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Gelatin nanoparticles as a vaccine delivery system

by

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ABSTRACT

The aim of this study was to characterize an efficient nanoparticle (NP) vaccine delivery system based on gelatin, a biodegradable, natural polymer for delivery to DCs *in vitro*. Gelatin NPs were labeled by fluorescent probes tetramethylrhodamine conjugated dextran (TMR-dextran) or Texas Red X. Murine bone marrow-derived DCs were incubated with TMR-dextran either in soluble form or in NPs. Triple color confocal laser scanning microscopy (CLSM) showed that gelatin NPs were phagocytosed by DCs and were localized in the cytoplasm, partly in lysosomes but not in the nucleus. Flow cytometry showed that NPs were efficiently taken up by DCs. Surface loading of immunomodulator on gelatin NPs was investigated using monophosphoryl lipid A (MPLA) and was efficiently loaded on gelatin NPs. These studies show that gelatin NPs are promising delivery systems for targeting macromolecules and immunomodulators to DCs and deserve further investigation as a vaccine delivery system.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AUC	Area under curve
AF4	Asymmetrical flow field-flow fractionation
APCs	Antigen presenting cells
BSA	Bovine serum albumin
CCl_4	Carbon tetrachloride
CLSM	Confocal laser scanning microscopy
CTL-P	Cytotoxic T lymphocyte precursor
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodimide hydrochloride
ER	Endoplasmic reticulum
FACS	Fluorescent activated cell sorting
FITC	Fluorescein-5-isothiocyanate
FSC	Forward Scatter
GM-CSF	Granulocyte macrophage-colony stimulating factor
HIV	Human immunodeficiency virus
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
i.m.	Intramuscular
i.v.	Intravenous
MHC	Major histocompatibility complex
MPLA	Monophosphoryl lipid A

MTT	[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]
NaOH	Sodium hydroxide
NPs	Nanoparticles
PBS	Phosphate-buffered saline
PCS	Photon correlation spectroscopy
PEG	Polyethylene glycol
PLGA	Poly (D,L-lactic acid-*co*-glycolic acid)
RES	Reticuloendothelial system
s.c.	Subcutaneous
SEM	Scanning electron microscopy
SSC	Side Scatter
TEAA	Triethylammonium acetate
TEM	Transmission electron microscopy
Th	T helper
TMR-	Tetramethylrhodamine conjugated

CHAPTER 1: INTRODUCTION

1.1 Nanoparticles

Optimal benefit of drug therapy requires selective drug delivery to its site of action. This would not only lower the total dose of drug that needs to be administered to achieve an optimal therapeutic response, but would also minimize any undesirable toxic effects. This aim can be achieved by developing novel drug delivery systems such as colloidal (dispersed or encapsulated systems of nanometers to micrometers in particle size) drug delivery systems that can lead to more efficient use of available drugs [1,2]. For the last four decades' extensive effort has gone into developing effective colloidal drug delivery systems. These include microspheres, liposomes, niosomes, nanoparticles (NPs), micelles and microemulsions. Each of the above delivery systems has their own advantages and disadvantages. Among the above delivery systems, liposomes have been studied the most because of their good bioacceptability, even though they have major stability problems because of their short shelf life and difficulties in large-scale manufacture that have lead to reduced development and commercialization [3]. On the other hand NPs offer a promising alternative as they possess better stability, that leads to their easy storage and also their preparation can be scaled-up [3,4]. Increase in the number of research articles, reviews and symposiums involving pharmaceutical uses of NPs in the last two decades gives us an indication that researchers have started to gain interest in NPs [5-10].

NPs are solid colloidal particles ranging from size 10-1000 nm. NPs are made up of macromolecular materials in which a drug or biologically active material are dissolved, entrapped, encapsulated, adsorbed or conjugated [7]. Two types of NPs that are used in

drug delivery are nanospheres and nanocapsules. Nanospheres and nanocapsules are differentiated by the structural differences that are caused by the difference in their preparation methods (Figure 1). Nanospheres are the NPs that consist of a continuous polymeric matrix that contains the active principle (drug or biologically active material). On the other hand nanocapsules are the NPs that have a capsule like structure which consists of a hydrophobic/oily phase or hydrophilic (core) in which the drug is entrapped and surrounded by a single polymeric membrane [6,11].

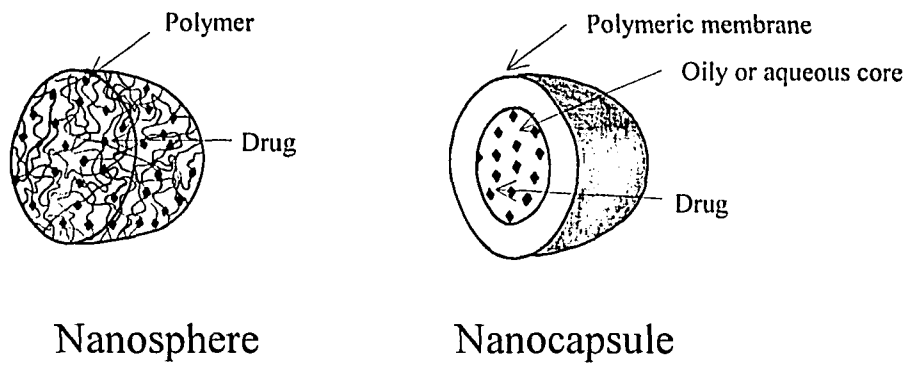

Figure 1. Nanoparticles: nanospheres and nanocapsules*.
***Modified from Brigger et al., 2002**

The concept of NPs for drug delivery was proposed by Speiser et al. based on Ehrlich's idea of drug loaded "magic bullets" that could be targeted to the specific sites in the body leading to an improved drug therapy [12]. Speiser et al. in early 1970's became the first group to manufacture NPs as a dosage form. This group was also the first to describe its use in drug delivery and targeting [3,4]. NPs have been used as drug carriers or as adjuvants for vaccine [7]. Due to their small size, NPs are able to pass through all capillaries after injection into the blood stream, and minimize irritant reactions at the

injection site. The small size of NPs also leads to increase in the interaction with the individual cells specifically antigen presenting cells (APCs) such as dendritic cells (DCs) and macrophages. Their small size also facilitates their escape from blood vessels through leaky fenestrations in tumors which can be used for drug targeting tumor sites [13]. They are specifically taken up by reticuloendothelial system (RES) (RES encompasses a range of cells capable of phagocytosis – macrophages, DCs and monocytes. They are either freely circulating within the blood or fixed to various connective tissues. Examples of the site of fixed cells includes pulmonary alveoli, liver sinusoids, skin, spleen and joints) *in vivo* so they are mostly accumulated in liver (60-90%), spleen (2-20%) and lung (varying amounts) and bone marrow (0.1-1%) after intravenous (i.v.) administration [7]. Function of RES is to remove senescent cells from circulation and provide phagocytic cells for immune responses. NPs specifically target RES and are therefore taken up by macrophages. Since macrophages act as reservoirs of the human immunodeficiency virus (HIV), delivery of anti HIV drugs using NPs may be beneficial to control Acquired Immune Deficiency Syndrome [14,15]. However the above advantage of RES targeting may be disadvantageous in drug delivery since they will result in rapid removal of NPs from the body and makes them inefficient in targeting other systems of the body. But the above problem had been overcome by coating the particles with surfactants or by polyethylene glycol (PEG) (ie. PEGylation of the NPs) that can lead to their increase circulation time in the blood and enhances their uptake in other organs [16]. Small size of NPs enables them to disperse in water forming a clear colloidal solution that leads them to be used as effective sustained-release solutions [4]. NPs can be administered via many routes. These include i.v., intramuscular (i.m.),

subcutaneous (s.c.), peroral, ophthalmic and even transdermal administration [3]. The most important ability of NPs is that they can be used as an efficient carrier system for anticancer drugs such as doxorubicin [17], mitoxantrone [18], 5-fluorouracil [19], mitomycin C [20], actinomycin D [21], methotrexate [22] and paclitaxel [23]. NPs can also be used to deliver drugs to the brain [24,25]. When administered intravenously, polysorbate 80 coated polybutyl cyanoacrylate NPs allowed delivery of encapsulated dalargin across blood brain barrier [26,27]. NPs are also being investigated as deoxyribonucleic acid (DNA) carrier system for gene delivery [28,29]. Studies in rats have shown that NPs can lead to the peroral delivery of drug such as insulin, which can only be injected as it gets destroyed in the gut in its soluble form when taken perorally. Peroral delivery of insulin via NPs has lead to a significant prolonged therapeutic effect in fasted diabetic rats [30]. NPs has also shown an improved peroral delivery of a poorly absorbable drugs such as vincamine [31], avarol [32] and cyclosporin A [33] that had lead to an increased oral bioavailability as compared to their solution form . NPs have a potential to be used as a diagnostic and imaging agent in disease such as cancer [5]. Many clinical studies are currently underway for the use of NPs as a drug delivery system. Research of NPs formulation for cosmetic industry for skin care has also been started and has shown great results [34,35]. Moreover NPs can be used to deliver antigens and adjuvants and therefore has an immense potential for the development of the vaccine delivery system. The use of NPs for vaccine delivery has recently been started in research and this delivery system needs to be improved and well characterized for vaccine delivery.

NPs can be made up of two types of material: synthetic polymers and natural compounds such as proteins, carbohydrates and lipids (Table 1). NPs made with either of the above materials have their own advantages and disadvantages. Monomers of the synthetic polymeric NPs may not be biodegradable and their byproducts may not be biocompatible and toxic residues such as monomers, and oligomers and catalyst may last *in vivo* [4]. This dissertation will focus on NPs that are made up of natural macromolecules (preferred for their low toxicity and biocompatibility) i.e. gelatin that offers a great promise as a biodegradable base for preparing NPs.

Table 1. Nanoparticles made of natural macromolecules and their size range

Natural macromolecule	Size range (nm)	Reference
Gelatin	100-800	[36]
Collagen	180-300	[37]
Human serum albumin	170-800	[38]
Bovine serum albumin (BSA)	132-200	[39]
Ethyl cellulose	190-1100	[40]
Casein	120-500	[3]
Dextran	500-2200	[41]
Mannan	500-2200	[41]
Lichenan	500-2200	[41]
Starch	500-2200	[41]
Chitosan	450-820	[42]
Lipid	180-300	[43]

1.2 Gelatin as a biodegradable material for preparing nanoparticles

Gelatin is a pure heterogeneous mixture of polypeptides originating from collagen contained in animal's skins and bones. The properties of gelatin depend upon its manufacturing method that can be acidic or basic in amino acid composition [36]. Gelatin is insoluble in ethanol, chloroform, fixed and volatile oils and ether but it is soluble in hot

water as well as mixture of glycerol and water. In cold water gelatin swells, gradually absorbing 5-10 times its weight of water [44]. Type-A gelatin is obtained by acid treatment of collagen and has an isoelectric point between 7.0 and 9.0. Type-B gelatin, on the other hand, is obtained by alkaline hydrolysis of collagen and has an isoelectric point between 4.6 and 5.2. Being a protein, gelatin is composed of a unique sequence of amino acids [45]. With the exception of tryptophan, gelatin contains all other seven amino acids essential to the human body [46]. Characteristic features of Type-A gelatin or Type-B gelatin are high content of the amino acids such as glycine (27%, almost 1 in 3 residues, arranged every third residue), proline (16%) and 4-hydroxyproline (14%) residues. Gelatin contains specific amounts of 18 different amino acids, which are joined together in sequences to form polypeptide chains to form primary structure. Conversion of collagen to gelatin yields wide range of fractions (α, β, δ and γ fractions) of gelatin with varying molecular weights ranging from a few thousand up to several hundred thousand daltons. The γ component is the one with a large amount of cross-linking consisting of δ fraction [47]. The factors affecting the molecular weight distribution of the gelatin polypeptides obtained during manufacture can be the raw material used , acid or basic treatment or the varying extraction conditions [48]. The molecular weight distribution of gelatin has a great effect on its physical properties and particularly affects its viscosity and gel strength values and also affect in the preparation of NP formulation that are prepared specifically by desolvation technique [36,44].

The biological origin, lack of bacterial contamintation, neutrality basic properties (film-forming, digestible, soluble in hot water, gelling, binding) of gelatin allow its wide use in the formulation of many medications and dietary supplements in the

pharmaceutical industry [49]. It has been used for preparing hard and soft capsules and can be used in other preparations like coating of vitamins and tablets. The protein nature of gelatin is important in its use as blood plasma substitutes [50].

Gelatin is easy to crosslink and chemically modified and therefore can be used for making wide variety of pharmaceutical formulations. The above properties of gelatin lead us to believe that it has an immense potential to be used in colloidal drug delivery systems such as microspheres and NPs. Gelatin-based colloidal drug delivery systems are usually produced by an emulsification process or by desolvating an aqueous gelatin solution [4,20]. Many important drugs or therapeutic agents can be loaded on gelatin particulate delivery systems. The controlled release of the drug or therapeutic agent from the above delivery system has lead researchers to gain interest in gelatin as a colloidal drug delivery system (Table 5). Gelatin is cheap, readily available in sterile, pyrogen free form and is a promising biodegradable base for developing NPs, and therefore has a significant advantage over the non-biodegradable synthetic polymers. One of the disadvantages of gelatin as a drug delivery system may be the breaking of this macromolecule into smaller peptide chains after prolonged heating. But it was found that gelatin NPs do not show any net change when they are autoclaved for 15 minutes at 121 °C [4].

