Preparation and Characterization of Phosphorylated Manno Oligosaccharide Nanoparticles and their Interaction with Mannose 6-Phosphate Receptors

Thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

By

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CERTIFICATE

This is to certify that this thesis entitled **"Preparation and characterization of phosphorylated manno oligosaccharide nanoparticles and their interaction with Mannose 6-phosphate receptors"** submitted to the University of Hyderabad by **Mr. ISMAIL KHAN,** for the degree of Doctor of Philosophy, is based on the studies carried out by him under my supervision. I declare to the best of my knowledge that this work has not been submitted earlier for the award of degree or diploma from any other University or Institution.

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DECLARATION

I hereby declare that the work presented in my thesis is entirely original and was carried out by me in the Department of Biochemistry, University of Hyderabad, under the supervision of **Prof. N. Siva Kumar** and co supervision of **Prof.M.Ramanadham**. I further declare that this work has not been submitted earlier for the award of degree or diploma from any other University or Institution.

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ISMAIL KHAN

CONTENTS

Publications

ABBREVIATIONS

BSA	Bovine Serum Albumin
DAPI	4', 6-diamidino-2-phenylindole
DE-52	Diethylaminoethyl cellulose
DMEM	Dulbecco's Modified Eagle Medium
DTT	Dithiothreitol
DVS	Divinyl sulfone
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme Linked Immunosorbent Assay
ER	Endoplasmic reticulum
et al	et alii (Latin: and others)
Fe_3O_4	Magnetite
FESEM	Field emission Scanning Electron Microscope
GH	Glycoside hydrolase
h/hr	Hour
HEPES	N-(2-hydroxyethyl)piperaine-N-2-ethane sulfonic acid
IGF-II	Insulin like growth factor-II
Kda	KiloDalton
LSD	Lysosomal storage disorders
M	Molar
Min	Minute
µm	Micrometer
mM	Milli molar
MPR	Mannose 6-phosphate receptor
MW	Molecular weight

ng	Nanogram
nm	Nanometer
NPT	Nanoparticles
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
pH	-log (H^+) concentration
PMC	Phosphomannan core
PMP	Penta mannosyl phosphate
R_f	Relative front
Rpm	Rotations per minute
SDS	Sodium dodecyl sulphate
SEM	Scanning Electron Microscope
TBS	Tris-buffered saline
TEM	Tranmission Electron Microscope
Tris	Tris(Hydroxymethyl)aminoethane
XRD	X-ray diffraction

Eukaryotic cells are highly complex in nature and contain a variety of specific organelles, large number of soluble and membrane proteins/glycoproteins that exhibit distinct properties and are required for normal cellular growth and function. One such organelle is the lysosome that contains a large number of acid hydrolases and constitutes an important organelle in cells. Their main function in the cell is the degradation of internalized material by lysosomal enzymes, and thus establishes communication between intracellular and extracellular medium by means of phagocytosis, endocytosis, and exocytosis (Castino *et al*., 2003). These enzymes are synthesized in the endoplasmic reticulum and during their maturation in the Golgi apparatus they acquire the mannose 6-phosphate (M6P) recognition marker (Reitman and Kornfeld, 1981, Waheed and Hasilik 1981, Varki and Kornfeld 1980, Waheed *et al.,* 1982). Most of these soluble hydrolases are transported to lysosomes through specific M6P receptors (Kaplan *et al.,* 1977). The receptor-enzyme complexes segregated in the *trans*-Golgi network are transferred to the lysosomal compartments *via* the secretory pathway through clathrin-coated vesicles.

1.1. Mannose 6-phosphate receptors

Mannose 6-phosphate receptors (MPRs) are proteins that bind newly synthesized lysosomal hydrolases in the *trans*-Golgi Network (TGN) and deliver them to pre-lysosomal compartments. There are two different MPRs, one with a molecular mass of 300 kDa (single polypeptide chain) and the second is a dimeric protein with molecular mass of 46 kDa (Hoflack and Kornfeld, 1985). The larger receptor is also known as the cation-independent mannose 6-phosphate receptor (CI-MPR) or mannose 6-phosphate/insulin-like growth factor- II receptor as it also binds human IGF-II (Kornfeld, 1992). The smaller

receptor is also known as CD-MPR as it requires divalent cations to efficiently recognize lysosomal hydrolases (Hoflack and Kornfeld, 1985).

In mammalian cells, the targeting of newly synthesized hydrolases, as well as others ligands with M6P residues on their N-linked oligosaccharides, to the lysosomes depends on their recognition by these two specific M6P receptors in the *trans*-Golgi network: These are the only members of the P-type lectin family and are type I transmembrane glycoproteins (Dahms and Hancock, 2002). They are considered as sorting receptors because of their targeting function. In the pre-lysosomal compartment, acidity induces release of enzymes from both receptors which are then recycled either to the TGN or to the plasma membrane while the enzymes are ferried to lysosomes.

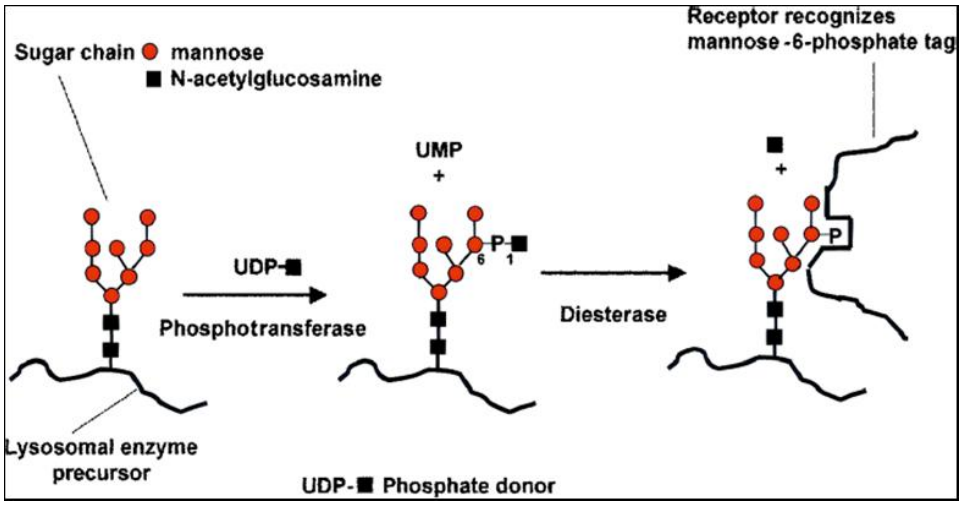


Figure 1.1. Formation of lysosomal recognition marker, mannose 6-phosphate.

It is well established that the CI-MPR, which participates in this cellular routing, is also anchored to the plasma membrane and can internalize extracellular ligands (Kornfeld, 1992, Munier *et al.,* 1996, Dahms, 1996). Taking advantage of this possibility, quite successful enzyme replacement therapies (ERT) were

designed for several lysosomal storage disorders. Before administration in patients, the exogenous missing enzyme must be modified with sufficient M6P to bind to the receptor with the appropriate affinity (Coutinho et al., 2012). While the major function of CI MPR on cell surface is to bind M6P containing ligands it also binds other ligands (*i.e.*, latent TGFβ precursor, urokinase-type plasminogen activator receptor, Granzyme B, growth factors, Herpes virus). Interestingly the CIMPR is known to selectively also bind human IGF-II (Kornfeld 1992) and hence is a multifunctional protein. This allows the degradation of IGF-II in lysosomes.

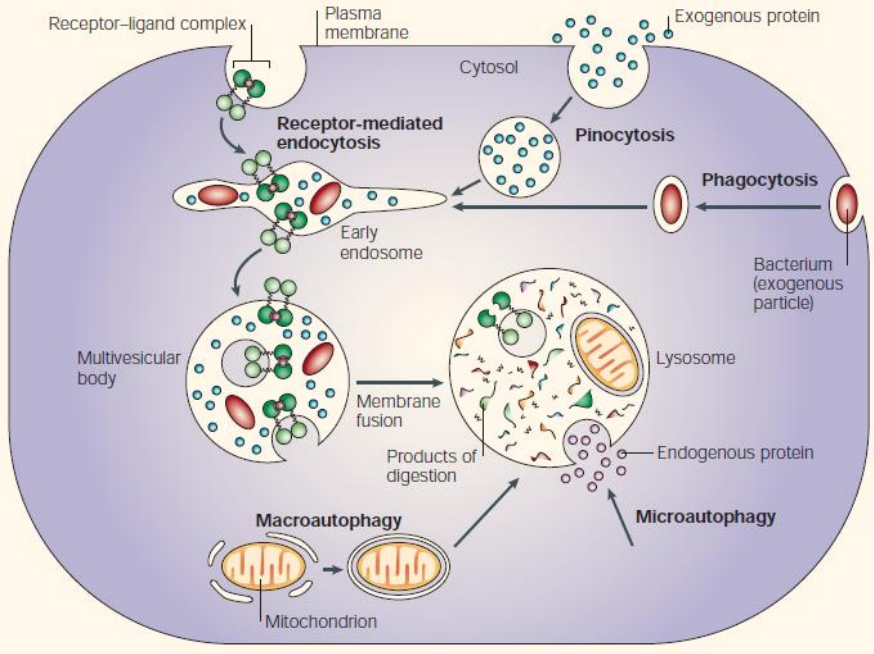


Figure.1.2. The four digestive processes mediated by the lysosome: 1) specific receptor-mediated endocytosis; 2) pinocytosis (nonspecific engulfment of cytosolic droplets containing extracellular fluid); 3) phagocytosis (of extracellular particles), and 4) autophagy (micro- and macroautophagy of intracellular proteins and organelles).

CI-MPR has been reported to be a potential tumour suppressor in 70% of hepatocarcinomas (De Souza *et al*., 1995) and 15-30% of breast cancers (Hankins *et al*., 1996, Chappell *et al.,* 1997, Oates *et al.,* 1998). Even though the CI-M6PR expression is decreased in some malignancies such as hepatocarcinoma, its over-expression in the majority of solid tumours, particularly in breast cancers (Berthe *et al.,* 2003) indicate that this receptor could be considered as a means to address cytotoxic drugs to lysosomes. The routing of cytotoxic drugs *via* CI-MPR may induce the lysis of lysosomes and then cell death. The higher specificity of CI-MPR targeting in cancer cells versus normal cells could be due to the higher expression of CI-MPR and its higher affinity at slightly acidic pH (pH 6-6.5) as found in the extracellular environment of solid tumors (Kleeberger and Rottinger, 1993, Gerweck *et al.,* 1999).

1.2. Nanotechnology in relevance to drug delivery

In recent years, most of the technological revolutions have focused on the ability to create products with smaller dimensions, but with higher precision. This concept is particularly true at the level of nanotechnology (deMello and Woolley, 2010). Thus, nanotechnology refers to the creation and use of devices, materials and systems through control of matter at the nanoscale as well as to create new materials with different functional characteristics of the common materials. Nanotechnology is a multidisciplinary field, combining different knowledge areas such as chemistry, physics, biology and engineering (Fatehi *et al.,* 2011, Roco, 2004). Broadly, nanotechnology is an interdisciplinary area with great potential to create new discoveries in main areas such as nanoelectronics, materials, information technology, medicine and healthcare as well as

biotechnology and also to bring together different fields of science (Amin *et al*., 2011, Bhushan, 2004, deMello and Woolley, 2010).

1.3. Nanoparticles: *What are nanoparticles?*

Nanoparticles are particulate dispersions or solid particles with a size in the range of 10-1000nm. Generally, nanometeric carriers are with various morphologies, including nanospheres, nanocapsules, nanomicelles, nanoliposomes, and nanodrugs, etc. Depending upon the method of preparation, nanoparticles, nanospheres or nanocapsules can be obtained (Jung, *et al.,* 2010, Reis, 2006).

1.4. The advantages of using nanoparticles as a drug delivery system

The ability of nanoparticles to manipulate the molecules and their structures has revolutionized the conventional drug delivery system. Nanoparticles have attracted a lot of attention of the pharmaceutical scientist in the drug delivery system due to versatility in targeting tissues, accessing deep molecular targets and controlling drug release (Nagpal *et al.,* 2010). Nanoparticle drug delivery systems are nanometeric carriers used to deliver drugs or biomolecules. Nanoparticle drug delivery systems have outstanding advantages (Jung *et al.,* 2010), they can pass through the smallest capillary vessels because of their ultra-tiny volume and avoid rapid clearance by phagocytes so that their duration in blood stream is greatly prolonged, they can penetrate cells and tissue gap to arrive at target organs such as liver, spleen, lung, spinal cord and lymph, they could show controlled release properties due to the biodegradability, pH, ion and/or temperature sensibility of materials, they can improve the utility of drugs and reduce toxic side effects; etc.

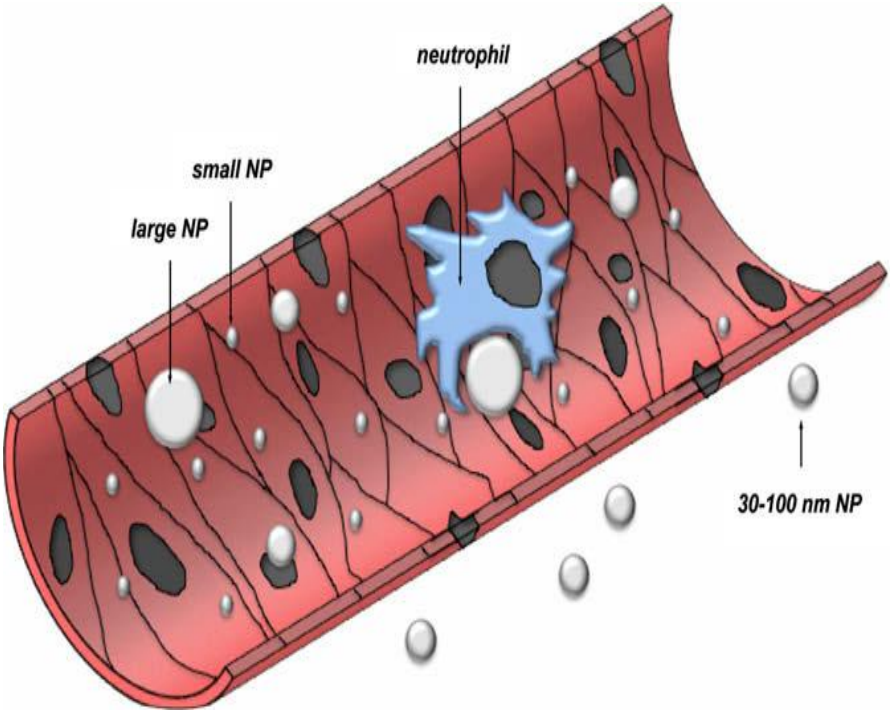


Figure.1.3. Fate of nanoparticles of different dimensions in blood stream; Very small nanoparticles, on the order of 1–20 nm, have long circulatory residence times with slow extravasation from the vasculature. Nanoparticles that are between 30 and 100 nm in diameter are small enough to avoid reticuloendothelial and phagocytic clearance, in contrast to larger nanoparticles, which are efficiently cleared (Faraji and Wipf, 2009).

As drug delivery system, nanoparticles can entrap drugs or biomolecules into their interior structures and/or absorb drugs or biomolecules onto their exterior surfaces. Presently, nanoparticles have been widely used to deliver drugs, polypeptides, proteins, vaccines, nucleic acids, genes and so on. Over the years, nanoparticle drug delivery systems have shown huge potential in biological, medical and pharmaceutical applications (Illum, 2007). Particle size and surface characteristics of nanoparticles can be easily manipulated to achieve both passive and active drug targeting after parenteral administration. Drug loading is relatively high and drugs can be incorporated into the systems

without any chemical reaction. They control and sustain release of the drug during the transportation and at the site of localization, altering organ distribution of the drug and subsequent clearance of the drug so as to achieve increase in drug therapeutic efficacy and reduction in side effects. The system can be used for various routes of administration including oral, nasal, parenteral, intra-ocular etc. Avoidance of coalescence leads to enhanced physical stability. Reduced mobility of incorporated drug molecules leads to reduction of drug leakage. Static interface solid/liquid facilitates surface modification. Site-specific targeting can be achieved by attaching targeting ligands to surface of particles or use of magnetic guidance. Particles with a very small size (<1000nm), low charge, and a hydrophilic surface are not recognised by the mononuclear phagocytic system (MPS) and therefore, have a long half life in the blood circulation which is essential for targeting NPs to target brain (Bagul et al ., 2012).

1.5. Ideal Properties of nanoparticle

Most nanoparticles that are being used need to be in one or the other following categories. They should be natural or synthetic polymers, inexpensive, nontoxic, biodegradable, Nonthrombogenic, Nonimmunogenic, non inflammatory. Further they should sustain for longer periods of time in circulation and also cause no platelet aggregation.

1.6. Characterisation of Nanoparticles

Characterization is done by using a variety of different techniques as indicated below.

Electron microscopy [TEM, SEM], Atomic force microscopy [AFM], Dynamic light scattering [DLM], X-ray photoelectron spectroscopy [XPS], Powder x-ray diffractometry [XRD] etc.

There are various types of nanoparticles like inorganic nanoparticles,liposomes, nanotubes, dendrimers, solid lipid nanoparticles, and polymeric nanoparticles etc., (Fig1.4).

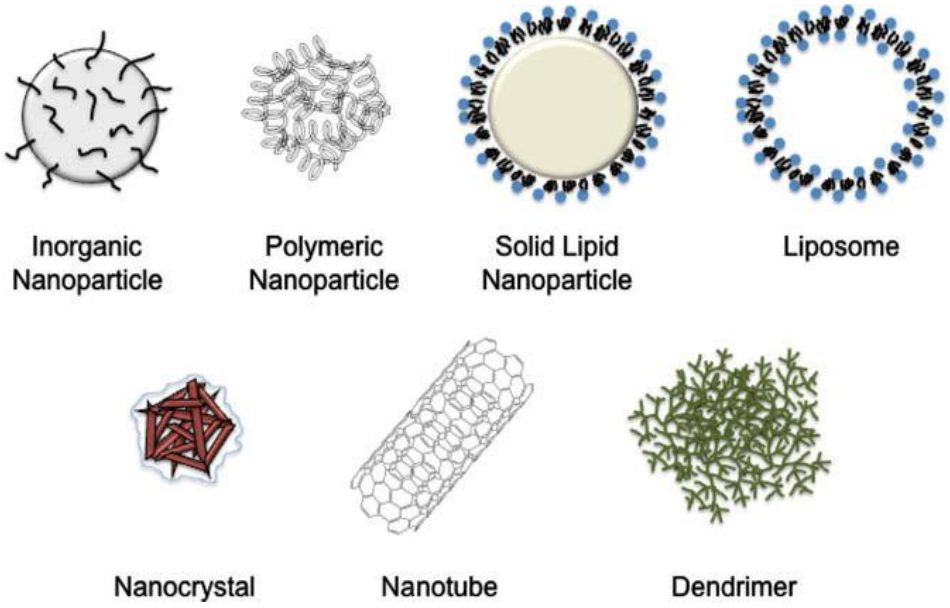


Figure.1.4. Various types of nanoparticles applied in drug delivery
(Faraji and Wipf, 2009)

1.7. Polymer nanoparticles

Most polymeric nanoparticles are biodegradable and biocompatible, include various natural or synthetic polymers and have been adopted as a preferred method for nanomaterial drug delivery. They also exhibit a good potential for surface modification via chemical transformations, provide excellent pharmacokinetic control, and are suitable for the entrapment and delivery of a wide range of therapeutic agents. Polymer nanoparticles can be divided into nanospheres, which build a continuous polymer matrix and can be referred as "drug sponges" and nanocapsules, which consist of a polymer layer enclosing a

fluid-filled cavity and are mimicking liposomes. Polymeric materials used for preparing nanoparticles for drug delivery are best suited if they are biocompatible and biodegradable. Therefore, a wide range of materials, such as natural or synthetic polymers, lipids, surfactants, dendrimers have been employed as drug delivery carriers and among these, natural polysaccharides, due to their outstanding merits, have received more and more attention in the field of drug delivery systems. Polysaccharides (particularly chitosan) seem to be the most promising materials in the preparation of nanometeric carriers (Liu *et al.,* 2008) and many other polymeric materials have been applied, including poly(lactic acid), poly(glycolic acid), polycaprolactone, poly(acrylic acid) family, proteins or polypeptides (such as gelatine) etc. In the present study phosphorylated mannooligosaccharide (polymeric compounds of mannose 6-phosphates) were used.

1.8. Nanoparticles as tools for drug delivery

Nanoparticles are widely used in drug delivery where they can increase drug solubility and, additionally, can lead to controlled release and/or drug targeting. They are used in anti-cancer treatment, gene delivery, asthma inhalers, hormone delivery through the skin, drug delivery through the eye and in oral and vaccine delivery systems. The accumulation of drugs in tumor tissue does not always guarantee successful therapy if the drug does not reach the target site of the tumor cell such as the cell membrane, cytosol, or nucleus. Therefore, a more effective mechanism should be employed such that the therapeutic agents are able to reach their molecular targets. Cancer cells often over-express some specific antigens or receptors on their surfaces, which can be utilized as targets in modern nanomedicine. Active targeting can be achieved by chemical

modification of nanosized drug carriers with targeting components that precisely recognize and specifically interact with receptors on the targeted tissue (Byrne *et al.,* 2008, Allen, 2002, Kim, 2007). In the initial experiments, researchers attempted direct conjugation of the targeting moiety to drugs. However, most clinical studies conducted for targeted drug conjugates failed to demonstrate their improved therapeutic effects on cancer treatment. This was due to a decrease in the biological activity of the drugs, compromised by conjugation of the targeting moiety. In addition, conjugation negatively affected the targeting molecule by disrupting receptor/ligand recognition (Tolcher, 1999). To circumvent this problem, researchers developed an efficient drug delivery system comprised of active chemotherapeutic drug, targeting moiety, and a nano-sized carrier made up of polymers or lipids. In this system, the therapeutic agents are physically entrapped in the carrier. This ternary system is very attractive over the ligand–drug conjugates for the following reasons: (i) the physically entrapped drugs can preserve their activity, (ii) a relatively large payload of drugs can be loaded into the hydrophobic cores of the carriers exceeding their intrinsic water solubility, (iii) the targeting moieties on the surface of the carriers can be precisely tuned to increase the probability of binding to the target cells, and (iv) owing to the small size of the carrier system, it can effectively infiltrate across the inflamed leaky disease vasculature but not at the normal vasculature (Allen, 2002). For successful active targeting, the specific receptors should be expressed exclusively on the cancer cells but not on the normal cells. Cancerous state is a highly stimulated environment of metabolically active cells. The cells under these conditions over express selective receptors for assimilation of factors essential for growth and

transformation. Such receptors would serve as potential targets for the specific ligand mediated transport of pharmaceutically active molecules. Several targeting moieties or ligands have been identified and successfully utilized for chitosan-based drug delivery systems. Folic acid, a low molecular weight vitamin, has a high affinity for folate receptors (FRs), which are frequently over-expressed in many types of human cancerous cells, particularly those found in the epithelial tumors of various organs such as colon, lung, prostate and ovaries. Therefore, folate-conjugated drugs or carriers can be rapidly internalized into cancer cells via receptor-mediated endocytosis. You et al. synthesized folate-conjugated stearic acid-grafted chitosan oligosaccharides (CSOSA) by reacting them with folic acid in the presence of carbodiimide coupling agents (You, *et al*., 2008). The cellular uptake of Fa-CSOSA nanoparticles containing anticancer drug, PTX via receptor-mediated endocytosis was tested. The authors demonstrated that HeLa cells expressing a large amount of FRs on the cell membrane rapidly could uptake the Fa-CSOSA nanoparticles, in comparison to a FR-deficient A549 cell lines. Another such ligand is transferrin (Tf), an 80-kDa glycoprotein, is found abundantly in the blood. The main function of Tf is to transport iron to cells with the transferrin receptors (TfRs). Transferrin is a well-studied ligand for the tumor targeting (Qian *et al.,* 2002, Singh, 1999) and gene delivery (Mishra *et al.,* 2009). Since TfRs are over-expressed in malignant tissues, Tf can be used as a ligand for tumor targeting. It has been confirmed that Tf-mediated drug delivery systems can overcome drug resistance because they can be internalized by avoiding the membrane-associated drug resistance proteins such as p-glycoprotein (Li and Qian, 2002). Targeted delivery of an anticancer drug doxorubicin, loaded with apotransferrin nanoparticles was

tested for its anticancer activity with minimum side effects through receptor mediated endocytosis (Krishna et al., 2009).

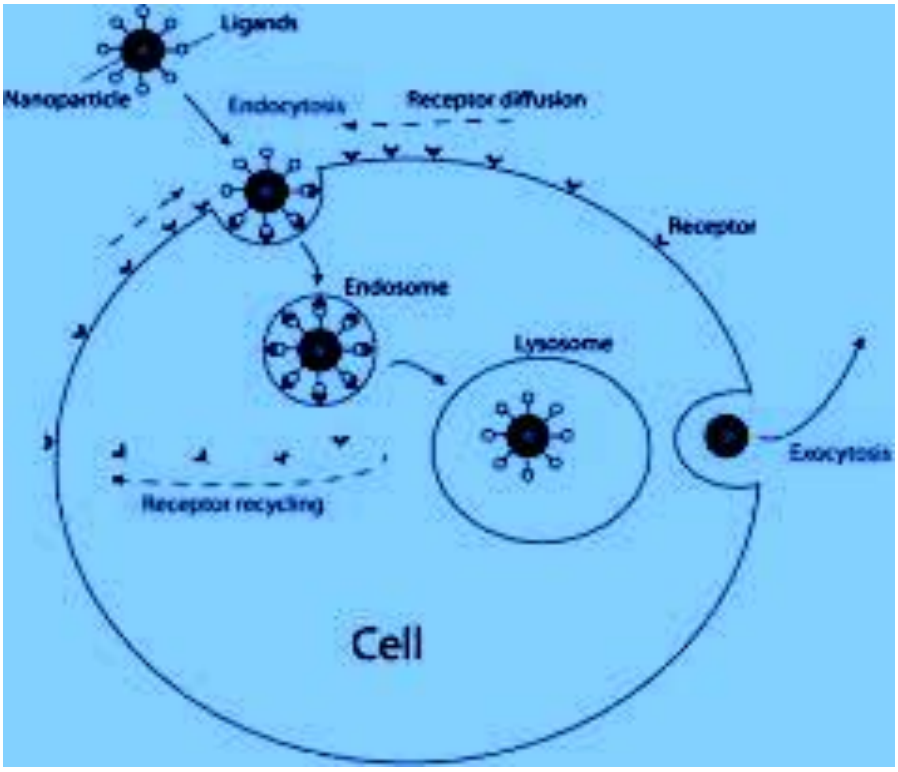


Figure.1.5. A schematic representation of the nanoparticle uptake through receptor mediated endocytosis is shown (Tran and Webster, 2013**)**

1.9. Magnetic (MNP) nanoparticles

In the last couple of years, interest in the use of nanomaterials, as special nanoparticles has increased markedly because these materials combine the fields of material science and biology and offer a wide technological application such as biocatalysis and bioseparation (De *et al.,* 2008). The growing interest in nanoparticles comes from the fact that these materials possess unique properties dependent on their size and can also be modified with different chemicals, materials and biomolecules (Liu, 2006, De *et al.,* 2008). A considerable emphasis has been given to Magnetic Nanoparticles (MNPs) due to

their properties, especially the superparamagnetic phenomena which have found remarkable applications in different areas (Safarik and Safarikova, 2009). Iron oxide nanoparticles are an important class of inorganic nanomaterials. The most promising materials of this class are the magnetite (Fe_3O_4) and maghemite (γ-Fe_2O_3) which are also biocompatible (Gupta and Gupta, 2005, Wu *et al.,* 2008; Boyer *et al.,* 2010). MNPs attract attention due to their distinguished features such as superparamagnetism, non corrosive nature and high magnetic susceptibility. These superparamagnetic properties correspond to an intermediate state between paramagnetic and ferromagnetic, which means that these materials are magnetized when exposed to a magnetic field but they lose the magnetic properties once the magnetic field is removed. Apart from their magnetic proprieties, MNPs are also biocompatible, easy to synthesize and subsequently can be coated and functionalized. They also present unique versatility provided by the high surface area to volume ratio, which provides a high loading. Being one of the most abundant metals in nature, iron has high magnetic, conductive properties. Magnetite (Fe_3O_4) and Maghemite (γ-Fe_2O_3), among sixteen pure phases, are the most extensively iron oxides used in many advanced applications at critical situations (Mohapatra and Anand, 2010). Bulk synthesis of iron oxide especially at nanoscale is still a challenge due to its instability. Although both physical and chemical methods can be employed for their synthesis chemical methods are the most preferred and widely used for preparing iron oxide nanoparticles. It is well-known that magnetite (Fe_3O_4) is not very stable and it is sensitive to oxidation and aggregation (Mohapatra and Anand, 2010, Islam et al., 2012). Magnetic iron oxide NPs have a large surface-to volume ratio and therefore possess high surface energies. Consequently, they

tend to aggregate so as to minimize the surface energies. Moreover, the naked iron oxide NPs have high chemical activity, and are easily oxidized in air (especially magnetite), generally resulting in loss of magnetism and dispersibility. Therefore, providing proper surface coating and developing some effective protection strategies to keep the stability of magnetic iron oxide NPs is very important. These strategies comprise grafting of or coating with organic molecules, including small organic molecules or surfactants, polymers, and biomolecules (Wu *et al.,* 2008). The coating of MNPs consists in the protection of the core structure with a layer through encapsulation or hydrophobic interactions, in order to isolate the core against harsh conditions, which improve biocompatibility of the particles and increase functionalization for further modifications (Lu *et al.,* 2007; Fang and Zhang, 2009; Dias *et al.,* 2011). The coatings most commonly used include: organic coatings such as surfactants and polymers or inorganic coatings as silica, carbon and precious metals (Lu *et al.,* 2007). For the surface coating of MNPs different strategies can be applied, since in-situ coatings to post-synthesis coatings. In the last years the coating of MNPs with polymers, particularly biopolymers such as polysaccharides, attracted attention of researchers. Surface functionalized magnetic iron oxide nanoparticles (NPs) are a kind of novel functional materials, which have been widely used in the biotechnology and catalysis. In order to implement the practical application, the particles must have combined properties of high magnetic saturation, stability, biocompatibility, and interactive functions at the surface. Moreover, the surface of iron oxide NPs could be modified by organic materials or inorganic materials, such as polymers, biomolecules, silica, metals, etc. Surface functionalization makes these particles more stable and applicable

(Wu *et al.,* 2008). Magnetic NPs are of great interest for researchers from a broad range of disciplines, including magnetic fluids, data storage, catalysis, and bioapplications (Patel *et al.,* 2008, Zhao, *et al.,* 2003, Mornet *et al.,* 2006, Stevens *et al.,* 2005, Jun *et al.,* 2007). The superparamagnetic properties of MNPs find interesting applications in the biomedical and biotechnological areas (Safarik and Safarikova, 2009; Dias *et al.,* 2011). In the biomedical area, MNPs have tremendous potential in cancer treatment by hyperthermia, magnetic resonance imaging (MRI), cellular therapy and labeling, tissue engineering, gene and drug delivery (Dias *et al.,* 2011). In terms of biotechnological applications, MNPs are applied in bioseparation processes, biosensing, biocatalysis, bioremediation and magnetofection (Dias *et al.,* 2011). The major benefits of using these particles in the biotechnological and bioengineering areas are the low-cost, speed, scalability and compatibility with complex biological suspensions (Batalha *et al.,* 2010). Currently the main research focus are the synthesis of water-soluble type functionalized iron oxide NPs with water solubility (good disperse in aqueous environment, cannot produce agglomeration) and biological compatibility. Water dispersable functionalized iron oxide NPs can be widely utilized in bioseparation and biodetection. Several reactions were employed for obtaining the water soluble functionalized iron oxide NPs. One method is to directly add the biocompatible small organic molecules such as amino acid (Sousa et al., 2001), citric acid (Sahoo et al., 2005, Morais et al., 2003), vitamin (Mornet *et al.,* 2004, Xie *et al.,* 2005), cyclodextrin (Banerjee and Chen, 2007, Hou *et al.,* 2005, Bonacchi *et al.,* 2004) etc. during the synthesis. Even though most studies have focused on the development of small organic molecules and surfactants coated iron oxide nanoparticles, recently

polymers functionalized iron oxide NPs are receiving more and more attention, due to the advantages of polymers coating will increase repulsive forces to balance the magnetic and the van der Waals attractive forces acting on the NPs. In addition, synthetic and natural polymers coating on the surface of iron oxide NPs offer a high potential in the application of several fields. Moreover, polymer functionalized iron oxide NPs have been extensively investigated due to interest in their unique physical or chemical properties. Currently, there are two major developing directions to form polymers functionalized iron oxide NPs; one is for the purpose of expanding application range by introducing the functional polymers. Another is for the purpose of manufacturing a monodisperse NPs with well-defined shape and a controlled composition (Gupta and Wells, 2004).

Water solubility and surface functionalization of nanomaterials is crucial for bioapplication (Chan *et al.,* 1998, Bruchez *et al.,* 1998, Robinson *et al.,* 2005, Grancharov *et al.,* 2005). The development a facile method for the preparation of water soluble and functionalized (coupled with amino or carboxylic groups) magnetite nanomaterials, especially for the template-free synthesis of amine-functionalized hollow magnetite spheres, is still an essential but yet challenging step as they have much potential in biological and medical fields, such as the immobilization of proteins, peptides, and enzymes (Xu *et al.,* 2004, Dyal *et al.,* 2003, Mirzabekov *et al.,* 2000) bioseparation (Gu *et al.,* 2003), immunoassays (Wang *et al.,* 2004).

1.10. Magnetic Nanoparticles for protein separation

Affinity chromatography is the most widely used technique for protein purification, but presents some limitations when using crude feeds related to the column operation and clogging of the packed bed adsorbents. This requires

a clarification step before introduction of the sample in the column, which translates into increased costs and process complexity. One option that has been used to overcome these problems is the bed expanded system. However, this system also suffers limitations of low capacities due to the diffusion limitations and the short contact times for capture protein between the adsorbent beads and the proteins (Bucak *et al.,* 2003; Roque *et al.,* 2004; Ditsch *et al.,* 2006; Roque *et al.,* 2007). MNPs appear as a promising support for magnetic-based separations, since they present minimum diffusion limitations and permit rapid and easy removal of the functionalized MNPs from complex heterogeneous reaction mixtures, without being necessary previously filtration or centrifugation. In addition these supports are of easy manipulation which makes them a good choice for a downstream process as shown in Figure 1.6. Bucak et al., 2003; Roque et al., 2004; Ditsch et al., 2006; Horák et al., 2007; Roque et al., 2007).

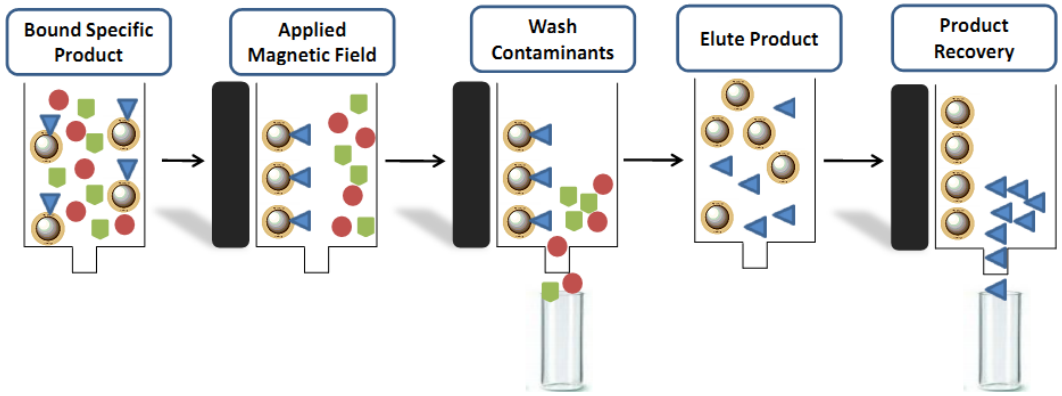


Figure.1.6. Schematic representation of a bio separation process using MNPs as adsorbent.

