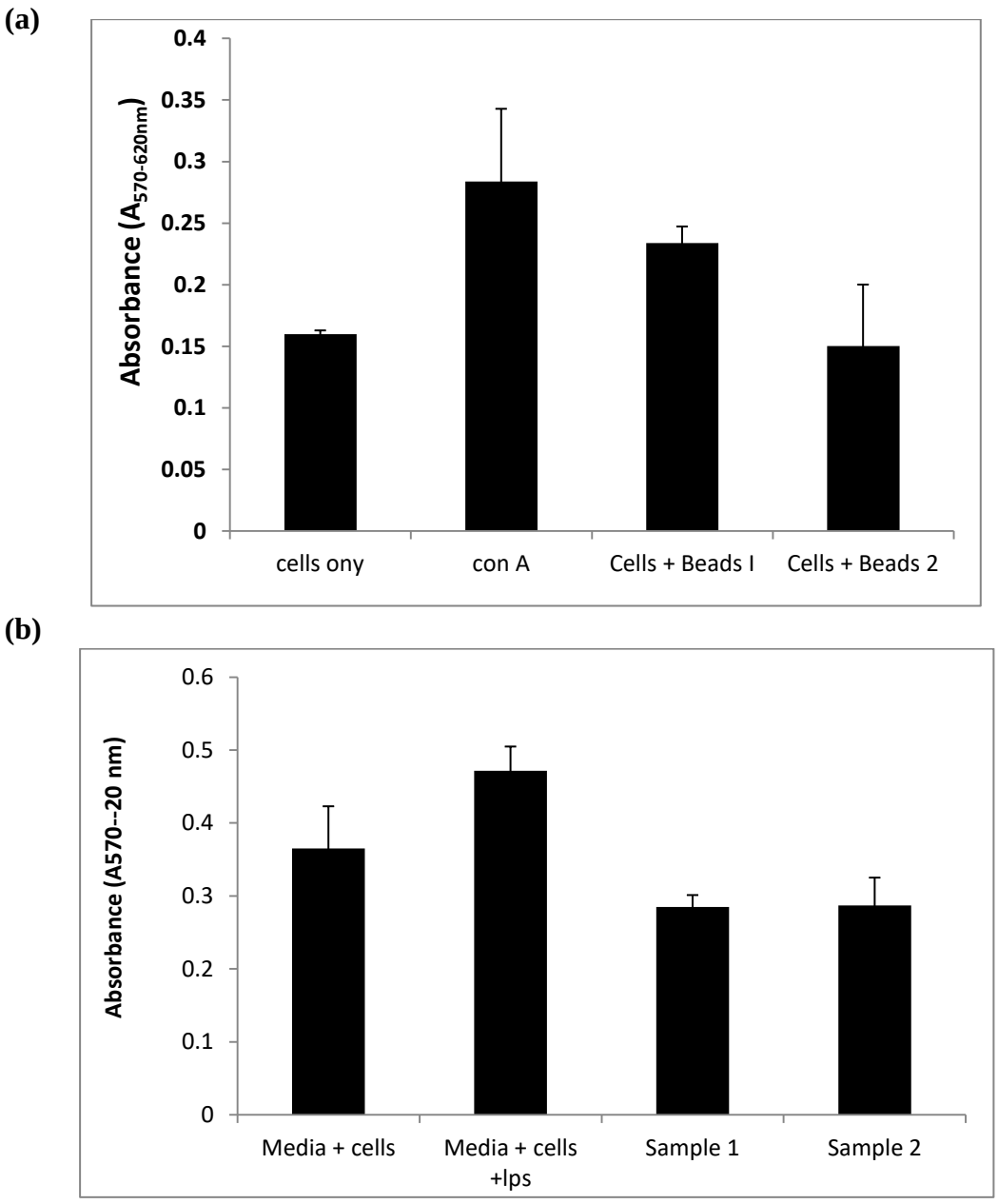

Fig 4.2 (a) Effect of beads on the growth of peripheral blood mononuclear cells (PBMC(Cell + beads 1: chitosan/sodium alginate beads, Cells + beads 2: sodium alginate beads). (b) Effect of beads on the growth of Raw 264.7 cell line (Sample 1: chitosan/ sodium alginate beads, Sample 2: sodium alginate beads).