1.3 Gelatin nanoparticles

Gelatin NPs are biodegradable and biocompatible NPs made up of gelatin. Gelatin can be used as a coating material to form gelatin nanocapsules or can be used to form the entire matrix to form gelatin nanospheres.

1.3.1 Preparation methods

Until now gelatin NPs have been prepared by two basic techniques i.e. desolvation and emulsification. Other modified techniques to prepare gelatin NPs can be salt induced complex coacervation, emulsification solvent diffusion and reverse micellisation.

1.3.1.1 Desolvation technique

Gelatin NPs were firstly prepared by Marty et al. by desolvating gelatin using a desolvating agent causing salting out phenomenon [4]. This process of making gelatin NPs was derived from the coacervation method of microencapsulation. The basic idea behind this process was to desolvate gelatin in a controlled manner by the use of desolvating agent and controlling the desolvation process such that it can be terminated just outside the coacervate region to obtain gelatin NPs [44]. The particles obtained can be stabilized by hardening them by a crosslinking agent such as glutaraldehyde. Glutaraldehyde forms chemical crosslinks by linking the free amino groups on the surface of the NPs (Figure 2). These particles can then be freeze dried and stored for later use. The desolvating agents that can be used in the above process are mainly 95 % v/v ethanol, 20 % w/v aqueous solution of sodium sulphate or acetone. The choice of desolvating agent to be used depends mainly upon the drug to be attached to the NP. In some cases surfactants must be added to solubilize some drugs and they also help in resuspending the freeze dried particles into a fine dispersion [4]. Preparing gelatin NPs by the above method leads to precipitation or coacervation, therefore a resolvating agent such as ethanol or isopropanol is added until the turbidity disappears. If the above system is hardened in the coacervated state, much larger particles, corresponding to the size of

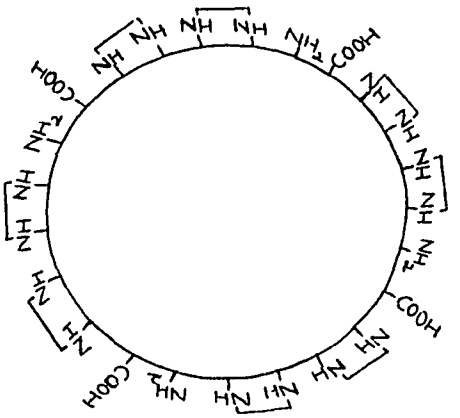

Figure 2. Schematic illustration of crosslink formed by glutaraldehyde on the surface of gelatin nanoparticle *. ( ): Glutaraldehyde molecule.
***Modified from Leo et al., 1997**

the coacervate droplets are produced. Crosslinking by glutaraldehyde should also be stopped before the particles get linked together to form large aggregates due to interparticulate crosslinking by the aldehyde. Crosslinking can be stopped by adding sodium sulfite or sodium metabisulfite [4,36]. This method of preparing gelatin NPs was very tiring and particles prepared were usually unstable and formed irreversible aggregates during crosslinking [36]. This was the main reason that we have not seen enough research papers on gelatin NPs for the last 21 years after they were first made by Marty et al, 1978.

Coester et al. have developed an improved technique of preparation of gelatin NPs using a two-step desolvation method [36]. In this method, an aqueous solution of gelatin was prepared under heating followed by magnetic stirring. A desolvating agent such as acetone was then added. After a short time the gelatin solution was sedimented. Supernatant containing desolvated and undesolvated gelatin was discarded and the sedimented gelatin was re-dissolved in water under heating and the pH was adjusted to

2.5 (for gelatin A) by adding hydrochloric acid (HCl) or adjusted to 12 (for gelatin B) by adding sodium hydroxide (NaOH), and the above system was magnetically stirred. The gelatin was again desolvated by dropwise addition of acetone until signs of turbidity were seen. After a few minutes of stirring, 25 % aqueous solution of glutaraldehyde was added as a crosslinking agent. The above dispersion was stirred until almost all of the acetone was evaporated. Finally, the above obtained NPs dispersion was purified by 3-fold centrifugation and redispersion in 30% acetone/water solution. The final redispersion was done in purified water and the NPs dispersion was stored at 4 °C. This new method of preparing gelatin NPs is much better than the one step desolvation technique as it is a very easy method and particles tend to form less aggregates. The particles prepared by this method are very stable and can be easily purified by centrifugation. The basic reason for using the two step desolvation method to produce stable NPs was found due to discarding of the supernatant after first desolvation step that contains small molecular weight gelatin that has a great influence on the stability of particles [36].

1.3.1.2 Salt-induced complex coacervation

Truong-LE et al. employed a salt-induced complex coacervation technique to prepare gelatin NPs for gene delivery [28]. The particles obtained by this method had size distribution of 200-700 nm. In this method 100 μL of 5% gelatin solution was mixed with 100 μL of 0.2 μg/μL of plasmid DNA solution containing 45 mM sodium sulphate. The above reaction was carried out at 55 °C and the above system was stirred for 1 minute on a vortex mixture that was set at the maximum speed. The above mixture was then centrifuged on a 100 μL sucrose step gradient at 40,000 X g for seven min. After that the sucrose fraction containing the NPs (55%) was diluted with water to 200 μL. The NPs

formed above were then crosslinked and conjugated with transferrin. The crosslinking was done by adding NPs solution containing 20 μg of transferrin to 22 μL of a 0.2 M morpholineethanesulfonic acid buffer solution with 4.5 pH, containing 1-ethyl-3-(3-dimethylaminopropyl) carbodimide hydrochloride (EDC, 0.1 mg/mL). This reaction was carried out at room temperature for 30 minutes. The reaction was then stopped by adding 0.2 M glycine. The addition of EDC leads to the activation of carboxylic group of the gelatin NPs that would lead to an increase in the zetapotential (surface charge) of the gelatin NPs. The gelatin NPs formed above could then be used to load many bioactive agents by ionic interaction with the gelatin matrix, physical entrapment or covalent conjugation on the surface of the NPs [28].

1.3.1.3 Emulsification technique

The emulsification technique has also been used earlier to prepare gelatin NPs as the desolvation technique for preparing NPs was not optimized during that time. In this method, basically emulsification of an aqueous solution of gelatin is done in oil. Yoshioka et al. was the first research group that were successful in preparing gelatin NPs having an average diameter of 280 nm [20]. They emulsified 0.3 mL of a 30% gelatin solution containing about 1.8 mg of drug in 3 mL of sesame seed oil using 6.6% sorbitan sesquioleate and 1.5% polyoxyethylene derivative of hydrogenated castor oil as emulsifiers. The emulsion prepared above was then cooled in an ice bath, resulting in complete gelation of gelatin droplets leading to the stabilization of the unhardened particles. The above emulsion was then diluted with acetone and was filtered through a membrane filter with a pore size of 50 nm. In order to remove the oil phase, the resulting particles were washed with acetone and then hardened with 30 mL of a 10% solution of

formaldehyde in acetone for 10 minutes, followed by washing with acetone and air drying, to remove oil from the NP formulation. Hardening on the filter lead to cross-linking of aggregated NPs and the resultant free flowing powder could be dispersed in saline solution with 1% polysorbate 80. Hardening of particles increased the stability of the particles but at the same time it increased the diameter of the NPs six fold as compared to the particles formed before hardening. Particles formed above had started to coalesce upon removal of oil and emulsifiers, and therefore the speed of coalesce had to be monitored to control the size of the NPs [20]. The above method was followed by many research groups in preparing gelatin microspheres. Tabata et al. used the above method by using 1:1 mixture of chloroform and toluene as the organic phase, sorbitan monooleate as an emulsifier and glutaraldehyde as a crosslinking agent [51,52]. In 1996 Li et al. came up with modified water-in-oil (w/o) emulsion method for preparing gelatin NPs. This group prepared NPs by dissolving 50 mg of gelatin in 2 mL of phosphate-buffered saline (PBS) by heating in a water bath at 90 °C. The gelatin solution was then placed in a water bath set at 40 °C and then the drug (BSA) solution (5mg/mL) was warmed to 40 °C in another water bath. As both of the above solutions reached the same temperature of 40 °C, they were mixed (2:1 v/v). The resulting solution was added to 100 mL corn oil (preheated to 40 °C). The above biphasic system was then homogenized at 20,000 X g for 12-15 minutes to form a w/o emulsion. After obtaining the particles with approximately 1 μm in size, the emulsion was cooled in a refrigerator. When the temperature of the emulsion fell below gelling point of the gelatin, the globules of the aqueous phase containing gelatin and BSA were converted to gelatin hydrogel particles that lead to the entrapment of BSA inside. The emulsion was then changed to a

suspension. Carbon tetrachloride (CCl_4) was then added to the above system (1:1 CCl_4:suspension) to reduce the viscosity of the suspension. The diluted suspension was then vacuum filtered using nylon membrane filter having a pore size of 100 nm. The filtrate was then washed using CCl_4 to remove the remaining oil. The particles then collected were vacuum dried overnight to obtain free flowing powder of gelatin NPs with an average diameter of 840 nm [53]. This technique was easy and better than the technique followed by Yoshioka et al., as in this technique there was no use of an emulsifier that may be harmful to some fragile drugs such as proteins or peptides, if the drugs are co-emulsified during the process. But stability is an issue for this formulation, as in this method there is no addition of crosslinking agent that is essentially required to obtain a stable formulation. So still there is a need for improvement in the above method. The above method may become better by using a crosslinking agent once the particles are formed that will lead to an enhancement in the stability of the particles.

1.3.1.4 Emulsification solvent diffusion

In 2002, El-shabouri followed the emulsification solvent diffusion method for preparing gelatin NPs [33] as developed by Niwa et al. in 1993 (for preparing Poly ($_{D,L}$-lactic acid-co-glycolic acid) (PLGA) nanospheres). In this method, the drug (Cyclosporin A) to be encapsulated or entrapped, and emulsifier (lecithin) were dissolved in methylene chloride and then mixed with acetone. The solution prepared above was then injected into an aqueous solution containing poloxamer 188 as an emulsifier in the presence of gelatin and the above system was magnetically stirred. The above system was then homogenized at high pressure for 5 minutes at a 1000 bar resulting in the formation of emulsion. Methylene chloride was then removed from the emulsion using rotavapor at room

temperature and then the whole dispersion was filtered through 1.0 μm filter to separate microspheres or other aggregates within the system. The resulting filtrate consisting of NPs was diluted with 50 mL of water to allow complete diffusion of acetone into the aqueous phase. The dispersion was then purified by centrifugation and redispersion to remove the remaining acetone. NPs obtained above had mean particle diameter of 139 nm [33].

1.3.1.5 Reverse micellisation

In 1983, Luisi et al developed a new method of preparing gelatin microspheres and NPs, by forming reverse micelles. A 9.5% solution of gelatin in water (containing

Table 2. Preparation methods for gelatin nanoparticles

Type of gelatin used	Preparation method	Reagents used	Average diameter (nm)	Reference
Gelatin A	Single step desolvation	Sodium sulphate, ethanol, glutaraldehyde	200 nm	[4]
Gelatin B	Single step desolvation	NaOH, ethanol	232 nm	[48,55]
Gelatin A	Two step desolvation	Acetone, HCl, glutaraldehyde	280 nm	[36]
Gelatin A	Salt induced complex coacervation	Plasmid DNA, sodium sulphate, transferring, MES, EDC	~ 450 nm	[28]
Gelatin A	Emulsification	Sesame oil, sorbitan, sesquioleate, castor oil, formaldehyde, acetone, polysorbate 80	280 nm	[20]
Gelatin A	Emulsification (emulsifier free)	PBS, corn oil, CCl_4	840 nm	[53]
Gelatin A	Emulsification solvent diffusion	Lecithin, methylene chloride, acetone	139 nm	[33]
Gelatin A	Reverse micellisation	Aerosol OT, isooctane, carbodiimide	—	[54]

drug) was injected into a 50 mM solution of the surfactant aerosol OT in isooctane at 40 °C. In a few minutes, a clear micellar solution was obtained. The above system was then crosslinked by using carbodiimide as a crosslinking agent. The NPs formed were then purified by dialysis and finally dispersed in an aqueous phase [54].

1.4 Characterization of gelatin nanoparticles

Gelatin NPs like any other colloidal drug delivery system can be characterized by their physicochemical nature, drug loading, degradation, release characteristics, biocompatibility and toxicity.