1.11. Scope of present investigation

Two receptors designated as the Mannose 6-phosphate receptors recognize the mannose 6-phosphate residues on all lysosomal enzymes and mediate their transport to lysosomes. Both receptors are transmembrane glycoproteins that are present in the *trans*-Golgi Network as well as on the plasma membrane and exhibit a molecular mass of 300 kDa (MPR300) and 46 kDa (MPR46) respectively. Interestingly the MPR300 protein on the cell surface can internalize ligands and hence is designated as the endocytosis receptor. Lysosomes in eukaryotic cells are very important for the digestive functions of the cells. These functions are catalyzed by a large number of acidic hydrolases which degrade biological molecules that are not required by the cell. The degraded material generated such as the free amino acids from proteins, sugars from glycans etc., can be reused by the cell. Absence of the lysosomal enzymes or the receptors that mediate their transport leads to severe lysosomal diseases that affect mankind. The present therapy available is the enzyme replacement therapy for some lysosomal disorders such as the Gauchers disease (glucocerebroside deficiency), Fabry's disease (α-galactosidase deficiency). Enzyme replacement therapy is a lifelong therapy. All products are administered intravenously either through a peripheral line or central access device, such as a Port-a-Cath. Infusions typically occur once every 2 weeks, except Aldurazyme and Elaprase which are administered weekly. Initially infusions should be performed in an outpatient clinic setting; although for some of the more medically fragile patients, an inpatient setting or short stay infusion unit may be more appropriate. Infusions are usually administered over 2-6

hours depending on the amount of drug that needs to be infused and the patient's previous adverse event history.

In view of this, alternate therapies would be useful for treatments of lysosomal diseases. Recent studies on the use of nanoparticles for drug delivery systems is gaining importance and several natural products are being used as effective nanoparticles for specific drug deliveries. Chitosan is one among them. The laboratory where this work was carried out has been interested for many years in understanding the structure-function relationships of lysosomal enzymes and their sorting receptors in the animal kingdom. By biochemical, immunological, molecular cloning, sequencing of cDNA's and Mass spectrometry analysis the laboratory has established that the lysosomal enzyme sorting receptors (MPR300 and 46) are evolutionarily conserved from fish to mammals (Siva Kumar and Amancha, 2010). More recent studies have also suggested that the lysosomal enzymes such as the α-fucosidase are also exhibit conserved amino acid sequence from mollusc to mammals. The critical feature of the receptors is their ability to interact with the mannose 6-phosphate containing ligands and in particular the MPR300 on the cell surface is capable of binding and internalizing the mannose 6-phosphate containing ligands into the cells. Phosphomannan core obtained from the acid hydrolysis of yeast *O*-Phosphonomannan is a polysaccharide consisting of phosphorylated mannooligosaccharides and therefore used as a potential ligand to affinity purify the Mannose 6-phosphate receptors from different animal tissues as well as from different cell lines (Yadavalli and Siva Kumar, 2008). Preparation of nanoparticles for the phosphorylated mannooligosaccharides and testing their ability to bind to cell surface receptor and its possible internalization would

allow us not only to establish the effectiveness of naturally occurring phosphorylatled mannoligosaccharides for nanoparticle preparations but also ascertain the specific role of the receptor in internalizing the nanoparticles. This basic phenomenon has been characterized in the present study in detail which can form the basis for using the nanoparticles prepared and the receptor on the cell surface for internalization of specific drugs coated to these nanoparticles. Other forms of nanoparticles such as the magnetite nanoparticles are also being used in modern biology. Magnetite nanoparticles were also prepared in the study and their potential applications were investigated. Phosphorylated mannooligosaccharide antibodies can be useful tools for detection, quantification of mannose 6-phosphate containing ligands. Therefore these have been also developed and their applications studied. Additionally, plant polysaccharide from *Moringa oliefera* seeds (mannan) was isolated in a preliminary study. The potentiality of this for use as a nanoparticle materials needs further experimentation.

2.1. Introduction

Newly synthesized lysosomal enzymes in eukaryotes acquire phosphate groups on mannose residues in the *trans*-Golgi Network (TGN) which is a prerequisite for their specific recognition and interaction with the mannose 6-phosphate receptors designated as MPR300 (Mr 300 kDa, the cation independent receptor which is also the receptor for IGF-II) and the MPR46 (Mr 46 kDa). In the TGN their function is to transport lysosomal enzymes to lysosomes. Both receptors are also localized also on the plasma membrane. However, only the MPR300 protein has been shown to exhibit specific interaction with the ligands containing mannose 6-phosphate as well as with IGF-II and selectively internalizes them for targeting to lysosomes and is thus an endocytosis receptor (Pohlmann *et al*., 1995). The vertebrate MPR300 receptor is also called as the Mannose 6-phosphate/IGF-II receptor (Yadavalli *et al.,* 2009). Additionally this receptor in mammals is also known to interact with other ligands such as the latent TGF precursor, urokinase-type plasminogen activator receptor, Granzyme *B*, Glycoprotein *D* of Herpes simplex virus and proliferin and is thus classed as a multifunctional protein (Nadimpalli and Amancha, 2010). Interest in the field of lysosomal enzymes and their receptors is largely due to a number of lysosomal disorders that have been identified in humans. However, there are only a few remedies for these disorders known such as the enzyme replacement therapy, gene therapy (Bailey., 2008). Recent studies have utilized nanoparticles as efficient tools for drug delivery into cells (Soppimath.K.S *et al.,* 2001). Nanoparticle delivery systems are essentially nano carriers (10–1000 nm size) having different morphologies such as the nanocapsules, nanomicelles, nanospheres and dendrimers that can be used for specific delivery of drugs,

biomolecules into cells (Soppimath *et al.,* 2001). Nanoparticle delivery systems have advantages, because of their very small size the clea rance of nanocarriers by phagocytes will be avoided so that their duration in blood stream is greatly prolonged, they can penetrate cells and tissue gaps to arrive at target organs (Svenson and Prud'homme 2012). Nanoparticles can be prepared from a variety of materials such as proteins, polysaccharides and synthetic polymers. Nanoparticles that are biodegradable offer better advantages as they would be non-toxic to the cells (Soppimath *et al*., 2001). Nanoparticles could be functionalized and can act as potential carriers for several biomolecules and drugs. Receptor-mediated targeting is a promising approach to selective drug delivery. Medical therapies have become more tailored to specific diseases and patients in recent years. Selective delivery of pharmaceutical agents into cells has been recently reviewed (Faraji *et al.*, 2009). Some naturally occurring polysaccharides such as those derived from chitin are available in abundance and have been shown to be biodegradable, nontoxic, and hence safe for use. More recently we successfully isolated and prepared nanoparticles for a galactomannan and galactan from the seeds of *Strychnos potatorum* (Saif *et al*., 2014). *Hansenula holstii* NRRL Y-2448 yeast produces a highly branched extracellular *O*-phosphonomannan that is composed of a core moiety to which oligosaccharide diester phosphate side chains are attached. Phosphomannan core is known to have amino groups (Probst *et al*., 2009). Mild acid hydrolysis of *O*-Phosphonomannan and ethanol precipitation separates the high molecular weight phosphomannan core (PMC) moiety and the low molecular weight pentamannosylphosphate fraction (PMP). Both PMC and PMP have become valuable tools in affinity purification and characterization of mannose 6-

phophate receptors from different vertebrates and invertebrates (Nadimpalli and Amancha, 2010). Mammalian cells express two lysosomal enzyme targeting receptors (MPR300 and 46) that have been extensively characterized (Pohlmann *et al.,* 1995).

Our initial research work in the field of lysosomal enzymes and receptors, focused on developing alternate affinity methods to purify the mannose 6-phosphate receptors from goat liver using phosphomannan core and pentamanosylphosphate as efficient ligands and raising antibodies to the purified goat receptors. Using goat as a mammalian model, we looked for homologous receptors in other vertebrate species and recent studies clearly established the evolutionary conservation of the receptors throughout the vertebrates (Nadimpalli and Amancha, 2010). Current studies are focused on the invertebrate receptors to further understand their evolutionary significance. Experiments with various cell lines earlier clearly demonstrated the efficient binding of several ligands (mannose 6-phosphate containing as well as human IGF-II) to MPR300 protein on the cell surface which then endocytosis these ligands into cells for targeting to lysosomes (Hille-Rehfeld. A, 1995). This suggests the possible utility of this specific MPR300 as a tool for drug/biomolecule delivery into cells. Since we affinity purified and extensively characterized different vertebrate MPR proteins, the present work was initiated with a long term objective to utilize the naturally occurring phosphorylated mannooligosaccharide nanoparticles as potential tools for binding to the receptor and their internalization into cells. The objectives of the present study are,

(i) To prepare nanoparticles of the natural ligands for the receptors such as the phosphomannan core, use specific lysosomal enzymes that contain mannose 6-phosphate such as the *β*-hexosaminidase, Cathepsin D and β-galactosidase,

(ii) Characterize the nanoparticles by atomic force microscopy, electron microscopy and

(iii) Analyze the ability of the nanoparticles prepared for binding, interaction and internalization by the MPR300 on the cell surface of NIH3T3 and CHO cells.

2.2. MATERIALS AND METHODS

2.2.1. Materials

O-Phosphonomannan is a generous gift of Dr.M.Slodki, USDA, Peoria, USA. DE-52 Cellulose is from Whatmann, Mannose 6-phosphate, P-nitro phenyl β-D-glucosaminide, DMEM, FITC, Pepstatin A and β-galactosidase are from Sigma. BCIP/NBT substrate is from Genei. Gold antifade reagent with DAPI is from Invitrogen. TEM Grids are from ICON analytical. Affigel-10 is from Bio-rad. Lamp1 antibody is from Abcam. CY5 conjugated secondary antibody is from Millipore. MPR-deficient mouse embryonic fibroblast cells ($mpr^{(-/-)}$ MEF cells, 23ISV) are kind gift of Prof.Dr.Regina Pohlmann, University of Muenster, Germany. NIH3T3 and Chinese Hamster Ovary (CHO) cells are available in the lab. All reagents and chemicals used in the study are of high purity and from Sisco Research Laboratories, Mumbai, India.

2.2.2. Hydrolysis of yeast O-phosphonomannan to phosphomannan core (PMC) and pentamannosylphosphate (PMP)

This was carried out according to Bretthauer et al., (1973). 2.5 g of *O*-phosphonomannan from the yeast *Hansenula holstii* was taken and suspended in 50 ml of water in a screw capped bottle and left overnight for swelling, 500

mg of KCl was added to the swollen phosphonomannan and the pH of suspension was adjusted to 2.4 with glacial acetic acid. The contents were then hydrolyzed in a boiling water bath at 100^{o}C for 60 min. The suspension was then cooled to room temperature and centrifuged at 10,000 rpm for 30 min. The solution was neutralized with saturated barium hydroxide solution. To this, an equal volume of 95% ethanol was added and left overnight at 4^{o}C. The precipitated phosphomannan core was collected by centrifugation at 10,000 rpm for 30 min, supernatant collected and saved. The precipitate was dissolved in water, made acidic with acetic acid, extensively dialyzed against water and lyophilized.

To the supernatant an equal volume of ethanol was added and was allowed to stand for one to two hours on ice. The suspension was centrifuged at 10,000 rpm for 15min. The pellet was redissolved in water with mild acidification. Penta Mannosyl Phosphate (PMP) was then desalted by passing through a Dowex 50 resin and the solution was then lyophilized to get the powder. The dried powder of PMC and PMP were stored at 4°C and used in all studies.

2.2.3. mpr$^{(-/-)}$ Mouse Embryonic Fibroblasts (231SV) cell culture and isolation of β-hexosaminidase from the secretions

The mpr $^{(-/-)}$ mouse embryonic fibroblast cells were cultured as described earlier (Yadavalli and Nadimpalli 2008). These cells lack both receptors and the newly synthesized lysosomal enzymes are secreted into the medium. Secreted medium from several culture flasks was collected and the total proteins were precipitated by ammonium sulphate (0-80% saturation), centrifuged at 12000 rpm for 20 min, the pellet obtained was dissolved in 25mM Tris-HCl buffer pH

7.4 (buffer A) and extensively dialyzed against the same buffer. The clear solution obtained after dialysis was used for the purification of the lysosomal enzyme β-hexosaminidase. The activity of β-hexosaminidase was assayed in the concentrated protein sample obtained above using *P*-nitrophenyl *N*-acetyl-β-D-glucosaminide as described (Amancha, et al 2009).

2.2.3.1. Ion exchange chromatography on DE-52

The protein containing the enzyme activity obtained above was passed through the DE-52 cellulose gel (30 ml) that was equilibrated with buffer A. The gel was washed extensively with buffer A to remove the unbound proteins. The bound sample was eluted with 50 mM NaCl in buffer A. Fractions were collected and aliquots were analyzed for lysosomal enzyme activities with the respective substrates. The enzyme containing fractions were pooled and concentrated by amicon concentrator to further obtain the β-hexosaminidase in a homogeneous form.

2.2.3.2. Affinity chromatography on Human MPR 300 affigel

The DE-52 eluted sample was dialyzed against MPR300 column buffer, 50 mM imidazole-HCl pH 7.0, 150 mM NaCl, 5 mM Sodium β-glycerophosphate, 5 mM EDTA, 0.05% Triton X-100 (buffer B). The dialyzed protein was applied on a human MPR300 receptor affigel (generous gift from Prof.Dr. K.von Figura, Goettingen University, Germany) that was pre-equilibrated with buffer B. The gel was washed extensively with buffer B to remove unbound proteins. The bound protein was eluted with 5 mM mannose 6-phosphate in buffer B. The column eluates from the ion exchange gel as well as from the MPR300 receptor-affigel were analyzed by SDS-PAGE as described below.

2.2.3.3. SDS-PAGE and western blotting

Protein concentrations in the extracted samples and column fractions were determined by the dye-binding method using bovine serum albumin (1.0 mg/ml) as the standard (Bradford, 1976). 10% SDS-PAGE analysis was carried out for column eluates according to Laemmli (1970). The column eluates from the ion exchange gel as well as from the MPR300 receptor-affigel were separated on a 10% SDS-PAGE under reducing conditions and the proteins were detected by silver staining (Oakley *et al.,* 1980). Glycoprotein nature of the purified protein (hexosaminidase) from MPR300 receptor gel was observed by periodic acid schiff's staining (Zacharius *et al.,* 1969).

2.2.4. Isolation and affinity purification of a lysosomal cathepsin D from Unio (Lamellidens corrianus)

Cathepsin D (EC 3.4.23.5) is one of the well characterized lysosomal enzymes among vertebrates, especially in mammals and has been shown to be involved in protein degradation *in vivo*. This lysosomal enzyme is an aspartyl proteinase and a glycoprotein that has been shown to also interact with MPR proteins. More recent studies in our laboratory confirmed that in invertebrates such as the echinoderm (starfish) and mollusc (Unio: *Lamellidens corrianus*) also contain cathepsin D that could be affinity purified on pepstatin-affigel and is capable of binding to MPR proteins (Merino and Siva Kumar 2012; Venugopal and Siva Kumar, 2013).

2.2.4.1. Preparation of Pepstatin A affinity gel

10 mg of Pepstatin A was dissolved in 1mL of absolute alcohol and boiled at 60^{o}C in a water bath for 10 min. The prepared Pepstatin A was then coupled to 2.0 mL of Affigel-10 (Biorad) following manufacturer's instructions. The gel was

washed with 10 mM sodium-phosphate buffer, pH 7.4, containing 150 mM NaCl (PBS) and stored at 4^{o}C until use.

2.2.4.2. Extraction and affinity purification of cathepsin D from Unio

All operations were carried out at 4˚C unless otherwise stated. Fifty grams of the whole animal tissue was homogenized with 4 volumes of 25 mM Tris-HCl buffer pH 7.4 (buffer A). The homogenate was allowed to stir overnight and centrifuged (26892 x g, 30 min). The pellet was discarded and the supernatant re-centrifuged for 15 min as described before. Ammonium sulphate was added to the supernatant to attain 80% saturation and the suspension stirred for 3 h at 4˚C. The precipitated protein was collected by centrifugation as described above, dissolved in a small volume of buffer A and dialyzed extensively against the same buffer. The dialyzed sample was briefly centrifuged and the clear supernatant was subjected to affinity chromatography on 30 ml Seralose-lactose column pre-equilibrated with buffer A (to remove the lactose lectin from the extracts). The unbound sample was collected and dialyzed against 50 mM sodium acetate buffer, pH 3.5, containing 0.2 M NaCl (buffer B). The dialysed sample was briefly centrifuged and the clear supernatant was applied to a 2 mL Pepstatin A affinity gel (1.5 x 9 cm) pre-equilibrated with buffer B. The unbound fraction was recycled several times through the column. The non-specific proteins were washed off with buffer B and elution was carried out with 50 mM Tris-HCl buffer pH 8.0 containing 0.2 M NaCl (buffer D) at a flow rate of 25 ml/h.

2.2.4.3. SDS-PAGE and western blotting

Protein concentrations in the extracted samples and column fractions were determined by the dye-binding method using bovine serum albumin (1.0

mg/mL) as the standard (Bradford, 1976). 10% SDS-PAGE analysis was carried out for column eluates under reducing conditions (Laemmli, 1970). The proteins were boiled for five minutes in the SDS sample buffer, separated on a gel and was detected by coomassie staining. Initially, the authenticity of the purified enzyme was analysed by western blot with starfish cathepsin D antiserum (1:500). The enzyme band was detected by incubating the membrane with the secondary antibody (goat anti-rabbit IgG conjugated to alkaline phosphatase) and developed with substrate BCIP/NBT**.**

2.2.5. Preparation of Phosphomannan core (PMC) nanoparticles

20 mg of PMC in 550 µl of sodium borate buffer pH 9.0 was tagged with FITC prior to nanoparticle preparation. 100 µl of fluorochrome solution of fluorescein-isothiocyanate (5 mg/ml in DMSO) was added to the PMC sample, mixed well and stored at 4°C overnight in dark. The free FITC was removed by passing the sample through Sephadex G-25 gel and FITC labelled protein was collected.

Nanoparticle preparation was carried out according to Krishna *et al* (2009) with a minor modification. 5 mg of phosphomannan core was dissolved in 500µl of 1xPBS, pH 7.4 and the mixture was incubated on ice for 5 min. To the mixture 15 ml of olive oil was added with continuous dispersion by gentle manual vortexing. The sample was sonicated at 4˚C with a probe for 15 times each with 30 sec, with a gap of 1 min between each successive step. The resulting mixture was immediately frozen in liquid nitrogen for 10 min. Then it was transferred to ice and incubated for 4hr. The particles were collected by

centrifugation at 8000 rpm for 10 min, the pellet was washed with diethyl ether, dispersed in PBS pH 7.4 and stored at 4 °C.

2.2.6. Preparation of β-hexosaminidase, cathepsin D and β-galactosidase nanoparticles

The purified enzymes β-hexosaminidase, cathepsin D and β-galactosidase were labelled with FITC prior to nanoparticle preparation separately. Nanoparticles of these enzymes were prepared as described for PMC nanoparticles and were dispersed in PBS, pH 7.4, stored at 4^{o}C until further use.

2.2.7. Microscopy of PMC, β-hexosaminidase, cathepsinD and β-galactosidase nanoparticles

Nanoparticles structure and morphology was investigated using Atomic Force Microscope (AFM), Scanning Electron Microscope (SEM) and Transmission Electron Microscope (TEM) as described below.

Atomic Force Microscopy (AFM) **-** Nanoparticle sample was uniformly dispersed on a clean glass cover slip using a spin coater and dried in a dust free zone. The unit containing the sample is kept in SPA-400, size and morphology of particles was studied following manufacturer's instructions (SPA 400 of Seiko Instruments Inc., Japan).

Scanning Electron Microscopy (SEM) **-** SEM-PHILIPS FEIXL, ESEM (USA) Operated at 10kV. Nanoparticle sample was uniformly dispersed on a clean glass cover slip using a spin coater and dried. Metal stubs were coated with double-sided adhesive tape, the cover slip with nanoparticle sample was kept on the sticky surface and sample was coated with gold in Sputter Coater. Specimens were stored in dry, dust free environment during the analysis.

Transmission Electron Microscopy (TEM) - Nanoparticles were processed for electron microscopy by air-drying in a dust free zone, a small drop of sample solution was placed on carbon-coated copper electron microscopy grids. The grids containing air dried samples were incubated with 2% (w/v) aqueous uranyl acetate solution for 10 min at room temperature and washed 3–4 times with distilled water. Nanoparticle morphology was examined using TEM (Model FEI Technai G2 S- Twin operated at 100 Kv).

2.2.8. Endocytosis of PMC nanoparticles and co localization with MPR300

The PMC nanoparticle sample was dispersed in incomplete DMEM. NIH3T3 cells were grown to 80% confluency on sterile glass cover slips in complete DMEM medium. The medium was discarded and the cells were washed with sterile 1xPBS for three times each 15 min. The healthy confluent cells were then incubated with nanoparticles that were dispersed in incomplete DMEM for 1 hour at 37°C to follow endocytosis. Following incubation with nanoparticles, cells were washed with PBS three times and then were fixed in 4% paraformaldehyde for 20 min at room temperature. The fixed cells were washed, permeabilised with 0.02% Triton X-100 for 30 seconds and were washed with PBS followed by blocking with 1% BSA in PBS for 30 min at room temperature. The cells were incubated with MPR300 antibody [This antibody was raised for purified goat MPR300 protein and recognizes the MPR300 protein from fish to mammals (1:200dilution in 1% BSA in PBS)]. The cells were washed 3 times with PBS and incubated with cy5 (red) conjugated secondary antibody for 1 hour at room temperature. Finally the cells were washed extensively, stained with DAPI and observed under confocal microscope.

2.2.9. Receptor (MPR300) binding studies of the enzyme nanoparticles

NIH3T3 and CHO cell types, cultured under standard conditions and which are known to contain the cell surface MPR300 protein were used in the study to analyze specific binding of nanoparticles to the cell surface receptor, localization of the endocytosed lysosomal enzyme nanopartricles and subcellular localization of the β-hexosaminidase nanoparticles as described below.

*2.2.9.*1 *Cell surface binding of enzyme nanoparticles*

The enzyme nanoparticle sample was dispersed in incomplete DMEM in three separate studies for each enzyme nanoparticles. NIH3T3 and Chinese Hamster Ovary (CHO) cells were grown to 80% confluence on sterile glass cover slips in complete DMEM medium. The medium was discarded and the cells were washed with sterile 1xPBS for three times each 15 min. The healthy confluent cells were then incubated with β-hexosaminidase nanoparticles that were dispersed in incomplete DMEM for 1 hour at 4°C to follow the cell surface binding. Following incubation with nanoparticles, cells were washed with PBS for three times and then were fixed in 4% paraformaldehyde for 20 min at room temperature. The fixed cells were washed, permeabilised with 0.02% Triton X-100 for 30 seconds and washed with PBS followed by blocking with 1% BSA in PBS for 30 min at room temperature. The cells were incubated with MPR300 antibody [This antibody was raised for purified goat MPR300 protein and recognizes the MPR300 protein fish to mammals (1:200 dilutions in 1% BSA in PBS)]. The cells were washed with PBS 3 times and incubated with cy5 (red) conjugated secondary antibody for 1 hour at room temperature. Finally the cells

were washed extensively, stained with DAPI and observed under confocal microscope.

2.2.9.2. Endocytosis of enzyme nanoparticles and co localization with MPR300

Endocytosis of β-hexosaminidase, cathepsin D and β-galactosidase nanoparticles was investigated as described above except that the cells were incubated at 37°C for 1 hr.

2.2.9.3. Subcellular localization of endocytosed β-hexosaminidase and cathepsin D nanoparticles with Lamp1

After the cells attained confluency in the complete DMEM, they were washed with PBS three times and incubated with nanoparticles of β-hexosaminidase and cathepsin D separately for 1 hr at 37°C, the cells were washed with PBS for three times and were fixed in 4% paraformaldehyde for 20 min at room temperature. The fixed cells were washed, permeabilised with 0.02% Triton X-100 for 30 sec and were washed with PBS followed by blocking with 1% BSA in PBS, pH7.4 for 30 min at room temperature. The processed cells were incubated with Lamp-1 antibody (this antibody from Abcam is known to interact with Lamp-1 from human and mammalian cell lines, 1µg/ml dilution in 1% BSA in PBS) overnight at 4˚C. The cells were washed with PBS three times and incubated with cy5 (red) conjugated secondary antibody for 1 hr at room temperature. Finally the cells were washed extensively, stained with DAPI and observed under confocal microscope.

2.2.9.4. Inhibition study of β-hexosaminidase nanoparticle internalization

In a separate experiment, the NIH3T3 cells were pre incubated with 5, 10, and 20 mM mannose 6-phosphate for 40 min at 4°C prior to the incubation with

enzyme nanoparticles as described above and the cells were visualized microscopically.

In another experiment 231SV (cells devoid of both the mannose 6-phosphate receptors) were incubated with the hexosaminidase nanoparticles for 1h at 37°C.

2.3. Results

In the present study four distinct types of nanoparticles have been prepared.

(i) PMC, a natural ligand for the mannose 6-phosphate receptors which is used for the affinity purification of the receptors and the following lysosomal enzymes.

(ii) β-hexosaminidase nanoparticles

(iii) Cathepsin D nanoparticles

(iv) β-galactosidase nanoparticles.

2.3.1. Purification, SDS-PAGE analysis and western blotting of β-hexosaminidase

mpr $^{(-/-)}$ MEF secretions were processed as described under the methods. After ion exchange chromatography, analysis of an aliquots of the enzyme containing fractions revealed predominantly β-hexosaminidase and α-mannosidase activity. Aliquots were also analyzed by SDS-PAGE which revealed the presence of a 66 kDa protein and some other proteins . To further obtain a homogeneous preparation of the β-hexosaminidase, the salt eluted fractions obtained above were pooled and subjected to affinity chromatography on MPR300 receptor-affigel as described under methods. SDS-PAGE analysis of the 5 mM Mannose 6-phosphate specific elution from the receptor gel revealed a single band corresponding to a molecular mass of ~66 kDa (Fig.2.4.1 A). The glycoprotein

nature of the affinity purified hexosaminidase was analyzed by Periodic Schiff's staining method and the enzyme was shown to be glycosylated (Fig.2.4.1B). Further in a separte experiment probing this protein with an antibody to goat liver hexosaminidase revealed a cross-reactivity confirming the authenticity of the enzyme puriried to be β-hexosaminidase (Fig.2.4.1.C).

2.3.2. Purification, SDS-PAGE analysis and immunoblotting of cathepsin D from Unio

The *Unio* tissue was processed as described under methods. The protein solution obtained after dialysis with buffer B was applied onto the pre-equilibrated Pepstatin A-affigel for purifying the enzyme. From 50 g of the tissue, 450μg protein could be obtained in the final step. An aliquot of cathepsin D eluate was subjected to 10% SDS-PAGE in the presence of reducing agent, which revealed a single protein band with an apparent molecular mass of 45kDa, indicating that the enzyme is a monomeric protein (Fig.2.4.2A). The purified protein also cross-reacted with the starfish cathepsin D antiserum suggesting antigenic similarities among the invertebrate enzymes (Fig. 2.4.2 B).

2.3.3. Microscopy of PMC nanoparticles

Our attempts to prepare PMC nanoparticles using the protocol described under the methods were successful. When these were characterized using AFM, SEM and TEM, the sizes of the particles were in the range of 100–300 nm (Fig.2.4.3.A,B,C). The particles were distinct and spherical with smooth surface morphology.

2.3.3.1. Endocytosis of FITC-Conjugated PMC nanoparticles

From Fig. 2.4.7 it is apparent that the nanoparticles of naturally occurring phosphorylated manno polysaccharides can be prepared and used for nanoparticle internalization. In a separate experiment as a negative control, cells were pre incubated with mannose 6-phosphate to block the MPR300 receptor on the cell surface as explained above for the enzyme nanoparticles.

(Kindly Note - All confocal images described are in the order described under methods and denoted as : Bold : *β –hexosaminidase,* Underline-Cathepsin *D* *and Normal font* – β-galactosidase).

2.3.4. Microscopy of ***β -hexosaminidase*** *Nanoparticles*

The nanoparticles for the purified enzyme were also prepared as described above and their size varied from 60–100 nm as determined by AFM, SEM and TEM **(Fig.2.4.4.A, B, and C).**

2.3.5. Microscopy of cathepsin D nanoparticles

Nanoparticles of the *Unio* (mollusc) lysosomal enzyme cathepsin D could also be prepared successfully and were characterized microscopically by AFM, SEM and TEM as shown in Fig.2.4.5.A.B.C.

2.3.6. Microscopy of β-galactosidase nanoparticles

β-galactosidase nanoparticles prepared were in the size range of 20–50 nm (Fig.2.4.6.A.B.C.). The variation in the size range of the three different enzyme nanoparticles prepared in our study might be due to the difference in the molecular masses of the enzymes used.

2.3.7. Cell surface binding of ***β-hexosaminidase,*** *cathepsin D and* β-galactosidase *nanoparticles*

The labelled enzyme nanoparticles efficiently bind to the cell surface receptor MPR300 as shown in ***Fig.2.4.8. (β-hexosaminidase),*** Fig.2.4.9. (*cathepsin D*), Fig.2.4.10. (β-galactosidase). When NIH3T3 cells were incubated with the nanoparticles, the particles were localized to the cell surface and the receptor was also detected using an antibody to the receptor. Similar results were obtained with Chinese hamster ovary cells (CHO). The effect of different concentrations of the mannose 6-phosphate on binding of the enzyme nanoparticles to the MPR300 in NIH3T3 cells was studied as described under methods. Only at very high concentration of 20 mM mannose 6-phosphate there was complete inhibition of the binding of the enzyme nanoparticles to the receptor.

2.3.8. Localization of endocytosed FITC-conjugated β-hexosaminidase, cathepsin D and β-galactosidase nanoparticles

The ability of the cell surface receptor to internalize the enzyme nanoparticles was studied as described under methods and the results were presented in ***Fig2.4.11. (β-hexosaminidase),*** Fig.2.4.13. (cathepsin D), Fig.2.4.15. (β-galactosidase)**.** From these figures it is apparent that the fluorescently labelled enzyme nanoparticles on incubation with both cell types used, were internalized which were also detected by using the antiserum to MPR300. These results suggest the internalization of the nanoparticles is through the cell surface MPR300 protein. Similar results were obtained for the three different lysosomal enzyme nanoparticles.

2.3.9. Subcellular localization of endocytosed β-hexosaminidase and cathepsinD nanoparticles with Lamp1

The subcellular localization of the internalized β-hexosaminidase nanoparticles in NIH3T3 cells was also investigated using Lamp1 antibody. The results shown in ***Fig.2.4.12. (β-hexosaminidase)*** and Fig.2.4.14. (cathepsin D) merged image D in these figures, suggest that the enzyme nanoparticles that are internalized are co-localized with Lamp-1 protein, a known lysosomal marker protein.

*2.3.10. Inhibition studies of **β-hexosaminidase** nanoparticles*

Pre-incubation of the cells with different concentrations of the mannose 6-phosphate prior to the addition of the nanoparticles showed complete inhibition of the binding of the nanoparticles to the cell surface only at 20 mM concentration of the sugar while the 5 mM mannose 6-phosphate and 10 mM mannose 6-phosphate did not show any inhibitory effect. This high concentration of the sugar required to cause complete inhibition might possibly be due to the large aggregates of nanoparticles that are binding to the cell surface receptor (Fig.2.4.17.). Similarly pre-incubation of the cells with the antibody of the MPR300 protein also showed complete inhibition of binding suggesting the non-availability of the cell surface receptor for the nanoparticle binding as shown in Fig.2.4.18. At the same time there was no binding and endocytosis of the nanoparticles in 231SV cell lines which are devoid of the mannose 6-phosphate receptors (Fig.2.4.16.).

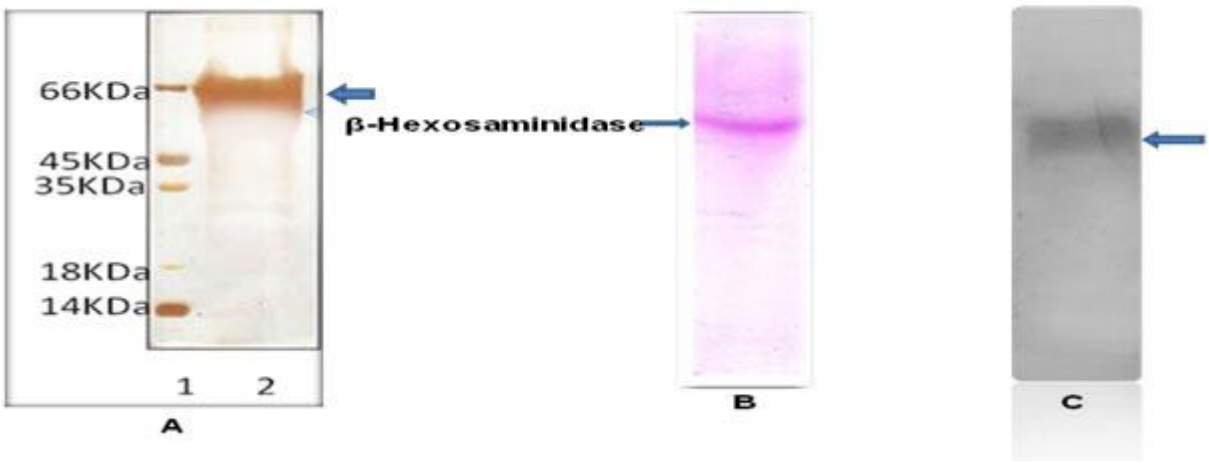


Figure.2.4.1. Electrophoresis of β-hexosaminidase from 23ISVcell secretions (A) Purified β-hexosaminidase was separated on SDS-PAGE under reducing conditions, followed by silver staining. Lane1: marker, Lane 2: β-hexosaminidase

(B) Periodic Acid Schiff staining of purified enzyme β-hexosaminidase

(C) Western blotting of β-hexosaminidase (using antibody of Goat liver lysosomal β-hexosaminidase)

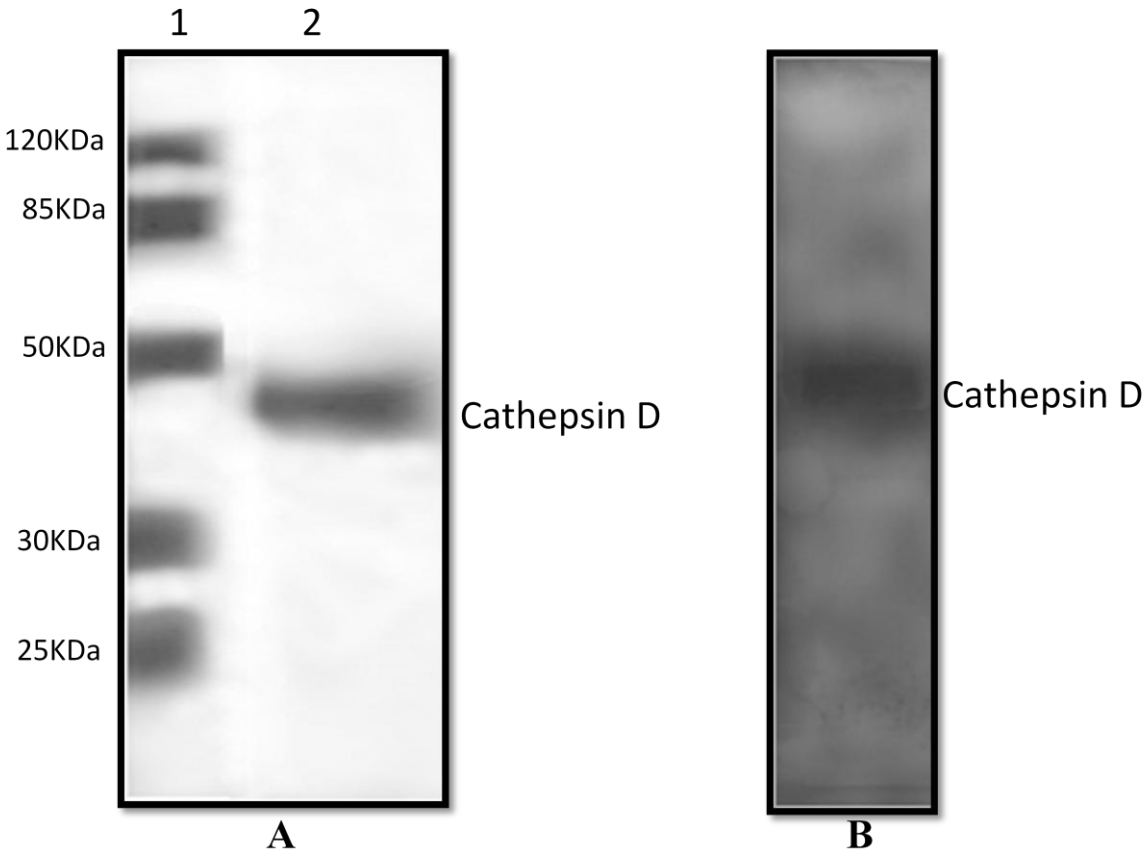


Figure.2.4.2.