4.3 Effect of peptide Encapsulated Chitosan/Sodium Alginate Beads on PBMC

Peptide was encapsulated in chitosan/sodium alginate beads and the assessed for their effect on PBMC. Encouraging results were obtained from the PBMC proliferation assay. It was found that PBMC interacted and proliferated more efficiently in the presence of peptide encapsulated in chitosan/sodium alginate beads as the absorbance value was found to be more than cells only and peptide treated cells. Hence, it can be said that chitosan/sodium alginate beads may acts as good adjuvant.

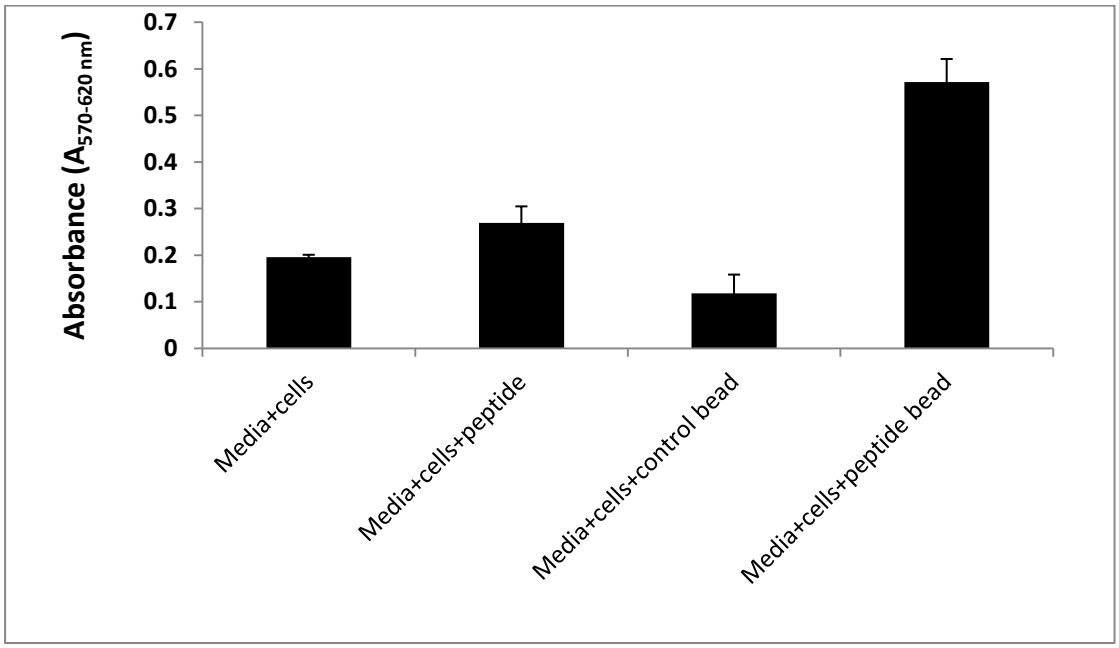

Fig 4.3 Effect of peptide encapsulated chitosan/sodium alginate beads on PBMC.

CONCLUSIONS

Adjuvants are compounds incorporated into vaccines to enhance the immune response and the development of these molecules has become an expanding field of research in the last decades. Adding an adjuvant to a vaccine antigen leads to several advantages, including dose sparing and the induction of a more rapid, broader and strong immune response.

In the present research polymeric carrier using sodium alginate / chitosan beads were formed in which the peptide was encapsulated. RAW bead interaction assay, MTT assay were performed in order to characterize the peptide loaded beads. Therefore, the effect of pristine chitosan/sodium alginate beads (with and without peptide) on peripheral blood mononuclear (PBMC) and RAW 264.7 cell line were observed. Polymeric beads were found to have no effect on PBMC as the absorbance value was found similar to that for cells. While, chitosan mixed sodium alginate beads was found to have little higher absorbance than cells only. When the effect of the beads was evaluated on Raw 264.7 cell line, it was observed that the absorbance is close to cells only for both beads. Hence, these beads have no cytotoxic effect on peripheral blood mononuclear cells (PBMC) and RAW 264.7 cell line. In peptide encapsulated sodium alginate/ chitosan beads PBMC interacted and proliferated more efficiently in the presence of peptide encapsulated in chitosan/sodium alginate beads as the absorbance value was found to be more than cells only and peptide treated cells. Conclusively, chitosan/sodium alginate beads can be utilized as a promising delivery vehicle after incorporating peptide based nanoadjuvants for enhancing immune response. As per the results obtained in the current study, it is expected that under specific conditions and parameters, peptide encapsulated beads could be more efficient as compared to administration of peptide alone. This topic is of importance as the specific mechanism of action of each single adjuvant may have different effects on the course of different diseases.

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