1.4.1 Physicochemical characteristics

Physicochemical properties by which gelatin NPs can be characterized include particle size, density, shape, molecular weight, surface charge, surface element analysis, zetapotential, water content, hydrophobicity and hydrophilicity of the particles. Among the above properties, particle size is the most important characteristic of NPs as it is crucial for targeting of the NP formulation within the human body. Other properties such as density and molecular weight may cause changes in their drug-release profile and degradation. Surface characteristics of the particles such as surface charge, zetapotential, hydrophobicity and hydrophilicity will greatly effect their interaction within the biological environment *in vivo* and will influence their biodistribution [3]. The most common and the fastest method used to measure the size of NPs is photon correlation spectroscopy (PCS). This technique can be used to determine the mean size as well as the polydispersity index of the NPs (by using correlators), and therefore provides us with the size distribution of the particles. Malvern Inc. has been making many instruments based

on the above technique that provide us with precise measurements of mean size and size distribution of the NPs. Using PCS, it has been found that gelatin NPs made in an oil emulsion have a size distribution between 100 nm and 600 nm [20], whereas gelatin NPs made by desolvation technique usually tend to have distribution between 150 nm and 550 nm [4,36]. PCS does not determine the actual diameter of the particles but basically determines their hydrodynamic diameter via brownian motion. For this reason, the size measured by this technique is influenced by the interaction of the particles with the surrounding liquid medium. Therefore, the exact viscosity of the surrounding liquid medium must be known in order to determine the exact diameter of the particles. Aggregates or dust particles in the NP formulation can lead to false results. Therefore, it is always better to confirm the results by the use of scanning electron microscopy (SEM). In one instance the same particles prepared by the desolvation technique were found to have surface diameter with a range of 50 nm to 100nm (Figure 3) using SEM [36] as compared to 150-800 nm with PCS technique. SEM not only determines the surface diameter but also allows us to know the shape of the particles. Many researchers have followed SEM and found gelatin NPs to be spherical in shape with smooth surface [55]. Transmission electron microscopy (TEM) is also an alternative to determine the size of the gelatin NPs. This technique also provides some indication of the interior of the NPs. Zhao et al. (2004) characterized gelatin NPs by TEM and found that NPs were ball-like with size distribution range of diameter from 20 to 200 nm [56]. Recently it has been shown that size distribution of gelatin NPs can be determined by asymmetrical flow field-flow fractionation (AF4) technique, coupled with multiangle light scattering detection [57]. The results obtained by AF4 were quite consistent when

compared with SEM and PCS. Size of gelatin NP can be influenced by changing parameters during their manufacture. Preparation parameters such as crosslinking time,

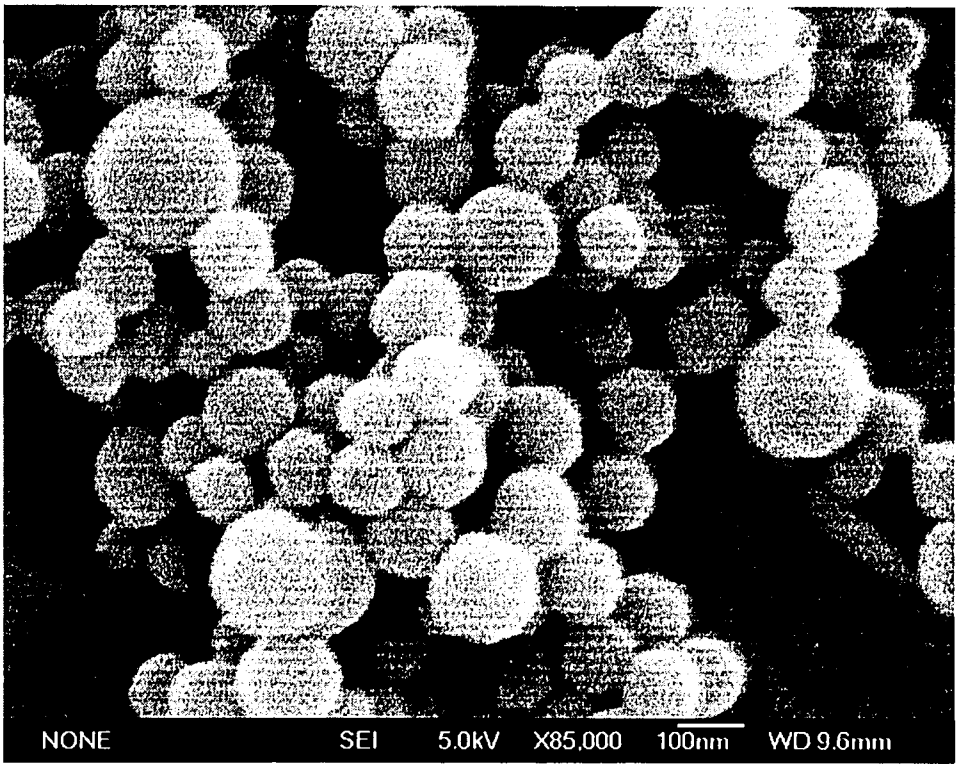

Figure 3. Scanning electron microscope picture of gelatin nanoparticles (Coester et al., manuscript in preparation, Ludwig-Maxmilians University, Munich, Germany).

amount of crosslinking agent, speed of stirring, time of stirring, speed of adding desolvating agent, homogenization time, and homogenization speed are some important crucial factors that can influence the particle size to a great extent. Modification of above parameters has allowed researchers to develop gelatin NPs of different size distribution that are required for a given experiment.

The molecular weight of the gelatin NPs cannot be determined by the common techniques available (i.e. gel permeation chromatography), as for gel permeation chromatography, it is necessary to dissolve the polymer [3]. Surface element analysis and

the identification of chemical functional groups of 100 A-thick surface layer of gelatin NPs can be done using electron spectroscopy for chemical analysis. This can lead to better loading of the drugs that can be conjugated to the chemical functional groups on the surface of the NPs [55]. The surface charge and zetapotential of NPs is determined by its electrophoretic mobility. Zetapotential values over a varied pH range for NPs [29,33] give us valuable information about the stability of the NPs formulation. Malvern Zetasizer can be used to determine the zetapotential of gelatin NPs dispersion at a particular pH or a range of pH. Considering gelatin NPs as a hydrogel drug delivery system, it is important to determine the water content of the particles that can be helpful

Table 3. Physicochemical characterization for gelatin nanoparticles

Physicochemical Property	**Methods of analysis**	**Reference**
Particle Size	PCS, SEM, TEM, AF4	[4,36,56,57]
Elemental and chemical functional group analysis on the surface	Electron spectroscopy for chemical analysis	[55]
Zetapotential	Electrophoresis	[33,57]
Water content	Using nylon filter membrane	[53]

in knowing the water uptaking capacity and permeability of the hydrogel. Water content of gelatin NPs can be measured by nylon filter membrane and can be calculated using the following equation: Water content (%) = 100 x (Wt – Wm – Wd)/ (Wt – Wm); where 'Wt' is the total weight of the moistened filter membrane, the swollen gelatin NPs, and the weighing bottle, 'Wm' is the total weight of the moistened filter membrane and weighing bottle and 'Wd' is the dry weight of gelatin NPs. It has been shown that water content of gelatin NPs is linearly related to temperature and it can also be

influenced by the degree of crosslinking of the gelatin [53]. Hydrophobicity of the gelatin NP surface could be determined by the available methods such as water contact angle measurements or by hydrophobic interaction chromatography [3]. The experiment for measuring hydrophobicity of the gelatin NPs still needs to be done as it may provide us with some valuable information about the biodistribution of the particles after i.v administration.

1.4.2 Drug Loading

Drugs may be loaded onto gelatin NPs either during NP preparation [17] or by addition of drugs to already prepared empty particles [58]. Both of the above methods can lead to a solid solution or solid dispersion of the drug in gelatin or surface adsorption or chemical conjugation of the drug to gelatin. The availability of functional groups such as amino and carboxyl groups on the crosslinked gelatin NPs surface enables the ease of surface modification like coupling of drugs to gelatin NP matrix [17,59]. The surface of Gelatin NPs can also be easily modified with sulfhydryl groups and later can be used for the covalent attachment of drugs or protein such as avidin to free sulfhydryl groups. Coupling between biotin and avidin is very rapid and stable, and leads to the formation of stable NPs [58]. Therefore the above system can be used as a universal carrier for biotinylated compounds. Positively charged gelatin NPs have shown promise as a DNA carrier system [28,29]. Amount of drug loading, the type of interaction of the drug with gelatin and binding of the drug to the gelatin NPs depend upon the amount of the drug added during or after particle preparation, amount and type of gelatin (gelatin A or B) used and preparation parameters.

Drug loading in gelatin NPs can be determined by separation of gelatin NPs from unloaded drug by centrifugation or ultracentrifugation [29] and following quantitative analysis of the drug after degradation or acidic hydrolysis or alkaline hydrolysis of the pelleted gelatin NPs [60]. Other useful separation methods for gelatin NPs can be ultrafiltration and gel filtration. As gelatin NPs of size 100-900 nm settles down completely by centrifuging particle dispersion at 20,000 X g for 25 minutes, therefore drug loading can be determined by centrifugation of the gelatin NPs dispersion and analyzing the supernatant using analytical techniques available such as high performance liquid chromatography (HPLC), size exclusion chromatography and ultraviolet spectrophotometry, fluorescent spectroscopy. The amount of the drug loaded on particles can then be calculated by subtracting the amount of the drug in the supernatant from the total amount of the drug present in the dispersion. Recently it has been shown that drug loading efficiency in gelatin NPs can also be determined by AF4 [57].

1.4.3 Degradation of gelatin nanoparticles

Degradation of gelatin NPs can occur by enzyme assisted degradation and acid hydrolysis [60]. Methods that permit degradation of gelatin without degradation of drugs will permit quantification of the encapsulated drugs in gelatin NPs. Many enzymes have been tried to degrade gelatin NPs such as trypsin, α-chrymotrypsin and protease. All above enzymes work faster initially and within two minutes, they are able to degrade 50% of the particles. Among these enzymes gelatin NPs were completely degraded by trypsin, as it worked for a longer time [17]. Studies have shown that trypsin appears to be more specific than collagenase for degrading gelatin. Therefore, trypsin is usually used to digest the gelatin matrix of NPs in order to perform the *in vitro* release studies [17,28].

Degradation of gelatin NPs caused by an enzyme can be determined by measuring the turbidity of gelatin NPs at room temperature by the absorbance at 546 nm using a ultraviolet spectrophotometer (as shown in a preliminary study that at 546 nm the absorbance was a linear function of the NP concentration in the range of 0.5-3 mg/mL) [17]. Narayani et al. found that gelatin NPs can also be degraded by acidic hydrolysis by heating NPs in HCl for 20 minutes at 60 °C [61]. Degradation of gelatin NPs by acidic hydrolysis is thought to be caused by breaking of gelatin into peptides and then further breaking of these peptides into amino acids.

Table 4. Degradation methods for gelatin nanoparticles

Mode of degradation	Material used	Time taken for degradation (hours)	Percentage of degradation (%)	Reference
Enzymatic	α-chymotrypsin	0.5	70	[17]
Enzymatic	Protease	0.5	80	[17]
Enzymatic	Trypsin	5.0	100	[17]
Acidic hydrolysis	HCl	0.3	100	[60]

1.4.4 Release of drug from gelatin nanoparticles

Drug release from gelatin NPs may occur by desorption of surface bound drug, diffusional release through the NP matrix, NP matrix erosion or a combination of the above processes. The main factors affecting drug release rate are: mechanism of release, the diffusion coefficient, rate of biodegradation of gelatin NPs and the biological environment to which NPs are exposed. Release of drug from NPs in the *in vivo* environment may be very different from the *in vitro* environment [3]. But it is always essential to do an *in vitro* release study of the drug from the NPs for characterization and quality control purposes [3]. The methods that have been followed for the determination

of *in vitro* release of the drug from the NPs are basically ultracentrifugation and ultrafiltration. For doing *in vitro* release studies, gelatin NPs with encapsulated drug or adsorbed drug were suspended in PBS with and without enzyme in a shaker bath at 37 °C [17]. At predetermined time intervals, the sample from the shaker bath is withdrawn and centrifuged, and a specific volume of supernatant was then quantified for the release of drug using various analytical techniques including ultraviolet spectrophotometry, fluorescence spectrophotometry, HPLC etc. PBS (with same volume that had been used for the analysis of supernatant) was then added to the centrifuged particles and then particles were resuspended and returned back to the shaker bath for further release studies. Trypsin was generally used as an enzyme in the above studies. The release of drug from the NPs that were manufactured by emulsification had shown a biphasic release profile. Initially the drug, which was surface-bound, showed a burst release followed by a typical diffusion controlled release mechanism. It was thought that in the presence of enzyme, the release of the drug was due to the degradation of the gelatin [55]. The drugs, which were chemically conjugated with the gelatin matrix were not released in a saline solution and the release of the drug for these particles was possible only in the presence of enzyme in the saline solution [17,28,55], as these drugs could only be released by the degradation of the gelatin. The *in vitro* release studies can also be performed in simulated gastric fluid (0.1 N HCl, pH 1.2) [60] for characterizing the release of the drug from the particles, if NPs requires to be administered orally.

1.4.5 Biocompatibility and toxicity

Biocompatibility and toxicity of NP are very important issue for using these NP formulations *in vivo*. Uptake of gelatin NPs by phagocytic cells can be completely

different in "*in vitro*" conditions as compared to "*in vivo*" conditions. NP formulations at large doses can be toxic to a variety of cells. So there is a need to determine the maximum dose of NPs that would not be toxic to the cells and tissues. Dye exclusion test can be carried out to determine the toxicity of the NP formulation by determining the percentage of the viable cells. In the above test, the vital dye can penetrate only into dead cells. But this test may give false results as NPs may adsorbed these vital dyes and can easily be taken up by cells leading to false results. Cytotoxic assays provide alternate methods to determine the toxic profile of gelatin NPs. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] cytotoxicity assay can be performed on gelatin NPs with various cells to determine their toxicity [55].

There is a need to determine the endotoxin level in NP drug formulations. Limulus amebocyte lysate test can be performed to determine the endotoxin level in NPs. There is also a need to determine the antigenicity of parenterally administered gelatin NPs. Parenteral administration of gelatin-based products in humans may not cause immunogenicity problems. However antibodies to gelatin have been found in humans who have never been injected with gelatin [61]. Therefore a test for antibodies following administration of a gelatin-based formulation is open to criticism. Marty et al. (1977) found that weekly i.v. injections of drug free gelatin microspheres (formed by crosslinking) into mice over a 12-week period caused no major adverse effects [62]. As only a few studies have been done for determining the biocompatibility and toxicity of the gelatin-based particulate delivery systems *in vivo*, more experiments are required for determining the biocompatibility of the gelatin NP formulation.