Figure.2.4.2. (A) Purified cathepsin D was separated on SDS-PAGE under reducing conditions, followed by coomassie staining. Lane1: marker, Lane 2: Cathepsin D**(B)** Immunoblot of Cathepsin D (using starfish cathepsin D antibody)

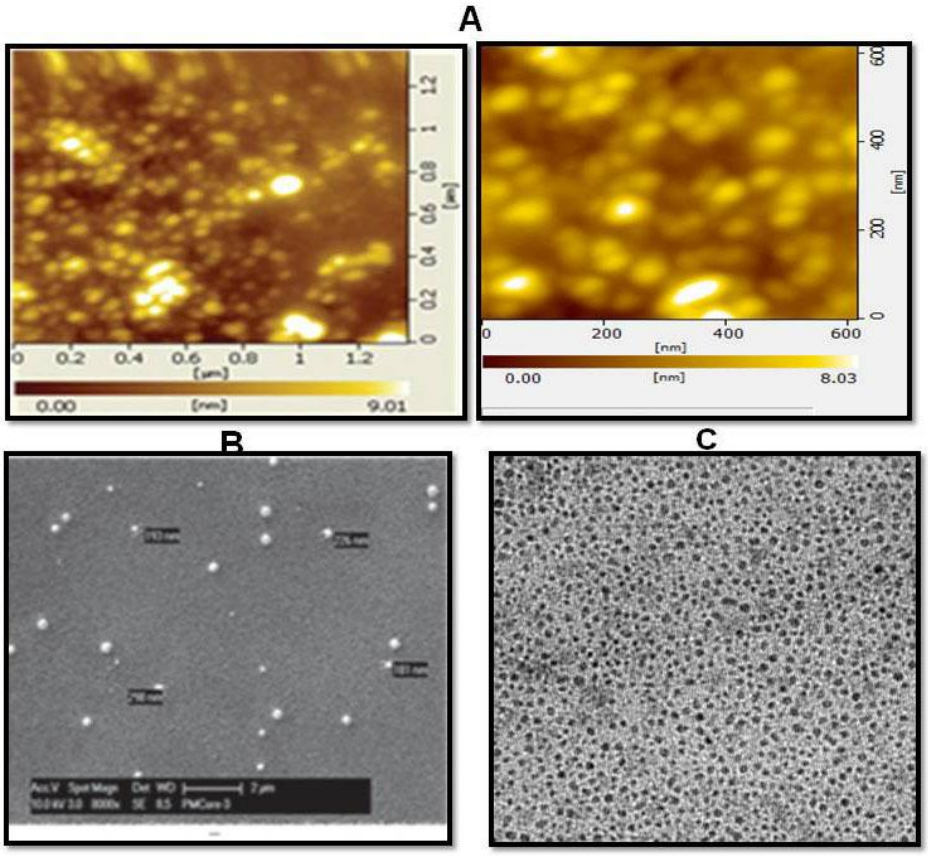


Figure.2.4.3. Microscopy of PMC nanoparticles

(A) Atomic Force Microscopy (AFM) **(B)** Scanning Electron Microscopy (SEM) and **(C)** Transmission Electron Microscopy (TEM)**.** Size of the nanoparticles in the range of 100-300nm

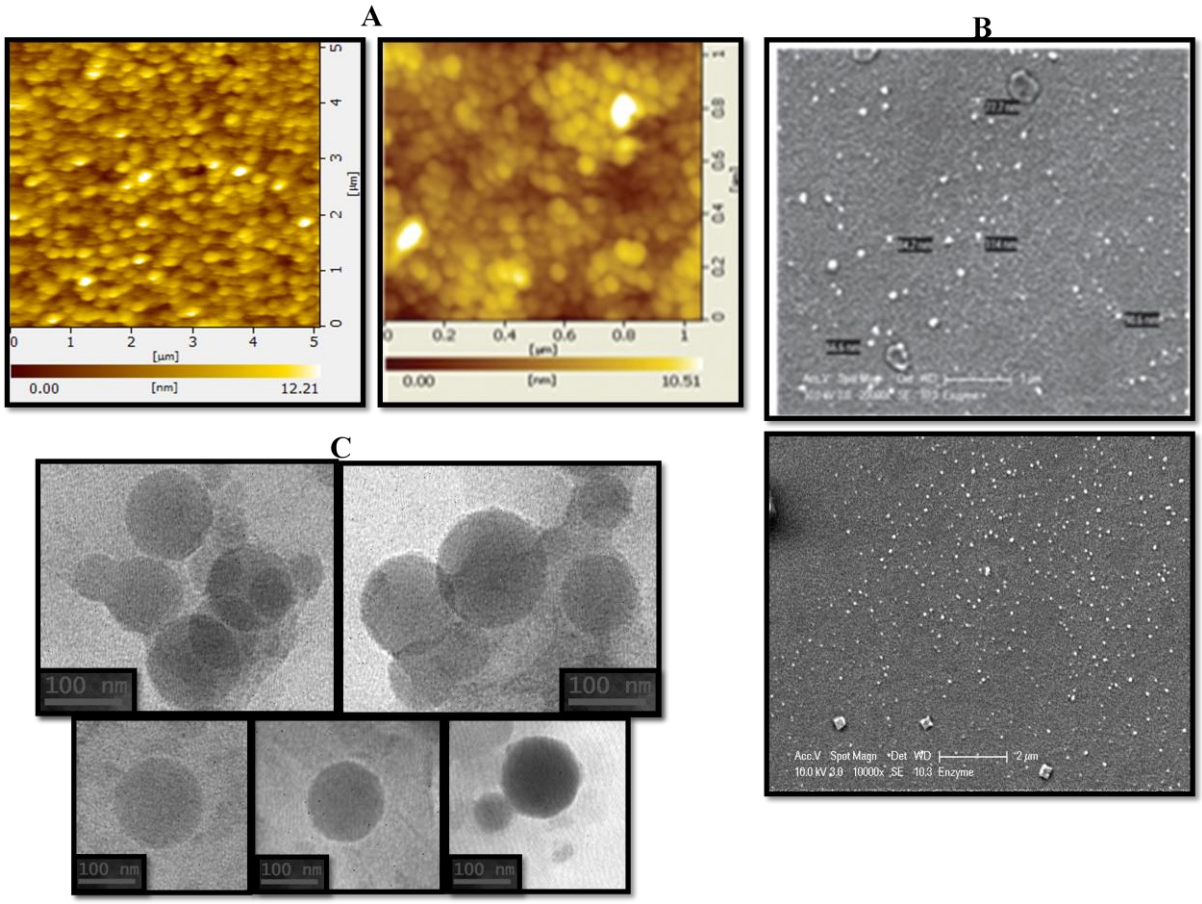


Figure.2.4.4. Microscopy of β-hexosaminidase nanoparticles

(A) Atomic Force Microscopy (AFM), **(B)** Scanning Electron Microscopy (SEM), **(C)** Transmission Electron Microscopy (TEM). Size of the nanoparticles in the range of 60-100nm

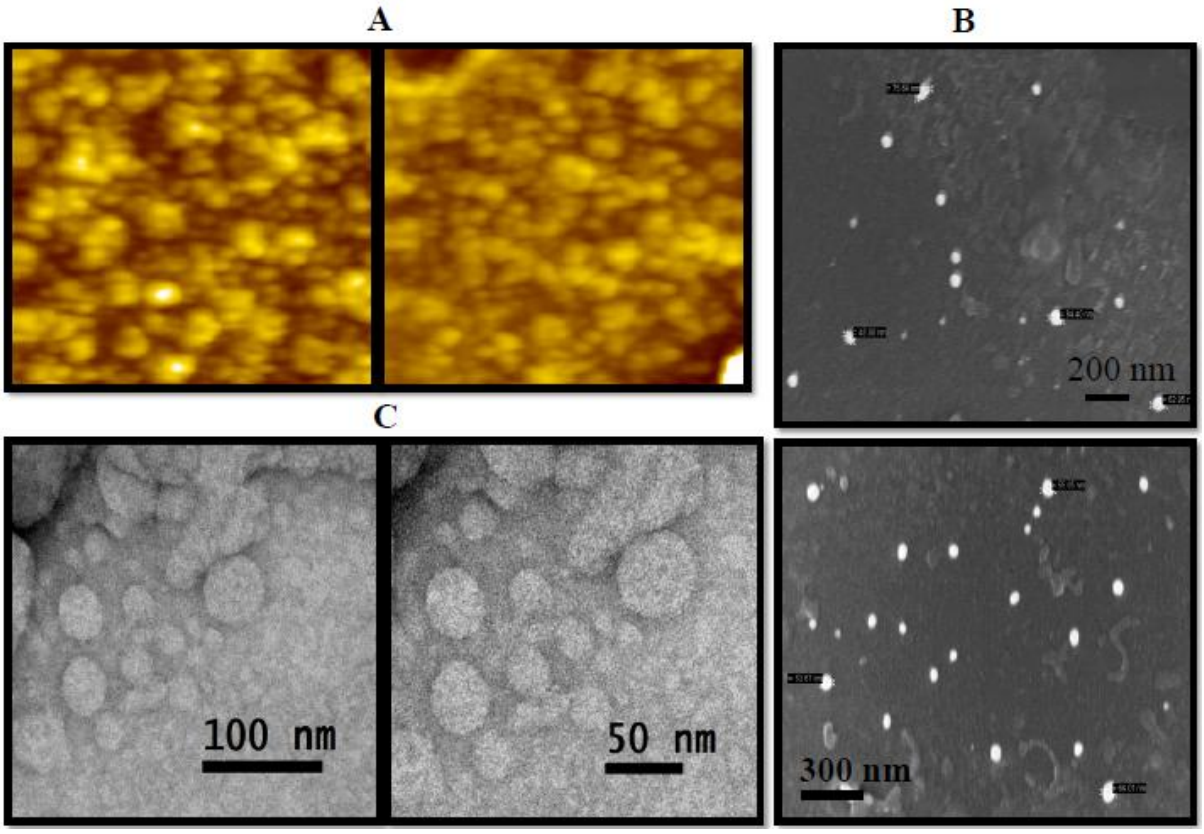


Figure.2.4.5. Microscopy of cathepsin D nanoparticles (A) Atomic Force Microscopy (AFM) **(B)** Field Emission Scanning Electron Microscopy (FESEM), **(C)** Transmission Electron Microscopy (TEM). Size of the nanoparticles in the range of 40-60 nm

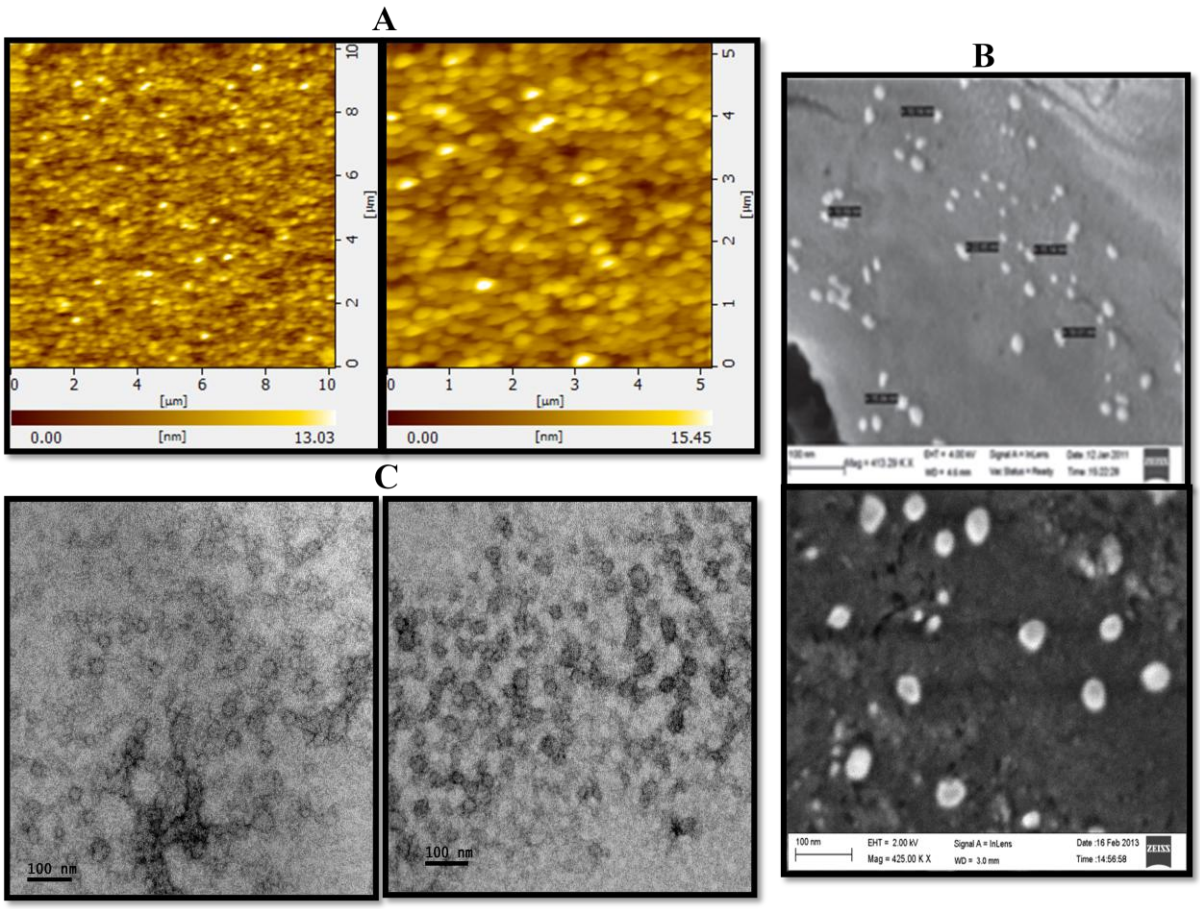


Figure.2.4.6. Microscopy of β-galactosidase nanoparticles(A) Atomic Force Microscopy (AFM), **(B)** Field Emission Scanning Electron Microscopy (FESEM), **(C)** Transmission Electron Microscopy (TEM). Size of the nanoparticles in the range of 20-50nm.

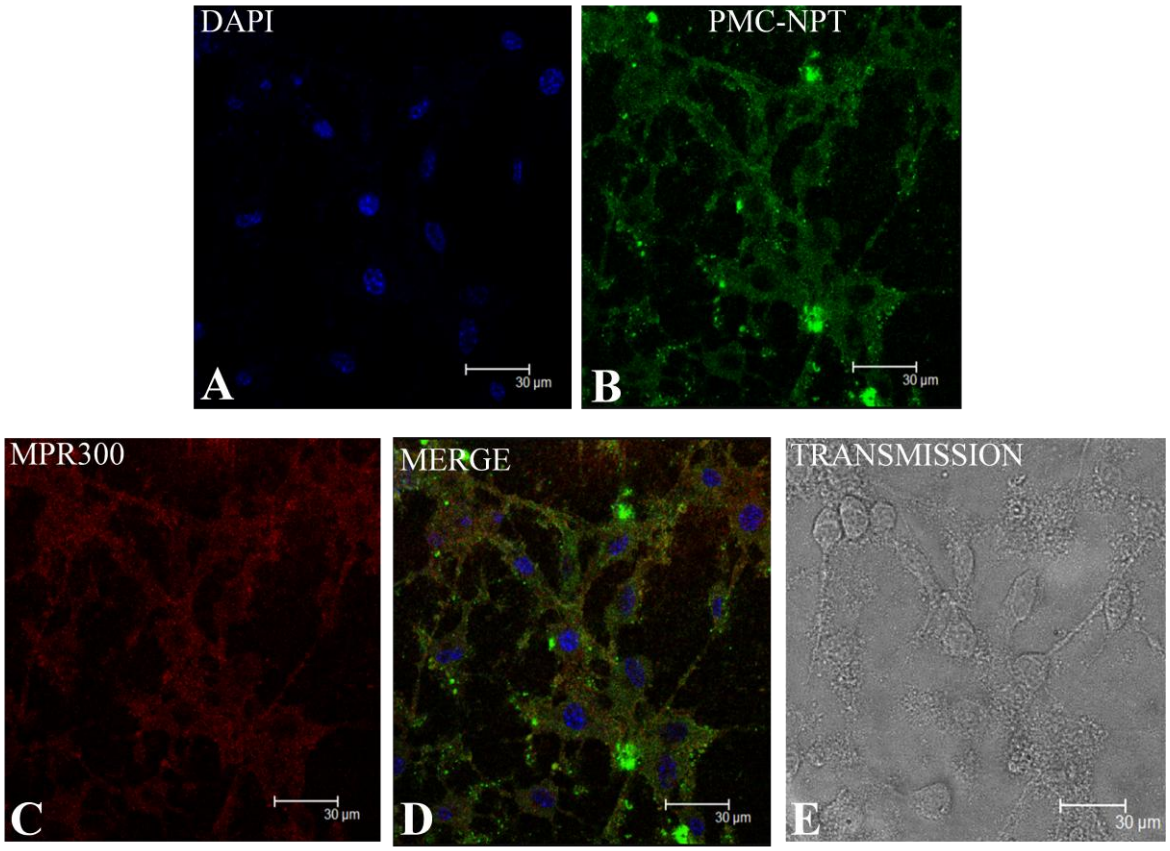


Figure.2.4.7. Intracellular localization of Phosphomannan core nanoparticles (A) is DAPI, **(B)** Incubation with FITC nanoparticles (PMC-NPT) for I hour at 37^{o}C, **(C)** Incubation with MPR300 antibody followed by cy5(red) conjugated secondary antibody, **(D)** is merged images of **A, B, C**. Cell line type is NIH3T3. Bar in the panel is 30µm.

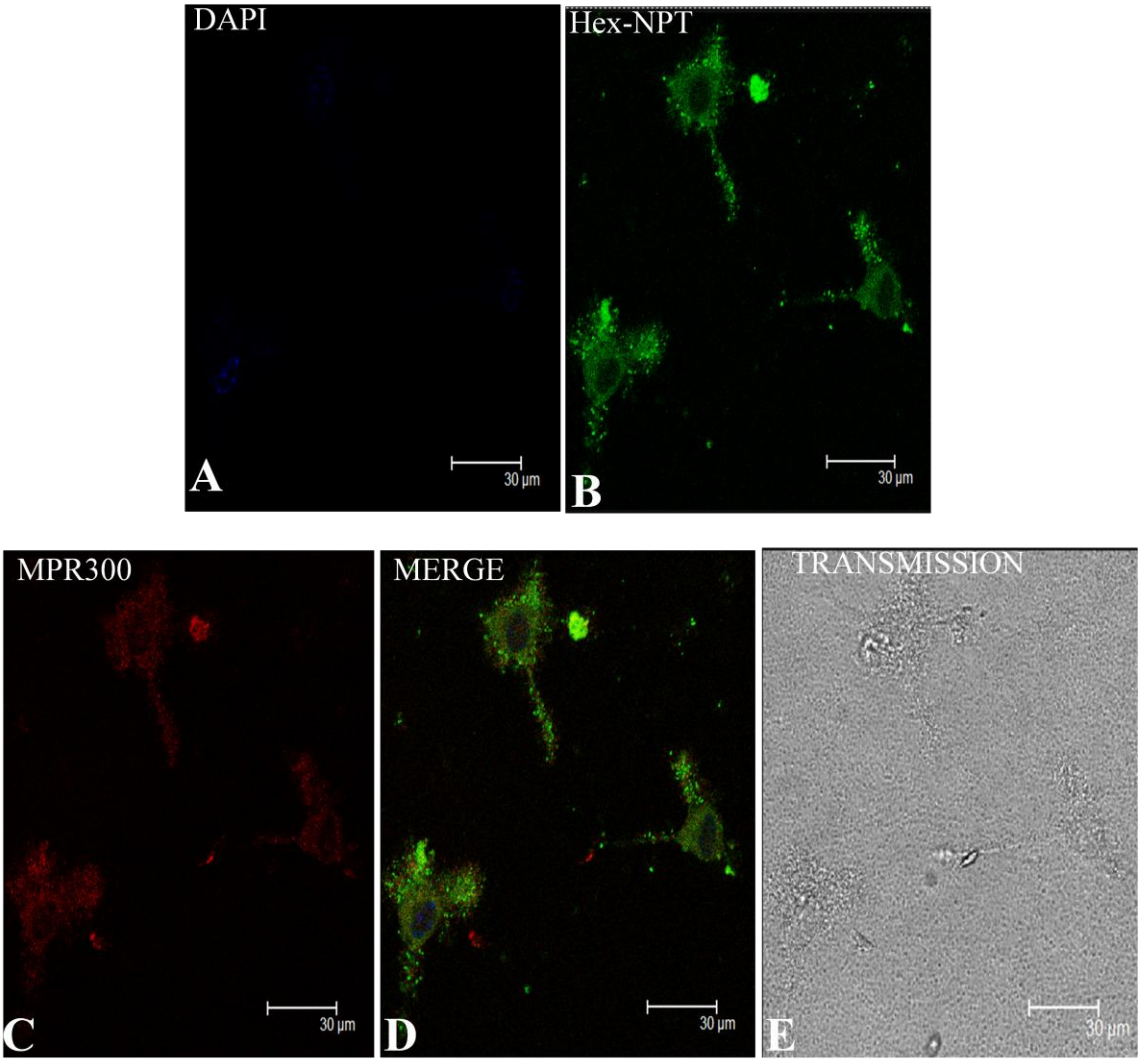


Figure.2.4.8. Surface colocalization of nanoparticles with the MPR300 protein, (A) is DAPI, (B) is incubation with β-hexosaminidase nanoparticles (Hex-NPT) for 30 min at 4^{o}C, (C) Incubation with MPR300 antibody followed by cy5 conjugated secondary antibody. (D) is merged image of A, B, C. Bar in the panel is 15µm, cell line type NIH3T3.

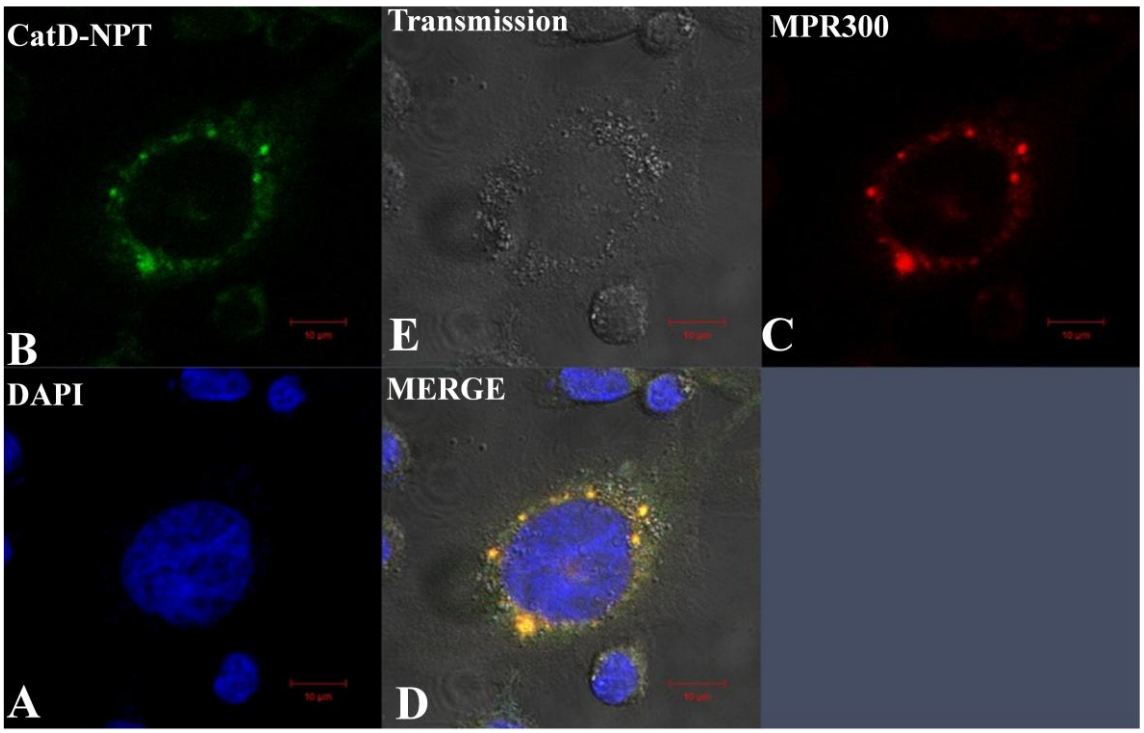


Figure.2.4.9. Surface colocalization of nanoparticles with the MPR300 protein, (A) is DAPI, (B) is incubation with cathepsin D nanoparticles (CatD-NPT) for 30 min at 4^{o}C, (C) Incubation with MPR300 antibody followed by cy5 conjugated secondary antibody. (D) is merged image of A, B, C. Bar in the panel is 15µm, cell line type Chinese Hamster Ovary (CHO).

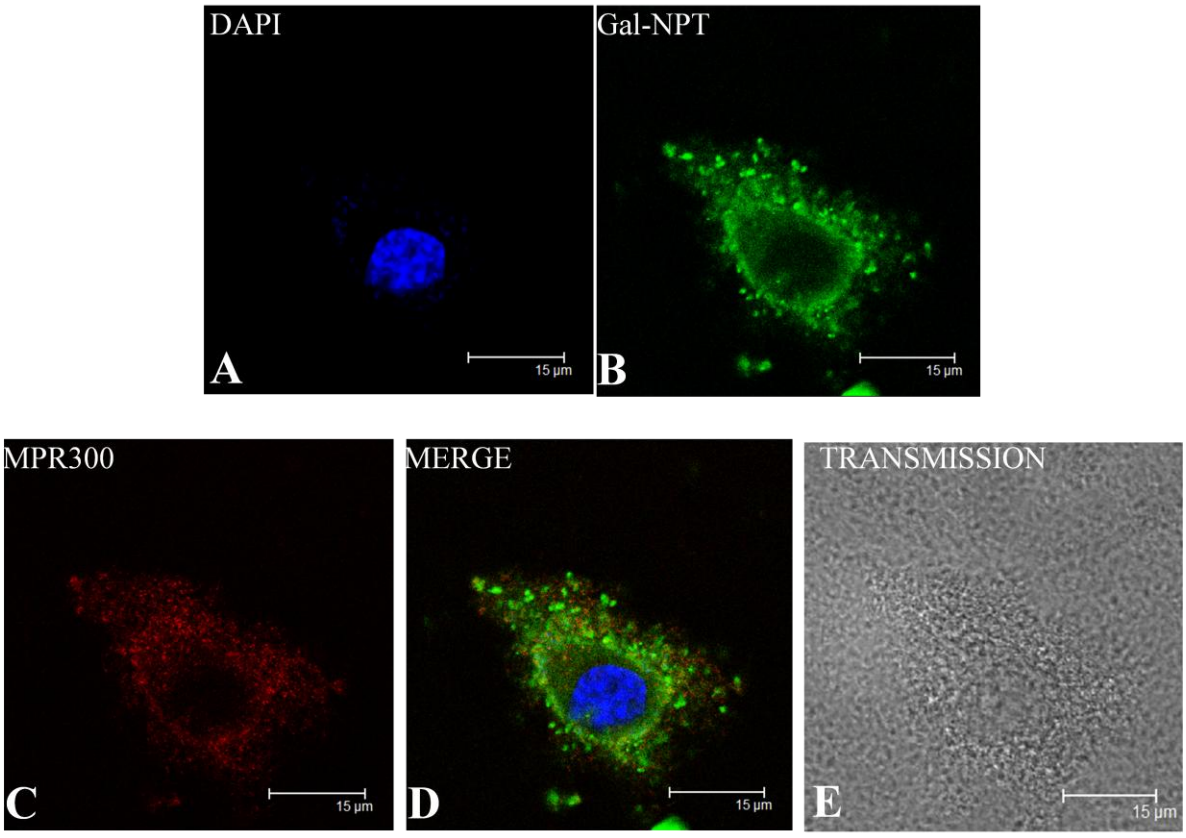


Figure.2.4.10. Surface colocalization of nanoparticles with the MPR300 protein, (A) is DAPI, (B) is incubation with β-galactosidase nanoparticles (Gal-NPT) for 30 min at 4^{o}C, (C) Incubation with MPR300 antibody followed by cy5 conjugated secondary antibody. (D) is merged image of A, B, C. Bar in the panel is 15µm, cell line type NIH3T3.

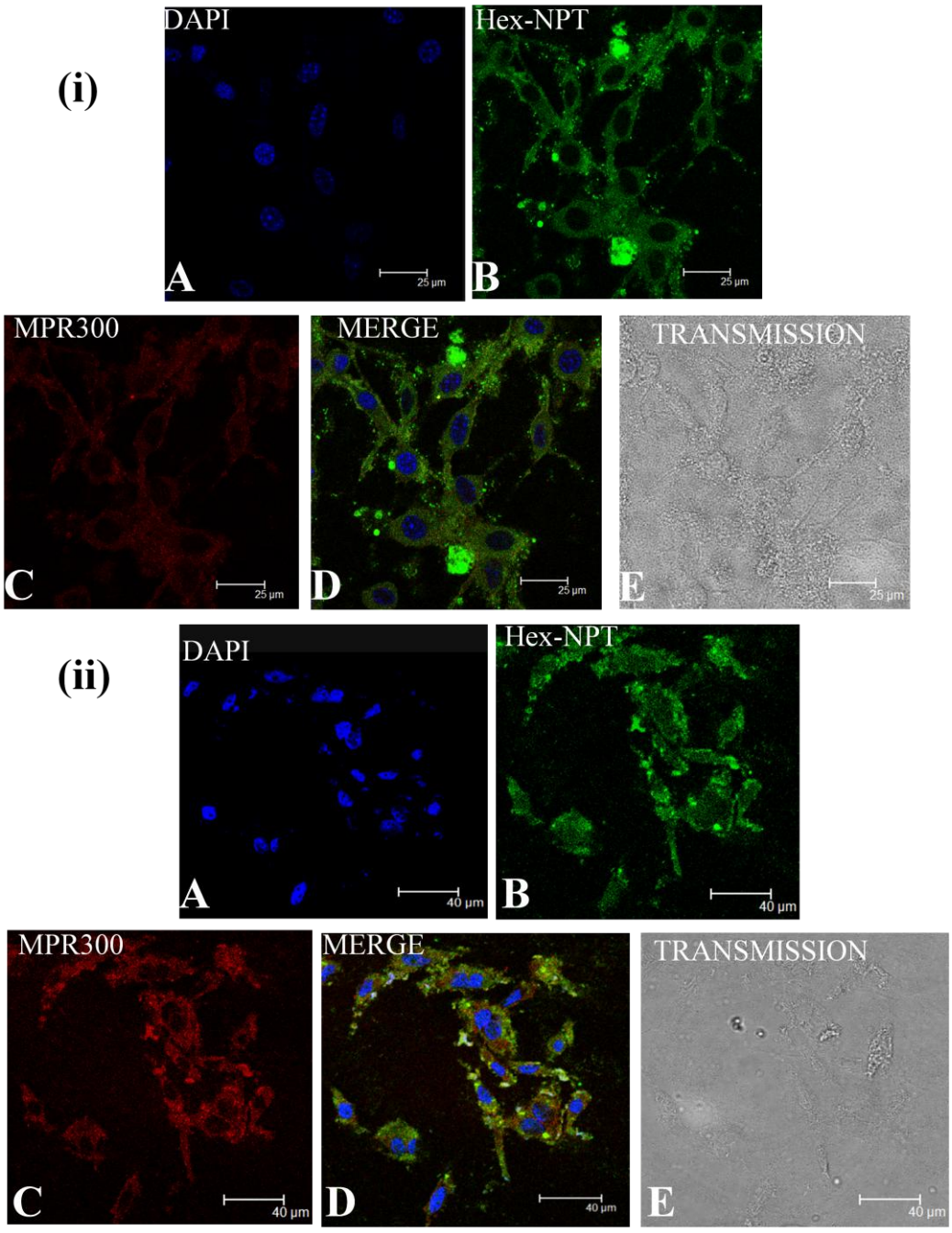


Figure.2.4.11. Intracellular localization of β-hexosaminidase nanoparticles in (i) NIH3T3 and (i) CHO cells: (B) Incubation with FITC nanoparticles (Hex-NPT) for I hour at 37^{o} C, (C) Incubation with MPR300 antibody followed by cy5(red) conjugated secondary antibody, (*D)* is merged images of A, B, C. Bar in the panel is 25 µm for NIH3T3 and 40 µm for CHO cells.

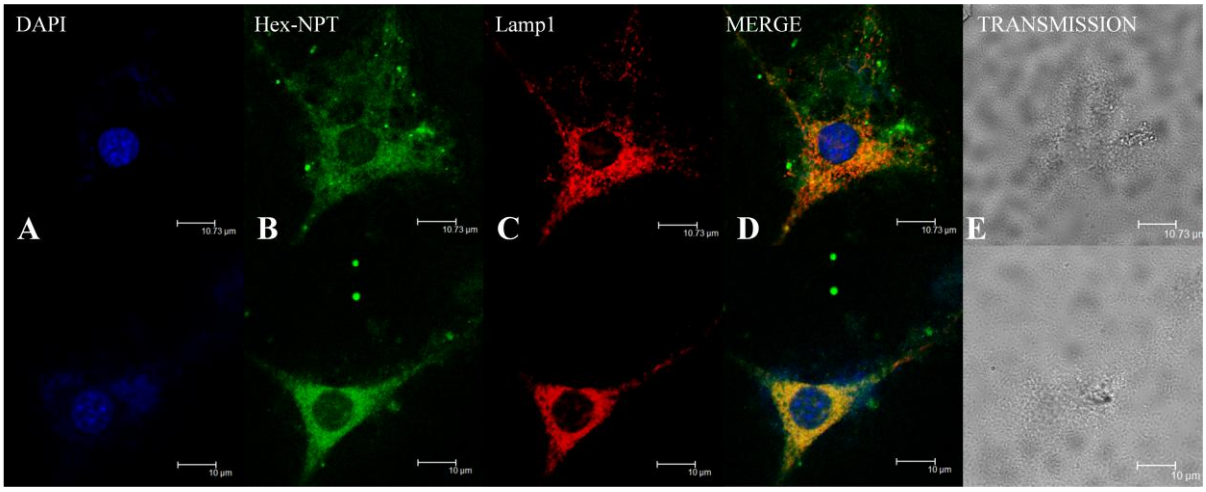


Figure.2.4.12. Sub cellular localization of *β*-hexosaminidase nanoparticles in NIH3T3 cells with Lamp1, (A) incubation with DAPI, (B) Incubation with nanoparticles (Hex-NPT) for 1 hour at 37^{o}C, (C) incubation with Lamp-1 antibody followed by cy5 (red) conjugated secondary antibody, (D) is merged image of A, B, C. Cell line type is indicated at the left side of the panel. Bar in the panel is 10.73μm (top panel) and 10µm (bottom panel).

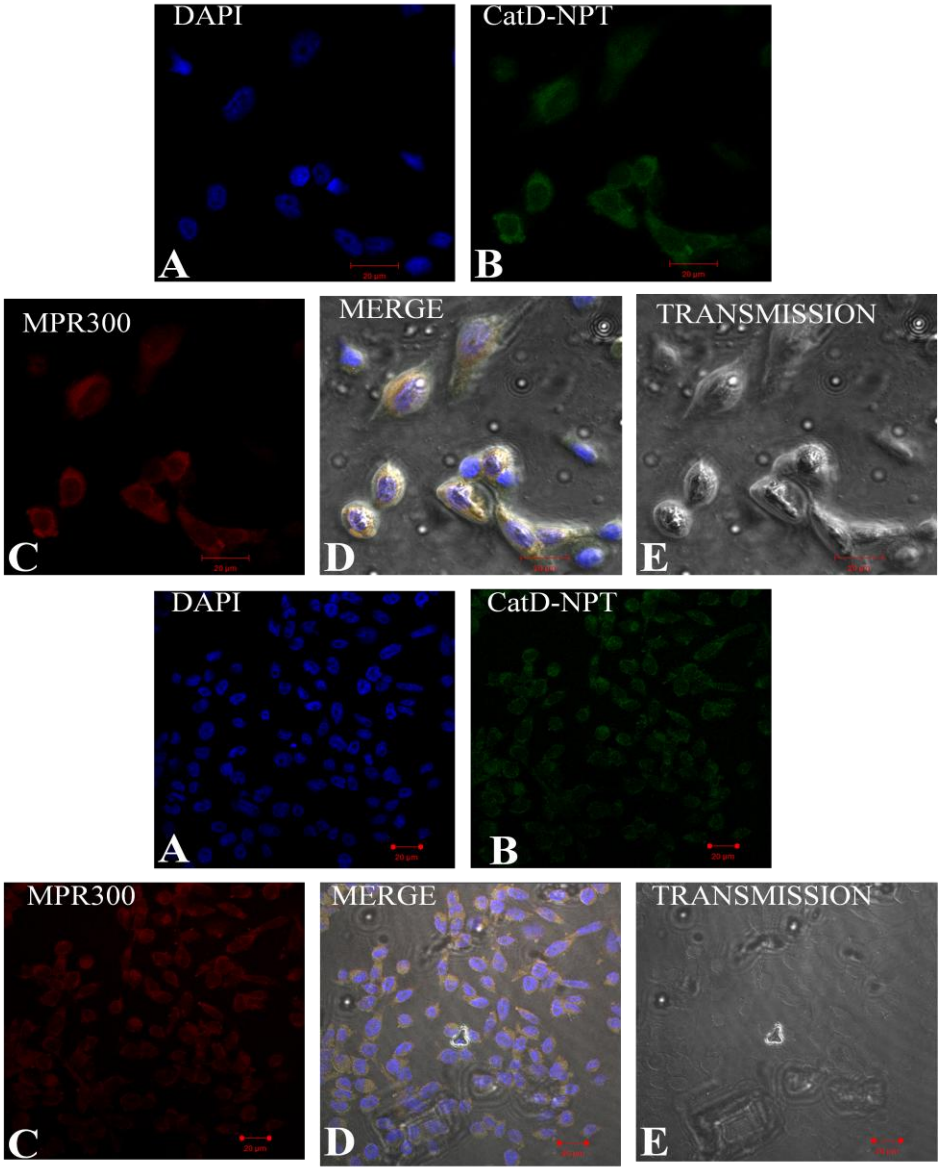


Figure.2.4.13. Intracellular localization of cathepsin D nanoparticles in CHO cells: (B) Incubation with FITC nanoparticles (CatD-NPT) for I hour at 37^{o} C, (C) Incubation with MPR300 antibody followed by cy5(red) conjugated secondary antibody, (*D)* is merged images of A, B, C. Bar in both the panels is 20 µm.

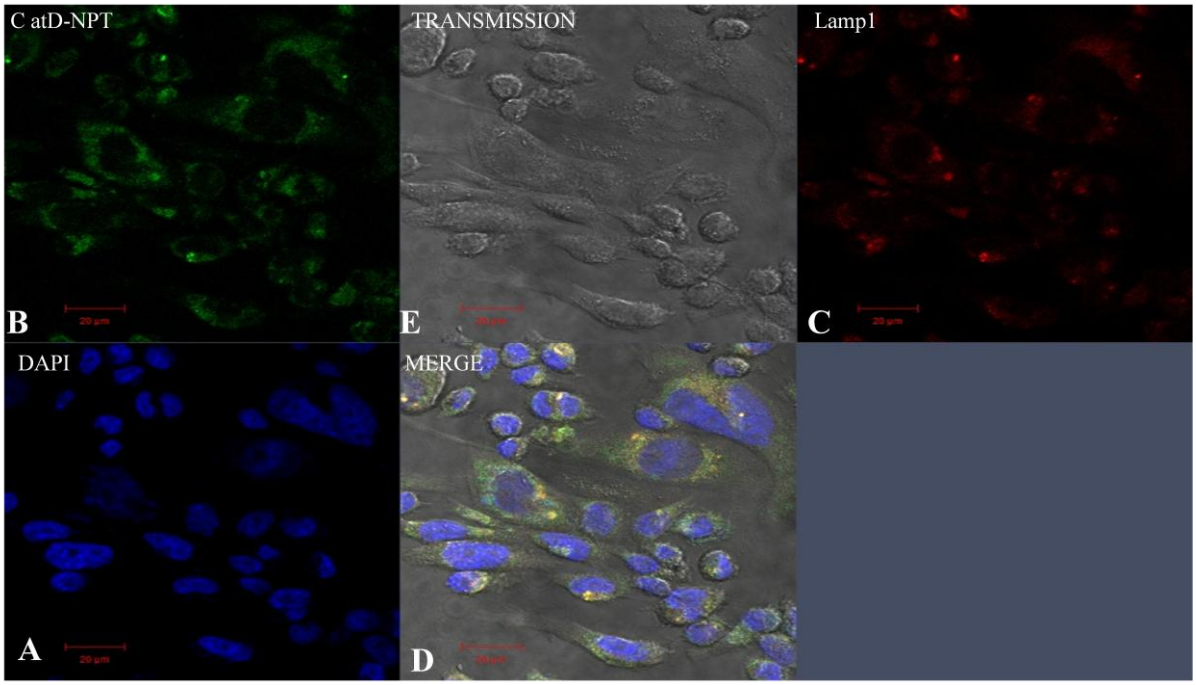


Figure.2.4.14. Sub cellular localization of cathepsinD nanoparticles in CHO cells with Lamp1, (A) incubation with DAPI, (B) Incubation with nanoparticles (Hex-NPT) for 1 hour at 37^{o}C, (C) incubation with Lamp-1 antibody followed by cy5 (red) conjugated secondary antibody, (D) is merged image of A, B, C. Cell line type is indicated at the left side of the panel. Bar in the panel is 10.73μm (top panel) and 10µm (bottom panel).

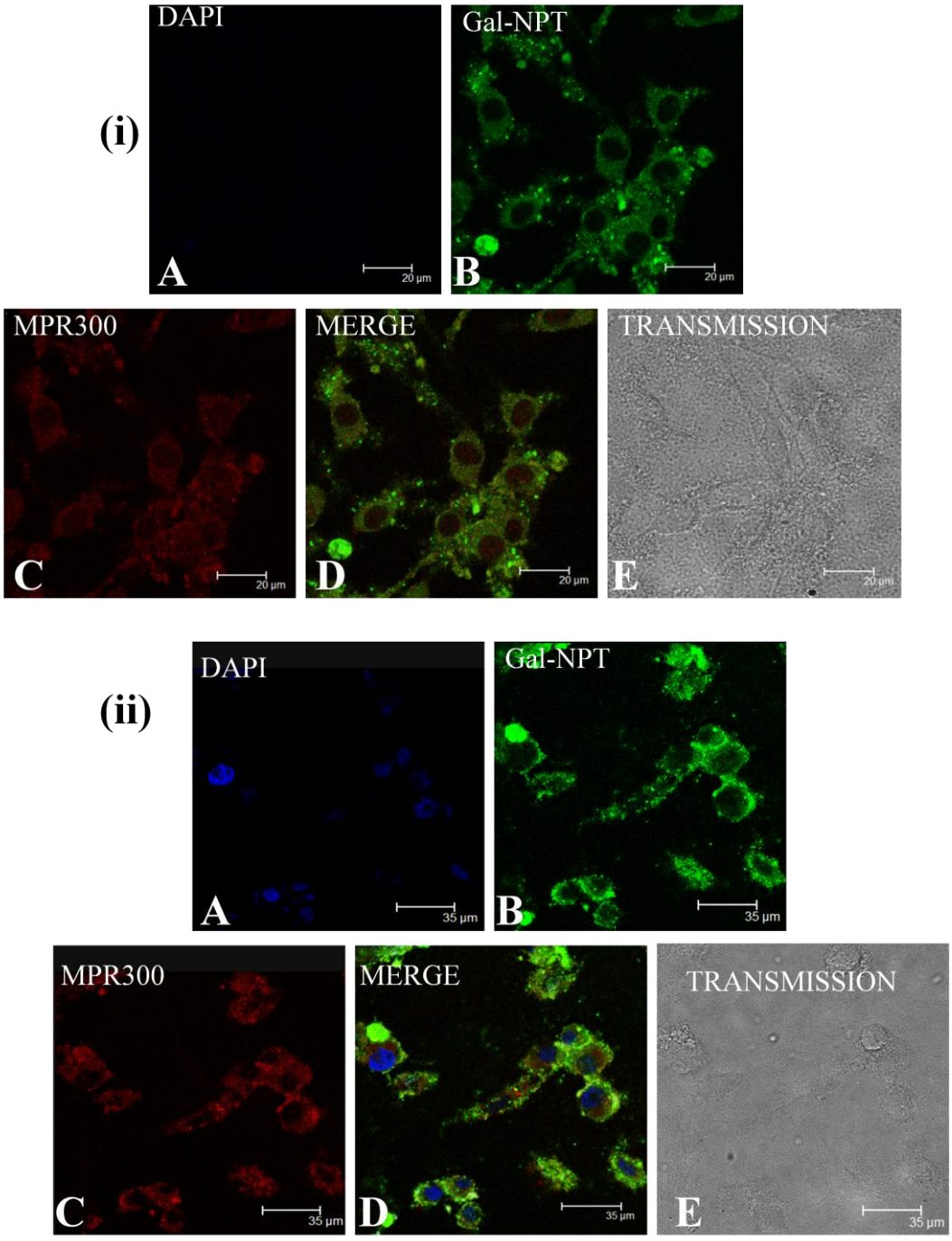


Figure.2.4.15. Intracellular localization of β-galactosidase nanoparticles in (i) NIH3T3 and (i) CHO cells: (B) Incubation with FITC nanoparticles (Gal-NPT) for I hour at 37° C, (C) Incubation with MPR300 antibody followed by cy5(red) conjugated secondary antibody, (*D)* is merged images of A, B, C. Bar in the panel is 20 µm for NIH3T3 and 35 µm for CHO cells.

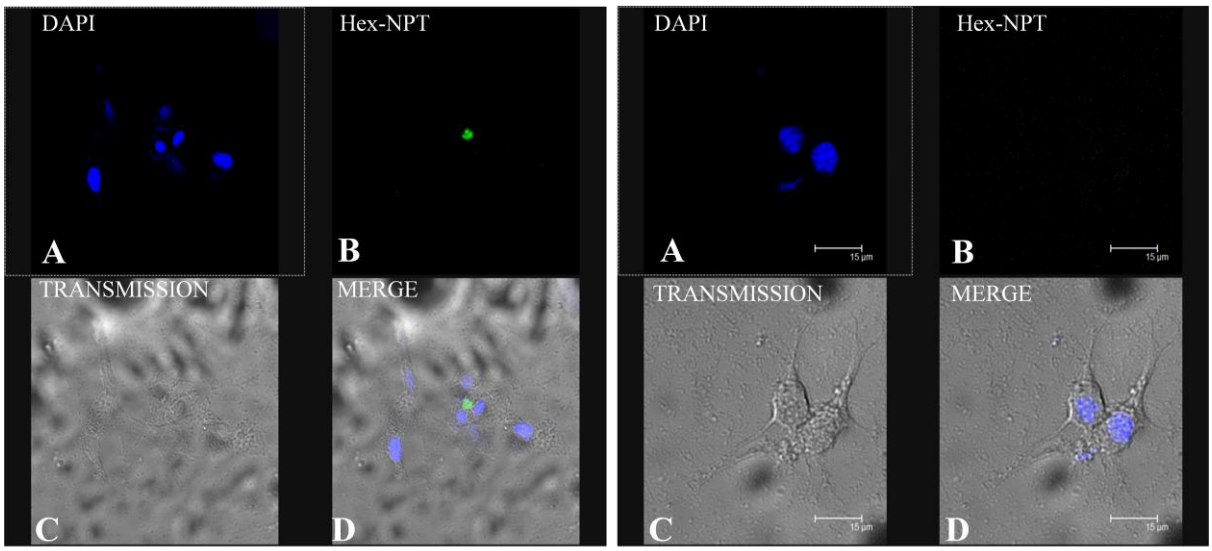


Figure.2.4.16. Negative control experiment of β-hexosamindase nanoparticle internalization in 231SV cell lines which are devoid of the mannose 6-phosphate receptors. (A) DAPI, (B) β-hexosamindase nanoparticles, (D) merged image of A&B, (C) is the transmission image. Cell line is 231SV. Bar in the right panel is 15μm.

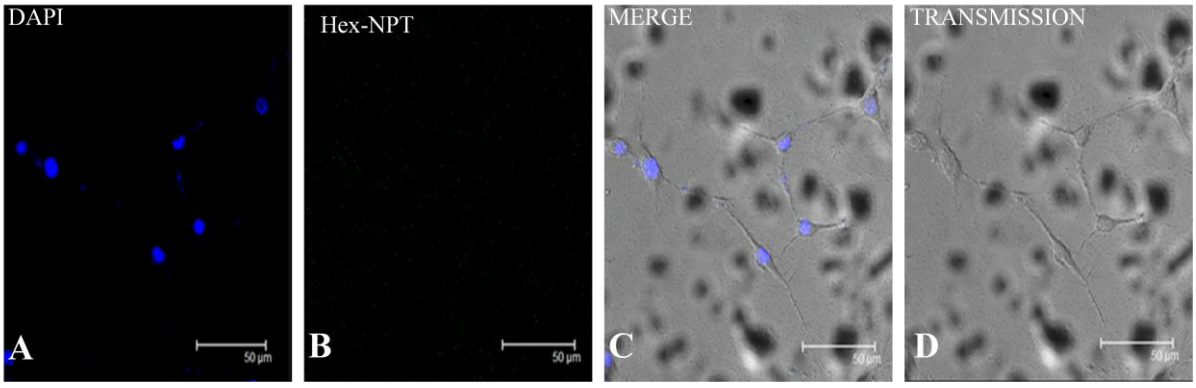


Figure.2.4.17. Inhibition of β-hexosamindase nanoparticles uptake. (A) DAPI, (B) blocking receptor with 20mM mannose 6-phosphate followed by incubation with nanoparticles (Hex-NPT), (C) merged image of A&B, (D) is the transmission image. Cell lines are NIH3T3; Bar in the panel is 50μm.

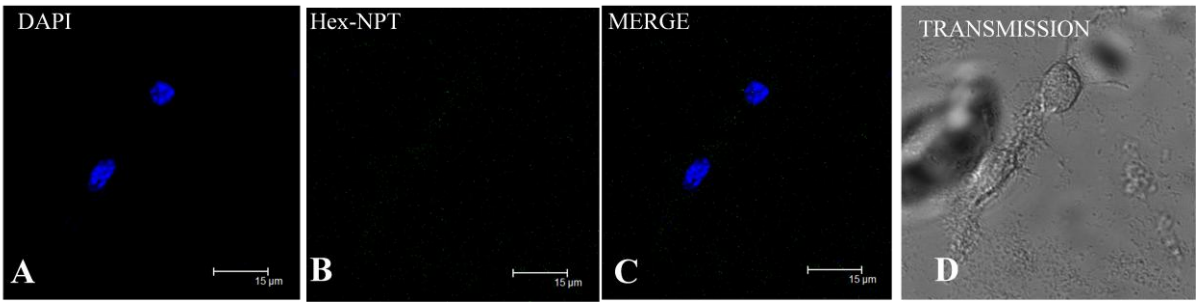


Figure.2.4.18. Inhibition of β-hexosamindase nanoparticles uptake. (A) DAPI, (B) blocking receptor with MPR300 antibody followed by incubation with nanoparticles (Hex-NPT), (C) merged image of A&B, (D) is the transmission image. Cell lines are NIH3T3; Bar in the panel is 15µm.

2.5. Discussion

Receptor mediated endocytosis is one of the mechanisms for the internalization of ligands into cells. Our laboratory has been working on two homologous but distinct mannose 6-phosphate receptor proteins, MPR300 and MPR46 that are known to specifically interact in cells with the lysosomal enzymes that harbour a mannose 6-phosphate signal. Extensive studies carried out using different cell lines, animal models revealed that both receptors are evolutionarily conserved among vertebrates from fish to mammals (Nadimpalli and Amancha, 2010). More recent studies on the invertebrate receptors and enzymes revealed the existence of lysosomal enzymes in invertebrates such as the echinoderm (starfish) and mollusc (*Unio*) which exhibit biochemical properties similar to the vertebrate enzymes and also show interaction with MPR proteins (Yadavalli and Nadimpalli, 2008, Venugopal and Sivakumar, 2013). These studies reveal possible conservation of the enzymes and receptors also in invertebrates. Though both receptors are also present in the plasma membrane, only MPR300 alone has been described as an endocytosis receptor in mammals as well as in some non-mammalian vertebrates. This receptor has been shown to be a multifunctional protein capable of binding several mannose 6-phosphate containing ligands as well as some non-mannose 6-phosphate containing ligands. Several lysosomal disorders that effect humans are known and it is important to develop methods that would eventually be useful for curing the diseases or to help combat the diseased condition. Nanoparticles of naturally occurring polysaccharides such as the chitosan have gained more attention as specific drug delivery carriers (Krishna Sailaja *et al.,* 2010). Although the chitosan based nanoparticles have been used for drug delivery, there are no

reports on the preparation of other types of oligosaccharide nanoparticles such as the mannooligosaccharides or phosphomannooligosaccharides. Since our laboratory has been working on Mannose 6-phosphate receptors and lysosomal enzymes that contain mannose 6-phosphate residues, we wanted to first explore the possibilities of preparation and characterization of the mannooligosaccharides or phosphomannooligosaccharides (PMC) as well as three lysosomal enzymes that constitutively carry mannose 6-phosphate residues in order to test whether they can be recognized and internalized into cells by the MPR300 protein, a known receptor for these ligands. Second we wanted to analyze if these nanoparticles prepared can bind to the cell surface MPR300 protein in different cell lines and therefore used specific cell lines NIH3T3 and the CHO cells that are known to contain this receptor. Our results suggest that the PMC nanoparticles as well as the enzyme nanoparticles prepared in the study show specific binding to the MPR300 on the cell surface as evidenced by confocal microscopy. Further confirmation of this binding has been established by the reactivity of the MPR300 antibody with the cell surface bound PMC, enzyme nanoparticles and MPR300. The specificity of this interaction has also been ascertained in two separate experiments. Pre incubation of the cells with different concentrations of the mannose 6-phosphate prior to the addition of nanoparticles, showed complete inhibition of the binding of the nanoparticles to the cell surface only at 20 mM concentration of the sugar while the 5 mM mannose 6-phsophate and 10 mM mannose 6-phosphate did not show any inhibitory effect. This high concentration of the sugar required to cause complete inhibition might possibly be due to the large aggregates of nanoparticles that are binding to the cell surface receptor.

Similarly pre incubation of the cells with the antibody of the MPR300 protein also showed complete inhibition of binding suggesting the non-availability of the cell surface receptor for the nanoparticle binding.

Third we wanted to analyze if the nanoparticles bound on the cell surface can be internalized and therefore did the incubation experiments at 37°C. Nanoparticles used in this study can be internalized by two different cell types that harbour the MPR300 protein. The localization of the nanoparticles together with the receptor in the cells was also visualized by using an antibody to the MPR300 protein (the antibody is directed against the purified MPR300 protein from goat liver and from the antiserum, specific IgG was affinity purified on MPR300-receptor-affigel was used in the study. This antibody can recognize the MPR300 protein from mammals to molluscs as evidenced from our studies). Furthermore the sub cellular localization of the nanoparticles/receptor together with the Lamp1 protein, a known lysosomal associated membrane protein, strongly suggests and supports the specificity of the interaction between the nanoparticles used and the receptor and the role of the receptor in specific targeting of the nanoparticles. This internalization phenomenon is further supported by our recent studies using mollusc cell lines where we showed that the receptor can internalize mannose 6-phosphate containing lysosomal enzymes (Amancha *et al.,* 2009).

Our long term goals are to establish suitable drug delivery systems for various therapeutic disorders that effect mankind with a preference for lysosomal disorders. As a first step, we prepared the phosphorylated mannooligosaccharide (phosphomannan core) nanoparticles, as well as three lysosomal enzyme nanoparticles and characterized them by AFM, SEM and TEM

with respect to their sizes. The nanoparticles prepared were in different size ranges as shown in the results and this difference in their sizes might possibly be due to the difference in their molecular masses.

Our results clearly establish the preparation of novel reagents (nanoparticles of PMC and lysosomal enzymes) that specifically interact with the MPR300 protein. Having established this, our future works aims at preparation of a variety of these nanoparticles in large scale and use them as efficient tools for tagging drugs/biomolecules which will have the capability of binding to the cell surface MPR300 protein and can be specifically delivered into the cells, thus making available the specific biomolecules/drugs in the cell. Furthermore, due to the presence of a wide variety of glycosidase enzymes in the cells, the nanoparticles would be biodegradable and would possibly not have any toxic effects to the cells.

The MPR300 expression is decreased in some malignancies such as the hepatocarcinoma. However, it's over expression in the majority of solid tumours particularly in breast cancers, indicates that this receptor could be considered as a tool for specific drug delivery through the nanoparticles that contain mannose 6-phosphate residues. Use of the different nanoparticles prepared in this study together with bioconjugation of specific anti-cancer drugs, in different cancer cell lines and their ability to interact with cell surface receptor should pave a new way to use the nanoparticles for their potential applications in treating diseases would be an independent study which is beyond the scope of the present investigation.

3.1. Introduction

Affinity chromatography is a method of selectively isolating macromolecules based on their biological specificity. A ligand is immobilized on to a solid support and the sample to be separated has biological affinity to the ligand. The interaction between the target protein and the ligand is not based on general properties of the biomolecule to be separated, such as the isoelectric point (pI) or hydrophobicity, which are more commonly used in adsorption chromatography, but exclusively dependent on the biological affinity/specificity. Some examples are the interaction of antibodies with antigens, enzymes with substrate analogues, nucleic acid with nucleic acid binding proteins, and hormones with receptors etc., (Paul Kutlar, 2004). To avoid any steric hindrance for the interaction to occur, normally the ligand is immobilized to a suitable matrix through a spacer arm which not only facilitates better interaction of the ligand with the biomolecule to be isolated but also allows a stable interaction between the two. Once prepared, the matrix can be used to purify its specific target from a suitable biological extract (Ostrove, 1990). An extract or partially purified sample containing the target protein is then passed through this affinity matrix on which the protein binds by virtue of its affinity for the ligand. Affinity chromatography to purify lectins exploits the selective binding between members of this class of proteins and particular carbohydrates. It has therefore found widespread use both in the isolation of glycoproteins and in removal of glycoprotein contaminants from other proteins. Following the development of methods for increased protein characterization including posttranslational modifications, the affinity isolation of proteins based on glyosylation **(**Caron *et al.,* 1998**)** or phosphorylation **(**Holmes and

Schiller 1997) is now possible. The matrix of choice should show physical and chemical properties required for adsorption chromatography techniques **(**Groman and Wilchek 1987 and Narayanan and Crane 1990**)**. Although the most commonly used matrix support is agarose, a range of other matrix supports, including silica and polyacrylamide are available. The matrix has to be activated with a reagent. Then a specific ligand can be coupled to the matrix and used for chromatography. Conventionally, agarose matrices are covalently coated with ligands and packed in chromatographic column, the source sample will be passed through the affinity matrix in the appropriate buffers and elution is achieved by varying the solvent conditions or introducing a solute that binds strongly either to the ligand or to the protein itself (Dean *et al.,* 1985).

In the current study an attempt was made, to replace the conventional column based nonmagnetic matrices with suspension form of affinity matrices having strong magnetic property. In this case a suspension of the ligand coated matrix particles is mixed, incubated with the source protein sample in the appropriate column buffer and after the binding and subsequent washing to remove unbound proteins, specific elution was done using a ligand and the eluted sample separated from the magnetite under the influence of strong magnetic field.

3.2. Materials and methods

3.2.1. Materials

$FeCl_3.6H_2O$ (SDFCL), Ethyleneglycol(SRL), 1,6-hexanediamine(Sigma Aldrich), Anhydrous sodium acetate (Qualigens), Imidazole (SRL), HCl (Qualigens), $MnCl_2$ (SRL), Triton X-100 (Sigma), Sodium chloride Qualigens, Sodium β-glycerophosphate (SRL), Mannose 6- phosphate, Divinyl sulfone (Sigma), $CaCl_2$,

PMSF, Lactose (SRL), BCIP/NBT substrate (Genei), MSC1 antibody (kindly provided by Prof.K.von Figura, Goettingen, Germany). All other chemicals and reagents were of analytical grade and procured from repute firms.

3.2.2. Methods

3.2.2.1 Synthesis of amine functionalized Fe_3O_4 nanoparticles

Magnetic nanoparticles with functional amine groups were synthesized by a method described earlier by Wang *et al*, (2006). In brief, 1.0 g of $FeCl_3.6H_2O$, 2.0 g of sodium acetate, 6.5 g of 1, 6-hexanediamine were added to 30 ml of ethylene glycol and stirred vigorously at 50^0C to get a clear solution. This solution was transferred to a Teflon-lined autoclave (50 ml) and incubated at 200^0C for 6 h. The magnetic nanoparticles were rinsed with deionised water and ethanol to effectively remove solvent, unbound 1, 6-hexanediamine. During each rinsing step, the nanoparticles were separated from the supernatant by using magnetic force. After washing, particles were dried at 50^oC before characterization and application.

3.2.2.2. Transmission Electron Microscopy (TEM)

A small drop of alcoholic suspension of the nanoparticles was dispersed on carbon-coated copper grids. Nanoparticles morphology was examined using the Transmission electron microscope (Model FEI Technai G2 S- Twin) operated at 100 kV.

3.2.2.3. Powder X-Ray diffraction (XRD)

X-ray diffraction (XRD) patterns were recorded from 10 to 80° using Cu Kα as the x-ray source (λ=1.54 Å); Bruker's AXS Model D8 Advance System was used to carry out the XRD experiments for identification of the crystal phase,

particles size and degree of agglomeration, chemical composition, and magnetic properties.

3.2.2.4. Raman spectroscopy

Raman spectra of the functionalized nanoparticles were recorded with micro-Raman (WiTec ALPHA 300 instrument) with a continuous wave Nd:YAG laser at 532 nm. Laser power of 40 mW was used. Acquisition time for recording was 5 s. Raman spectra were collected at 5 different position of the sample and their average has been considered. All spectra are calibrated with Raman peak of silicon wafer at 520 cm^{-1}. In the Micro Raman spectrometer laser beam was focused on to the sample using an objective lens (100X) and the theoretical beam waist estimated was ~700 nm. Raman signals were collected in back scattering geometry.

3.2.2.5. Hydrolysis of yeast O-phosphonomannan, separation of phosphomannan core (PMC) and pentamannosylphosphate (PMP)

O-Phosphonomannan (a generous gift from Dr. M.E. Slodki, USA) was hydrolyzed with acid and separated into larger fragment of phosphomannan core (PMC) and smaller fragment of pentamannosylphosphate (PMP) as described in chapter 2.

Preparation of phosphomannan affinity matrix and purification of MPR46 from goat liver

3.2.2.6. Activation of magnetite nanoparticles with Divinyl sulfone and coupling of phosphomannan core (PMC) to the activated magnetite

5 ml suspension of the amine functionalized nanoparticles were washed thoroughly with distilled water and suspended in 0.5M sodium carbonate/bicarbonate buffer pH11.0. 0.5 ml of divinyl sulfone was added and

the suspension was gently shaken at room temperature for 70 min and washed thoroughly with distilled water. The activated particles obtained above were washed with 0.5M sodium/bicarbonate buffer pH 10.0 and the particles were suspended in 5 ml carbonate buffer pH 10.0 containing 20 mg of PMC. Coupling was allowed to proceed in cold (4^{o}C) for 48h. At the end of coupling the particle pellet was washed with deionized water and suspended in 0.5M sodium bicarbonate buffer pH 8.5 containing 50 µl of β-mercaptoethanol and mixed at room temperature for 3 h. Finally, the particles were washed with distilled water and stored at 4^{o}C in column buffer until further use. The extent of PMC coupled to magnetite was determined as mannose equivalents as described (Dubois *et al*., 1956).

3.2.2.7. Preparation of acetone powder

Reagents required: (All steps were performed at 4^{o}C)

- 0.5M $CaCl_2$ and 1mM $NaHCO_3$
- 4 N acetic acid
- Chilled acetone
- Diethyl ether

Acetone powder was prepared following the protocol of Siva kumar (1996). Fresh goat liver tissue was homogenized in a waring blender with 1.6 volumes of 0.5 mM $CaCl_2$, 1 mM $NaHCO_3$ and pH was adjusted to 5.0 by the drop wise addition of 4N acetic acid. The suspension was centrifuged for 15 min at 9000 rpm and the pellet was resuspended in 2.4 volumes of the same buffer. The pH was adjusted to 5.0 and centrifuged at 9000 rpm for 15 min. The pellet was collected and homogenized for 1min in a waring blender with 6 volumes of

chilled acetone. The suspension was filtered rapidly through a Whatmann 3 MM filter paper placed over Buchner funnel. The reddish brown cake obtained was washed with cold diethylether and finally air dried. The dry powder obtained was stored at -80^{o}C until use.

3.2.2.8. Extraction of membrane proteins and purification of MPR46 (CD-MPR)

All operations mentioned below were performed at 4^{o}C.

Buffers used:

Buffer A: 50 mM imidazole-HCl, pH7.0, 150 mM NaCl, 0.5 mM $CaCl_2$ and 0.1mM PMSF

Buffer B: 50 mM sodium acetate, pH 4.6, 150 mM NaCl and 0.5mM $CaCl_2$.

Buffer C: 50 mM imidazole-HCl, pH7.0, 5 mM β-glycerophosphate and 150 mM NaCl.

Buffer D: Buffer C containing 0.05% Triton X-100 and 2 mM PMSF.

Buffer E: Buffer C containing 0.05% Triton X-100 and 10 mM $CaCl_2$.

The fresh acetone powder prepared above was homogenized with 6 volumes of buffer A in a waring blender and stirred overnight. The suspension was centrifuged at 9000 rpm for 15 min and the supernatant discarded. The pellet obtained was suspended in 6 volumes of buffer B, homogenized and centrifuged as above. The pellet was suspended in 6 volumes of buffer C, homogenized and the suspension was kept stirring in cold to which deoxycholate and Triton X-100 were added to a final concentration of 0.1% and 1% respectively. The suspension was allowed to stir overnight. The suspension was then centrifuged

at 4000 rpm for 15 min, the clear supernatant containing the membrane proteins was collected and 10 mM $MnCl_2$ was added. This served as the source for purifying MPR46. The suspension obtained was stirred for 60 min and centrifuged at 9000 rpm for 45 min. The clear supernatant obtained was then subjected to affinity chromatography employing appropriate buffers as given below.

3.2.2.9. Purification of MPR46 by magnetic affinity chromatography

The membrane proteins extracted above were mixed with Fe_3O_4-NH_2-PMC nanoparticles that were equilibrated with buffer E for the purification of MPR46 alone and the suspension (40 ml) was kept for rotation at 4^oC overnight. The following day the magnetite nanoparticles were subjected to magnetic separation using a strong magnet and the unbound protein sample was discarded. The magnetic pellet was washed extensively with the buffer E several times. Elution was performed with 5 mM mannose 6-phosphate in buffer E.

3.2.2.10. SDS-PAGE and western blotting

Protein concentrations in the extracted samples and column fractions were determined by the dye-binding method using bovine serum albumin (1.0 mg/mL) as a standard (Bradford, 1976). 10% SDS-PAGE analysis was carried out for eluates according to Laemmli (1970) in the presence of reducing agent. The proteins were boiled for five minutes in the SDS sample buffer, separated on the gel and were detected by silver staining. The authenticity of the purified receptor was analyzed by western blot experiment with MSC1 (MSC1-antibody-recognizes MPR46 from molluscs to mammals and it is MPR46 c-tail peptide

antibody). The MPR46 band was detected by incubating the membrane with the secondary antibody (goat anti-rabbit IgG conjugated to alkaline phosphatase) followed by incubation with the substrate, BCIP/NBT (Bangalore Genei, India).

Preparation of Lactose affinity gel and purification of lactose specific lectin from Unio

3.2.2.11. Activation of magnetite nanoparticles with Divinyl sulfone and Coupling of lactose to the activated magnetite

Lactose was coupled to the activated magnetite nanostructures as described above for the PMC coated magnetite nanoparticles, except that 0.5g of lactose was coupled to divinyl sulfone activated magnetic nanoparticles.

3.2.2.12. Extraction and isolation of lactose specific lectin from Unio

All operations were carried out at 4^{o}C unless otherwise stated. Fifty grams of the whole animal tissue was homogenized with 4 volumes (200 mL) of 25 mM Tris-HCl, pH 7.0 (buffer A). The homogenate was stirred overnight and centrifuged (12,000 rpm, 30 min; 4^{o}C). The pellet was discarded and the supernatant (200 ml) was recentrifuged for 15 min. The supernatant was subjected to 0-80% ammonium sulphate fractionation for 3h at 4^{o}C and was centrifuged at 12,000 rpm for 30 min. The pellet was collected, dissolved in minimum volume of 25 mM Tris buffered saline (buffer B) and dialyzed against the same buffer. 50 ml of the soluble extract was mixed with lactose coupled magnetite nanoparticles and kept for rotation at 4^{o}C overnight. The following day the particles were separated from the sample by applying magnetic field, washed with buffer B and the bound lectin was eluted with 0.2M lactose in buffer B.

3.2.2.13. SDS-PAGE and western blotting

Protein concentration in the extracted sample was determined as described above. The purified lactose specific lectin by magnetite nanostructure was analysed in 10% SDS-PAGE according to Laemmli (1970) and proteins were detected by silver staining. The authenticity of the purified lactose lectin was analyzed by western blot with lactose-specific lectin antibody. The lectin bands were detected by incubating the membrane with secondary antibody (goat anti-rabbit IgG conjugated to alkaline phosphatase) followed by incubation with the substrate, BCIP/NBT (Bangalore Genei, India)

3.3. Results

3.3.1. Preparation of amine-functionalized magnetic nanoparticles

Amine functionalized magnetite nanoparticles could be successfully prepared by facile one Pot synthesis using $FeCl_3.6H_2O$ as a sole iron source and 1, 6-hexanediamine as a source of amine groups.

3.3.2. Characterization of amine-functionalized magnetic nanoparticles

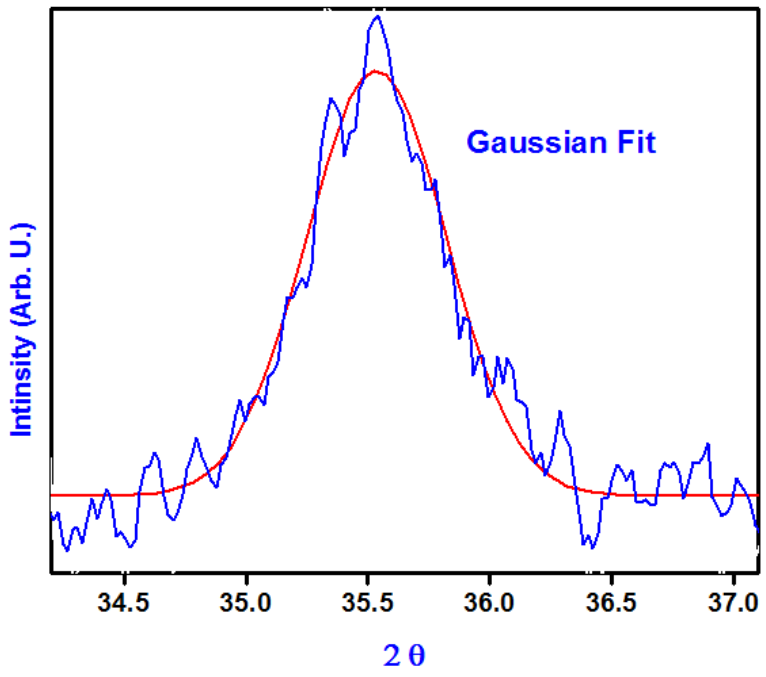
As part of characterization transmission electron microscopy, X-ray diffraction and Raman spectroscopy experiments were done

3.3.2.1. Transmission Electron Microscopy (TEM)

A small drop of alcoholic suspension of the nanoparticles was dispersed on carbon-coated copper grids. Nanoparticles morphology was examined using the Transmission electron microscope (Model FEI Technai G2 S- Twin) operated at 100 kV (Figure.3.4.1.)

3.3.2.2. Powder X-Ray diffraction (XRD)

X-ray diffraction (XRD) patterns were recorded from 10 to 80° using Cu Kα as the x-ray source (λ=1.54 Å); Bruker's AXS Model D8 Advance System was used to carry out the XRD experiments for identification of the crystal phase, particles size and degree of agglomeration, chemical composition, and magnetic properties. X-Ray Diffraction pattern of the prepared Magnetite (Fe_3O_4-NH_2) nanoparticles is shown in Figure 3.4.2. The peak positions appear at 30.3°, 35.5°, 43.3°, 57.1° and 62.8° can be indexed as (220), (311), (400), (510), (440) crystal planes of Fe_3O_4 respectively. From the XRD data, we can estimate the particles size using a Debye- Scherrer equation. Calculations start by drawing the Gaussian fitting for the highest intensity peak and measuring the values of (2θ) and peak width. The analysis is performed using Origin 6 software and the results are d= 2.55 which are in good agreement with the diameters measured from the TEM images.



B(2θ) = 0.57409 (taken at one point on graph)

K= 0.9

2θ = 35.534

λ = 1.54 nm.

$$d = \frac{k\lambda}{B(2\theta).Cos\theta} = \frac{0.9 * 1.54}{0.57 * \cos(17.76)} = 2.55\ nm$$

Where B(2θ) is the peak width and can be determined using Gaussian curve. However, the constant of proportionality, K (the Scherrer constant) depends on how the width is determined, the shape of the crystal, and the size distribution and usually taken between 0.9-0.94). Finally λ=1.54nm (constant)

3.3.2.3. Raman spectroscopy

Raman spectra of the functionalised nanoparticles were recorded with micro-Raman (WiTec ALPHA 300 instrument) with a continuous wave Nd:YAG laser at 532 nm (Fig.3.4.3.) Laser power of 40 mW was used. Acquisition time for recording was 5 s. Raman spectra were collected at 5 different position of the sample and their average has been considered. As shown in Figure.3.4.3, the peaks correspond to that of the Fe_3O_4 and NH_2 groups.