1.5 Biodistribution of gelatin nanoparticles

Like other colloidal drug delivery system, gelatin NPs must be taken up by RES specifically liver, spleen and bone marrow. Gelatin NPs can be labelled with radionucleotide- ^{99m}Tc. Oppenheim et al. (1978) performed i.v. administration of ^{99m}Tc labelled gelatin NPs in mice. A rapid uptake was found in the liver (i.e. 50% of the dose) and the rate of liver accumulation of ^{99m}Tc NPs following i.m. and intraperitoneal administration was much slower as compared to i.v. administration [44]. Gelatin NPs can be coated or modified with PEG [55] and therefore can be targeted to other parts of body. *In vivo* studies should be performed for determining the biodistribution of the PEG coated gelatin NPs. Gelatin NPs can also be labelled by fluorescent dyes such as Tetramethylrhodamine conjugated (TMR-) dextran, Texas Red and fluorescein isothiocynate. These fluorescently labelled gelatin NPs can be used to determine their uptake up by tumor cells line BT-20 (a human breast cancer cell line) [55].

1.6 Advantages and therapeutic applications of gelatin nanoparticles

Gelatin is a natural macromolecule and is a promising biomaterial and biocompatible for preparation of NPs. Its biodegradability and biocompatibility provide significant advantages over non-biodegradable synthetic polymers. These particles are easy to produce and can be manufactured at a large scale at very low cost. [4]. Gelatin NPs can easily be loaded with anticancer drugs such as doxorubicin [17], dactinomycin [37], hypocrellin B [56], methotrexate [63] and mitomycin C [20]. *In vitro* release studies have shown a controlled release of these drugs from the particles. More *in vivo* studies are required in order to see the efficacy of this delivery system. In addition to anticancer drugs, these particles can also be loaded with other drugs such metronidazole [64],

muramyl dipeptide [52], primaquine [64] and triamcinolon [65] (Table 4). Gelatin NPs can also be loaded by fluorescent dyes such as TMR dextran [55], Texas Red succinyl chloride [36] and fluoresceinamine [36], and therefore can be used for cellular uptake studies. Modification with sulfhydryl groups on the gelatin NP surface can be further

Table 5. Drugs and biological agents loaded on gelatin nanoparticles and their uses or applications

Drugs or biological agents loaded or encapsulated on Gelatin NPs	Possible Uses or Applications	Reference
Doxorubicin	Anticancer	[17]
Interferon	Treatment of chronic hepatitis B and C	[51]
Hypocrellin B	Anticancer	[56]
Mitomycin C	Anticancer	[20]
Dactinomycin	Anticancer	[37]
Methotrexate	Anticancer	[63]
Metronidazole	Antibacterial and antiprotozoal	[64]
Muramyl dipeptide	Immunomodulator	[52]
Primaquine	Antimalarial	[64]
Triamcinolon	Treatment of Asthma, Multiple Sclerosis, Psoriasis	[65]
BSA	Antigen delivery	[28,53]
Tetramethyl rhodamine dextran	Cellular uptake studies	[55]
Texas Red X	Cellular uptake studies	[36]
p42-clacZ DNA	Gene delivery	[28]
PcRELuc DNA	Gene delivery	[28]
RNA (sperm whale)	Antispasmatic	[28]
Interleukin 4	Treatment of Kaposi's sarcoma	[28]
Chloroquine	Antimalarial, Increased transfection level	[28]
Cyclosporin-A	Immunosuppressant	[33]
Oligonucleotides	Antisense therapy	[29]
Fluoresceinamine	Cellular uptake studies	[36,65]
Peptide nucleic acid	Antisense therapy	[59]
Plasmid DNA	Vaccine development	[29]
Pilocarpine hydrochloride	Glaucoma	[67]
Hydrocortisone	Immune and allergic disorders	[67]

used for the covalent attachment of avidin that can further be used as a carrier system for antisense peptide nucleic acid against HIV [59]. Gelatin NPs can also be used as a DNA carrier system for gene therapy [28, 66]. As these particles are capable of loading protein and peptide drugs [53], therefore they may have a potential to be used as a vaccine delivery system.

1.7 The immune system in cancer

The immune system provides defense mechanism against infectious agents. The two major components of immune system are innate immunity and adaptive immunity. Innate immunity refers to nonspecific defence mechanisms that come into play immediately or within hours of an antigen's appearance in the body. These mechanisms include physical barriers such as skin, chemicals in the blood, and immune cells that attack foreign cells in the body. The innate immune response is basically antigen independent and is stimulated by mediators released through inflammation or tissue damage. On the other hand adaptive immune response involves specificity, diversity and memory. Adaptive immune system consists of cell mediated immunity (mediated by T cells) and humoral immunity (involves antibody i.e. the product of B cells). Humoral immunity is required for the elimination of extracellular pathogens whereas cell mediated immunity is required for the removal of intracellular pathogens as well as cancer. There are two possible pathways for antigen processing and presentation to the immunogenic cells namely major histocompatability complex (MHC) class I and MHC class II pathways. MHC class I pathway involves the processing and presentation of endogenous antigens as well as exogenous antigens (by transport of internalized exogenous antigens

from endosomes or phagosomes to endoplasmic reticulum (ER) by phagosomes and ER fusion, then to cytosol termed as cross priming) [68-72] (Figure 4) consequently leads to the cytotoxic T lymphocyte (CTL) activation, whereas MHC class II pathway involves the processing and presentation of exogenous antigens that further leads to T helper (Th) cell activation. The two important subsets of Th cells are: Th1 and Th2 cells. Th1 cells activate mainly cell-mediated immune responses and stimulate the production of opsonizing and complement-activating antibodies. Th cells can activate APC's, which will up regulate their co-stimulatory molecules. At the same time Th cells produce IL-2. Thus antigen:MHC I recognition, in combination with co-stimulatory molecules from APC and IL-2 from Th cells will activate naive CD8 T cells to proliferate and differentiate into cytotoxic T cells. On the other hand Th2 cells provide help to the humoral responses [73,74]. So there is a need to activate $CD4^+$ Th cells in such a way that leads to the activation of Th1 pathway and help in providing antitumor activity. The activation of $CD4^+$ Th1 cells is influenced by the delivery system that delivers antigen to the DCs and antigen presentation by DCs to T cells. Therefore cancer vaccines are designed now-a-days to induce Th1 specific immune responses against defined cancer antigens [75,76] or to a pool of undefined antigens present in tumor cell lysates. MHC class I restricted antigens recognized by CD8 T cells have been investigated for specific immunologic effects in clinical studies [77]. Peptide vaccines derived from the cancer testis antigens (MAGE, GAGE etc.), melanocyte differentiation antigen Melan A/MART-1, CEA and gp100 have already been clinically tested and has shown promising immunological results [78,79]. Cancer associated antigens such as p53, HER-2/neu and MUC1 mucin which are overexpressed in cancer have also been shown as important

targets for immune attack. Researchers have been trying to achieve strong specific immune responses against cancer by using vaccines with specific antigenic peptide derived from cancer antigens alone or with adjuvant or with cytokines. A liposomal vaccine formulated with MUC1 mucin peptide has already been developed in our research group [75] and has completed Phase II clinical trials. Our group has also developed novel vaccine delivery systems such as PLGA microspheres for delivering peptides derived from tumor Further investigations on these delivery systems are ongoing [76,80].

1.8 DCs as a target for vaccine delivery

DCs are the key professional APCs responsible for stimulation of T lymphocytes against pathogens and cancer [81]. They are also the only APCs that have the ability to induce a primary immune response in resting naïve T lymphocytes [81]. In addition to this, DCs play a major role in establishing memory by maintaining B cell functions and recall responses. They are also responsible for induction of T cell tolerance against 'self molecules' thus avoiding autoimmune responses [82]. DCs originate from hemopoietic progenitors in the bone marrow consisting of both lymphoid and myeloid lineages [83,84]. DCs that have never encountered an antigen are called immature DCs, and are phagocytotic in nature [84]. These immature DCs can phagocytose exogenous (soluble or particulate) antigens leading to the maturation and upregulation of the expression of molecules such as CD11c, CD 40, CD80, CD83, CD86, MHC II and IL-12 [85]. These mature and activated DCs that are non-phagocytotic in nature, then migrate to draining lymph nodes via afferent lymphatics and home to T cell areas, where they present

processed antigens in association with MHC class I and class II molecules to the naïve T cells resulting in T cell activation [86].

Toll-like receptors on DCs play an important role in pathogen recognition and initiation of inflammatory and immune responses [87,88]. The recognition of the molecular structure on the invading pathogens such as lipopolysaccharide on bacteria, mannan on fungi, and surface glycoproteins on viruses is one of the ways that allows the immune system via DCs to distinguish between self and nonself. The stimulation of Toll-like receptors by microbial products leads to the activation of signalling pathways that result in the maturation of DCs. This leads to the induction of costimulatory molecules and also increases antigen-presenting capacity of DCs. Thus, microbial recognition by Toll-like receptors helps to direct adaptive immune responses to antigens derived from microbial pathogens [89]. Though DCs are very rare in our body, they can be generated in large numbers from $CD34^+$ bone marrow precursors or from $CD14^+$ monocytes. This has led researchers to study these cells in detail which was not possible earlier in the 1980's [90-92].

In addition, researchers have shown that DCs can induce antigen-specific tolerance in central lymphoid organs and in the periphery [93]. In the thymus DCs generate tolerance by deleting self-reactive T cells. DCs can also induce peripheral immune tolerance by taking up dying cells [94]. In absence of infection and acute inflammation, the targeting of immature DC with low doses of antigens leads to deletion of the corresponding T cells and the suppressed T cells can not be restimulated by antigenic rechallenge with or without adjuvants [95]. Many strategies are currently being followed for the generation of tolerogenic DCs for the prevention of allograft rejection.

One of the recently invented techniques used mitomycin C treated DCs that selectively decrease adhesion molecule (intercellular adhesion molecule [ICAM]-1) and co-stimulatory molecules (CD80, CD86). T cells suppressed in the above study could not be restimulated indicating that mitomycin C treated DCs induce tolerance [96].

1.9 Monophosphoryl lipid A as an immunomodulator

For the last two decades many adjuvants have been used in the vaccine formulations for mediating and promoting the induction of wide range of immune responses. Adjuvants such as monophosphoryl lipid A (MPLA) obtained from lipopolysaccharide of gram negative bacteria (Salmonella minnesota) [97] has shown promise as an important immunomodulator in vaccine formulations. The mechanism of action of MPLA is not well understood. But, MPLA has been shown to induce the synthesis and release of cytokines particularly IFN-γ that leads to the generation of Th1 responses [98]. MPLA may act directly on T cells, likely through their Toll-like receptors (TLR-4 ligand), by increasing their intracellular calcium and up-regulating their CD40 ligand expression. This shows that MPLA enhances T cell responses by having an impact on DC and T cells. Calcium mobilization, mitogen-activated protein kinase activation, and the NF-κB transcription factor were induced after high dose MPLA (100 μg/mL) stimulation of DC. MPLA at low doses (5 μg/mL) had no impact on DC maturation [99,100]. Formulating MPLA in colloidal drug delivery systems such as liposomes, microspheres and nanospheres have been shown to enhance immune responses as compared to its soluble form [75,76,80,101,102]. Particulate delivery of MPLA along with antigen leads to a significant increase in the T cell responses as compared to its soluble form. Low amount of MPLA incorporated in particulate delivery systems can

achieve the same level of immune responses as achieved by large doses of MPLA in a soluble form [80,102]. Liposomes containing MPLA (up to 2.2 mg) have been utilized in a phase I clinical trial of a proposed malaria vaccine and resulted in very high levels of antibodies against the malarial antigen, and was non pyrogenic and safe for human use [103].

1.10 Gelatin nanoparticles as a vaccine delivery system

Researchers have shown earlier that soluble forms of antigens have very low efficiency of targeting APCs. Many studies have shown that particulate forms of delivering antigen as compared to its soluble form, leads to higher uptake of antigen by APCs and has shown higher immune responses as compared to its soluble form [76,79,104,105]. This may be due to the difference in the mechanism of the uptake of particulate (via phagocytosis) and soluble forms (pinocytosis) by DCs [106]. So there is a need to develop an efficient particulate delivery system that can selectively target APCs, specifically DCs for generating effective immune responses. The successful vaccination strategies can be those that favor the uptake and presentation of antigens by DCs. This can be accomplished by developing efficient particulate vaccine delivery systems that can enhance the *ex vivo* loading of DCs with antigens or by targeting these particulate delivery systems loaded with antigens to DCs *in vivo*. The microenvironment of antigen capture and antigen presentation by DCs controls the direction and magnitude of immune responses. Two approaches can be followed for using particulate delivery systems to induce immunity against cancer antigens. The first approach is to directly inject the particulate formulation loaded with cancer antigen that will target RES, that will further capturing of antigen by immature DCs that have a high level of phagocytic property.