3.3.3. Affinity purification of goat liver MPR46 using Magnetite nanoparticles derivatized with PMC

The membrane extract (40 ml) from the goat liver tissue was subjected to magnetic affinity separation in the presence of 10 mM $MnCl_2$ for the purification of MPR46 alone as described under methods. Scheme for the purification is shown in Fig.3.4.4. After washing the particles extensively, the bound protein was eluted with 5 mM mannose 6-phosphate in column buffer. From 0.52

mg/ml of crude extract, 0.006 mg of purified receptor could be obtained. The eluted protein was concentrated using centricon concentrator and was analyzed by 10% SDS gel electrophoresis which revealed a band with a molecular mass of ~ 46 kDa, representing the MPR 46 protein (Fig.3.4.5. A and B). The authenticity of the receptor was further confirmed by using an antibody MSC1 that specifically recognizes MPR46 protein from mollusc to mammals.

3.3.4. Affinity purification of lactose specific lectin from Unio using Magnetite – lactose gel

The soluble extract (50 ml) of *Unio* tissue was subjected to magnetic affinity separation in 25 mM Tris buffered saline. Scheme for the purification is shown in Fig.3.4.4. After washing the particles extensively, the bound protein was eluted with 0.2M lactose in the same buffer. From 1.6mg/ml of crude extract, 0.05mg of purified lectin could be obtained. The eluted protein was concentrated using centricon concentrator and was electrophoresed on 10% SDS gel electrophoresis (Fig.3.4.6. A and B).

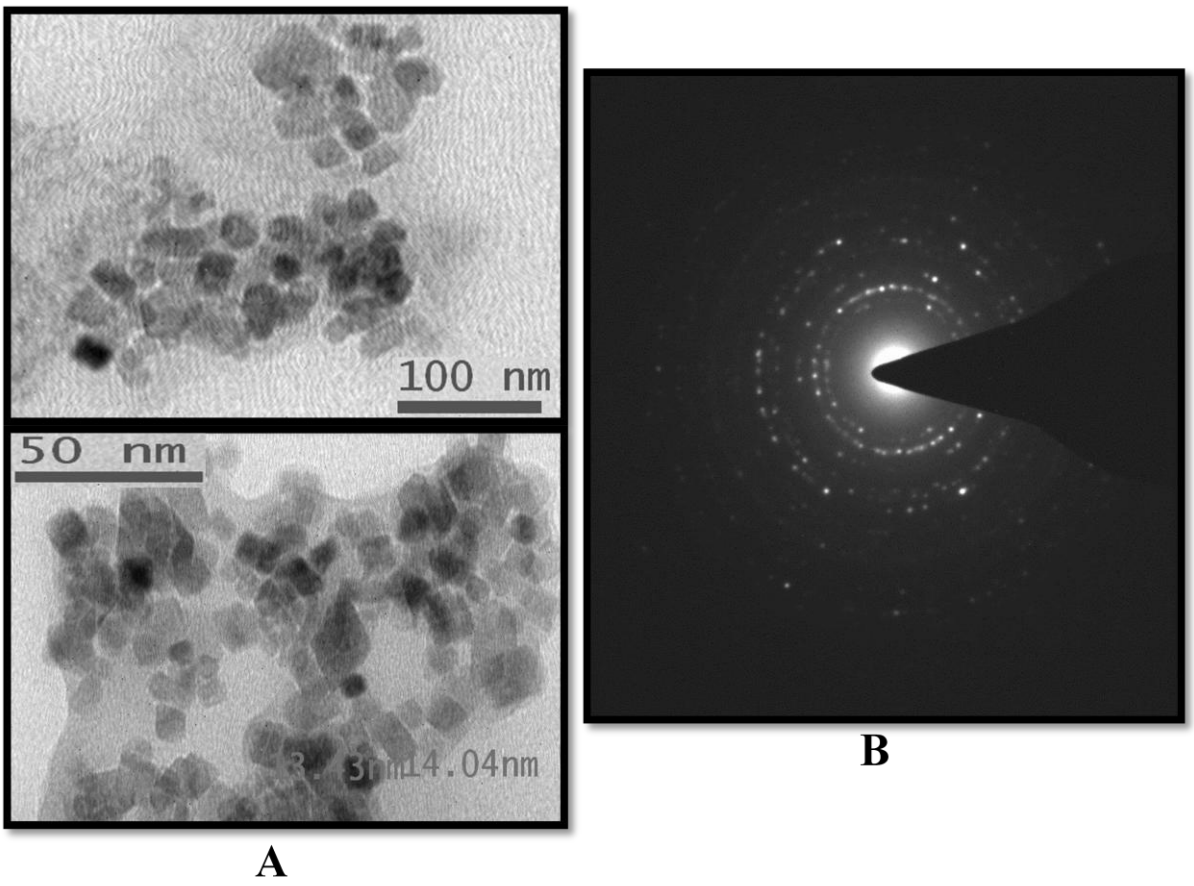


Figure.3.4.1. TEM images of the amine functionalized magnetite (Fe_3O_4-NH_2) nanoparticles with different sizes (A) and Diffraction pattern in TEM (B), size of the particles 10-25nm.

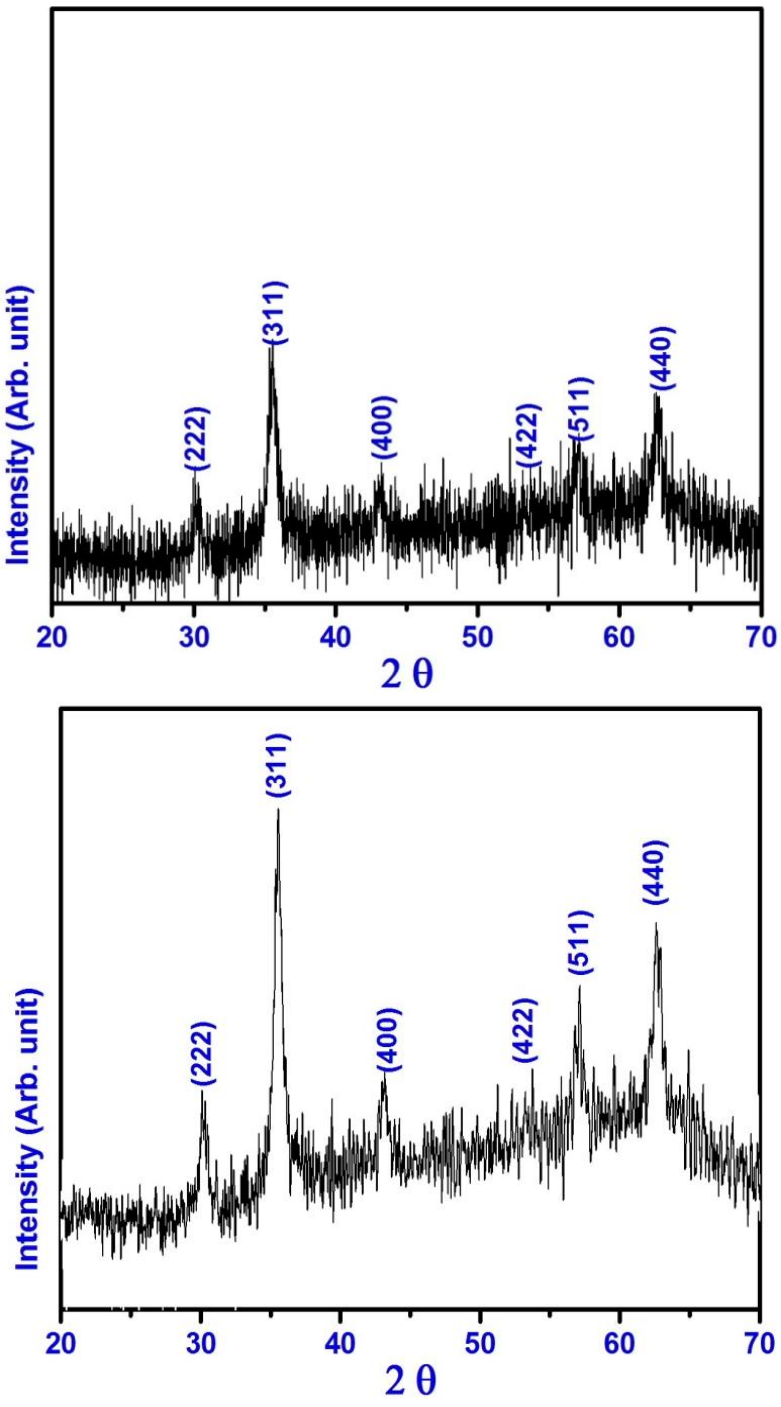


Figure.3.4.2. Powder X-Ray diffraction pattern of amine functionalized Fe_3O_4nanoparticles. (The peaks correspond to that of magnetite)

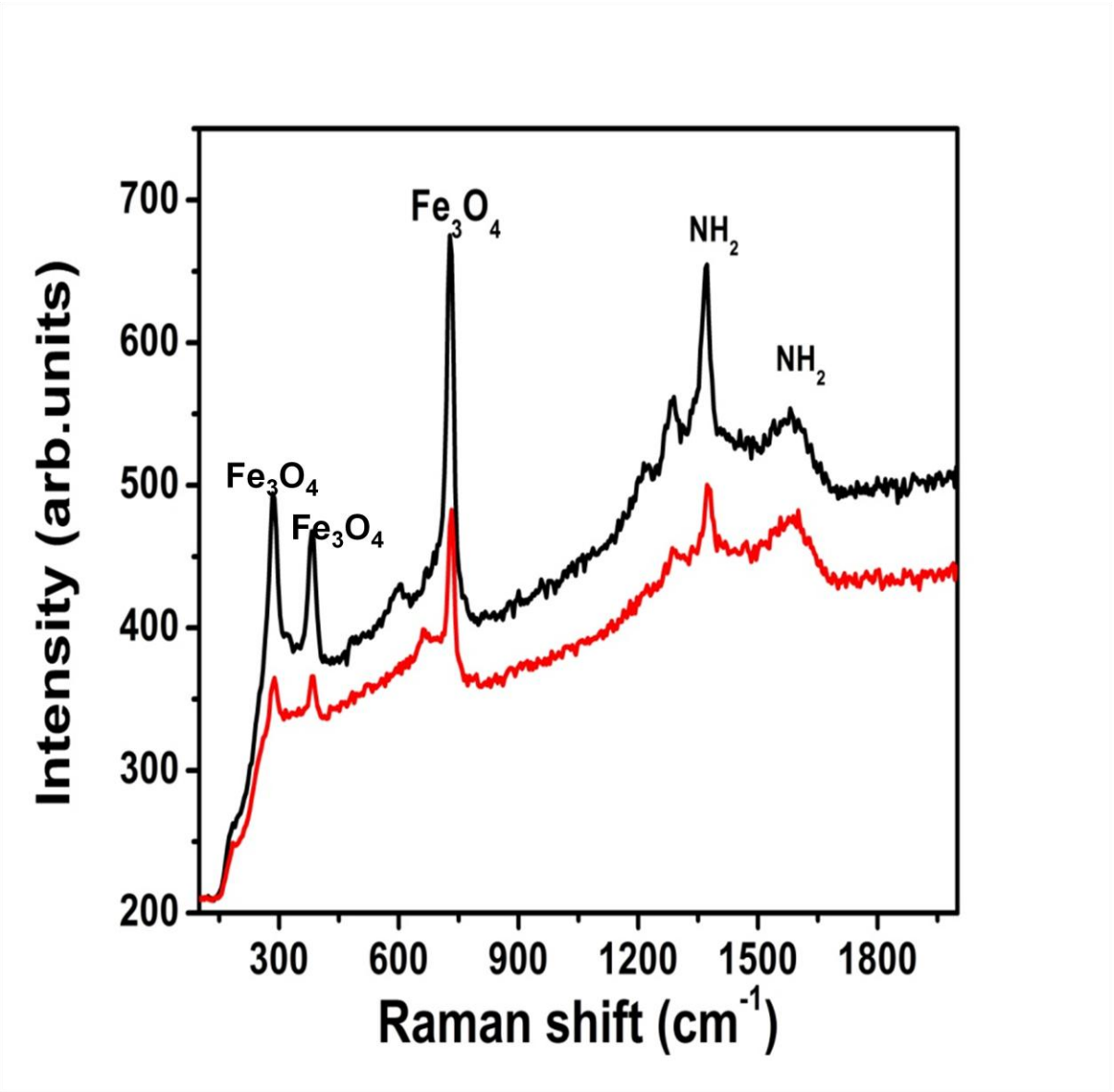


Figure.3.4.3. Raman spectroscopy of amine terminated magnetite (Fe_3O_4) nanoparticles

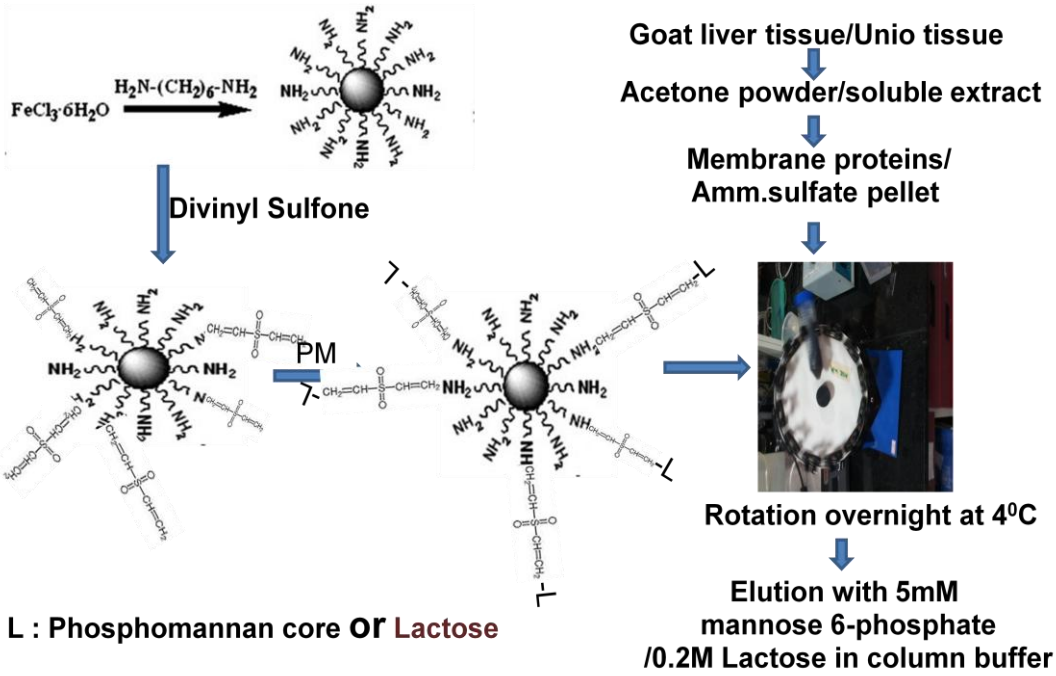


Figure.3.4.4. Schematic representation of the steps involved in the magnetic affinity purification of MPR 46 from goat liver and Lactose-specific lectin from Unio

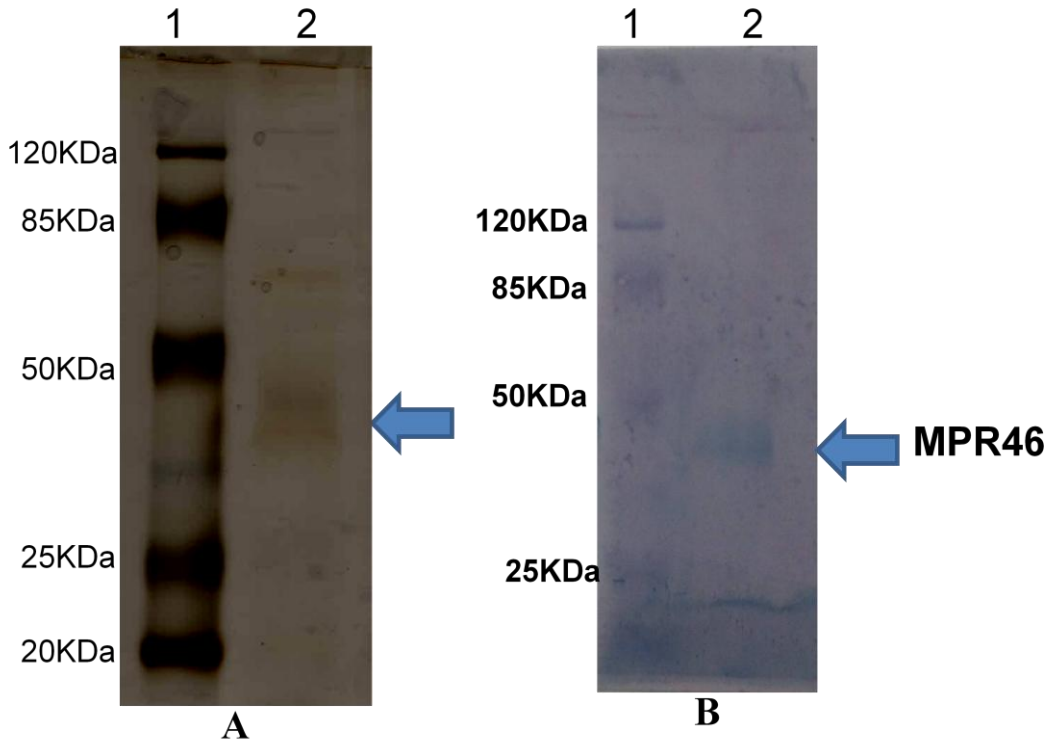


Figure.3.4.5. SDS-PAGE and western blotting of purified MPR46, (A) Purified MPR46/CD-MPR was separated on 10% SDS-PAGE under reducing conditions, followed by silver staining, **(B)** Immunoblot of MPR46 with MSC1 antibody. (Lane1: Prestained marker, Lane 2: MPR46/CD-MPR).

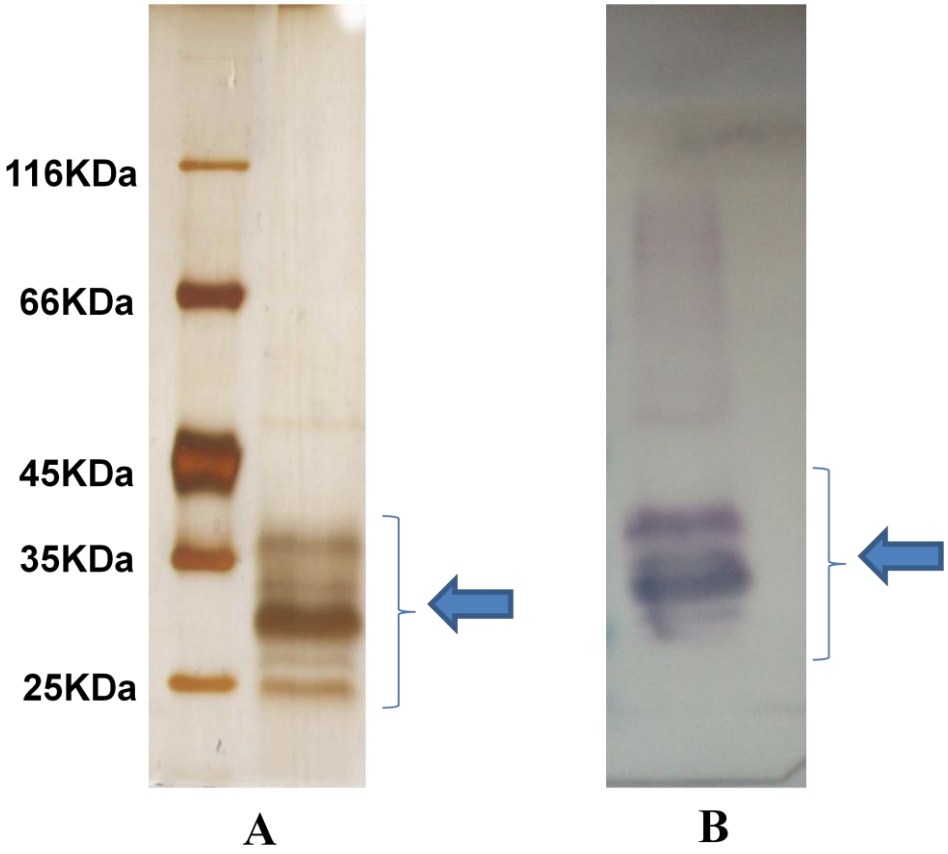


Figure. 3.4.6. SDS-PAGE and western blotting of purified lactose specific lectin, (A) Purified lectin was separated on 10% SDS-PAGE under reducing conditions, followed by silver staining, **(B)** Immunoblot of lectin with antibody. (Lane1: marker, Lane 2: lactose lectin).

3.5. Discussion

Nanoparticles have recently sparked many applications in various fields of biology and medicine (Wang *et al.,* 2003). Magnetic particles are widely studied and have applications in various fields of biology and medicine such as magnetic resonance imaging (Perez et al., 2002), immunoassays, RNA and DNA isolation (Neuberger *et al.,* 2005), waste water treatment (Khodabakhshi *et al.,* 2011, Liu *et al.,* 2009) etc., Most promising nanoparticles for these applications are superparamagnetic nanoparticles based on a core consisting of iron oxide (SPION) that can be contacted through external magnets. SPION is coated with biocompatible materials and can be functionalized with drugs, proteins or plasmids (Neuberger *et al.,* 2005). Iron oxide nanoparticles are the particles with diameters between 1 and 100 nm. The two main forms are magnetite (Fe_3O_4) and its oxidized form maghemite (γ-Fe_2O_3). Among these two forms, magnetite is more magnetic. They have attracted extensive interest due to their super magnetic properties and potential applications in many fields. Applications of magnetic nanoparticles are not limited to traditional electrical, optical and magnetic areas but also expand to some new applications, including magnetically assisted bioseparation and biocatalysis (Yu *et al.,* 2009).

Surface functionalization of nanomaterials is crucial for bioapplication (Chan *et al.,* 1998, Bruchez et al., 1998, Robinson et al., 2005, Grancharov *et al.,* 2005). Modification of the surface of organic molecules is an important event that will allow stabilization of the nanoparticles in biological samples both in terms of pH (7.4) and high salt concentration. The available surface molecules can be further derivatized (Neuberger et al., 2005). Proteins are the central elements of cell machinery and their study is essential for better understanding of the cellular

functions. Protein purification has been a fundamental requisite in advances made in biotechnology. The potentiality of these prepared matrices can be ascertained by their ability to purify proteins. In some traditional methods more than one step is required for protein purification and in biotechnology, affinity protein purification using antibody-based separation or a matrix with specific tags for binding target protein are commonly used methods. The challenge is to use a common matrix for purification of different proteins. Magnetic nanoparticles can be used as a novel, versatile and quick method for the efficient capture of selected target biomolecules in the presence of other suspended solids even for a small sample volume. The use of magnetic nanoparticles such as superparamagnetic iron oxide nanoparticles for purification of biomolecules in general and proteins in particular have many advantages such as high surface area to volume ratio which allows the efficient binding of desired protein to the surface of the ligand coated particles and get separated using a simple magnetic field (Okoli *et al.,* 2011). In the current study, magnetic nanocrystals were functionalized with amino groups by facile one-pot synthetic process (Wang *et al.,* 2006) and specific ligand coupled affinity matrices with Fe_3O_4-NH_2 as the core were prepared and investigated their ability to purify two biologically important and well characterized proteins i.e. cation Dependent mannose 6-phosphate receptor (MPR46/CD-MPR) of goat liver and a lactose specific lectin from *Unio* (a mollusc) with a long term objective to use these materials for the affinity purification of different proteins of our interest in future. Currently Seralose and affigel based affinity matrices are under use for purifying glycosidases and different lectins from various sources in our laboratory.

A new method to purify MPR46/CD-MPR and a lactose specific lectin from goat liver and *Unio* tissue respectively using magnetic nanoparticles is reported. The method is based on ligand coated on amine functionalized magnetic nanoparticles prepared from $FeCl_3.6H_2O$, that are able to capture proteins in a simple and efficient way and which could promote large-scale purification. Both the target proteins were captured onto the magnetic nanoparticles via a ligand interaction mechanism. In contrast to the conventional method, purification of both the proteins suggests a simple and cost effective method for purifying target proteins. It is proposed that this method be extended to the purification of proteins with similar characteristics and possibly other biomolecules.

4.1. Introduction

O-Phosphomannan is composed of a highly branched high molecular weight phopshomannan core (PMC) and penta mannosyl phosphate (PMP) (Jeans, *et al.,* 1961). Both PMC and PMP have been used in the affinity purification of mannose 6-phosphate receptors (Lesley *et al.,* 1996, Siva Kumar, 1996, Nadimpalli and Amancha, 2010, Yadavalli *et al.,* 2009, Nadimpalli and von Figura 2002, Yerramalla and Nadimpalli, 1996). Antibodies to purified proteins, receptors and lysosomal enzymes have been used extensively to understand the functions of lysosomal enzymes and their sorting receptors MPR300 and MPR46 (Merino and Nadimpalli, 2012 and the references therein). In an earlier study mannose 6-phosphate receptors from different tissues were quantified using the affinity purified receptor specific antibodies by an ELISA method (Suresh *et al.,* 2002).

The common feature of all lysosomal enzymes is the presence of phosphorylated oligosaccharide attached to the enzyme which is recognized by the MPR proteins that facilitates their transport within the cell or internalization by the MPR300 protein from the cell surface into the cells. However there has been no report on the development of antibodies to these phosphorylated manno oligosaccaharides. Availability of such an antibody would be important to not only screen for lysosomal enzymes but also phosphorylated mannose residues containing glycoproteins/oligosaccharides. This part of the study was therefore initiated to develop polyclonal antibodies for these ligands, affinity purify the specific IgG and study their specific interaction with the receptors and lysosomal enzymes.

4.2. Materials and methods

4.2.1 Materials

O-Phosphonomannan was a generous gift from Dr. Slodki, USDA, IL and USA, Affigel-10 (Bio-Rad laboratories), p-nitro phenyl phosphate, Manganese chloride (SRL), Imidazole, Sepharose-4B, Acrylamide, Bisacrylamide (SRL), BSA, HEPES, Tween-20, TritonX-100, Divinyl sulfone, Freund's complete/incomplete adjuvant, Mannose 6-phosphate(Sigma Aldrich), Acetone, Acetic acid, Calcium chloride, EDTA, Formaldehyde, Glycine (Qualigens), 96 well microtiter plates (Himedia). Bovine DNase 1 and bovine liver lysosomal β-galactosidase were purchased from Sigma Aldrich. All other chemicals and reagents used in the study were of high quality and purchased from reputed firms.

4.2.2. Methods

4.2.2.1. Hydrolysis of yeast O-phosphonomannan and separation of PMC and PMP

This experiment was done as described in chapter 2. The acid hydrolysed components were lyophilized separately and stored at 4^0C until further use.

4.2.2.2. Raising antibodies for PMC and PMP

Rabbits were housed and handled at the University of Hyderabad animal house. Polyclonal antiserum was raised against PMC and PMP in two separate rabbits. 2 mg of PMC in 1 ml 1xPBS buffer pH7.4 (buffer A) was mixed with 1.0 ml Freund's complete adjuvant and injected subcutaneously into a rabbit. After three weeks, a booster injection was given with 2 mg of the PMC in 0.5 ml buffer A mixed with 1 ml Freund's incomplete adjuvant. Two weeks after the booster injection, blood was collected by ear vein puncture from the rabbit into a falcon tube and allowed to clot at room temperature. Antiserum was collected by

centrifugation and stored at −20 °C. Similarly a polyclonal antibody was raised against PMP after it was conjugated with hemocyanin. Typically penta mannosyl phosphate (hapten) and haemocyanin (carrier protein) were mixed in 1:2 ratio in bicarbonate buffer of pH 11.0 and coupled using divinyl sulfone as a coupling agent overnight on a rotator and the preparation (immunogen) was dialyzed against buffer A. 0.5 ml of the above preparation in buffer A was mixed with 1.0 ml Freund's complete adjuvant and injected subcutaneously into a rabbit following the protocol described for PMC. Two weeks after the booster injection, blood was collected by ear vein puncture from the rabbit into a falcon tube; serum was collected by centrifugation and stored at −20 °C.

4.2.2.3. Preparation of phosphomannan core-divinyl sulfone-Seralose, PMP-divinyl sulfone-Seralose affinity matrices and affinity purification of antibodies

The affinity matrices containing PMC and PMP were prepared separately as described earlier (Siva Kumar *et al*., 1997). The antisera collected for PMC and PMP hemocyanin was passed through respective columns that were pre-equilibrated with 25 mM Tris Buffered Saline, pH 7.4 (buffer B) several times and the bound antibody was eluted with 100 mM Glycine-HCl buffer pH 2.65 (buffer C). The eluates were immediately neutralized with 2 M Tris, concentrated by centricon and stored at 4^0C.

4.2.2.4. SDS-PAGE and Western blot analysis

To check the purity of PMC and PMP specific IgGs, an aliquot of the eluate was subjected to 7.5% SDS-PAGE under non reducing conditions and specific IgG was identified by silver staining. In a separate experiment PMP-hemocyanin was electrophoresed on a 7.5% SDS-PAGE under non reducing conditions and

the conjugate transferred to a nitrocellulose membrane. The membrane was incubated with the purified PMP antibody. The blot was probed by incubating the membrane with goat anti rabbit IgG-ALP (Genei, Bangalore) and developed with BCIP/NBT substrate

4.2.2.5. Immunoprecipitation

To confirm the specificity of the purified antibody, aliquot of the PMP-hemocyanin was taken in two separate tubes and incubated with pre immune serum (control) and affinity purified PMP-hemocyanin (10µg) respectively overnight at 4°C with rotation in buffer A containing 0.05%Tween 20 (PBST) making a total volume of 500µL. The antigen-antibody complexes were adsorbed to protein A-agarose beads (Genei, India) and were incubated for 1 h. Beads were collected by centrifugation at 3502xg and the supernatants were discarded carefully. The pellet was washed extensively with PBST. The immunoprecipitate was solubilised under reducing conditions, analyzed by SDS-PAGE and silver stained. Since PMC is a high molecular weight polysaccharide unlike PMP-hemocyanin (PMP and protein conjugate) immunoprecipitation could not be done**.**

4.2.2.6. Ligand blot analysis of starfish MPR300 with PMC and PMP

The specificity of the antigens used was studied in a ligand blot assay using starfish MPR 300 protein. The purified MPR300 from starfish was separated on SDS-PAGE under reducing conditions in two separate lanes and the proteins transferred to a nitrocellulose membrane. The membrane was cut and each lane containing the receptor was separately incubated for 16 h with PMC and PMP-hemocyanin (100µg) in blocking buffer (PBST containing 3% BSA) and the blot

processed as described in earlier chapter. Subsequently the membrane strips were probed with purified PMC and PMP antibodies (10µg). The receptor was visualized by incubating the membrane with goat-anti rabbit ALP conjugate and developed using BCIP/NBT substrate (Bangalore Genei).

4.2.2.7. Preparation of the total membrane extracts from Goat liver tissue

The whole animal tissue was used to prepare the acetone powder as described earlier (Siva Kumar *et al*., 1997). All operations were carried out at 4^{o}C. 50 g of the acetone powder prepared (as described in chapter 3). After processing the clear membrane extract was used for quantification by ELISA method.

4.2.2.8. Quantification of Mannose 6-phosphate receptors (MPR300&MPR46) by ELISA method

Enzyme linked immunosorbent assay is commonly used procedure for quantifying protein antigens and antibodies. As the negatively charged polysaccharides do not attach to the microtiter plates, they were first coated by Poly L-lysine at a concentration of 5µg/ml in buffer A at 28^{0}C, 4h (Maija and Frasch 1982). Subsequently wells were coated with PMC and PMP at a concentration of 2 µg/ml in buffer A overnight at 28^{0}C. Wells were washed with washing buffer (0.01% Tween-20 in buffer A) and were incubated with the membrane protein extract (goat liver) in buffer A for 2h at 37^{0}C in the concentration range of 1µg to 5µg per well. After the wells were blocked, they were incubated with PMC and PMP in blocking buffer (1%BSA in buffer A, 250ng of affinity-purified antibody in blocking buffer) at 37^{0}C. Wells were incubated with the respective affinity purified primary antibodies and subsequently with ALP conjugated secondary antibody and then with substrate

p-nitro phenyl phosphate (PNPP, 1mg/ml in 0.1M Tris-HCl, 0.1M NaCl, 2mM MgCl2, pH9.5) for 40 minutes and the absorbance measured at 405 nm.

4.3. Results

4.3.1. Affinity purification of anti-PMC and anti-PMP antibodies

In the present study, PMC and PMP were used to generate polyclonal antibodies separately. PMC and PMP specific IgGs were obtained from the antiserum developed by passing the antiserum on the Seralose-PMC and PMP affinity gels respectively as described under methods. Elution profiles of both PMC and PMP specific IgGs from are shown in Fig 4.4.1[A] and Fig.4.4.2 [A]. To check the homogeneity of the specific IgGs eluted from the gels, an aliquot of the eluates was subjected to 7.5% SDS-PAGE under non-reducing conditions (lane 2 from Fig 4.4.1[B] and Fig.4.4.2 [B]). From 20 ml of the PMC antiserum 3.9 mg antibody was obtained and from 10 ml of the PMP antiserum 0.55 mg antibody was obtained. The specificity of both the purified antibodies for the respective antigens could be confirmed by their ability to bind to the respective antigen-Seralose matrices. The specificity of the PMP-hemocyanin antibody obtained was further tested in a western blot experiment. From Fig 4.4.3[A], it is apparent that the PMP-hemocyanin antibody recognizes the antigen. PMP-hemocyanin conjugate was immunoprecipitated using the PMP specific antibody as described under methods. The precipitated protein was then subjected to SDS-PAGE and visualized by Western blotting as shown in Fig.4.4.3 [B]. Under the same condition pre-immune serum did not show any reactivity with the antigen suggesting the immunoprecipitation to be specific.

4.3.2. Ligand blot analysis of starfish MPR300 with PMC and PMP

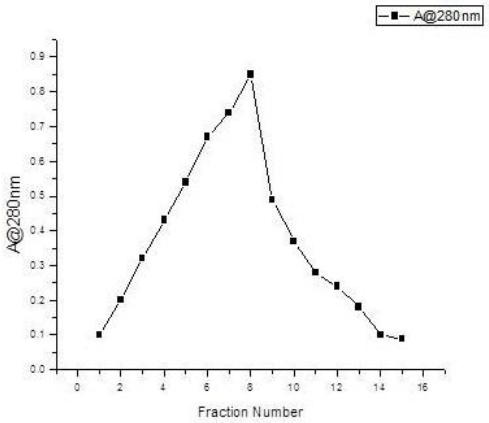
Specific interaction of the antigens to the antibodies was also analyzed in a ligand blot assay as described under methods. The membrane strips containing the proteins was probed with PMC and PMP separately , followed by incubation with goat anti rabbit-ALP secondary antibody, washed and developed with BCIP/NBT reagent. Protein bands were visualized showing a molecular mass of 300 kDa. From Fig 4.4.4. (A) with PMC and (B) with PMP, it is apparent that the band representing starfish MPR 300 showed binding to PMC and PMP. This suggests that there is an interaction between the receptor and the antigens PMC and PMP.

4.3.3. Detection of mannose 6-phosphate (M6P) containing ligands with PMC specific IgG

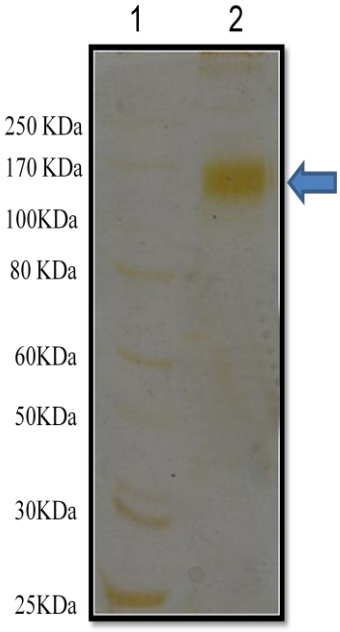
The antibodies which were developed in the present study are specific for the mannose 6-phosphate oligosaccharides. So to further elucidate the applications of the purified antibodies, we have chosen a few ligands that are known to contain mannose 6-phosphate residues, such as bovine DNase1, β-galactosidase from bovine liver (commercially procured), β-hexosaminidase from secretions of mouse cell lines devoid of the mannose 6-phosphate receptors and immunoblotted with the PMC specific IgG as shown in Fig.4.4.5 and Fig.4.4.6. The M6P containing proteins could be successfully detected. In another experiment the cross-reactivity of the PMC antibody with the PMP-hemocyanin conjugate was investigated in an immunoblot to further support our earlier findings with the M6P containing enzymes and we could see the cross-reactivity of PMC specific antibody with PMP-hemocyanin conjugate (Fig.4.4.7).

4.3.4. Mannose 6-phosphate receptors (MPR300&MPR46) quantification by ELISA

Using the affinity purified antibodies; an ELISA method was developed to quantify the mannose 6-phosphate receptors from goat liver tissue. The crude receptor was sandwiched between the PMC and PMP in an ELISA experiment where the receptor bound ligands (i.e. PMC and PMP) could be detected by the respective affinity purified antibodies as shown in Fig.4.4.8.[A]-ELISA with PMC and [B] ELISA with PMP

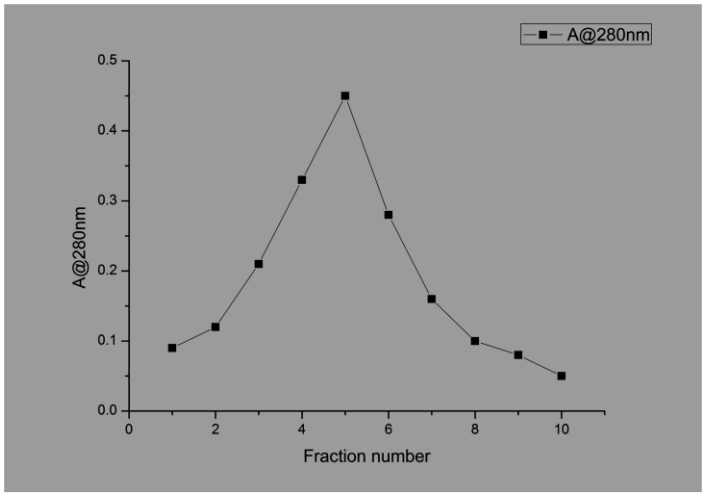


A

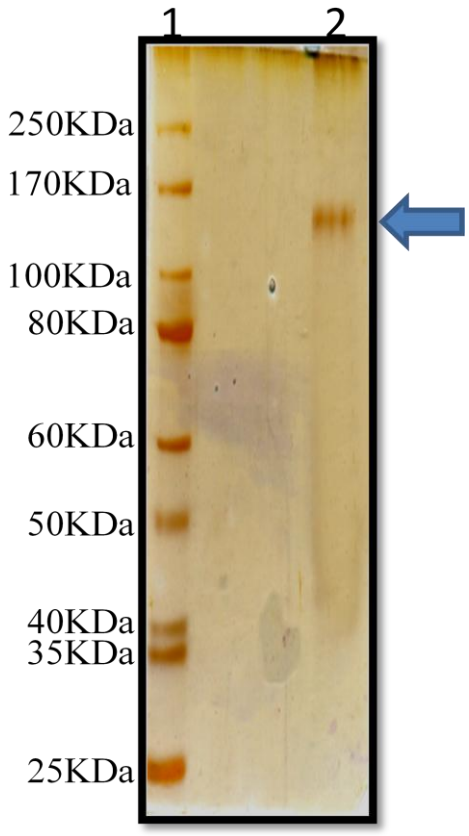


B

Figure.4.4.1. Purification of PMC specific IgG.
(A) Elution profile of PMC specific IgG from PMC-Seralose gel. **(B)** 7.5% SDS-PAGE analysis (non-reducing conditions) of PMC specific IgG eluted from PMC-Seralose gel column with 100 mM glycine-HCl buffer, pH 2.65. Arrow indicates IgG band.



A



B

Figure.4.4.2. Purification of PMP specific IgG.
(A) Elution profile of PMP specific IgG from PMP-Seralose gel. **(B)** 7.5% SDS-PAGE analysis (non-reducing conditions) of PMP specific IgG eluted from PMP-Seralose gel column with 100 mM glycine-HCl buffer, pH 2.65. Arrow indicates IgG band

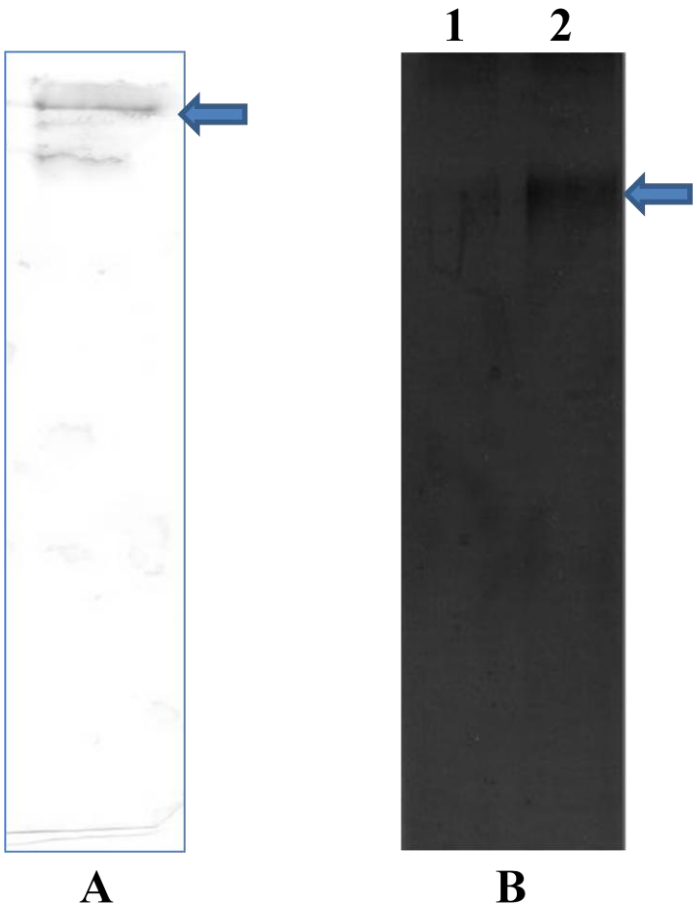


Figure.4.4.3. Interaction of the purified PMP-IgG with PMP-hemocyanin (A) Immunoblotting of the PMP-hemocyanin. **(B)** Immunoprecipitation of PMP-hemocyanin Lane1, PMP-hemocyanin and pre-immune serum, Lane 2, PMP-hemocyanin and immune serum.

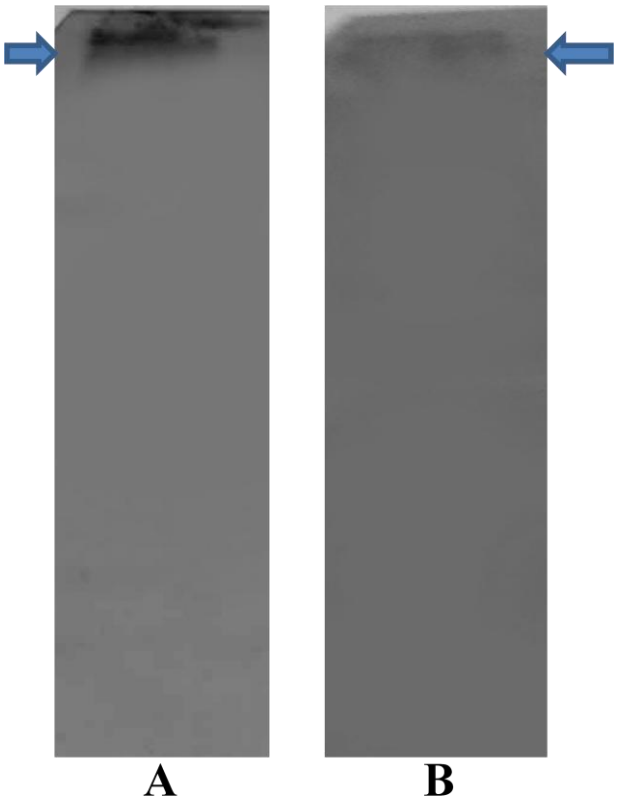


Figure.4.4.4. Ligand blotting of starfish MPR300 with (A) PMC and (B) PMP. Starfish MPR 300 was transferred to PVDF membrane, incubated with PMC and PMP followed by detection with the specific antibody as described under methods.

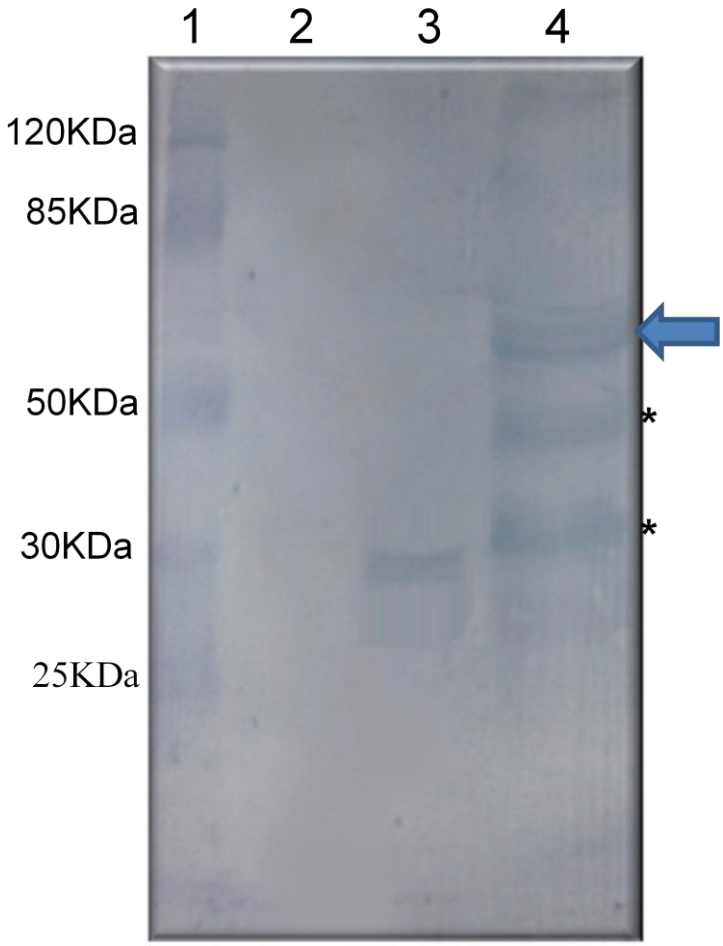


Figure.4.4.5. Blotting of mannose 6-phosphate containing ligands with PMC antibody. Lane 1: Marker, Lane 2: BSA, Lane 3: Bovine DNase1, Lane 4: β-galactosidase, *-Possible degraded protein.

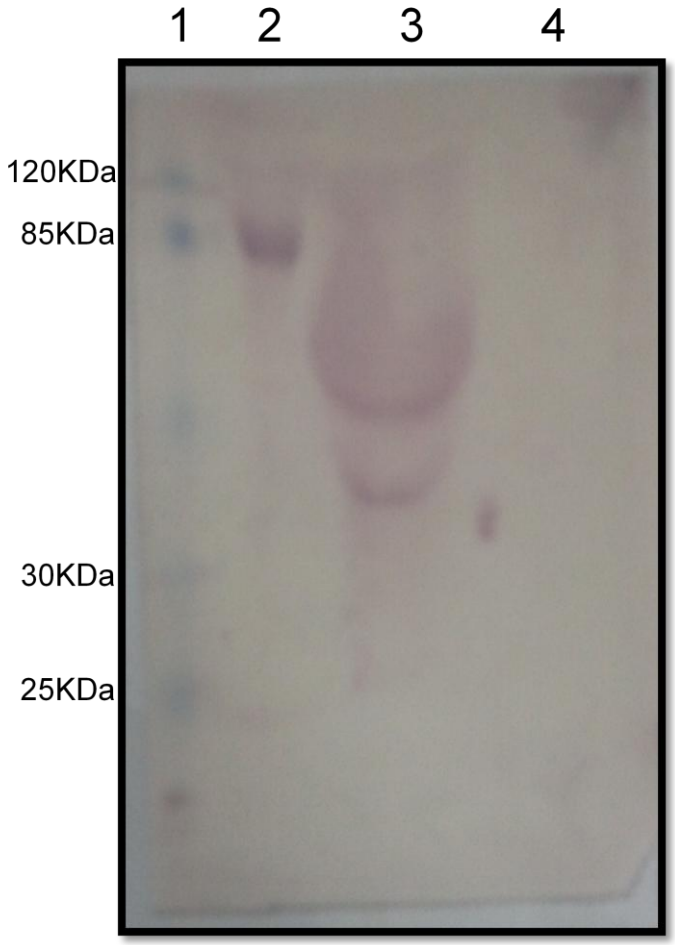
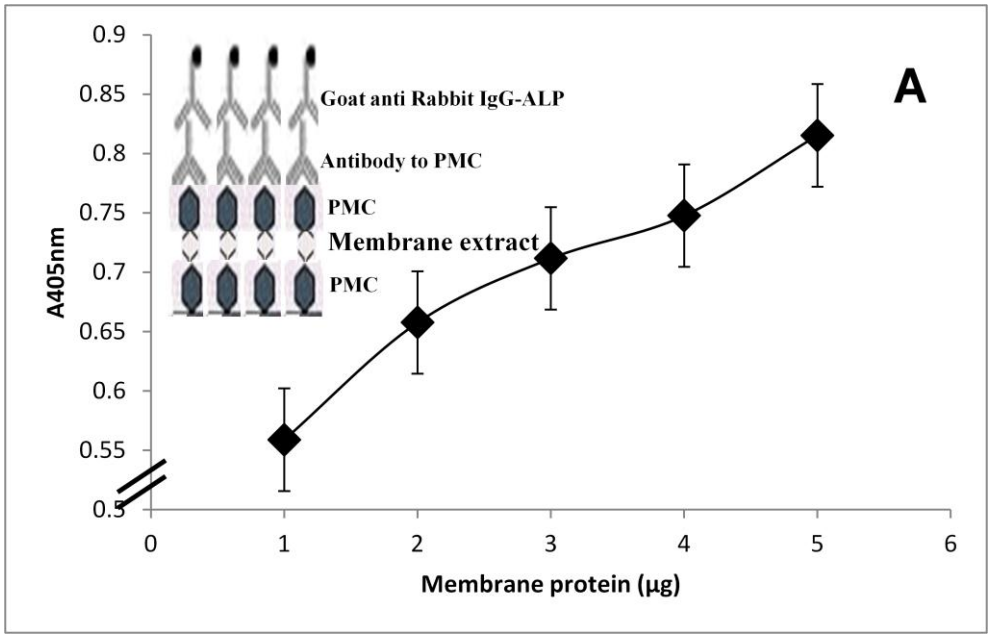


Figure.4.4.6. Blotting of mannose 6-phosphate containing ligands with PMC antibodies Lane 1: Marker, Lane 2: *Unio* soluble extract, Lane 3: β-hexosmainidase from MEF mpr-/- secretions, Lane 4: BSA.

Figure.4.4.7. Immunoblotting of PMP-hemocyanin with PMC specific IgG. PMP-hemocyanin was separated on 10%SDS-PAGE, transferred to PVDF membrane and detected with PMC specific antibody.



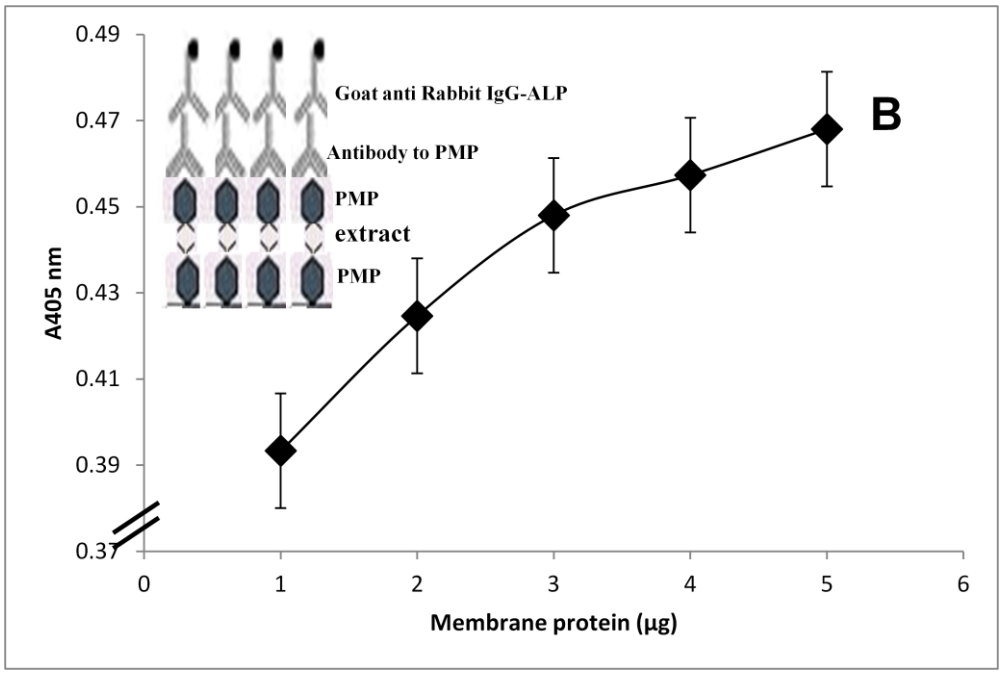


Figure.4.4.8. ELISA to detect the MPR proteins in the liver membrane extract of goat. The assay was done as described under materials and methods and schematic representation of the respective assays is shown in both the panels where the ligands are PMC (panel A) and PMP (panel B). A representative experiment, from a total of three is shown.

4.5. Discussion

Phosphomannan core (high molecular weight) and pentamannosylphosphate (low molecular weight) are two sugar phosphate containing compounds that are obtained by the acid hydroloysis of *O*-Phosphonomannan from the yeast *Hanseula holstii.* In the present study polyclonal antibodies were developed for PMC as an antigen. However for obtaining PMP antibodies, PMP was conjugated to hemocyanain and this served as an antigen. From the antiserum both PMC and PMP specific IGs were affinity purified using Seralose-PMC and Seralose-PMP gels respectively. The specificity of these antisera were analyzed by various methods. The long term objective of the study is to use these antisera to quantify the receptors among different tissues/cell extracts and also test their ability to interact with mannose 6-phosphate containing ligands. Further in literature there are no reports on the availability of such antisera and therefore this part of the work was initiated and the results obtained have given sufficient basic information to support the specificity of these antibodies to recognize important biological macromolecules. Several lines of evidence support this. First from the antisera developed, PMC and PMP specific IgG's could be affinity purified on PMC and PMP gels. Second, the immunoblotting and immunoprecipitation experiments with PMP-hemocyanin, the antigen could be recognized. However pre immune serum didnot show any reactivity indicating the specificity of the antigen-antibody interaction. Similar experiment with PMC could not be shown, as the PMC being a high molecular weight polysaccharide, doesnot enter the gel and therefore difficult to precisely analyze through this experimentation (However the purification of the specific IgG from PMC antiserum on PMC gel is already suggestive of the production of

the specific antisera). Third, in a ligand blot assay using starfish MPR300 [which is known to exhibit similar molecular mass as the vertebrate MPR300 protein (Merino and Siva Kumar, 2012)] specific recognition by both the PMC and PMP IgGs strongly suggest the utility of these antisera on one hand and their specificity on the other. Fourth, in a western blot experiment, other mannose 6-phosphate containing ligands such as the Bovine DNase 1, β-galactosidase (Nishikawa et al., 1997) could also be recognized by the PMC antiserum developed. These data suggest the possible utility of this antisera developed. Fifth, western blot analysis of PMP-hemocyanin separated in SDS-PAGE was detected using PMC antiserum suggesting that the PMC antiserum is capable of recognizing PMP antigen containing phosphorylated manno oligosaccharides. Sixth, an ELISA method was developed to quantify the receptors in crude extracts using the antisera developed in the study. In a previous study in our laboratory, we have used antibodies from purified goat MPR300 protein and found that these antisera can be used to quantify the receptors in the crude extracts of goat and chicken tissues. Interestingly the antisera developed in this study have similar potential applications. Thus these antisera can be used to identify and quantify the mannose 6-phosphate containing ligands from different sources.

5.1. Introduction

Moringa oleifera is a tropical plant and is distributed throughout tropics and subtropics; it belongs to the genus Moringaceae. *M.oleifera* is a highly valued tree with most of its parts (leaves, flowers, fruits, wood and seeds) having pharmacological and nutritional properties. Its leaves and fruits are edible and very rich in ascorbic acid. Many studies have been done on the performance of *M.oleifera* seeds as a natural coagulant in water treatment (Jahn, 1988, Ndabigengesere *et al.,* 1995, Muyibi and Evison, 1996). Its utility as a biological absorbent has also been extensively described (Sharma *et al.*, 2006). Very recently different lectins have been purified from this plant which has the coagulant and insecticidal properties (Coelho *et al.,* 2009, Oliveira *et al.,* 2010).

Classification of the genus ***Moringa*** *is* as follows:

Kingdom : Plantae

Phylum : Angiosperms

Class : Eudicots

Order : Brassicales

Family : *Moringaceae*

Genus : *Moringa oleifera*

Species : *Moringa oleifera* Lam

Variety : Periyakulam 1(PKM1) [*The variety used in the present study*]

Moringa oleifera, commonly referred to as Sajina in India and also as, the drumstick tree (describing the shape of its pods), horseradish tree (describing the taste of its roots) is widely distributed. Seeds of some *Moringa* varieties are consumed by humans after roasting and taste like groundnuts (Ramachandran

et al., 1980). The high concentrations of ascorbic acid, oestrogenic substances and β-sitosterol, iron, calcium, phosphorus, copper, vitamins A, B and C, α-tocopherol, riboflavin, nicotinic acid, folic acid, pyridoxine, β-carotene, protein, and in particular essential amino acids such as methionine, cystine, tryptophan and lysine present in *Moringa* leaves and pods make it virtually an ideal dietary supplement (Makkar and Becker, 1996). The sulphur-containing amino acids in kernel proteins are present at higher concentrations, but other essential amino acids are deficient. Among all the naturally occurring coagulant removal sources *M.oleifera* (MO) seed is one of the most commonly known natural coagulants that can be used for water purification (Jahn, 1988).

Mannan polysaccharides serve as storage reserves in seeds and as structural elements in cell walls, but they may also perform other important functions during plant growth. In addition to cellulose, hemicelluloses and pectic polysaccharides, polysaccharides containing β-1,4-linked mannosyl residues, referred to as mannan polysaccharides, are widespread and found as cell wall components in angiosperms and gymnosperms (Bacic *et al*., 1988).

The mannan, an important component of the hemicelluloses family, can be classified into four different polymers such as linear mannan, glucomannan, galactomannan, and galactoglucomanan (Petkowicz et al. 2001). Each of these polysaccharides presents a β-1,4-linked backbone containing mannose or a combination of glucose and mannose residues (Liepman *et al*., 2007). The mannans from ivory nuts can be separated into two components: A and B (Meier 1958; Petkowicz *et al.* .2007). In addition, the mannan backbone can be substituted with side chains of α-1,6-linked galactose residues.

Two types of polysaccharide from the mature pods of *Moringa oleifera* have been reported recently. One among them is a α-(1→4) linked imunoenhancing glucan and second one is a water-soluble heteropolysaccharide containing D-galactose, 6-O-Me-D-galactose, D-galacturonic acid, L-arabinose, and L-rhamnose in a molar ratio of 1:1:1:1:1 isolated from hot water extracts of mature pods of *Moringa oleifera* and were well characterized (Mondal *et al*., 2004, Roy *et al*., 2007). In the current study we have started our work with the mature seeds of *Moringa oleifera* (PKM_1 variety) which were ground and processed further as described under materials and methods and identified the same as mannan.

5.2. Materials and methods

5.2.1. Materials

Dry mature seeds of *Moringa oleifera* (MO) PKM 1 variety were purchased locally. Acetone, ammonium sulphate, Dialysis membrane, Ethanol, SephadexG-100, Phenol, Sulfuric acid, Whattman paper No.3, Butanol, Pyridine, Water, Aniline, Phthalic acid and all other chemicals, reagents procured from reputed firms and were used without further distillation.

5.2.2. Methods

The total seeds obtained were ground using a kitchen blender. Seed powder was defatted using acetone and the solids were dried at room temperature. The biomolecules from 50 g of seed powder were extracted overnight with 500 ml of 25 mM PBS pH (7.0). The suspension was clarified by centrifugation and filtered through Whatmann paper No. 3. The filtrate is termed as the crude

extract. The protein was precipitated from aqueous filtrate by the addition of solid ammonium sulphate to 80% saturation. The precipitated protein was separated by centrifugation and was discarded. The supernatant solution was dialysed through dialysis tubing with a molecular weight cut-off of 14 kDa. Total volume of supernatant that was devoid of most of the proteins was 500 ml.

5.2.3. Ethanol precipitation of the polysaccharide

Chilled ethanol was added to 150 ml of the above obtained supernatant after ammonium sulphate fractionation in the ratio of 1:10 (v/v) and incubated overnight at 4^{o}C. Next day the white precipitated polysaccharide fraction was separated from the supernatant by centrifugation at 10,000 rpm (4^{0}C) for 30 min, and was washed with chilled ethanol several times. The sample was air dried and used for further studies. From 150 ml of the protein free crude material, 1.3g of polysaccharide could be precipitated.

5.2.4. Gel filtration chromatography on Sephadex G-100

The above obtained polysaccharide fraction after ethanol precipitation was further purified using Sephadex G-100 (60 ml) column pre-equilibrated with water. 100 mg of the dried polysaccharide was dissolved in 1ml of deionized water and passed through the SephadexG-100 at a flow rate of 10 ml/h. 20 fractions each of 2 ml were collected immediately after the void volume. The column fractions were assayed for carbohydrate using phenol sulphuric acid method (Dubois, et al., 1956) using glucose as standard. Peak fractions which were positive for phenol sulphuric acid test were pooled and lyophilized. From 100 mg of the dry powder 50 mg of the purified polysaccharide could be obtained and was used for acid hydrolysis followed by paper chromatography analysis to check the composition of the purified polysaccharide.

5.2.5. Acid hydrolysis of the polysaccharide and paper chromatography

An aliquot of the lyophilized polysaccharide was subjected to acid hydrolysis using concentrated hydrochloric acid in a closed test tube and heated at 100^0C for 2h to obtain the constituent monosaccharides. The monosaccharide composition of the polysaccharide was monitored on paper chromatography, using solvent of n-butanol/ pyridine/ water 6: 4: 3 v/ v/ v. Stained with Aniline-phthalate regent.

5.2.6. Nuclear Magnetic Resonance Spectroscopy (NMR)

A small quantity of the isolated polysaccharide in its native form was studied by NMR (Bruker Topspin 400MHz) using D_2O as solvent.

5.3. Results

Total polysaccharides were extracted from the *Moringa oleifera* seed powder. The extracted polysaccharide was chromatographed on Sephadex G-100 gel equilibrated with water and the same was also used for the elution. Fractions were monitored for carbohydrate using phenol sulphuric acid method. Figure 5.4.1. shows the elution profile of the polysaccharide on a Sephadex G-100 column. Fractions positive for carbohydrate (fractions 1-8) were separately pooled and lyophilized for further analysis. The lyophilized powder obtained was hydrolyzed with acid and the hydrolysate was processed as described under methods.

Paper chromatography

The mannan component was hydrolyzed yielding only mannose sugars as evidenced by the paper chromatography using *n*-butanol /pyridine / water 6: 4: 3 v / v / v, as solvent. Subsequent staining with Aniline-phthalate, showed the sugar spots. Comparing the standards mobility to those in the experimental

samples, it can be concluded that the isolated polysaccharide was mannan (Figure.5.4.2)

NMR spectroscopy

The shifts (3.44ppm to 4.9ppm) obtained in the spectra are corresponding to the sugar D-Mannose with a small shift, indicating that the functional groups of mannose are involved in linkage formation, which have to be further elucidated (Figure.5.4.3.). The chemical shift at 4.76ppm is that of solvent D_2O. The chemical shifts at 1.8ppm and 2.2ppm are that of O=C-O-CH3 group. The anomeric proton (H-1) did show a chemical shift at 4.8ppm which is for a β glycosidic linkage. From this we can say that the anomeric carbon is involved in β glycosidic linkage which is a common type of linkage in known plant mannans.

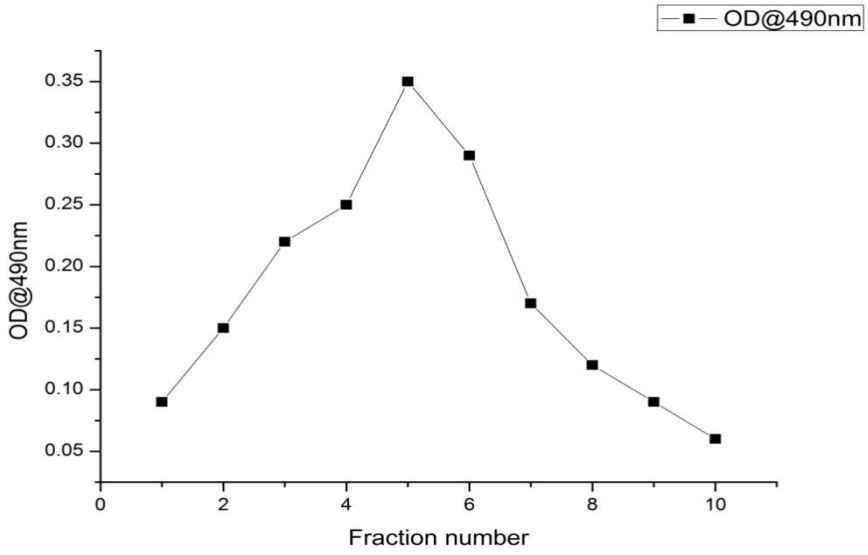


Figure.5.4.1. Elution profile of Mannan from SephadexG-100 (determined by phenol sulphuric acid test)

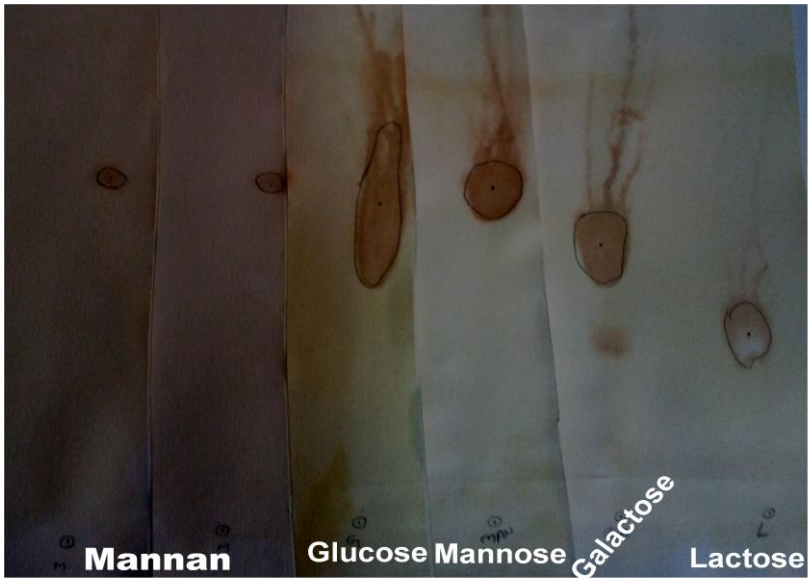
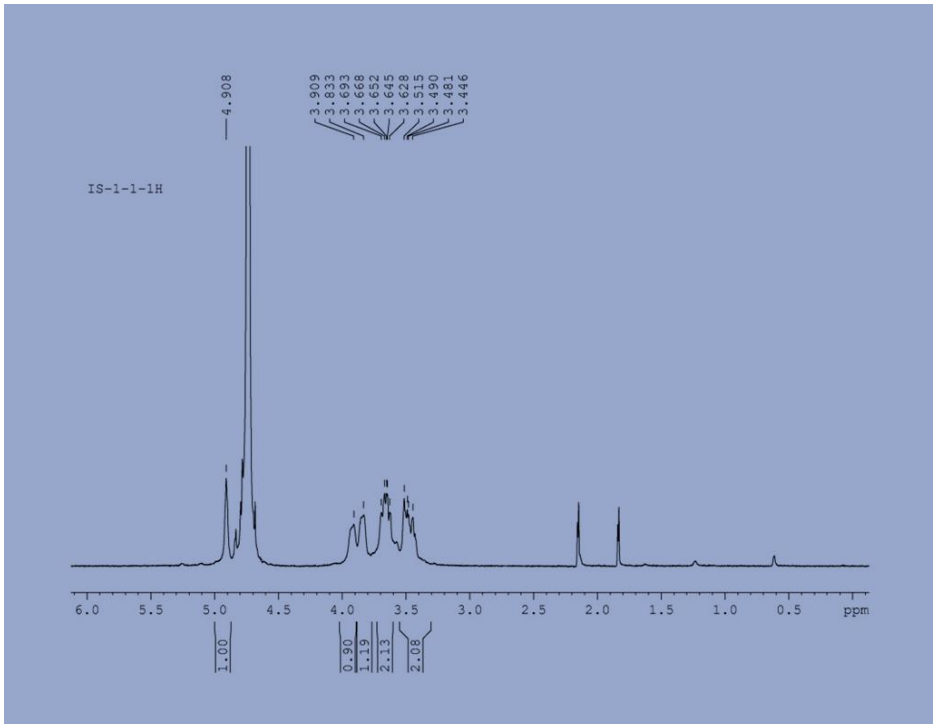


Figure.5.4.2. Paper chromatogram showing reference compounds (glucose, mannose, lactose and galactose) and the products of acid hydrolysis mannan, isolated from *Moringa oleifera* seeds. The solvent used is *n*-butanol/ pyridine/ water 6: 4: 3 v/ v/ v. Stained with Aniline phthalate reagent.



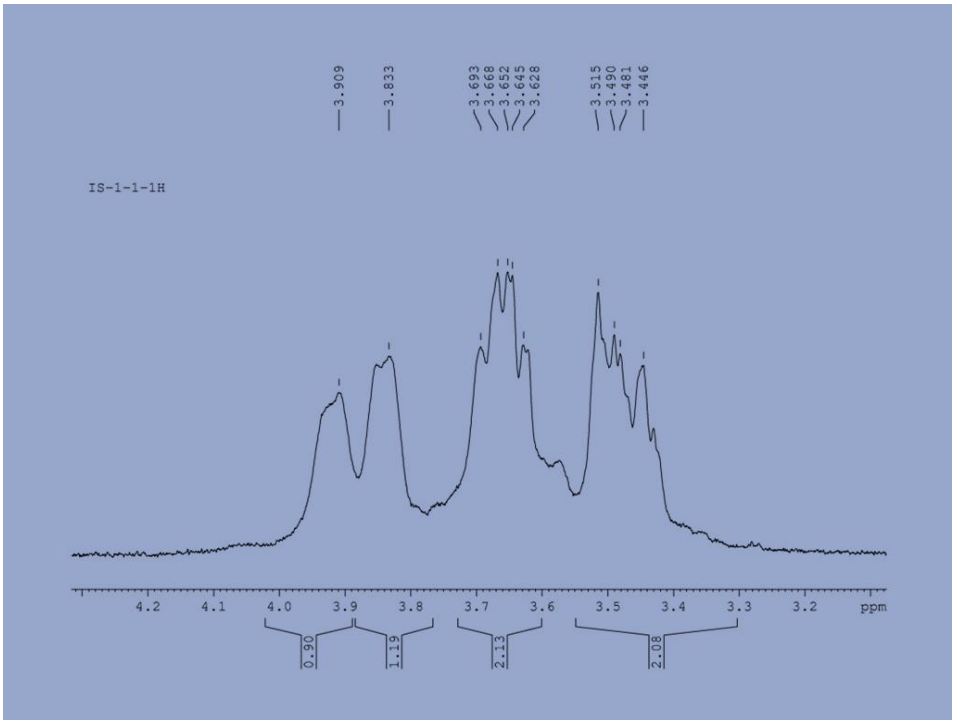


Figure.5.4.3. 400MHz ^{1}H NMR spectrum of the polysaccharide obtained from the seeds of *Moringa oleifera* recorded at $27^{0}C$ using D_2O as solvent.

5.5. Discussion

Plant mannan has β-(1→4) linkages and it is a form of storage polysaccharide (Moreira and Filho, 2008). So far there are only a few reports on mannan. One such mannan is from ivory nut (Meier 1958, Petkowicz *et al.*, 2007), this mannan is the source of Mannan-oligosaccharide based nutritional supplements (MOS). Mannans have many applications such as MOS which are widely used as prebiotics in animal husbandry and nutritional supplements as a natural additive. MOS has been shown to improve gastrointestinal health as well as overall health. Most MOS products, particularly those that have been scientifically reviewed, derive from the cell wall of the yeast, *Saccharomyces cerevisiae*.

As some aspects of polysaccharide occurrence in *Moringa oleifera* seeds have not been elucidated, this study was aimed at exploring the polysaccharide depositions in the mature seeds of this plant. In a recent study we have isolated galactomannan and galactan polysaccharides from the seeds of *Strychnous potatorum* which have the potential to purify plant galactose specific lectins (Mohammed et al., 2014). The long term objective of the study was to understand the nature and structural properties of the polysaccharide component of the seeds and study their applications. The present study shows that the seed material contains distinct mannan polysaccharides that can be separated by ethanol precipitation followed by gel filtration in contrast to the earlier studies. The isolated polysaccharide was indeed mannan and was confirmed by acid hydrolysis of the separated polysaccharides and separation of the hydrolysate by paper chromatography into the constituent

monosaccharides which identified the separated polysaccharides to be mannan and also by NMR spectroscopy. Molecular mass of the isolated polysaccharide is approximately in the range of 70-80 kDa. The isolated mannan from *M.oleifera* seeds need to be tested for its applications as source material for preparation of nanoparticles for drug delivery into cells and as potential agent for purification of mannose binding lectins.