These immature DCs then migrate to the secondary lymphoid organs as mature DCs and lead to present antigen to naïve T cells in context with MHC I and II molecules that will further lead to antigen specific T cell responses (Figure 4) [107]. These T cell responses, specifically Th1 responses that lead to cell-mediated immunity are required to fight viral infections and cancer [74]. Second and a very common approach uses DCs loaded with particulate antigen delivery systems *ex vivo* and then injected back *in vivo* to present

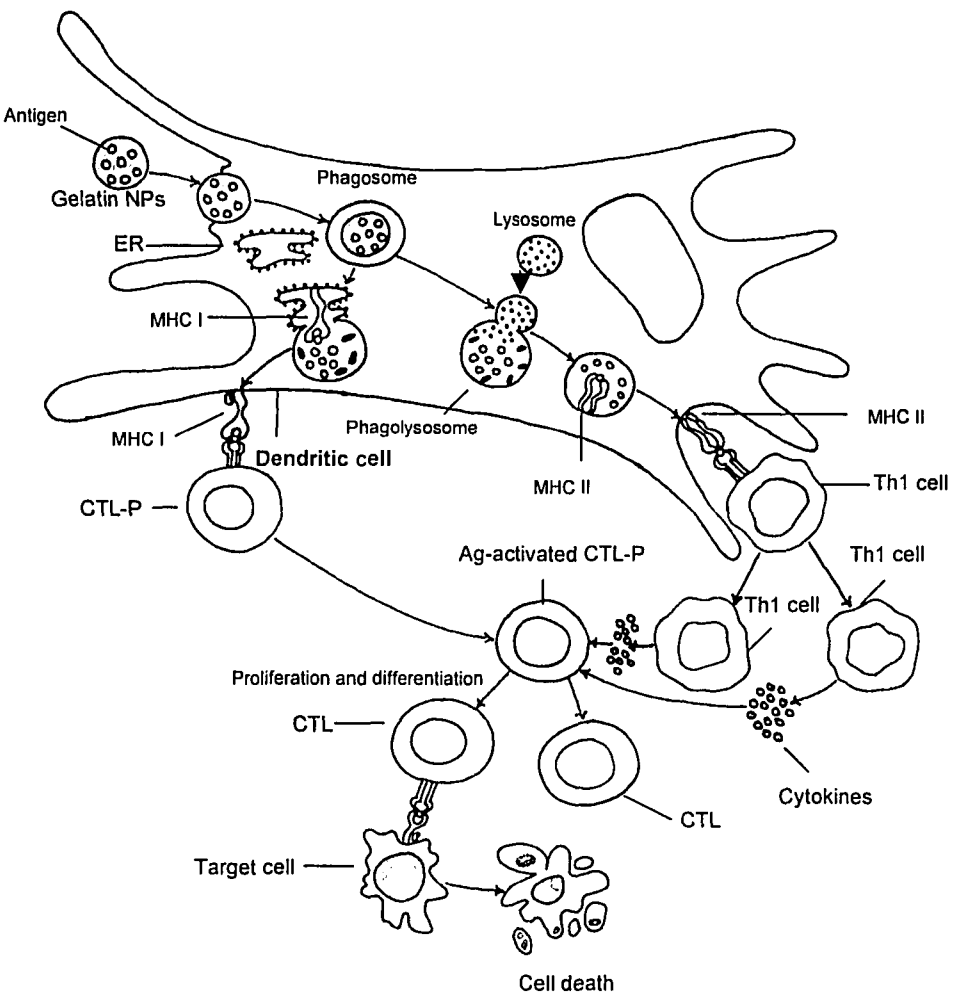

Figure 4. Proposed mechanism for gelatin nanoparticles as a vaccine delivery system based on DC.

tumor-associated antigens and thereby generating tumor-specific immune responses. Pre-clinical data have demonstrated that a response can be triggered against cancer cells, both *in vitro* and *in vivo*, following incubation of DCs with tumor antigen [108,109]. Researchers have reported that the antigen pulsed DC therapy approach to breast cancer treatment is safe and feasible. Clinical trials of DC based vaccination are ongoing in many institutions world wide for the treatment of melanoma, breast cancer, lung cancer, colon cancer, prostate cancer, renal cell carcinoma and B-cell lymphoma and has shown very encouraging results against cancer [85]. So, it is very important to develop an efficient vaccine delivery system that can safely and efficiently deliver antigens to DCs. Biodegradable NPs are among the most promising carriers for the delivery of protein antigens to DCs as they are specifically taken up by RES because of their small size [13]. Protein/peptide antigens can be encapsulated in biodegradable NPs for a sustained release of antigen to DCs, providing a long-term antigen supply to the immune system. Biodegradable nanospheres are an efficient vaccine delivery system for delivering immunogenic materials to DCs that will further lead to enhancement and prolonged specific immune responses [80,110]. For the above reasons, we have selected a biodegradable NP delivery system based on gelatin for DC delivery. Gelatin is a promising candidate for preparing biodegradable NPs. It is cheap and readily available possessing low antigenicity and a great experience exists for its use in parenteral formulations [111,112]. Gelatin NPs have shown their tendency to encapsulate protein/peptide drugs [53]. All this leads us to infer that gelatin NPs can be used as an efficient vaccine delivery system for delivering antigens to DCs that will further lead to

the enhancement and prolonging of specific immune responses. Further studies need to be done for characterization of this system *in vivo*.

CHAPTER 2: OBJECTIVE, HYPOTHESIS AND SHORT TERM OBJECTIVES

2.1 Aim

To characterize an efficient NP vaccine delivery system based on gelatin, a biodegradable, natural polymer for delivery to DCs *in vitro*.

2.2 Hypothesis

1) Gelatin NPs loaded with fluorescent probes would be taken up by DCs and thereby provide a means for studying cellular uptake of Gelatin NPs *in vitro*.
2) Particulate delivery of TMR-dextran to DCs will be more effective than its soluble form.
3) Gelatin NPs will be efficiently loaded with immunomodulator such as MPLA.

2.2 Short term objectives

a) To load molecular fluorescent probes- TMR-dextran and Texas Red X in gelatin NPs.
b) To determine the *in vitro* release of fluorescent probes from gelatin NPs that are encapsulated and surface loaded on gelatin NPs.
c) To determine the amount of TMR-dextran loaded NP uptake by the murine bone marrow DCs ($CD11c^+$ and MHC II^+) and to determine the NP uptake per DC using flow cytometry and comparing this uptake with the soluble form of TMR-dextran.
d) To determine the intra-cellular location and the mechanism of uptake of NPs by DCs using confocal laser scanning microscopy (CLSM).
e) To determine the loading efficiency of MPLA on gelatin NPs.

CHAPTER 3: METHODS AND MATERIALS

3.1 Preparation of gelatin nanoparticles

The unlabelled gelatin NPs were prepared by a two-step desolvation method [36]. In this method, 1.25 g of gelatin type A (source: porcine skin, 175 bloom strength) (Sigma Chemical Co., St Louis, USA) was dissolved in 25 mL of water and the solution was heated to around 50 °C until a clear solution of gelatin was formed. The gelatin was then desolvated by adding 25 mL of acetone and sedimented for a short time. The supernatant containing gelatin and desolvated gelatin was then discarded and the sediment was then re-dissolved in water under heating and the pH of the solution was made acidic (2.5 pH) by adding 200 μL of 1M NaOH. The gelatin was then again desolvated by dropwise addition of 50-60 mL of acetone. After 5 minutes of stirring, 400 μL of glutaraldehyde (25%) was added to the above system to crosslink the particles. The above dispersion was stirred overnight until most of the acetone was evaporated. Dispersion was then centrifuged at 20,000 X g for 28 minutes. NPs were then purified by threefold centrifugation and redispersion in 30% acetone in Milli-Q water. After third centrifugation, the particles were redispersed in purified water.

3.2 Preparation of TMR-dextran loaded gelatin nanoparticles

In this method, 0.625 g of gelatin type A was dissolved in 12.5 mL of water and the solution was heated to around 50 °C until a clear solution of gelatin was formed. The gelatin was then desolvated by adding 12.5 mL of acetone and sedimented for a short time. The supernatant containing gelatin and desolvated gelatin was then discarded and the sediment was then re-dissolved in water under heating and the pH of the solution was

made alkaline by adding 100 μl of 1M NaOH. 2.5mg/100 μl of TMR-dextran (mol. Wt-40000 daltons) (Molecular probes, Eugene, Orlando, USA) in phosphate-buffered saline (PBS) was added to the above system and then gelatin was desolvated again by dropwise addition of acetone (30 mL) until it formed a dark pink colored precipitate. The colorless supernatant was removed and the colored precipitate was re-dissolve in 12.5 mL of water under heating and the pH of the coloured solution was made acidic by adding 120 μl of HCl. After that, again 2.5 mg/100 μL of TMR-dextran was added and gelatin was again desolvated by dropwise addition of acetone (30-35 mL). After 5 minutes of stirring 200 μL of 25% solution of glutaraldehyde was added to crosslink the particles. After 2-3 hours of stirring, until most of the acetone was evaporated, the dispersion was centrifuged at 20,000 X g for 28 minutes. TMR-dextran labeled particles were then purified by centrifugation and redispersion in 30% acetone in Milli-Q water until we got a colorless supernatant. And after we had obtained colorless supernatant, particles were redispersed in Milli-Q water.

3.3 Preparation of Texas Red X loaded gelatin nanoparticles

In this method [36], 1.25 g of gelatin type A was dissolved in 25 mL of water and the solution was heated to around 50 °C until a clear solution of gelatin was formed. The gelatin was then desolvated by adding 25 mL of acetone and sedimented for a short time. The supernatant containing gelatin and desolvated gelatin was then discarded and the sediment was then re-dissolved in water under heating to around 45 °C. The above dispersion was stirred and 1 mg of Texas Red X (mol. Wt- 816.94) (Molecular Probes, Eugene, Orlando, USA) was added to the above system. The dispersion was stirred for 90 minutes and in between the dispersion was kept warm after regular intervals of 20

minutes, until all the dye was dissolved into it. After 90 minutes of stirring, the dispersion was heated to 50 °C and acetone was added around 65-75 mL until the dispersion turned milky pink. After two minutes of stirring, 400 μL of glutaraldehyde (25%) was added to crosslink the particles and the dispersion was stirred overnight until most of the acetone was evaporated. Dispersion was then centrifuged at 20,000 X g for 28 minutes. Texas Red X labeled particles were purified in the same way as TMR-dextran loaded NPs.

3.4 Characterization of nanoparticles

Particle size for unloaded particles and fluorescently labeled particles was then determined by PCS using Zetasizer 3000 (Malvern, UK). 100 μl of the NP dispersion was mixed with 3.0 mL of Milli-Q water in the measuring cuvette for conducting size measurement studies. Concentration of NPs was determined by drying 500 μl of NPs in oven at 60 °C for 12 hours.

3.5 TMR-dextran loading

To calculate the amount of loading of TMR-dextran in NPs, 1 mL (20.1 mg) of TMR-dextran loaded NPs were sedimented by centrifugation at 20,000 X g and the sedimented particles were mixed with 1.0 mL of 0.2 M NaOH solution until a clear solution was observed (~ 48 hours) at room temperature. The resulting solution was again centrifuged at 20,000 X g for 28 minutes and the fluorescent intensity of supernatant was measured by exciting the fluorescent probe at 552 nm excitation wavelength and measuring the fluorescence intensity at 577 nm emission wavelength using fluorescence spectrophotometer (FluoroMax spectrofluorometer, Spex Industries Inc., New Jersey, USA). We also used 0.05 % Trypsin-EDTA (Invitrogen Canada Inc., Burlington, Ontario,

Canada) in a similar way to calculate the loading. Sedimented TMR-dextran loaded NPs were resuspended in 1.0 mL of Trypsin-EDTA and Milli-Q water (1:1) and particles were then incubated at 37 °C for 24 hours until a clear solution was observed. The encapsulation efficiency and probe loading weight % of TMR-dextran was calculated by comparing the standard curve of TMR-dextran in PBS (pH 7.3). Encapsulation efficiency of TMR-dextran is defined as the percentage of TMR-dextran added during the preparation of gelatin NPs that is encapsulated (Encapsulation efficiency = $(W_E / W_{TD}) * 100$, where 'W_E' is weight of TMR-dextran encapsulated in gelatin NPs and 'W_{TD}' is weight of TMR-dextran added during the preparation of the gelatin NPs) whereas probe loading weight % of TMR-dextran is defined as the weight percent of the TMR-dextran encapsulated in the gelatin NPs (Probe loading weight % = $(W_E / W_N) * 100$, where 'W_E' is weight of TMR-dextran encapsulated in gelatin NPs and 'W_N' is dry weight of gelatin NPs).

3.6 Texas Red X loading

The same method for calculating the amount of Texas Red X loaded on NPs was employed here as mentioned above for TMR-dextran loaded NPs. The fluorescent intensity of supernatant was measured by exciting the fluorescent probe at 588 nm excitation wavelength and measuring the fluorescence intensity at 607 nm emission wavelength using fluorescence spectrophotometer. The surface loading efficiency and probe loading weight % of Texas Red X was calculated by comparing the standard curve of Texas Red X in water. Surface loading efficiency of Texas Red X is defined as the percentage of Texas Red X added during the preparation of gelatin NPs that is surface loaded to the NPs (Surface loading efficiency = $(W_{SL} / W_{TRX}) * 100$, where 'W_{SL}' is

weight of Texas Red X surface loaded on gelatin NPs and 'W_{TRX}' is weight of Texas Red X added during the preparation of the gelatin NPs) whereas probe loading weight % of Texas Red X is defined as the weight percent of the Texas Red X surface loaded on the gelatin NPs (Probe loading weight % = (W_{SL} / W_N) * 100 , where 'W_{SL}' is weight of Texas Red X surface loaded on gelatin NPs and 'W_N' is dry weight of gelatin NPs).