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Mannose 6-Phosphate Containing Nanoparticles: Preparation, Characterization and Interaction with Cation Independent Mannose 6-Phosphate/IGF-II Receptor (MPR300)

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The cation independent mannose 6-phosphate/IGF-II receptor (MPR300 : Mr 300 kDa) on the plasma membrane is an important protein that selectively interacts with lysosomal enzymes, mannose 6-phosphate containing ligands as well as IGF-II and by endocytosis targets them to lysosomes. Phosphomannan core (PMC) and pentamannosylphosphate derived by acid hydrolysis of yeast *O*-phosphonomannan are also efficient ligands for specific binding to the cation independent mannose 6-phosphate/IGF-II receptor (MPR300) protein. We prepared nanoparticles for the phosphomannan core as well as to purified lysosomal enzymes β-hexosaminidase and β-galactosidase. Characterization of these by AFM and SEM revealed their sizes to be in the range of 100 to 300 nm for the PMC nanoparticles, 60 to 100 nm for the β-hexosaminidase nanoparticles and 20–50 nm for the β-galactosidase nanoparticles. Employing NIH3T3 cells and CHO cells that are known to contain the MPR300 protein, we confirm that different nanoparticles prepared specifically interact with the cell surface MPR300 and the receptor is also involved in the internalization of these nanoparticles. Preincubation of the cells with mannose 6-phosphate prevents internalization confirming the specificity of interaction. The novel tool of nanoparticles developed in this study possibly would have applications for targeted delivery of the drugs and biomolecules into the cells through receptor mediated endocytosis.

Keywords: MPR300, Lysosomal Enzymes, Nanoparticles, Phosphomannan Core, Confocal Microscopy, Receptor.

1. INTRODUCTION

Newly synthesized lysosomal enzymes in eukaryotes acquire phosphorylation of their mannose residues which is a prerequisite for their specific recognition and interaction with the mannose 6-phosphate receptors designated as MPR300 (Mr 300 kDa, the cation independent receptor which is also the receptor for IGF-II) and the MPR46 (Mr 46 kDa), the cation dependent receptor which mediate their transport to the lysosomes. Both receptors are also localized in the plasma membrane. However only the MPR300 protein has been shown to exhibit specific interaction with the ligands containing mannose 6-phosphate as well as with IGF-II and selectively internalizes them for targeting to lysosomes and is thus an endocytosis receptor.[1] Additionally this receptor in mammals is also known to interact with other ligands such as the latent TGF precursor, urokinase-type plasminogen activator receptor, Granzyme *B*, Glycoprotein *D* of Herpes simplex virus and proliferin and is thus classed as a multifunctional protein.[2] Interest in the field of lysosomal enzymes and their receptors is largely due to a number of lysosomal disorders that have been identified in humans. However there are only a few remedies for these disorders known such as the enzyme replacement therapy, gene therapy.[3] Recent studies have utilized nanoparticles as efficient tools for drug delivery into cells. Nanoparticle delivery systems are essentially nanosized carriers (10–1000 nm size) having different morphologies such as the nanocapsules, nanomicelles, nanospheres and dendrimers that can be used for specific delivery of drugs, biomolecules into cells. Nanoparticle delivery systems have advantages, because

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of their very small size the clearance of nanocarriers by phagocytes will be avoided so that their duration in blood stream is greatly prolonged, they can penetrate cells and tissue gaps to arrive at target organs. Nanoparticles can be prepared from a variety of materials such as proteins, polysaccharides and synthetic polymers. Nanoparticles that are biodegradable offer better advantages as they would be non-toxic to the cells. Nanoparticles could be functionalized and can act as potential carriers for several biomoloecules and drugs. Receptor-mediated targeting is a promising approach to selective drug delivery. Medical therapies have become more tailored to specific diseases and patients in recent years. Selective delivery of pharmaceutical agents into cells has been recently reviewed.[4]

Natural polysaccharides are nontoxic, biodegradable, safe and are available in abundance. The yeast *Hansenula holstii* NRRL Y-2448 produces a viscous, highly branched extracellular *O*-phosphonomannan that is composed of a core moiety to which oligosaccharide diester phosphate side chains are attached. Mild acid hydrolysis of *O*-Phosphonomannan and ethanol precipitation separates the high molecular weight phosphomannan core (PMC) moiety and the low molecular weight pentamannosylphosphate fraction (PMP). Both PMC and PMP have become valuable tools in affinity purification and characterization of mannose 6-phophate receptors. Mammalian cells express two lysosomal enzyme targeting receptors (MPR300 and 46) that have been extensively characterized.[1]

Our initial research work in the field of lysosomal enzymes and receptors, focused on developing alternate affinity methods to purify the mannose 6-phosphate receptors from goat liver using phosphomannan core and pentamanosylphosphate as efficient ligands and raising antibodies to the purified goat receptors. Using goat as a mammalian model, we looked for homologous receptors in other vertebrate species and recent studies clearly established the evolutionary conservation of the receptors throughout the vertebrates. Current studies are focused on the invertebrate receptors to further understand their evolutionary significance. A large number of diversity of ligands bearing mannose 6-phosphate signal that bind to the endocytosis receptor, MPR300 suggests a possible utility of this protein for drug/biomolecule delivery into cells. Since we extensively studied the MPR proteins the present work was initiated with a long term objective to utilize the naturally occurring phosphorylated mannooligosaccharide nanoparticles as potential tools for drug delivery mediated by the MPR300 protein. The objectives of the present study were,

(i) to prepare nanoparticles of the natural ligands for the receptors such as the phosphomannan core,

(ii) use specific lysosomal enzymes such as the β-hexosaminidase and β-galactosidase to prepare nanoparticles,

(iii) characterize the nanoparticles by atomic force microscopy, electron microscopy and

(iv) analyze the binding of the nanoparticles prepared to cells that contain MPR300 and study their specific interaction and internalization.

2. MATERIALS AND METHODS

O-Phosphonomannan was a generous gift from Doctor Slodki, USDA, IL and USA. Olive oil, diethyl ether from and DE-52 cellulose were procured from Qualigens and Whattman respectively. Mannose 6-phosphate, *P*-nitro phenyl β-*D*-glucosaminide, DMEM, FITC and β-galactosidase were purchased from Sigma Aldrich. Enzyme grade ammonium sulphate and other reagents used in the study were purchased from SRL. Goat MPR300 antibody raised in rabbit and the specific IgG affinity purified in our laboratory. Rabbit Lamp1 antibody and other secondary antibodies used in the study were from abcam. Equipment such as the SEM-PHILIPS FEI-XL ESEM (USA), AFM-SPA400, Probe Sonicator, Confocal microscope (Leica TCS SP2 AOBS) were available in the central facilities of the University of Hyderabad..*Cells*: MPR-deficient mouse embryonic fibroblast cells ($mpr^{(-/-)}$ MEF cells) used in the present study are devoid of both receptors and were kindly provided by Professor Dr. Regina Pohlmann, University of Muenster, Germany, NIH3T3 and Chinese Hamster Ovary (CHO) cells, were available in the lab.

2.1. Hydrolysis of *O*-phosphonomannan and Preparation of Phosphomannan Core (PMC) Nanoparticles

O-phosphonomannan from the yeast *Hansenula holstii* was hydrolysed with acid and the phosphomannan core (PMC) was ethanol precipitated and lyophilized as described.[5] Phosphomannan core is known to have amine groups.[6] 20 mg of phosphomannan core was incubated with 600 μl of sodium borate buffer (50 mM, pH9.0) and 400 μl of fluorochrome solution of fluorescein-isothiocyanate (5 mg FITC dissolved in 1ml of DMSO) overnight at 4 °C. Free FITC was removed by desalting using a Sephadex G-25 gel. For the preparation of nanoparticles, the procedure described by Krishna et al. with slight modifications was adopted.[7] To the fluorescent labelled phosphomannan core, 15 ml olive oil was added drop wise with continuous stirring on a cyclomixer. It was sonicated 15 times, each time for 30 sec with 1 min gap. Immediately it was frozen in liquid nitrogen for 10 min. It was then kept at 4 °C for 4 hr and the nanoparticle pellet was collected by centrifugation at 8000 rpm for 10 min. The residual oil was removed by washing the pellet with chilled diethyl ether 3–4 times. The clear pellet was dissolved in PBS pH 7.4 and the nanoparticle sample was stored at 4 °C.

2.2. mpr$^{(-/-)}$ MEF Cell Culture, Purification of β-hexosaminidase by Ion Exchange Chromatography and MPR300 Affinity Chromatography

mpr$^{(-/-)}$ mouse embryonic fibroblasts lack both the receptor proteins that are involved in transport of lysosomal enzymes to lysosomes. The newly synthesized lysosomal hydrolases can not be targeted to the lysosomes. Therefore these cells secrete the newly synthesized lysosomal enzymes that have the M6P signal in their structure. The mpr$^{(-/-)}$ mouse embryonic fibroblast cells were cultured as described earlier[8] and from several culture flasks, the medium was collected and stored at −80 °C. From this the total proteins were precipitated by ammonium sulfate precipitation (0–80% saturation) and the pellet obtained was dissolved in 25 mM Tris-HCl buffer pH 7.4 (buffer A) and extensively dialyzed against the same buffer. The clear solution after dialysis served as the source of the enzyme for further purification. The activity of β-hexosaminidase was analyzed in the concentrated protein sample obtained above using *P*-nitro phenyl β-D glucosaminide (SIGMA) as described.[9]

The protein containing the enzyme activity obtained above was passed through the DE-52 cellulose gel (30 ml) that was equilibrated with buffer A. The gel was washed extensively with buffer A to remove the unbound proteins. Then the sample was eluted with 50 mM NaCl in buffer A. Fractions were collected and aliquots were analyzed for lysosomal enzyme activities. Only hexosaminidase and mannosidase activities were detectable. However hexosamindiase was relatively much higher than mannosidase. Therefore, the enzyme containing fractions were pooled and concentrated by amicon concentrator to further obtain the hexosaminidase in a homogeneous form. The concentrated sample was dialyzed against MPR300 column buffer 50 mM imidazole-HCl pH 7.0, 150 mM NaCl, 5 mM Sodium β-glycerophosphate, 5 mM EDTA, 0.05% Triton X-100 (buffer B). The protein was applied on a human MPR300 receptor affigel, (generous gift from Professor Doctor von Figura, Goettingen University, Germany), that was pre-equilibrated with buffer B. The gel was washed extensively with buffer B. The bound protein was eluted with 5 mM mannose 6-phosphate in buffer B. This experiment was done several times and the eluted enzyme fractions each time were pooled and concentrated by amicon concentrator. The column eluates from the ion exchange gel as well as from the MPR300 receptor-affigel were separated on a 10% SDS-PAGE under reducing conditions[10] and the proteins detected by silver staining.[11] Protein eluted from the MPR300 receptor gel was also processed for periodic acid –Schiff's staining to detect the glycoprotein nature of the hexosaminidase purified.

2.3. Preparation of Enzyme Nanoparticles and Their Characterization

5 mg of purified β-hexosaminidase in 550 μl sodium borate buffer of pH 9.0 is tagged with FITC prior to nanoparticle preparation. 100 μl of fluorochrome solution of flourescein-isothiocyanate (5 mg FITC dissolved in 1ml of DMSO) was added to the enzyme sample and mixed well and the sample stored at 4 °C overnight in dark. The following day it was passed through a Sephadex G-25 gel to remove free FITC. Nanoparticles were prepared as described for PMC nanoparticles. Similarly nanoparticles for the enzyme β-galactosidase (procured from Sigma) were also prepared.

The PMC nanoparticles, the β-hexosaminidase and β-galactosidase nanoparticles were all separately characterized as described below.

2.3.1. Atomic Force Microscopy (AFM)

Nanoparticle sample was uniformly dispersed on a clean glass cover slip using a spin coater and dried in a dust free zone. The unit containing the sample is kept in SPA-400 and size and morphology of particles was studied following manufacturer's instructions.

2.3.2. Scanning Electron Microscopy (SEM)

Operated at 10 kV. Nanoparticle sample was uniformly dispersed on a clean glass cover slip using a spin coater and dried. Metal stubs were coated with double-sided adhesive tape, the coverslip with nanoparticle sample was kept on the sticky surface and sample was coated with gold in Sputter Coater. Specimens were stored in dry, dust free environment during the analysis.

2.4. Confocal Microscopy

NIH3T3 and CHO cell types, cultured under standard conditions and which are known to contain the cell surface MPR300 protein were used in the study to analyze specific binding of hexosaminidase nanoparticles to cell surface receptor, localization of the endocytosed FITC conjugated hexosaminidase and β-galactosidase nanoparticles, localization and endocytosis of PMC nanoparticles as well as subcellular localization of the β-hexosaminidase nanoparticles as described below.

2.4.1. Cell Surface Binding of β-Hexosaminidase Nanoparticles

This was carried out as described. The enzyme nanoparticle sample was dispersed in incomplete DMEM. NIH3T3 and Chinese Hamster Ovary (CHO) cells were grown to 80% confluency on sterile glass cover slips in complete DMEM medium. The medium was discarded and

the cells were washed with sterile1xPBS three times in 15 min. The healthy confluent cells were then incubated with β-hexosaminidase nanoparticles that were dispersed in incomplete DMEM for 1 hour at 4 °C to follow the cell surface binding.. Following incubation with nanoparticles, cells were washed with PBS three times and then they were fixed in 4% paraformaldehyde for 20 min at room temperature. The fixed cells were washed, permeabilised with 0.02%Triton X-100 for 30 seconds and were washed with PBS followed by blocking with 1% BSA in PBS for 30 min at room temperature. The cells were incubated with MPR300 antibody (This antibody was raised for purified goat MPR300 protein and recognizes the MPR300 protein fish to mammals (1:200 dilution in 1% BSA in PBS). The cells were washed with PBS 3 times and incubated with cy5 (red) conjugated secondary antibody for 1 hour at room temperature. Finally the cells were washed extensively, stained with DAPI and observed under confocal microscope. In a separate experiment, the NIH3T3 cells were preincubated with 5, 10, and 20 mM mannose 6-phosphate for 40 min at 4 °C prior to the addition of the enzyme nanoparticles as described above and the cells were visualized microscopically.

2.4.2. Localization of Endocytosed FITC-Conjugated β-Hexosaminidase and β-Galactosidase Nanoparticles

In a separate experiment whether the β-hexosaminidase and β-galactosidase nanoparticles have been endocytosed by the two types of cells has been investigated. The protocol described above was used except that the cells were incubated at 37 °C for 1 hr.

2.4.3. Localization of Endocytosed FITC-Conjugated Phosphomannan Core (PMC) Nanoparticles

As the PMC is a potential ligand for the MPR300 interaction and has been used to affinity purify the receptor from tissues and specific cell types, PMC nanoparticles prepared in the study were also tested for their ability to bind the cell surface MPR300 protein. The ability of the cells to endocytose the PMC nanoparticles was tested as described for the enzymes above.

2.4.4. Sub Cellular Localization of Endocytosed β-Hexosaminidase Nanoparticles with Lamp1 Protein

For this experiment, NIH3T3 cells were incubated with nanoparticles and co localized with Lamp1 protein. Following incubation with nanoparticles for 1 hr at 37 °C, the cells were washed with PBS three times and then they were fixed in 4% paraformaldehyde for 20 min at room temperature. The fixed cells were washed, permeabilised with 0.02% Triton X-100 for 30 sec and were washed with PBS followed by blocking with 1% BSA in PBS for 30 min at room temperature. The processed cells were incubated with Lamp-1 antibody (this antibody from abcam is known to interact with Lamp-1 from human and mouse cell lines, 1 μg/ml dilution in 1% BSA in PBS) overnight at 4 °C. The cells were washed with PBS 3 times and incubated with cy5 (red) conjugated secondary antibody for I hr at room temperature. Finally the cells were washed extensively, stained with DAPI and observed under confocal microscope.

3. RESULTS

3.1. Preparation and Characterization of Nanoparticles

In the present study three distinct type of nanoparticles have been prepared.
(i) a natural ligand PMC for the MPR300 protein which is used for the affinity purification of the receptor.
(ii) β-hexosaminidase (purified lysosomal enzyme) nanoparticles and
(iii) β-galactosidase (lysosomal enzyme) nanoparticles.

3.1.1. PMC Nanoparticles

Our attempts to prepare PMC nanoparticles using the protocol described under the methods was successful. When these were characterized using AFM and SEM, the sizes of the particles were in the range of 100–300 nm Figure 1(A). The particles were distinct and spherical with smooth surface morphology.

3.1.2. β-Hexosaminidase Nanoparticles

The nanoparticles for the purified enzyme were also prepared as described above and their size varied from 60–100 nm as determined by AFM and SEM Figure 1(B).

3.1.3. β-Galactosidase Nanoparticles

This enzyme was commercially procured and nanoparticles for this enzyme have also been prepared following the protocol described above. When these were analyzed by SEM, they showed a size in the range of 20–50 nm Figure 1(C). The variation in the size range of the three types of nanoparticles prepared in our study might be due to the respective molecular sizes of the materials used

3.2. β-Hexosaminidase Purification From the Secretions of $mpr^{(-/-)}$ MEF Cells

When the ammonium sulfate precipitated proteins from the secretions obtained above was passed through DE-52 cellulose gel, the β-hexosaminidase activity was bound on the gel and was eluted with very low salt concentration. Analysis of an aliquot of the enzyme containing fraction

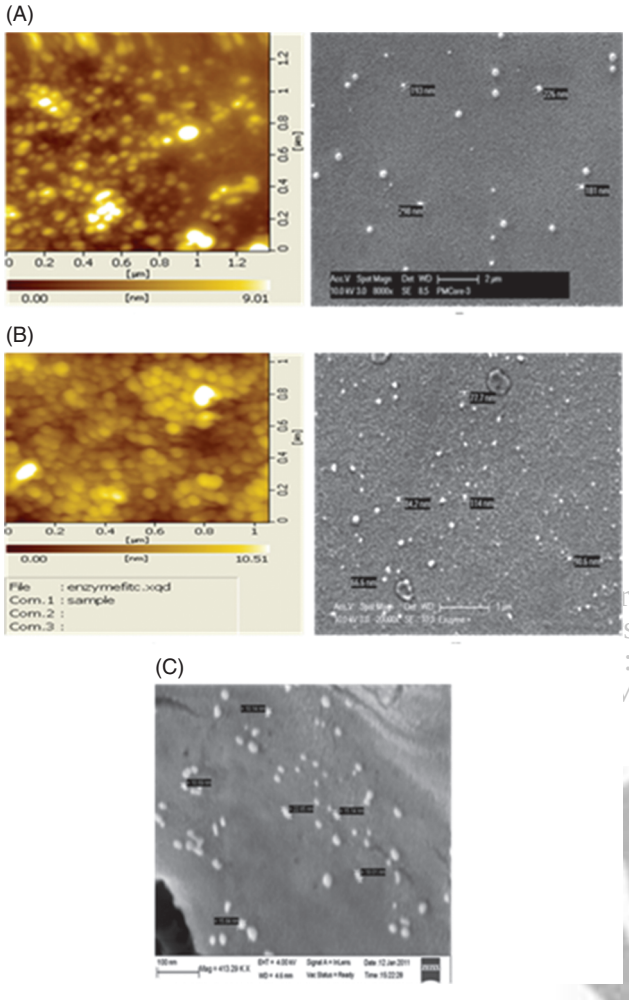

Fig. 1. (A) Characterization of nanoparticles. AFM (left) and SEM (right) micrographs of PMC nanoparticles, scan area is 1.2 μ and 2 μ respectively (B) AFM (left) and SEM (right) analysis of β-hexosaminidase nanoparticles, scan area is 1 μm (both). (C) FESEM micrograph of β-galactosidase nanoparticles.

by SDS-PAGE revealed the presence of a 66 kDa protein band, marked by arrow in Figure 2(A). To further obtain a homogeneous preparation of the β-hexosaminidase, the salt eluates obtained were subjected to affinity chromatography on MPR300 receptor-affigel as described under methods. The specific elutions from the gel when analyzed by SDS-PAGE revealed the presence of a single band corresponding to a molecular mass of about ~60 kDa that stained with silver staining (data not shown) and the same protein when tested for glycoprotein stained positive for the same Figure 2(B) suggesting the glycoprotein nature of the enzyme.

3.3. Confocal Microscopy

The ability of the nanoparticles prepared in the study to interact with the MPR300 protein on the NIH3T3 and CHO cell surface was investigated using confocal microscopy.

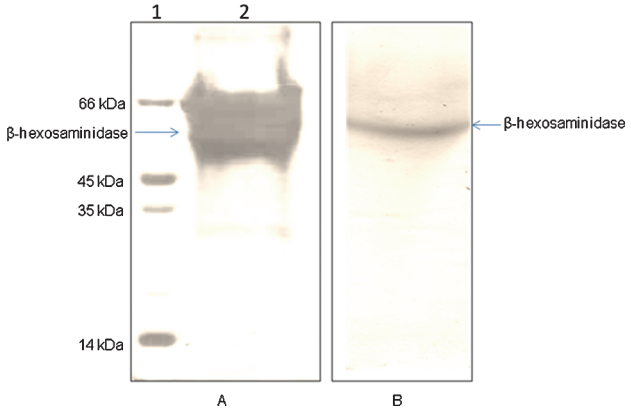

Fig. 2. (A) SDS-PAGE analysis. Purified β-hexosaminidase was separated on a 10% gel under reducing conditions, protein bands were detected by silver staining. Lane1, marker, Lane 2, β-hexosaminidase (B) Periodic Acid Schiff staining of purified enzyme β-hexosaminidase.

3.3.1. *Localization of Endocytosed FITC-Conjugated Phosphomannan Core (PMC) Nanoparticles*

From Figure 3, it is apparent that the nanoparticles of naturally occurring mannophosphorylated polysaccharides can be prepared and used for nanoparticle localization as shown. In a separate experiment as a negative control, cells were pre incubated with mannose 6-phosphate to block the MPR300 receptor on the cell surface as explained above for the enzyme nanoparticles. Additionally, when the cells were preincubated with MPR300 antiserum (1:500 dilution) and then the enzyme nanoparticles, added, there was no binding visualized (data not shown).

3.3.2. *Cell Surface Binding of β-Hexosaminidase Nanoparticles*

We could see the efficient binding of the fluorescently labeled enzyme nanoparticles to the cell surface receptor MPR300. These results are shown in Figure 4. When NIH3T3 cells were used, the nanoparticles were localized to the cell surface (i, panel *G*) and the receptor was also detected using an antibody to the receptor (i, panel *H*). When the CHO cells were used, similar results were obtained (ii, panel *B* and *C*). The effect of different concentrations of the mannose 6-phosphate on binding of the enzyme nanoparticles to the MPR300 in NIH3T3 cells was studied as described under methods. Only at very high concentration of 20 mM mannose 6-phosphate there was complete inhibition of the binding of the enzyme nanoparticles to the receptor (iii, panel B).

3.3.3. *Localization of Endocytosed FITC-Conjugated β-Hexosaminidase and β-Galactosidase Nanoparticles*

The ability of the cell surface receptor to internalize the hexosaminidase and galactosidase enzyme nanoparticles

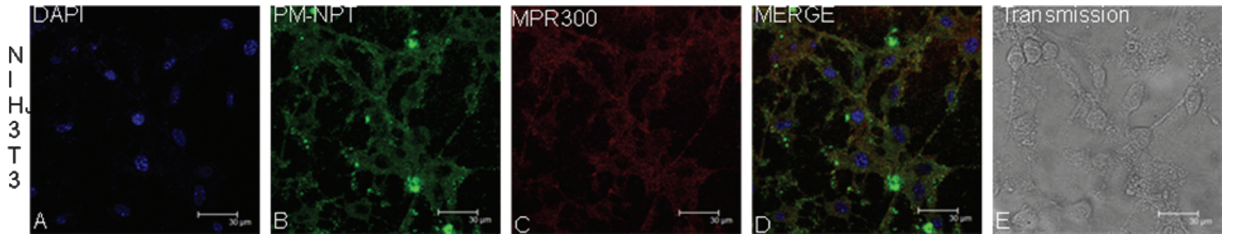

Fig. 3. Intracellular localization of Phosphomannan core nanoparticles in NIH3T3 cells (A) is DAPI, (B) Incubation with FITC nanoparticles (PM-NPT) for I hour at 37 °C, (C) Incubation with MPR300 antibody followed by cy5(red) conjugated secondary antibody, (D) is merged images of A, B, C. Cell line type is indicated at the left side of the panel. Bar in the panel is 30 μm.

was tested as described under methods and the results are presented in Figures 5(A and B) respectively. From Figure 5(A) it is apparent that the fluorescently labeled enzyme nanoparticles on incubation with both cell types used, were internalized (*B* and *G*) which were also detected by using the antiserum to MPR300 (*C* and *H*). These results suggest the internalization of the nanoparticles is through the cell surface MPR300 protein. Figure 5(B) shows the similar results obtained for the galactosidase enzyme nanoparticles.

3.3.4. Sub Cellular Localization of Endocytosed β-Hexosaminidase Nanoparticles with Lamp1 Protein

The subcellular localization of the internalized β-hexosaminidase nanoparticles in NIH3T3 cells was also investigated using Lamp1 antibody. The results shown in Figures 6(B and C) suggest that the enzyme nanoparticles that are internalized are co localized with Lamp-1 protein, a known lysosomal marker protein.

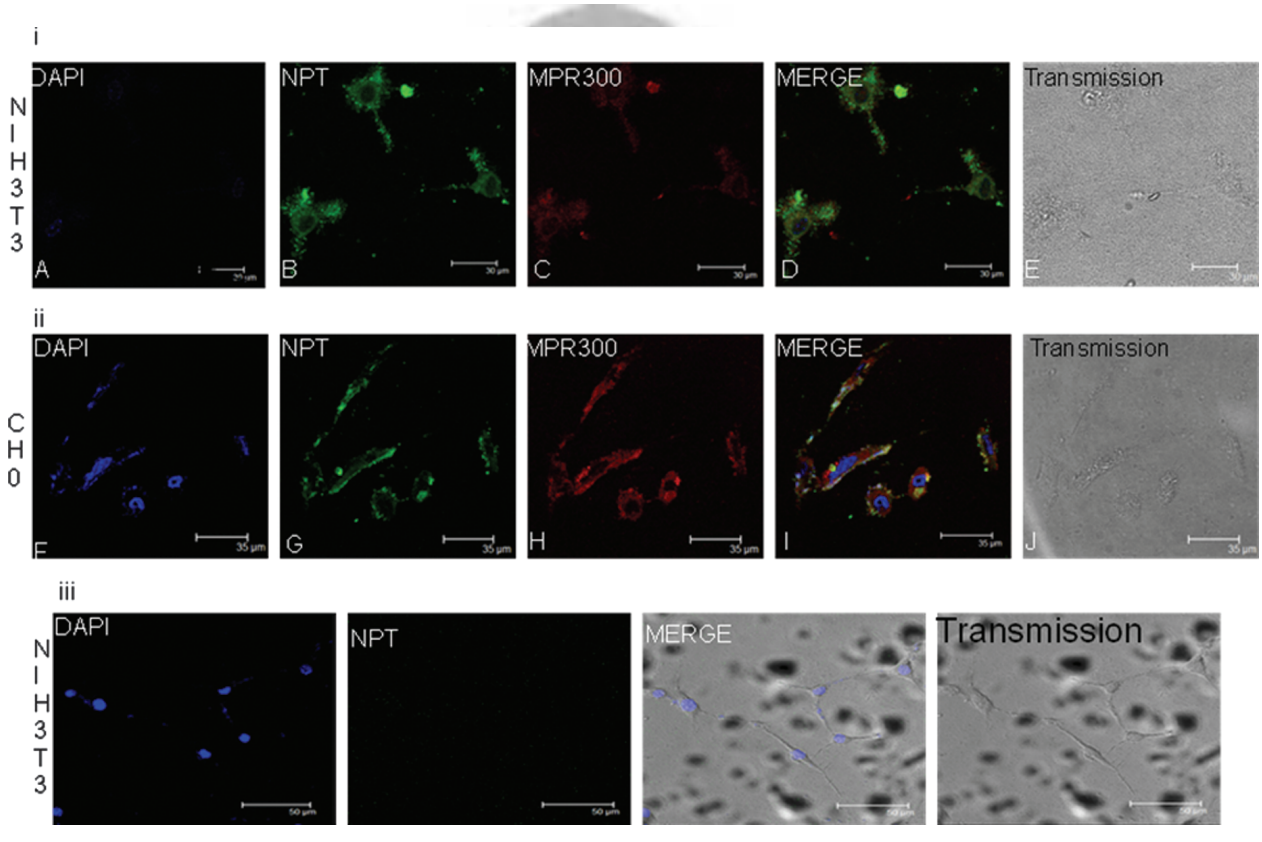

Fig. 4. Surface colocalization of nanoparticles with the MPR300 protein in (i) NIH3T3 cells and (ii) CHO cells and (B) and (G) incubation with hexosaminidase nanoparticles (NPT) for 30 min at 4 °C. (C) and (F) Incubation with MPR300 antibody followed by cy5 conjugated secondary antibody. (D) and (I) are merged images of A, B, C and F, G, H respectively. Cell line type is indicated at the left side of each panel. Bar in the panel is 30 μm (NIH3T3) and 35 μm (CHO). (iii) Inhibition of nanoparticle internalization by 20 mM mannose 6-phosphate. (A) DAPI, (B) blocking receptor with 20 mM mannose 6-phosphate followed by incubation with nanoparticles, (C) merged image of A&B, (D) is the transmission image. Cell line is NIH3T3; Bar in the panel is 50 μm.

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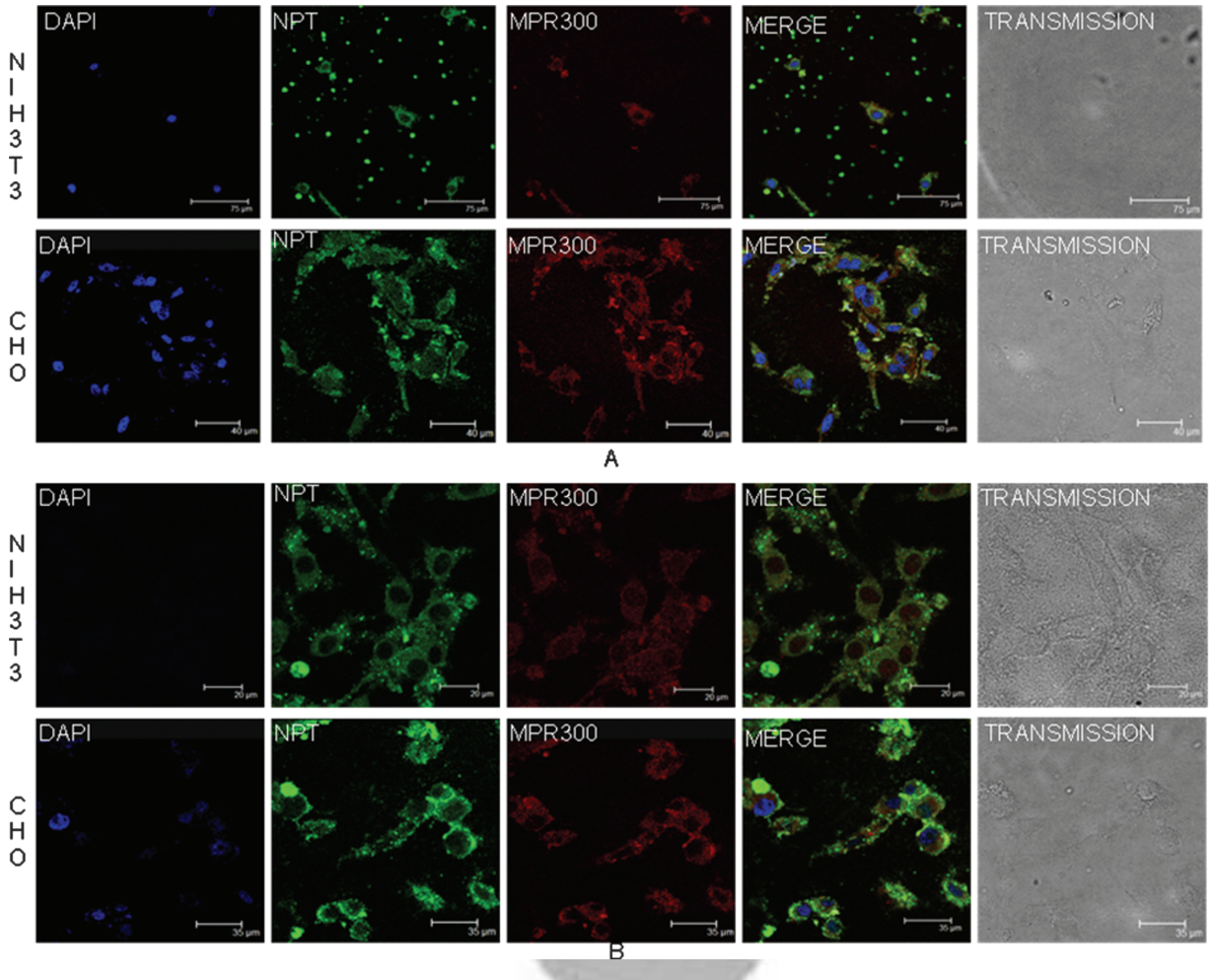

Fig. 5. Intracellular localization of β-hexosaminidase nanoparticles in NIH3T3 and CHO cells: (B) and (G) Incubation with FITC nanoparticles (NPT) for I hour at 37 °C, (C) and (F) Incubation with MPR300 antibody followed by cy5(red) conjugated secondary antibody, (*D*) and (*I*) are merged images of A, B, C and F, G, H respectively. Cell line type is indicated at the left side of each panel. Bar in the panel is 75 μm for NIH3T3 and 40 μm for CHO cells. (B) Intracellular localization of β-galactosidase nanoparticles in NIH3T3 and CHO cells: (B) and (G) Incubation with FITC nanoparticles (NPT) for I hour at 37 °C, (C) and (F) Incubation with MPR300 antibody followed by cy5 (red) conjugated secondary antibody, (D) and (I) are merged images of A, B, C and F, G, H respectively. Cell line type is indicated at the left side of each panel. Bar in the panel is 20 μm for NIH3T3 and 35 μm for CHO cells.

4. DISCUSSION

Receptor mediated endocytosis is one of the mechanisms for the internalization of ligands into cells. Our laboratory has been working on two homologous but distinct mannose 6-phosphate receptor proteins, MPR300 and MPR46 that are known to specifically interact in cells with the lysosomal enzymes that harbor a mannose 6-phosphate signal in order to gain new insights into the structure and function of these receptors and their evolutionary significance. Though both receptors are also present in the plasma membrane, only MPR300 alone has been described as an endocytosis receptor in mammals as well as in some non-mammalian vertebrates.[12] This receptor has been shown to be multifunctional protein capable of binding several mannose 6-phosphate containing ligands as well as some non-mannose 6-phosphate containing ligands. Several lysosomal disorders that effect humans are known and it is important to develop methods that would eventually be useful for curing the diseases or to help combat the diseased condition. Nanoparticles of naturally occurring polysaccharides such as the chitosan have gained more attention as specific drug delivery carriers.[13] Although the chitosan based nanoparticles have been used for drug delivery, there are no reports on the preparation of other types of oligosaccharide nanoparticles such as the mannooligosaccharides or phosphomannooligosaccharides. Since our laboratory has been working on Mannose 6-phosphate receptors and lysosomal enzymes that contain mannose 6-phosphate residues, we wanted to explore the possibilities of preparation and characterization of the mannooligosaccharides or phosphomannooligosaccharides and two lysosomal enzymes in order to test whether they can be recognized and internalized into

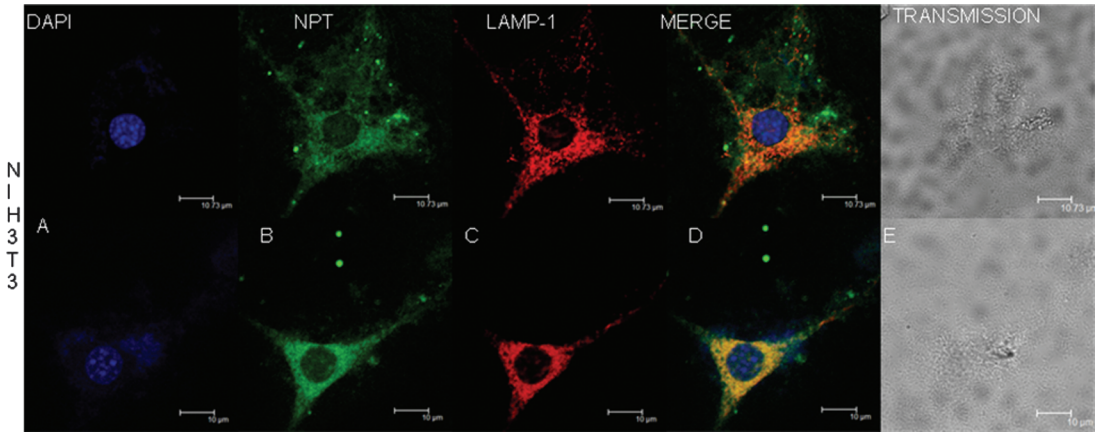

Fig. 6. Sub cellular localization of β-hexosaminidase nanoparticles in NIH3T3 cell lines, (A) incubation with DAPI, (B) Incubation with FITC nanoparticles (NPT) for 1 hour at 37 °C, (C) incubation with Lamp-1 antibody followed by cy5 (red) conjugated secondary antibody, (D) is merged image of A, B, C. Cell line type is indicated at the left side of the panel. Bar in the panel is 10.73 μm (top panel) and 10 μm (bottom panel).