3.7 *In vitro* release of TMR-dextran from gelatin nanoparticles

The *in vitro* release study of TMR-dextran from NPs was carried out in PBS, with and without Trypsin-EDTA (Invitrogen Canada Inc., Burlington, Ontario) as enzyme, at 37 °C in a shaking water bath. 20.1 mg of sedimented TMR-dextran loaded gelatin NPs were resuspended with 1.0 mL of PBS or 1.0 mL of PBS containing trypsin-EDTA (4:1 v/v). After different intervals of time, the particles were centrifuged and 500 μl of supernatant was removed. The PBS or PBS containing trypsin-EDTA was replaced every time in the vial containing NPs, to maintain the constant volume. The fluorescence intensity of the supernatant obtained at different intervals of time was measured using the same conditions as described above in the loading study and the cumulative amount released was calculated from appropriate calibration curves.

3.8 *In vitro* release of Texas Red X from gelatin nanoparticles

Similarly, the *in vitro* release study of Texas Red X from NPs was carried out as described above for the TMR-dextran loaded NPs, but with the use of its respective calibration curves.

For conducting fluorescent intensity reading for the loading studies as well as *in vitro* release studies, 100 μl of sample mixed with 3.0 mL of Milli-Q water in cuvette was

analyzed in fluorescence spectrophotometer. For all of the above *in vitro* release studies, from every fluorescent reading measurements, background fluorescence intensity reading (i.e. fluorescence intensity readings for the unloaded NPs treated in the similar way as TMR-dextran loaded NPs or Texas Red X loaded NPs) was deducted.

3.9 Isolation and culture of DCs

The method of generating bone marrow derived DCs with granulocyte macrophage-colony stimulating factor (GM-CSF) (Peprotech, Rockville, MA) was followed [113]. DCs were isolated from the bone marrow of mice by washing out the bone marrow 2-4 times and plating them in bacteriological grade 100 mm petridish at 2 x 10^6 cells per 10 mL of RPMI-1640 containing 200 ng of GM-CSF. The above petridish was then incubated at 37 °C, 5% CO_2 for three days. On day 3, 200 ng of GM-CSF was again added to the petridish and the culture media was replaced by fresh media, and petridish was again incubated at the same above condition. On day 6, we repeated the above step. Five petridishes (A, B, C, D and E) containing cells (2 x 10^6 cells) were prepared as shown above for conducting the phagocytic studies. It has been shown by previous studies in our lab that the day seven is the best for feeding the DCs with the formulation [79]. So, on day 7, petridish 'A' was fed with 200 μl of HBSS. Petridish 'B' was fed with 200 μl of TMR-dextran (80 μg) in HBSS. Petridish 'C' was fed by 200 μL (4.0 mg) of unloaded gelatin NPs in HBSS. Petridish 'D' was fed by 200 μL (4.0 mg) of TMR-dextran NPs in HBSS that were loaded with 80 μg of TMR-dextran.

3.10 Flow cytometry

After incubation for 24 hours, the above DCs were harvested by gentle pipeting and aliquoted at 2 x 10^5 cells in a microcentrifuge tube (one antibody per tube) containing cold staining buffer (PBS containing 10% FBS and 0.05% sodium azide). For staining the cells, monoclonal antibodies specific for CD11c (HL3 Purified Hamster Anti-Mouse CD11c monoclonal antibody) and MHC-II (2G9 Purified Rat Anti-mouse I-A/I-E monoclonal antibody) (Pharmingen, ON, Canada) along with their respective isotype controls G235-2356 Purified Hamster IgG1, λ1 Monoclonal Immunoglobulin Isotype Standard (anti-Trinitrophenol) and A95-1 Purified Rat IgG2b, κ Monoclonal Immunoglobulin Isotype standard were used at 1 μg per tube. After staining the cells, they were incubated at 4 °C for 30 minutes and washed three times and were resuspended in cold staining buffer. Fluorescein-5-isothiocyanate (FITC) conjugated secondary antibodies (G94-56 FITC-conjugated Mouse Anti-Armenian and Syrian Hamster IgG1 monoclonal antibody and G15-337 FITC-conjugated Mouse Anti-Rat IgG_{2b} monoclonal antibody) were added to the above cells that were stained with the primary antibody CD11c and MHC II respectively, and were allowed to incubate for 30 minutes at 4°C. After incubation the above cells were washed with staining buffer and transferred to Falcon tubes. Samples were analyzed by flow cytometry on a Becton Dickinson FACS scan (Franklin Lakes, NJ, USA).

3.11 Confocal laser scanning microscopy (CLSM)

DCs were transferred from petridish into Lab-Tek II 8 chamber slide (Nalgene Nunc Int., IL, USA) on day 7 in culture, at a concentration of 2 x 10^5 cells/300 μl. Control wells were pretreated by cytochalasin B (7.5 μg/mL) for 30 minutes. Wells

received many formulations such as 200 μl (20 μg) of unlabeled gelatin NPs in culture media, 500 μL (20 μg) of TMR-dextran loaded gelatin NPs in culture media as well as soluble form of 500 μL TMR-dextran in culture media. After 24 hours of incubation at 37 °C, supernatant was removed and the cells were washed three times with 500 μL of PBS buffer. Before fluorescent labeling of the cell membrane, cells were incubated for 30 minutes with lysotracker blue DND-22 (50 nM) i.e. freely permeant to cell membranes, (Molecular Probes, OR, USA) for labeling lysosomes. Cell membranes were then labeled by incubating the cells with 100 μL of 0.0005% Alexa fluor 488 - concanvalin A (Molecular Probes, OR, USA) in PBS buffer for 1-2 min or with CD11c-FITC. Cells were then washed 3 times with PBS and were then fixed with 100 μL of 4% paraformaldehyde in PBS for 10 min. For nucleus labeling, above fixed cells were washed with PBS and then incubated for 15 minutes with Hoechst 33342 dye (2 μM) (Molecular Probes, OR, USA). Cells are then finally washed with PBS, slides were prepared with a solution of 2.5% 1,4-Diazabicyclo [2.2.2.] octane (DABCO) (Aldrich Chemical Company Ltd., WI, USA) and 7.5% gelatin in 50:50 PBS:glycerol. Finally the slides were examined under confocal microscope Zeiss 510 LSMNLO (Carl Zeiss Microscope systems, Zena, Germany).

3.12 Loading of MPLA in gelatin nanoparticles

Standard curve of MPLA was made by analyzing 2.5 μg/100 μL, 5 μg/100 μL, 7.5 μg/100 μL, 10 μg/100 μL and 20 μg/100 μL solution of MPLA in chloroform:methanol (2:1). Injection volume for standard curve and loading studies was 100 μL. 100 μL of chloroform:methanol (2:1) without MPLA was used as a background

for the above measurements. Reverse phase HPLC (Waters 625 LC system, Millipore) was connected with UV detector (Waters 486) set at 260 nm. C18 reverse phase column was used for the analysis of MPLA and it was eluted using (a) 50 mM triethylammonium acetate (TEAA) in water and (b) 50 mM TEAA in acetonitrile as mobile phases. MPLA was eluted using linear gradient of 25% to 60% B over 40 minutes at a flow rate of 1 mL/minute. For loading MPLA (synthetic MPLA was supplied by Biomira Inc., Edmonton, Alberta, Canada) on gelatin NPs, already prepared and purified unloaded gelatin NPs (2.4 mg/500 μL) in water were mixed with 100 μg/200 μl of MPLA in chloroform:methanol (2:1) and 300 μL of Milli-Q water was added to make the final volume of 1 mL in eppendorf tubes. After mixing we incubate it at two different incubation times, i.e. 2 hours and 24 hours at 37 °C. After incubation, the NPs mixed with MPLA were freeze dried overnight. After freeze drying, the particles were resuspended in chloroform:methanol (2:1). Particles were then centrifuged down at 20,000 X g for 28 minutes. The supernatant containing free MPLA was collected and was analyzed using reverse phase HPLC. The surface loading efficiency of MPLA was then determined by subtracting the amount of free MPLA from the amount of MPLA added to the gelatin NPs. Surface loading efficiency of MPLA is defined as the percentage of MPLA added during the preparation of gelatin NPs that is surface loaded on gelatin NPs.

CHAPTER 4: RESULTS AND DISCUSSION

Fluorescent labelled gelatin NPs were prepared and characterized for their physicochemical properties. Two fluorescent macromolecular probes (TMR-dextran and Texas Red X) were preferred over radiolabeled probe due to its ease of handling. Fluorescent probes permit loading and release studies using fluorescence spectrophotometry. Fluorescent probes also permit visualization of the uptake of NPs by DCs using CLSM and flow cytometry. TMR-dextran was incorporated non-covalently by encapsulation and Texas Red X was incorporated by chemical conjugation to gelatin surface before second desolvation step.

4.1 Characterization of nanoparticles

4.1.1 Particle size analysis

The mean particle size of unlabeled gelatin NPs, TMR-dextran loaded gelatin NPs and Texas Red X loaded gelatin NPs was determined using PCS and it was found to be 225.6 nm, 244.9 nm and 200.4 nm with a narrow polydispersity index of 0.09, 0.04 and 0.05 respectively (Table 6 and Figure 5a, 5b and 5c).

Table 6. Particle size analysis and concentration of nanoparticles

NP type	Size (nm)	Polydispersity index	Concentration (mg/mL)
Unloaded gelatin	225.6	0.09	20.5
TMR-dextran loaded	244.9	0.04	20.1
Texas Red X loaded	200.4	0.05	20.0

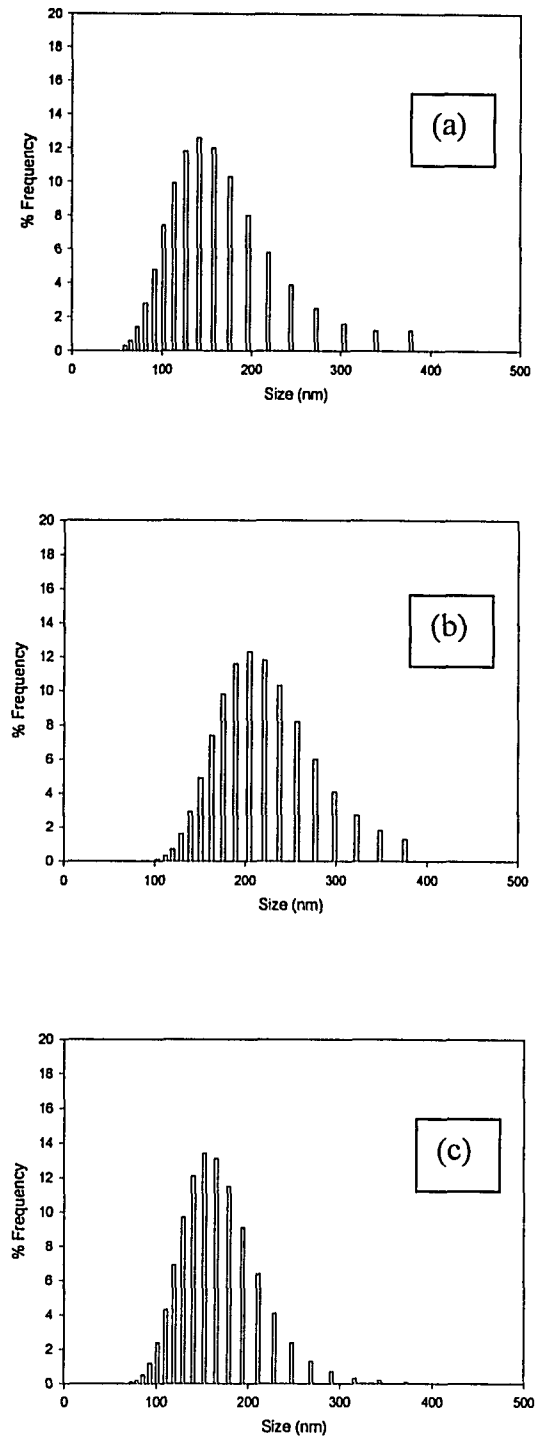

Figure 5. Size distribution of (a) Unloaded, (b) TMR-dextran loaded and (c) Texas Red X loaded gelatin nanoparticles by PCS using Zetasizer 3000.

4.2 Assay for fluorescent probes in gelatin nanoparticles

The loading efficiency of fluorescent probes TMR-dextran and Texas Red X was determined using fluorescence spectrophotometry. The loading efficiency was determined by two methods i.e. by alkaline hydrolysis of gelatin NPs and by digesting the NPs using enzyme (Trypsin-EDTA) as trypsin had earlier been shown to be the most effective enzyme for the degradation of gelatin NP [17]. Both of the above methods lead to the degradation of the gelatin NPs.

4.2.1 TMR-dextran loading and encapsulation efficiency

TMR-dextran loaded gelatin NPs were formed by forming strong interpenetrating networks of gelatin with dextran [114,115] and lead to the encapsulation of TMR-dextran after the last desolvation step. The encapsulation efficiency determined by two methods i.e. by alkaline hydrolysis (0.2 M NaOH) of gelatin NPs and by digesting the NPs using enzyme (Trypsin-EDTA) was calculated to be 96.16 % and 70.07 % respectively. Probe loading weight % for TMR-dextran determined by the above methods was calculated to be 1.87 % and 1.34 % respectively. Loading of TMR-dextran calculated by enzyme-induced digestion of NPs was less as compared to the alkaline hydrolysis method. This might be due the linking of dextran molecules with the amino acids of degraded gelatin and non-degraded gelatin that were still there after the degradation of the NPs.

4.2.2 Texas Red X loading and surface loading efficiency

Texas Red X loaded particles were made by forming stable amide bond between Texas Red X and gelatin particle surface. Hence, Texas Red X was surface loaded to the particles. Surface loading efficiency of Texas Red X loaded NPs determined by alkaline

hydrolysis and by enzyme (Trypsin-EDTA) assisted method was found to be 0.52 % and 0.48 % respectively. Probe loading weight % for Texas Red X, determined by the above methods was calculated to be 0.026 % and 0.024 % respectively.

Based on the loading studies, we found that loading studies done by alkaline hydrolysis method of degrading NPs gives us more precise values than by using the enzyme (Trypsin-EDTA) assisted method. Also, a high background for "enzyme assisted method of degradation" was observed, which was not observed for "alkaline hydrolysis method".

4.3 *In vitro* release studies

4.3.1 In vitro release of TMR-dextran from gelatin nanoparticles

The release of TMR-dextran was performed in PBS alone, and in presence of Trypsin-EDTA at 37 °C. Figure 6 shows that there was just 0.6 % release of the TMR-dextran after 12 hours shaking in a water bath; also after 24 and 48 hours there was only a very low release of about 1.0 % TMR-dextran. The results for diffusional release of TMR-dextran in our formulations were completely different as reported by Kaul et al., 2002 [55]. This difference can be due to a different preparation method of the TMR-dextran loaded gelatin NPs. One example is that there was no addition of crosslinking agents such as glutaraldehyde in the method of preparing gelatin NPs done by Kaut et al., 2002 [55]. The addition of glutaraldehyde, which forms an inter- and intra-molecular crosslinking on the surface of the gelatin NPs leading to a hardening that causes a lower swelling and possibly the decrease of the TMR-dextran release by diffusion. In contrary, a release of nearly 80% after 24 hours was achieved with the addition of trypsin EDTA. Therewith we can predict that the later described release in tissue culture is probably not

via passive diffusion; cellular enzymes must be responsible for the release. We have not seen a complete release of TMR-dextran even after 72 hours, as there might be some dextran that is still linked with parts of the gelatin/gelatin NPs as already discussed above.

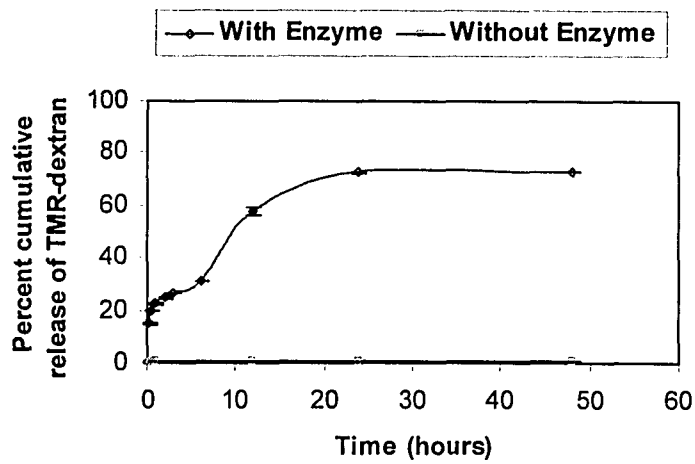

Figure 6. ***In vitro*** **release of TMR-dextran from gelatin nanoparticles in PBS with and without enzyme (trypsin-EDTA) at 37 °C (mean ± S.D.; n=3)**

4.3.2 In vitro release of Texas Red X from gelatin nanoparticles

In vitro release of Texas Red X from gelatin NPs was also carried out as above both in PBS alone, and PBS with enzyme (trypsin-EDTA) at 37 °C. Figure 7 shows that there was almost 3.4% release of Texas Red X after 12 hours of shaking in water bath. There was really no further release of Texas Red X after 24 and 48 hours. This release could be due to diffusional release. This result looks similar to the *in vitro* release of TMR-dextran. But when Texas Red X loaded NPs were shaken in PBS containing enzyme (trypsin-EDTA) at 37 °C, there was around 90% release of Texas Red X within 24 hours (Figure 7). This shows that the release of the Texas Red X here also is

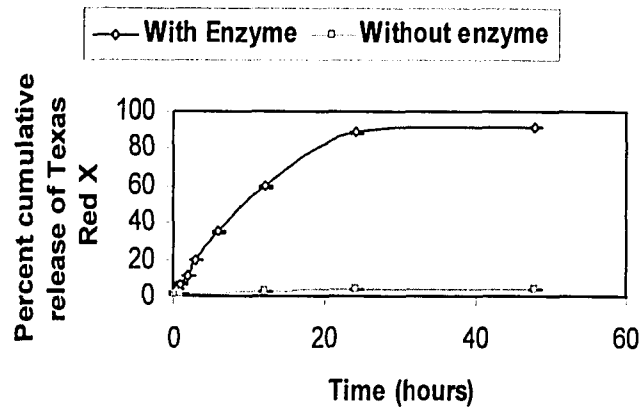

Figure 7. ***In vitro*** **release of Texas Red X from gelatin nanoparticles in PBS with and without enzyme (trypsin-EDTA) at 37 °C (mean ± S.D.; n=3).**

mainly induced by enzyme (trypsin-EDTA) digestion of the NPs as we have seen in the case of TMR-dextran loaded gelatin NPs. Here also we have not seen a complete release of Texas Red X after 48 hours, as there might be some Texas Red X that is still binding with the free amino groups of degraded gelatin.

4.4 Uptake of NPs by DCs: Quantitative study using flow cytometry

We tried to quantitatively analyze the phagocytosis using flow cytometry that helped us to provide information regarding the total number of phagocytic cells as well as the amount of uptake per cell. The NPs to be used for the DC uptake studies were tested for their endotoxin level using endotoxin testing kit (QCL-1000, Bio-Whittaker, MD, USA) and were found to be endotoxin free (data not shown). After exposure of TMR-dextran loaded gelatin NPs to DCs, DCs ingested NPs become granular. This can be seen in the flow cytometry dot plot by increased side scatter indicative of increased granularity (Figure 8). The cells that were gated for the changed granularity were found positive (98%) for TMR-dextran. No change in the cellular granularity was seen when DCs were fed with equivalent amount of soluble form of TMR-dextran, and only 4% of the cells

showed uptake of soluble form of TMR-dextran. There percentage of cells expressing CD11c (Figure 9) and MHC II (Figure 9) were dramatically increased when the cells were incubated with the TMR-dextran gelatin NPs (Figure 9). The number of CD11c cells were increased from 65% to 90 % and the number of MHC II cells were increased from 61% to 89% following incubation with NPs. Only 4% of DCs were double positive for CD11c and TMR-dextran that were fed with the soluble form of TMR-dextran. On the other hand 88% of DCs were double positive for CD11c and TMR-dextran that were fed with TMR-dextran gelatin NPs (Figure 9). A similar difference was seen for DCs that were positive for both MHC II and TMR-dextran that were fed with the soluble form of TMR-dextran (3%) as compared to TMR-dextran loaded gelatin NPs (87%) (Figure 9). Mean fluorescence intensity (MFI) obtained from the FACS data indicates the amount of NPs per cell. DCs that were double positive for MHC II and TMR-dextran and were fed

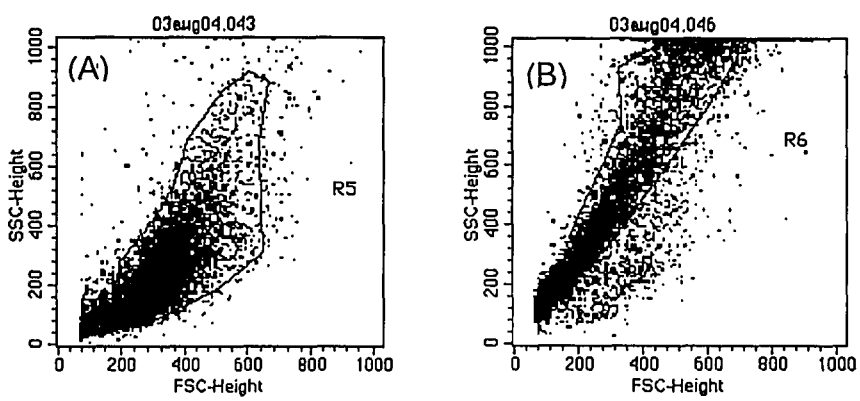

Figure 8. Dot plot for DCs fed with (A) PBS and (B) TMR-dextran loaded gelatin nanoparticles. Side scatter (SSC) indicates cell granularity or complexity and forward scatter (FSC) is an indication of the cell size.

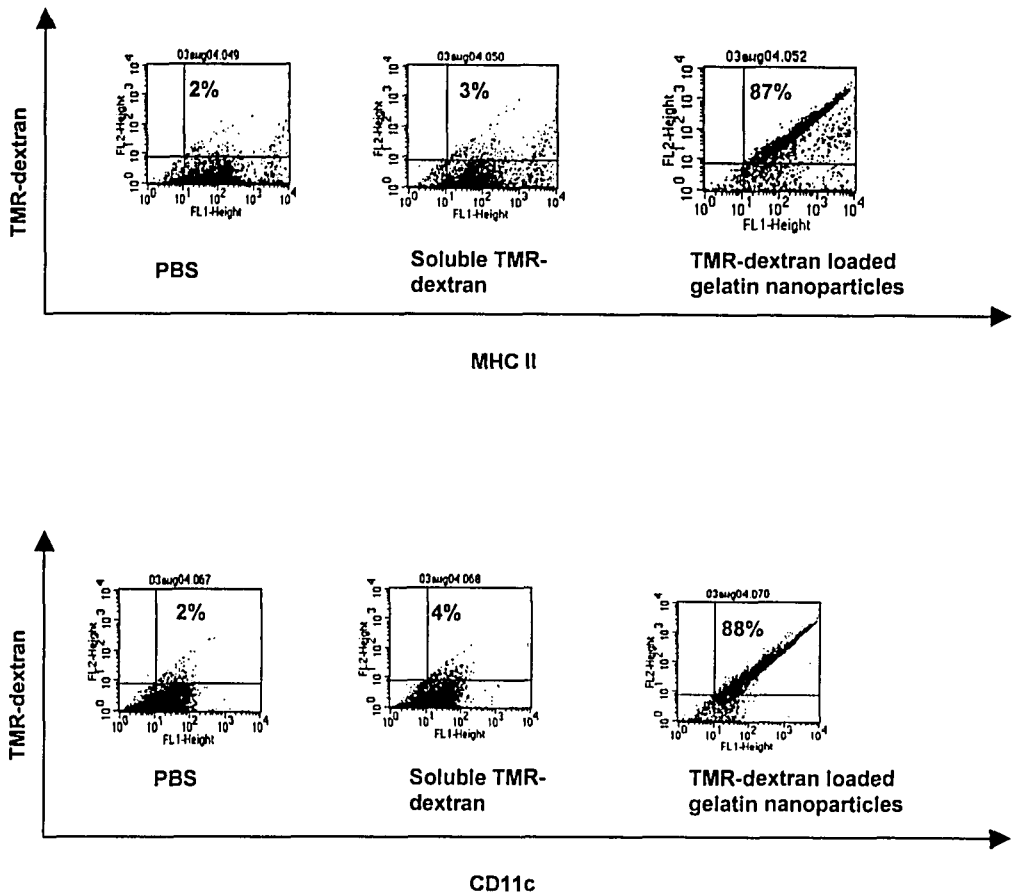

Figure 9. Percentage of DCs that were double positive for MHC II and TMR-dextran or double positive for CD 11c and TMR-dextran. DCs were incubated with different formulations at 37 °C with plain PBS, soluble form of TMR-dextran, and TMR-dextran loaded gelatin nanoparticles for 24 hours. The values shown in the above graph were made by the single determinations performed one time.

with TMR-dextran loaded gelatin NPs showed almost three times as high MFI of TMR-dextran (MFI of 178) as compared to DCs that were double positive for MHC II and TMR-dextran and fed with soluble form of TMR-dextran (MFI of 65). A similar result was found for the DCs that were $CD11c^{+}$.