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cells by the MPR300 protein, a known receptor for these ligands. Our long term goals are to establish a suitable drug delivery systems for various therapeutic disorders that effect mankind with a preference for lysosomal disorders. As a first step, we prepared the phosphorylated mannooligosaccharide (phosphomannan core) nanoparticles, as well as two lysosomal enzyme nanoparticles and characterized them by AFM and SEM with respect to their sizes. The nanoparticles prepared were in different size ranges as shown in the results and this difference in their sizes might possibly be due to the difference in their molecular masses.

Second we wanted to analyze if these nanoparticles prepared can bind to the cell surface MPR300 protein, and therefore used specific cell lines NIH3T3 and the CHO cells that are known to contain this receptor. Our results suggest that the PMC nanoparticles as well as the enzyme nanoparticles prepared in the study show specific binding to the MPR3000 on the cell surface as evidenced by confocal microscopy. Further confirmation of this binding has been established by the reactivity of the MPR300 antibody with the cell surface bound PMC, enzyme nanoparticles and MPR300. The specificity of this interaction has also been ascertained in two separate experiments. Preincubation of the cells with different concentration of the mannose 6-phosphate prior to the addition of the nanoparticles, showed complete inhibition of the binding of the nanoparticles to the cell surface only at 20 mM concentration of the sugar while the 5 mM mannose 6-phsophate and 10 mM mannose 6-phosphate did not show any inhibitory effect (data not shown). This high concentration of the sugar required to cause complete inhibition might possibly be due to the large aggregates of nanoparticles that are binding to the cell surface receptor. Similarly preincubation of the cells with the antibody of the MPR300 protein, also showed complete inhibition of binding suggesting the non-availability of the cell surface receptor for the nanoparticle binding.

Third we wanted to analyze if the nanoparticles bound on the cell surface can be internalized and therefore did the incubation experiments at 37 °C. The three types of nanoparticles used in this study can be internalized by two different cell types that harbor the MPR300 protein. The localization of the nanoparticles together with the receptor in the cells was also visualized by using an antibody to the MPR300 protein (the antibody is directed against the purified MPR300 protein from goat liver, and from the antiserum, specific IgG was affinity purified on MPR300-receptor-affigel and used in the study. This antibody can recognize the MPR300 protein from mammals to molluscs as evidenced from our various studies). Furthermore the subcellular localization of the nanoparticles/receptor together with the Lamp1 protein, a known lysosomal associated membrane protein, strongly suggests and supports the specificity of the interaction between the nanoparticles used and the receptor and the role of the receptor in specific targeting of the nanoparticles.

The MPR300 expression is decreased in some malignancies such as the hepatocarcinoma, however, it's over expression in the majority of solid tumors particularly in breast cancers,[14] indicates that this receptor could be considered as a tool for specific drug delivery through the nanoparticles that contain mannose 6-phosphate residues.

In summary, our results clearly establish the preparation of novel reagents (nanoparticles of PMC and lysosomal enzymes) that specifically interact with the MPR300 protein. Having established this, our future work aims at preparation of a variety of these nanoparticles in large scale and uses them as efficient tools for tagging drugs/biomolecules which will have the capability of binding to the cell surface MPR300 protein and can be specifically delivered into the cells, thus making available the specific biomolecules/drugs in the cell. Furthermore due to the presence of a wide variety of glycosidase enzymes in the cells, the nanoparticles would be biodegradable and would possibly not have any toxic effects to the cells.

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ARTICLE

Preparation and Characterization of *Strychnos potatorum* L. Seed Polysaccharide Nanoparticles and Affinity Matrices: Relevance to Biological Applications

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The genus *Strychnos* is well known for its phytochemistry and is distributed throughout the tropics. Experiments conducted with the *Strychnos potatorum* seed meal revealed the presence of coagulant proteins, glycosidases as well as some polysaccharides. In a recent study we confirmed that some proteins in the extract have the ability to bind cadmium. Additionally some glycosidase activities were also detected among which only α-mannosidase was predominant. Additionally polysaccharides such as the Galactomannan and Galactan are also found in these seeds which we isolated and purified by gel filtration to make nanoparticles as in recent years, naturally occurring polysaccharides in nanoparticle forms are being used in various fields of biology and medicine. Nanoparticles were prepared for the two isolated polysaccharides separately and characterized by AFM, SEM and TEM. The size of the nanoparticles ranged from 37–100 nm (galactan) and 45 nm to 110 nm (galactomannan). The polysaccharides prepared when immobilized to Seralose gel were found to specifically bind galactose specific lectins from *Dolichos lablab* seeds and snake gourd seeds.

KEYWORDS: Galactan, Galactomannan, Nanoparticles, Polysaccharides, *Strychnos potatorum*.

1. INTRODUCTION

Polysaccharides are a class of biopolymers that are building blocks of simple sugar monomers. They are abundant in nature, universally found in almost all living organisms. They are present in various tissues of seeds, stems and leaves of plants, body fluids of animals, shells of crustaceans and insects. They are also found in the cell walls and extra cellular fluids of bacteria, yeast and fungi and are thus renewable reservoirs for synthesizing high performance materials.[1,2] Polysaccharides have complex structures in which the monosaccharides are linked together by O-glycosidic linkages and diversification of the monosaccharides determines their properties.[3] Moreover polysaccharides are hydrophilic, biodegradable, non toxic, stable and safe to use, which suggests their use in targeted drug delivery systems. They can profoundly affect the immune system and therefore have the potential as immunomodulators with wide clinical applications.[4–6] Many of the polysaccharides have specific receptors on the cells so they can be easily taken by the cells, it was reported that Hyaluronic acid (HA) was taken by HA specific receptor-mediated endocytosis, and HA was suitable for the targeted drug delivery systems mediated by the specific receptor.[7,8] Some glycoproteins/glycosides such as asialoglycoproteins and galactosides were known to be suitable for the receptor-mediated drug delivery systems. It was reported that the mannosylated, fucosylated and galactosylated liposomes showed high accumulation in the liver via each specific receptor.[9] Polysaccharides have been successfully used as drug carriers due to their superior properties and biocompatibility.[10–15] Storage polysaccharides in seeds are mostly starches or galactomannans.[16] Galactomannans are heteropolysaccharides containing residues of galactose and mannose. They are an important group of polysaccharides produced in plants as cell wall storage polysaccharides.[17] Galactomannan serves as water retainer for the seeds due to their swelling property in addition to

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being food reserve for the germinating seeds.[18] A large group of galactomannans are produced from seeds of leguminoseae family. Thus, out of 163 legume seeds examined, 119 seed endosperm mucilage contained galactomannans as the major reserve polysaccharide constituent.[19] Seed galactomannans are heterogeneous polysaccharides consisting mainly of the monosaccharides mannose and galactose units. The mannose-elements form a linear chain consisting of $(1 \rightarrow 4)$-β-D-mannopyranosyl residues, with $(1 \rightarrow 6)$ linked α-D galactopyranosyl residues as side chain at varying distances, dependent on the plant of origin[20] according to Aspinall[21] galactomannans are those mannans that contain more than 5% of D-galactose. The major difference in galactomannans from different seed species is in the ratio of D-galactose to D-mannose. However, there is also some variation in molecular weight of the polysaccharides. The structural differences of these make them as versatile materials with applications in food, drug delivery, and health care products.[22, 23] In literature nanoparticles of galactomannan (Guar gum) from the seeds of *Cyamopsis tetragonoloba* have been prepared and characterized.[24] Recent studies have utilized nanoparticles as efficient tools for drug delivery into cells. Nanoparticles can be prepared from a variety of materials such as proteins, polysaccharides and synthetic polymers. The use of biomolecules as reducing agents for production of nanoparticles is gaining considerable momentum for use in cosmestics, medical, biological and industrial applications.[25] Nanoparticle delivery systems have advantages, because of their very small size the clearance of nanocarriers by phagocytes will be avoided so that their duration in blood stream is greatly prolonged, they can penetrate cells and tissue gaps to arrive at target organs. Nanoparticles that are biodegradable offer better advantages as they would be non-toxic to the cells. It is because of this reason that the polysaccharide nanoparticles have been the subject of study in recent years. Nanoparticles could be functionalized and can act as potential carriers for several biomoloecules and drugs. Recent work from our lab using yeast phoshpomannan core polysaccharide nanoparticles revealed their selective binding to the Mannose 6-phosphate receptor (MPR300) on the cell surface and nanoparticle internalization mediated by this receptor. Additionally, the natural ligands for the receptor, the known lysosomal enzyme nanoparticles were also internalized by this receptor.[26] Receptor-mediated targeting is a promising approach for selective drug delivery. Medical therapies have become more tailored to specific diseases and patients in recent years.[27] Since we isolated the two distinct polysaccharides from the *S. potatorum* seeds, our long term goals were to explore the use of these as potential nano-carriers for drug delivery; as a first step toward this, in the present study we prepared nanoparticles for the two isolated polysaccharides and characterized them. Additionally these polysaccharides were also tested for their ability to bind sugar specific lectins.

2. MATERIALS AND METHODS

2.1. Materials

All the chemicals and reagents used in the present study were of analytical grade. All the glassware used were washed with 10% (v/v) HNO_3 and subsequently rinsed several times with de-ionized distilled water. Bio-Gel-P60 (Polyacrylamide Gel) was procured from Bio-Rad Laboratories, USA. diethyl ether, D-Galactose are from SRL, India. Seralose, Divinly sulfone (DVS) from Sigma Aldrich (USA) Equipments such as AFM-(SPA-400, USA), Probe sonicator, SEM-(PHILIPS FEI-XL ESEM, USA), Spin Coater, TEM-JOEL JEM 1011, USA, were available in the central facilities and center for nanotechnology at University of Hyderabad.

2.2. Seed Material

S. potatorum L. seeds were obtained from the divisional forest, flying squad division, Rajahmundry, East Godavari district, Andhra Pradesh India. Seeds were dried at 40 °C for 2 days in hot air oven. Seeds were made into powder in Cyclotech 1093 sample mill (Tecator AB, Hoganas, Sweden). 5 g of the seed powder was used for every batch of polysaccharide extraction.

2.3. Methods

2.3.1. Extraction, Separation and Purification of Polysaccharides from S. potatorum Seed Powder

Extraction and purification of the polysaccharides from *Strychnos potatorum* seed powder was carried out as described earlier.[28] In brief 5 g of the seed powder was added to 250 ml of double distilled water, stirred for 15 min and then centrifuged at 10000 rpm for 10 min. The supernatant solution was filtered through Whatman GFC glass fiber discs (0.45 μm) followed by Whatman cellulose acetate filters. The filtrate was added to an equal volume of a 1:1 mixture of 10 mM pH 7.5 Tris-saturated phenol and chloroform, the emulsion was stirred at room temperature for 30 min followed by centrifugation at 10000 rpm for 10 min. The aqueous layer was removed, shaken with an equal volume of chloroform, centrifuged at 10000 rpm for 10 min, separated from the organic phase, and mixed with 2.5 volumes of ethanol. The polysaccharide fraction was precipitated overnight at −20 °C, washed in ethanol and dried in a vacuum dessicator. Each gram of seed powder yielded 100 mg of polysaccharides. The polysaccharides extracted above were dissolved in a minimal amount of sodium acetate buffer pH 5.2 and chromatographed on Bio-gel P-60 (Bio-Rad) column. The column was calibrated with sodium acetate buffer pH 5.2 and eluted with the same buffer. The column eluates were monitored for carbohydrate using phenol sulphuric acid method[29] and were resolved into two peaks A and B.

2.3.2. Acid Hydrolysis and Paper Chromatography

The peak A and peak B polysaccharides obtained were separately subjected to acid hydrolysis using concentrated hydrochloric acid in a sealed test tube and placed in an oven at 100 °C for 1 h. After hydrolysis the acid was removed by evaporation and the carbohydrate composition in the samples was monitored by paper chromatography using the solvent *n*-butanol/pyridine/water (6:4:3 v/v/v). Individual monosaccharides were detected by staining with alkaline silver nitrate after separation on the paper chromatogram.

2.3.3. Preparation of Galactomannan and Galactan Nanoparticles and Their Characterization

For the preparation of nanoparticles, the procedure described by Krishna et al. with minor modifications was adopted.[30] 20 mg each of lyophilized galactomannan and Galactan were separately dissolved in 500 μl of 1 $\times$ PBS buffer of pH 7.4 and kept on ice for 5 min. 15 ml olive oil was added drop wise with continuous stirring on a cyclomixer. It was sonicated 15 times, each time for 30 sec with 1 min gap. Immediately it was frozen in liquid nitrogen for 10 min. It was then kept at 4 °C for 4 h and the nanoparticle pellet was collected by centrifugation at 8000 rpm for 10 min. The residual oil was removed by washing the pellet with chilled diethyl ether 3–4 times. The clear pellet was dissolved in PBS buffer pH 7.4 and the nanoparticle sample was stored at 4 °C.

2.3.3.1. Atomic Force Microscopy (AFM). Nanoparticle sample was uniformly dispersed on a clean glass cover slip using a spin coater and dried in a dust free zone. The unit containing the sample was kept in SPA-400 and size and morphology of particles was studied following manufacturer's instructions.

2.3.3.2. Scanning Electron Microscopy (SEM). Operated at 10 kV. Nanoparticle sample was uniformly dispersed on a clean glass cover slip using a spin coater and dried. Metal stubs were coated with double-sided adhesive tape, the cover slip with nanoparticle sample was kept on the sticky surface and sample was coated with gold in Sputter Coater. Specimens were stored in dry, dust free environment during the analysis.

2.3.3.3. Transmission Electron Microscopy (TEM). Galactomannan and galactan nanoparticles were processed for electron microscopy by air-drying in a dust free zone a small drop of sample solution on carbon-coated copper electron microscopy grids. The grids containing air-dried samples were incubated with a 2% (w/v) aqueous uranyl acetate solution for 10 min at room temperature and washed 3–4 times with distilled water. Polysaccharide nanoparticle morphology was examined using the JOEL JEM 1011 100 kV electron microscope. Electron diffraction patterns were recorded from a selected area that is well occupied with polysaccharides nanoparticles in order to obtain high diffraction intensities. Particle size distributions were made by measuring diameters for polysaccharide nanoparticles.[31]

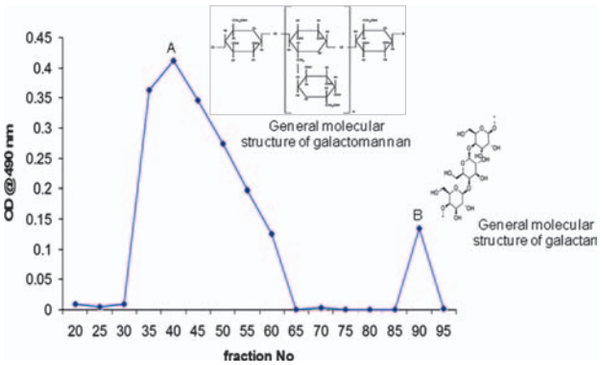

Fig. 1. Gel filtration of the polysaccharides isolated. The polysaccharide fraction was applied to a Biogel P-60 column equilibrated with 50 mM sodium acetate buffer pH 5.2 at a flow rate of 30 ml/h. Fractions of 0.5 ml were collected. Carbohydrate in the column fractions were onto red using phenolsulphuric acid method. Two distinct peaks, A (galactomannan) and B (Galactan) could be obtained which were pooled separately and lyophilized for further analysis.

2.3.4. Preparation of Seralose-Divinylsulfone-Galactomannan and Galactan Gels

5 ml of Seralose 4B was activated with divinyl sulfone, after activation, the gel was divided into two equal parts (2.5 ml each). One portion received 25 mg of isolated galactomannan and the other portion received 25 mg of galactan and these ligands were coupled and the gel processed as described earlier,[32] gel was finally washed with distilled water and was packed in 10 ml plastic columns for further use.

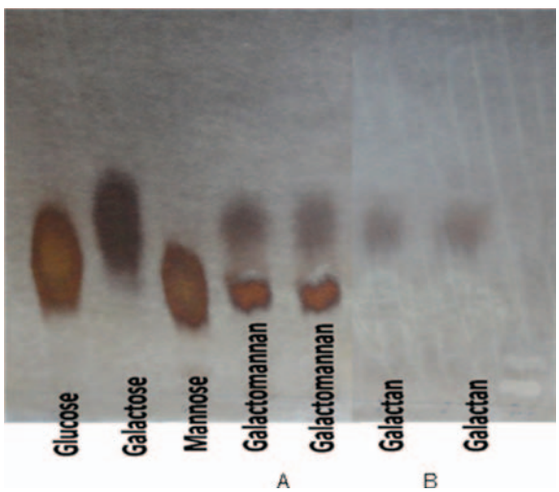

Fig. 2. Paper chromatogram showing reference compounds (glucose, mannose and galactose) and the products of acid hydrolysis galactomannan (A) and Galactan (B) isolated from Strychnos potatorum seeds. The solvent used is *n*-butanol/pyridine/water 6: 4: 3 v/v/v. Stained with alkaline silver nitrate.

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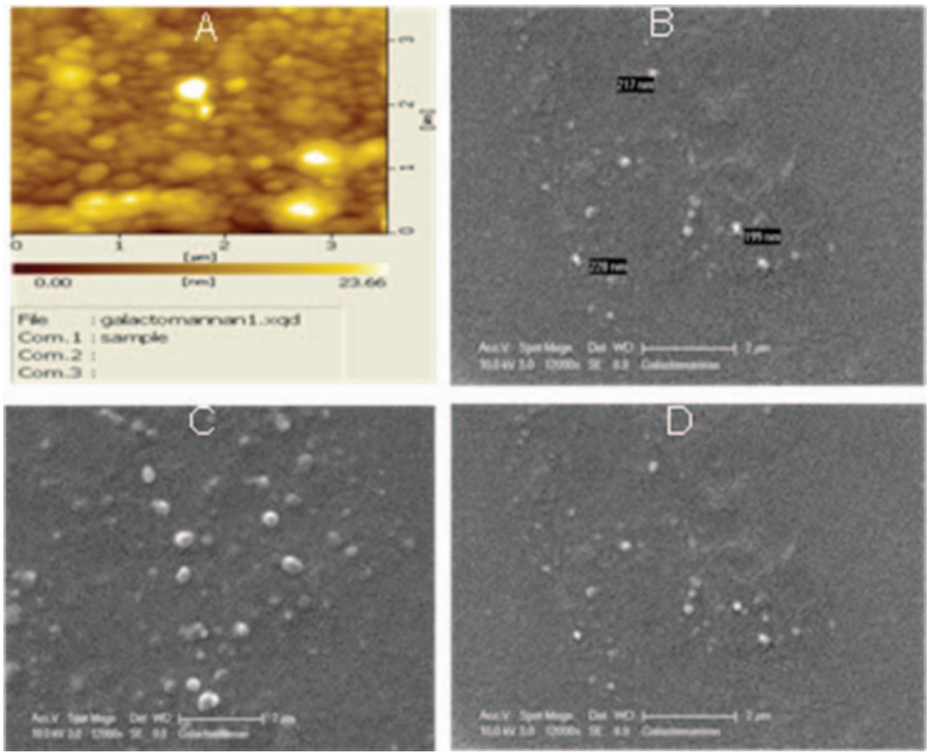

Fig. 3. (A) AFM image of galactomannan nanoparticles shows spherical shape of nanoparticles, (B)–(D) SEM images of galactomannan nanoparticles the system was operated at 10.0 and 25.0 kV. The size of the galactomannan nanoparticles were varying from 55 to 200 nm.

2.3.5. Analysis of Binding of Galactose Specific Lectin to Galactomannnan and Galactan Affinity Gels

2.3.5.1. Affinity Binding and Elution of Galactose Specific Lectin From Dolichos Lab Lab Seed Powder on Galactan Affigel. This was done as described earlier.[33] 100 g of seed powder was extracted overnight with 700 ml of 25 mM Tris Buffered Saline (TBS) pH 7.4 at 4 °C. The suspension was clarified by centrifugation (10,000 rpm at 4 °C) and the clear supernatant (crude extract) was subjected to 0–60% and 60–80% ammonium sulfate fractionation. The fractionation allowed the separation of the mannose/glucose lectin in the 0–60% fraction and the galactose lectin in the 60–80% fraction. The fraction containing the galactose lectin was dialyzed against TBS and passed through Seralose–mannose gel to deplete the fraction of any mannose/glucose lectin. To the unbound protein

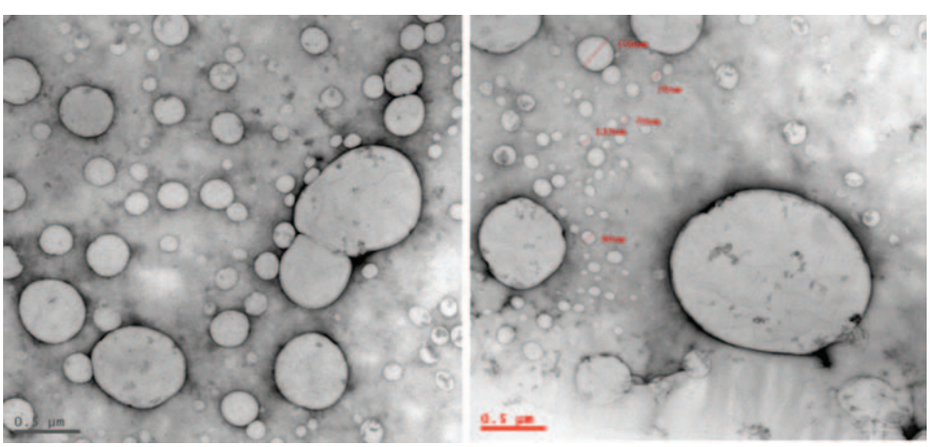

Fig. 4. TEM images of galactomannan nanoparticles, the electron microscopy grids containing air-dried samples were incubated with a 2% (w/v) aqueous uranyl acetate solution for staining at room temperature, then washed with distilled water, galactomannan nanoparticles images were examined using the JOEL JEM 1011 100 kV.

from Seralose–mannose gel 1.5 M ammonium sulfate was added and applied on a fresh Seralose-galactomannan and Galactan gels separately, that were equilibrated with TBS containing 1.5 M ammonium sulfate (column buffer). The gel was thoroughly washed with the column buffer until the A_{280} was 0.05 and the bound protein was eluted using 0.3 M galactose in column buffer and the protein was analyzed by coomassie staining as shown in the results. 1.5 M ammonium sulfate was required for the optimal binding of the galactose specific lectin on Seralose-galactose gel.

2.3.5.2. Affinity Binding and Elution of Galactose Specific Lectin from Snake Gourd Seeds on Galactan Affigel. To further validate our results we have checked the specific binding of another galactose specific lectin from snake gourd seeds to the galactan gel what we have prepared using a purified preparation of the lectin (kindly provided by Professor M. J. Swamy, School of Chemistry, University of Hyderabad, India). This experiment was done as described earlier.[34] In brief, the purified Galactose specific lectin in 1xPBS buffer pH 7.4 from snake gourd seeds was passed through the galactan gel that was pre-equilibrated with the column buffer (1 × PBS pH 7.4 containing 10 mM β-Mercaptoethanol) at 4 °C several times followed by extensive washing with column buffer and ligand specific elution with 0.1 M lactose in 1 × PBS pH 7.4. The lectin active eluted fraction was TCA precipitated and gel electrophoresis was performed on an aliquot of the eluate in 10% SDS-PAGE under reducing conditions.

3. RESULTS

3.1.

Total polysaccharideswere extracted from the *S. potatorum* seed powder and the extracted polysaccharides were chromatographed on a Bio-gel P-60 gel equilibrated with the column buffer (50 mM sodium acetate pH 5.2) as described under methods. Column fractions were monitored for carbohydrate using phenolsulphuric acid method. Figure 1 shows the separation of polysaccharides on Bio-gel-P60 column which resolved into two distinct peaks (A) and (B). Fractions corresponding to these two peaks were pooled separately and lyophilized for further analysis. Galactomannan and galactan polysaccharides peaks were subjected to acid hydrolysis and the hydrolysate processed for paper chromatography to identify the monosaccharides. Figure 2 shows the paper chromatography of peak (A) and peak (B) polysaccharides. It is evident from the figure that galactose and mannose spots for the product of the acid hydrolysis of peak (A) could be detected confirming the component isolated is indeed Galactomannan and one spot galactose for the product of acid hydrolysis of peak (B) could be detected confirming the component to be Galactan. Both peak (A) and peak (B) fractions were lyophilized separately and used in further studies as described below.

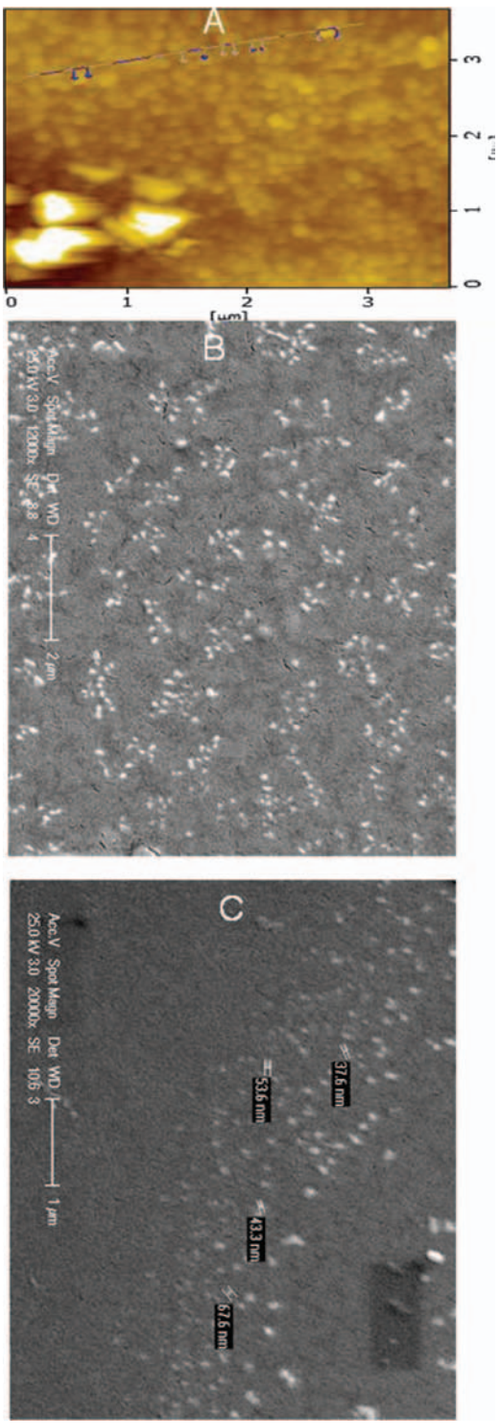

Fig. 5. (A) AFM and (B) and (C) SEM images of galactan nanoparticles.

3.2. Nanoparticle Characterization

The galactomannan and galactan polysaccharides after separation were also used for the nanoparticle preparation as described under methods. These were then characterized by AFM, SEM and TEM. Structure and

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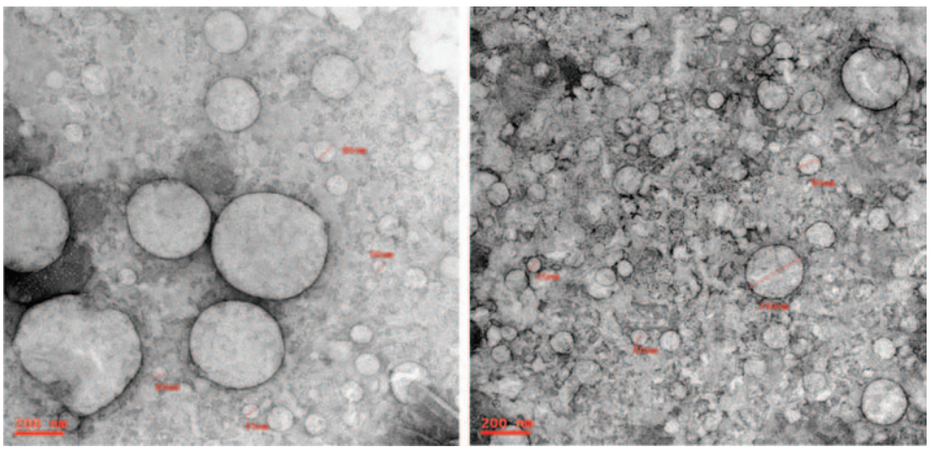

Fig. 6. TEM images of galactan nanoparticles, the electron microscopy grids containing galactan air-dried samples were incubated with a 2% (w/v) aqueous uranyl acetate solution for staining at room temperature, then washed with distilled water, galactan nanoparticles images were examined using the JOEL JEM 1011 100 kV.

morphology of the nanoparticles were investigated using Scanning electron microscope (Philips FEI-XL 30 ESEM, USA-operated at 20 kV), Transmission electron microscope (JOEL JEM 1011, USA-operated at 100 kV) and Atomic force microscope (SPA 400, USA); manufacturer's instructions were followed for sample preparation, data collection and analysis of particles as describe below. From Figures 3 and 4 we could confirm the size of

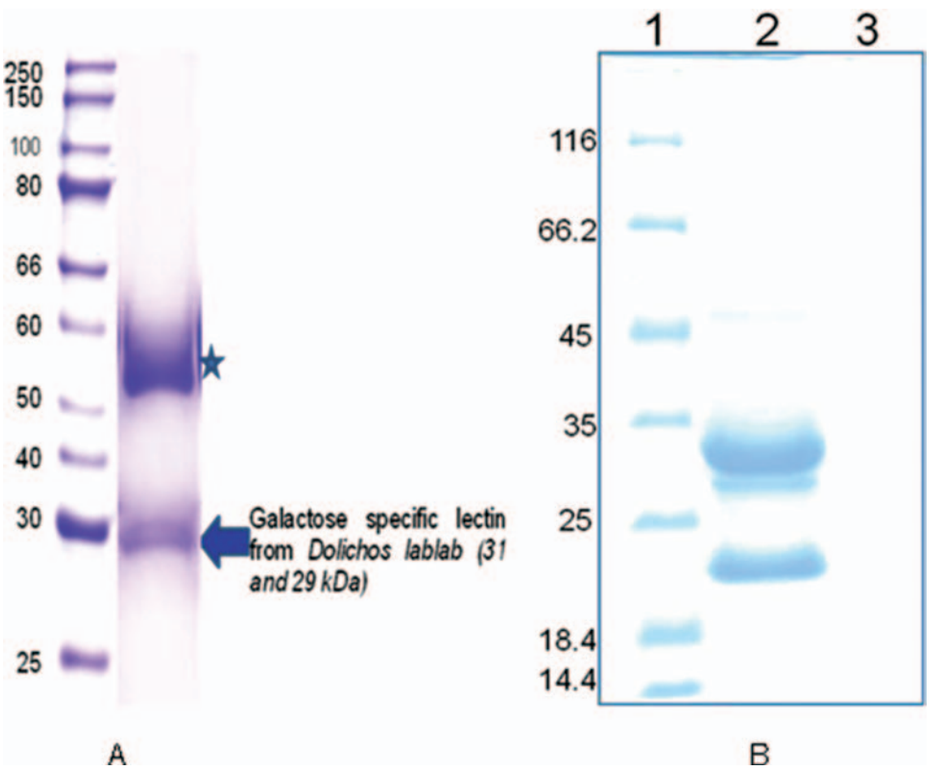

Fig. 7. (A) 10% SDS-PAGE analysis of the eluted Lectin after Affinity chromatography, Lane 1 molecular weight markers. Result shown for galactan gel elution (Galactose specific lectin from *Dolichos lablab*) containing sample applied on Galactan/galactomannan seralose column: Gel washed, and eluted with 0.3 M galactose. (★Represents possibly a storage protein that can be separated from the lectin by gel filtration after the affinity chromatography) (B) Gel electrophoresis of the eluted galactose specific lectin (SGSL) on 10% SDS-PAGE after Affinity chromatography, Lane 1 molecular weight markers. Lane 2 eluted lectin, Lane 3 unbound protein. Result shown for *galactan gel elution* (galactose specific lectin from *Snake gourd seeds*) containing sample applied on Galactan/galactomannan Seralose column.

galactomannan nanoparticles which varied from 45 nm to 110 nm. Figures 5 and 6 show the SEM and TEM images of Galactan nanoparticles, the size of Galactan nanoparticles was found to be in the range of 37 nm to 100 nm.

3.3.

The two polysaccharides were separately immobilized to Seralose 4B as described under methods and tested for their ability to bind galactose specific lectin in a sugar specific manner. This study shows that the gels can bind galactose specific lectins from the seeds of *Dolichos lablab* and Snake gourd seeds which can be bound and eluted specifically with galactose and lactose respectively as shown in Figures 7(A) and (B) respectively. Galactose specific lectin from *Dolichos lab lab* known to contain two subunits of molecular weights 31 and 29 kDa respectively and galactose specific lectin from snake gourd seeds is a dimer of 32 KDa and 23 KDa subunits which can be eluted also with lactose from the gel.

4. DISCUSSION

Plant polysaccharides particularly chitosan are being widely used as a potential agent in nanoparticle form for targeted drug delivery, one of the prime uses of nanoparticle research.[11] Recent literature report also cites use of other biomolecules as potential agents for targeted drug delivery. One of the important features for such nanoparticle based materials is their non-toxic nature to the cells and their easy biodegradability. Our laboratory has recently published the importance of proteins from the seeds of *Strychnos potatorum* seeds in binding cadmium and removing cadmium toxicity from aqueous solutions.[35] We found that these seed extracts also contain high concentrations of polysaccharides that were characterized in a preliminary study.[27] We wanted to explore and identify the other potential components of this seeds as biologically degradable material. Since polysaccharides such as chitosan have already been used in targeting drug delivery we wanted to explore the potentiality of the two distinct polysaccharides (galactomannan and galactan) from this seed materials for preparation of nanoparticles with a long term objective to possibly use them as potential tools for targeting drug delivery like other known polysaccharide nanoparticles. Therefore our results presented here is the first study on preparation and characterization of nanoparticles for both the polysaccharides isolated from the seeds of *Strychnos potatorum. L.*

The two polysaccharides (galactomannan and galactan) that were isolated from *S. potatorum* seeds were used for the nanoparticle preparation. Nanoparticles of both Galactan and Galactomannan were successfully prepared in separate experiments by Sol-oil chemistry method. From the microscopic characterization it is shown that the nanoparticles are spherical in shape for both the polysaccharides and the size of galactomannan nanoparticles varied from 45 nm to 110 nm and that of Galactan nanoparticles from 37 nm to 100 nm.

Since the *Strychnos potatorum* seed polysaccharides isolated are galactomannan and galactan respectively we wanted to explore their potentiality to bind and purify plant lectins that exhibit galactose specificity. Plant polysaccharides such as guar gum (galactomannan) have been extensively used for the isolation of galactose specific lectins.[36] Our laboratory has already coupled phosphomannan core containing phosphorylated manno oligosaccharides from yeast to Seralose-divinyl sulfone and used this matrix to purify mannose 6-phosphate receptors.[37] Therefore galactomannan and galactan isolated in this study were separately coupled to Seralose via divinvylsulfone. To test these gels for binding galactose lectins we have used extracts of *Dolichos lablab* seeds (that are known to contain the galactose specific lectin) and also a purified preparation of another galactose specific lectin from snake gourd seeds. Interestingly our results shown both these matrices had the potential to bind galactose specific lectins and hence can thus serve as very useful natural resources for the purification of galactose specific lectins.

In summary this is the first report on the preparation and characterization of galactomannan and galactan nanoparticles from the seeds of *Strychnos potatorum*. Thus these seed materials which have recently been shown by us to bind and remove metal ions such as cadmium from aqueous solutions have also potential applications in isolation of galactose binding lectins. Our future work aims at testing the efficiency of the prepared nanoparticles as alternate natural sources of polysaccharides that can be used for targeted drug delivery of biomolecules into cells.

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