4.5 Uptake of gelatin nanoparticles by DCs: Qualitative study using CLSM

Double and triple color CLSM was performed to determine the mechanism of uptake of the NPs by murine DCs and their intracellular location. Earlier studies with

human DCs have shown that NPs were taken up via phagocytosis [110]. Regarding murine DCs, we performed intensive CLSM-studies to confirm the uptake of different nanoparticulate formulations made of PLGA, human serum albumin (HSA) and gelatin, that were concentrated on different layers in the z-axis. The results of these studies confirmed that PLGA and gelatin NPs are taken up by murine DCs whereas NPs made of HSA tended to adhere on the cell membranes [116]. Having these former lab studies in mind, we now focused only on one layer in the z-axis that was more or less in the middle of the imaged cells, depending upon the cells size for figure 10A, 10B, 10C, 10D, 10E and 10F. Our first aim was to confirm the former results on human DCs [110] for murine cells. In figure 10A the murine DCs were only stained with Alexa 488 Concanavalin A. Figure 10B shows DCs incubated with TMR-dextran loaded gelatin NP but pretreated with the phagocytosis inhibitor cytochalasin B where no cellular uptake is visible. Incubating the DCs with soluble TMR-dextran shows just a minor uptake that correlates with our FACS data (Figure 10C). Finally, in figure 10D, DCs were incubated with TMR-dextran loaded gelatin NPs and the cellular uptake correlates also very well with the FACS analysis. Until here the study is a logical consequence of our earlier studies [110, 116] and somehow predictable. The challenging part of this study was now to perform a single cell imaging to achieve more details on the intracellular distribution of the TMR-dextran loaded gelatin NPs. For this study we used triple color staining. We have chosen two cellular compartments, nucleus (Figure 10F3) and the lysosomes (Figure 10E3), to stain in addition to the labeled NPs and the cell membranes. The population of murine DCs using the method of Lutz et al [112] leads to about 65% CD11c positive cells as shown in the FACS analysis (data not shown). Hence, for the CLSM imaging of

populations between 5 and 12 cells, as shown in figure 10A, 10B, 10C and 10D an unspecific membrane staining is reasonable and analysis with a specific murine DC marker is necessary. For single cell imaging this is not appropriate because there is still a small chance that it is not a DC image. For this reason we have chosen the more complicated primary/secondary antibody CD11c staining, along with the isotype controls, to confirm that the imaged cell is a murine DC. This is also the reason why the membrane staining in figure 10E2 and 10F2 is not as clear as in figure 10A, 10B and 10C. We have chosen to present the single cell imaging by showing each fluorescent channel separately (Figure 10E1, 10E2, 10E3 and 10F1, 10F2, 10F3) and then the overlay of all three (Figure 10E4 and 10F4) to demonstrate a good channel separation with just minor interferences. Discussing figure 10F in more detail we can clearly say that after an incubation of 24 hours TMR-dextran labeled gelatin NPs cannot be found in the nucleus. Looking at the overlay (figure 10F4) it can be assumed that some of the NPs taken up are in a very close surrounding area to the nucleus. Although the discussed results of figure 10F seem to be predictable too for a hydrophilic colloidal drug carrier system like gelatin NPs was never demonstrated this way before. Due to the common opinion that colloidal drug carrier systems taken up by phagocytosis are stuck in the endo-lysosomes we would predict that the finding in figure 10F should be different regarding the lysosomes. Hence, we would expect that there should be a co-localization of the TMR-dextran loaded gelatin NPs with the lysotracker stained lysosomes. From our studies this prediction is just partly correct. It is obvious that figure 10E1 and 10E3 show a co-localization of the blue stained lysosomes and the red labeled gelatin NPs. Having a closer look at the overlay (Figure 10E4) it shows also a clear blue fluorescence that is not co-localized with the red stained

NPs. This gives us the information that not all lysosomes are involved in the uptake process. But the most interesting finding is that there is also still red fluorescence that is not co-localized with the blue stained lysosomes. Although the red fluorescence in the upper part of the cell interferes with the green membrane staining and therewith is more

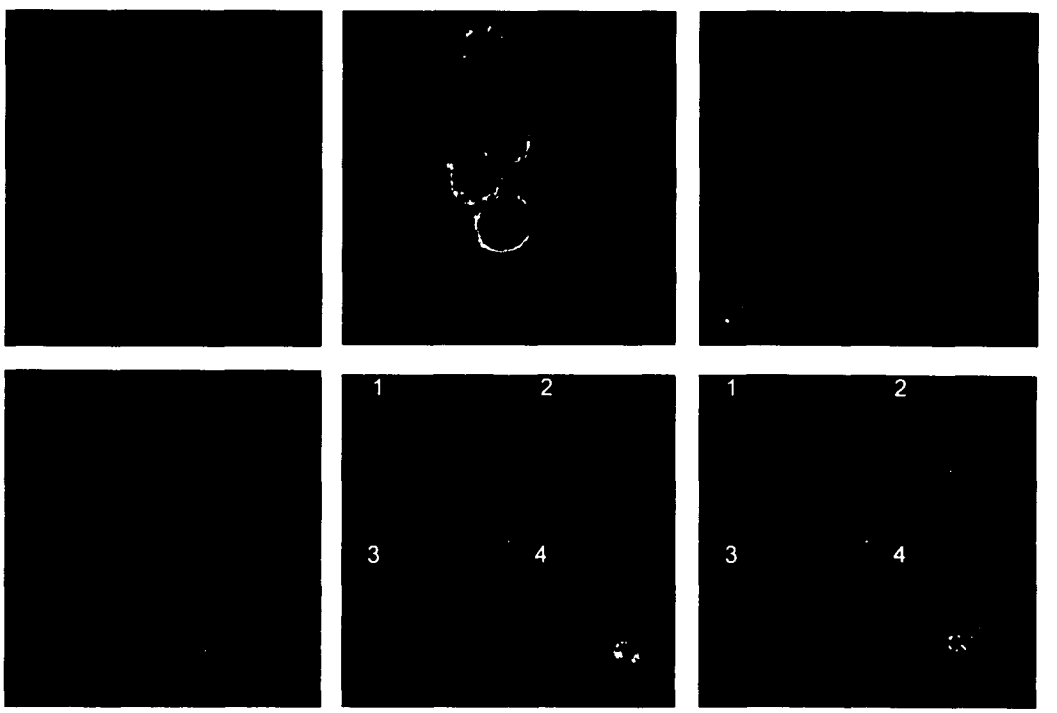

Figure 10. Intracellular location of TMR-dextran loaded gelatin nanoparticles in DCs. (A): DCs incubated with PBS; (B): Cytochalasin B pretreated DCs incubated with TMR-dextran loaded nanoparticles; (C): DCs incubated with soluble TMR-dextran; (D) DCs incubated with TMR-dextran loaded nanoparticles; DCs (A,B,C,D) membrane were stained with Alexa fluor 488 Con A (green); E(1) DC incubated with TMR-dextran loaded nanoparticles (red) and stained with cell membrane CD11c [E(2) green] and lysosome [E(3) blue] dyes; E(4): An overlay of E(1) to E(3); F(1) DC incubated with TMR-dextran loaded nanoparticles (red) and stained with cell membrane CD11c-FITC [F(2) green] and nucleus [F(3) blue] dyes; F(4): An overlay of F(1) to F(3).

orange than red in the overlay, from several single cell images we made we would predict that gelatin NPs are not completely stuck in lysosomes after an incubation of 24 hours.

The last issue to be discussed is the possibility that by an enzymatic degradation the TMR-dextran can be released during the incubation period of 24 hours and we detect free TMR-dextran and not labeled NPs. The forced enzyme assisted degradation study we performed would support this theory because we analyzed a release of about 80% TMR-dextran from NPs within 24 hours. We are still not able to finally answer the question whether we had detected TMR-dextran labeled gelatin NPs in the cytoplasm or that was a released fluorescent dye. More experiments are required to answer the above question. Several studies are in process to study the intracellular trafficking of colloidal drug carrier systems.

4.6 Loading of MPLA on gelatin nanoparticles

We loaded a potent immunomodulator, MPLA on gelatin NPs. For determination of MPLA loading on gelatin NPs, we developed an assay for MPLA in gelatin NPs. A

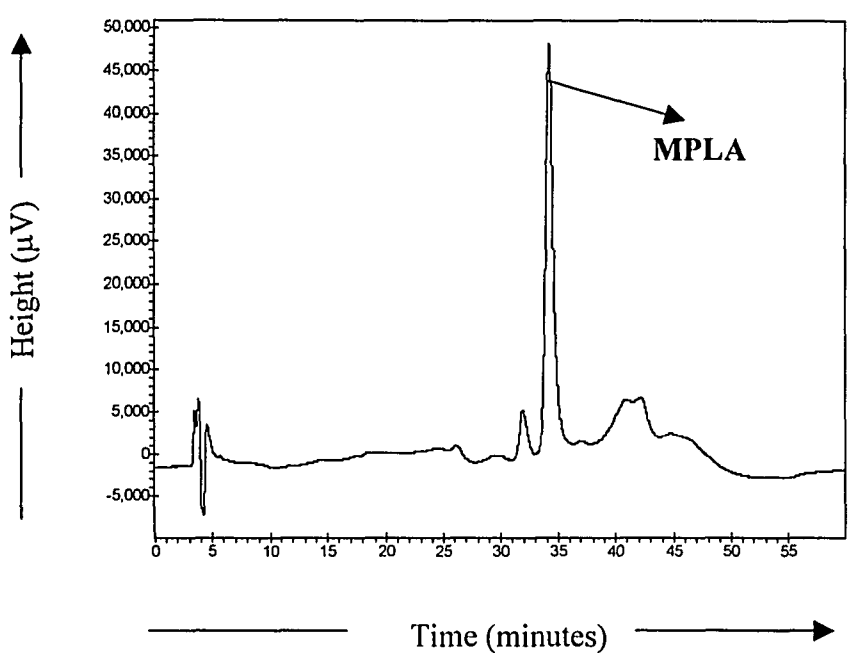

Figure 11. HPLC of MPLA.

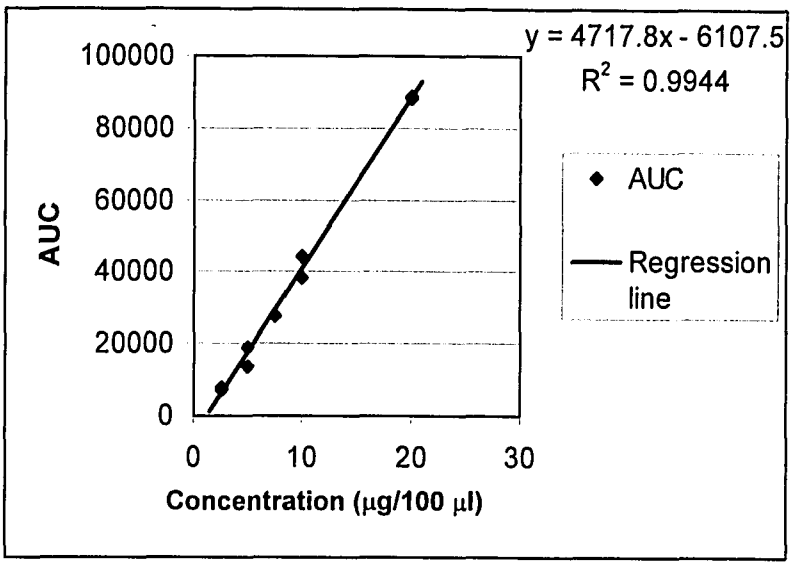

Figure 12. Standard curve of MPLA using reverse phase HPLC.

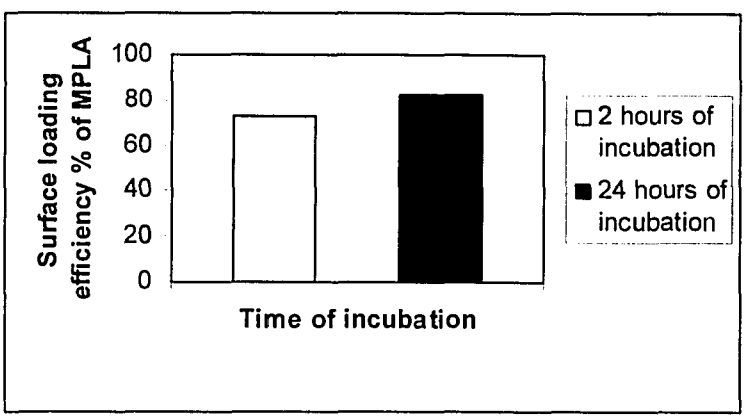

Figure 13. Surface loading efficiency of MPLA on gelatin nanoparticles. The values shown in the above graph were made by the single determinations performed one time.

sample of synthetic MPLA was analyzed by reverse-phase HPLC. The chromatogram showed a single distinct peak for MPLA that eluted at 34 minute (Figure 11). The minimum concentration that we were able to detect using HPLC was 2.5 μg of MPLA.

A standard curve of MPLA was made (Figure 12). MPLA was incorporated in gelatin NPs after the preparation of NPs by direct adsorption of MPLA on NPs by incubation at room temperature. The amount of MPLA loaded on NPs was determined indirectly by quantifying the MPLA in the supernatant. The analysis of supernatant was done using HPLC based on the standard curve. Surface loading efficiency was calculated to be 72.4% and 82.6% for 2 hours and 24 hours of hours of incubation time respectively for 2.4 mg/500 μL of gelatin NPs (Figure 13). 7.24 μg and 8.26 μg of MPLA was found to be adsorbed to 2.4 mg of gelatin NPs at 2 hour and 24 hour incubation time respectively. The results clearly indicate that MPLA can be efficiently loaded on gelatin NPs. Further studies may be required to confirm the loading of MPLA on gelatin NPs by degrading the NPs loaded with MPLA and quantifying the MPLA released from the NPs.

CHAPTER 5: CONCLUSIONS

Summarizing the facts of this study, we demonstrated that internalization of TMR-dextran loaded gelatin NPs by murine bone marrow DCs is higher as compared to its soluble form as shown by flow cytometry and double color CLSM. Also the percentage of CD11c cells and MHC II cells increased after incubation with NPs. Triple color single cell CLSM revealed that gelatin NPs were phagocytosed by DCs and confirmed to be localized partly in the lysosomes, as expected and most likely not in the nucleus. This is a novel study as no one has earlier shown the intracellular fate of the NPs within the DCs. An understanding of intracellular fate of NPs within DC may provide researchers to achieve selective targeting of antigens via NPs at the subcellular level, as this is very crucial in the outcome and efficiency of the immune responses. Summarizing the predictions of this study, we assume that either the TMR-dextran loaded gelatin NPs or released fluorescent dye can be localized in the cytoplasm and that this can lead to improved efficiency of vaccine loaded NPs targeting antigens to DCs for a safe enhanced immunotherapy for different cancer types. Moreover gelatin NPs can be efficiently loaded with MPLA an immunoadjuvant currently investigated in cancer vaccines. These studies show that gelatin NPs are promising delivery systems for targeting macromolecules and immunomodulators to DCs and deserve further investigation as a vaccine delivery system. However, further characterization of this delivery system for DCs is required *in vitro*. Such studies should include *in vitro* antigen presentation assays to determine the ability of DCs (loaded with antigens via gelatin NPs), to present antigen to naïve T-cells and analysis of the cytokine (IFN-γ, IL-4 and IL-10) secretion profile of

proliferating T cells to determine the type of T helper response and comparison of this delivery system with the soluble form of antigen.

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