Expert Opinion

Mannose-targeted systems for the delivery of therapeutics

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Background: The specific targeting of nanomedicines to mannose receptors, highly expressed in cells of the immune system, performs a useful strategy for improving the efficacy of vaccines and chemotherapy. *Objective*: This review discusses the potential of mannose-targeted drug/antigen delivery systems for vaccination and treatment of diseases localized in macrophages and other antigen-presenting cells. *Methods*: The first part of the review describes the characteristics, localization and functions of mannose receptors. The following sections are devoted to the description of different methods used to deliver therapeutic agents, including mannose conjugates and mannosylated carriers or particulates (i.e., liposomes, nanoparticles and niosomes). *Results/conclusions*: A general overview of published reports confirms the effectiveness of mannosylation strategies, although the optimization and full exploitation of mannose-targeted drug delivery systems would require a deeper understanding of the structure–activity relationship. In the near future, these nanomedicines have the potential to treat a number of diseases (including cancer) and improve the quality of life of patients.

Keywords: conjugates, dendritic cells, liposomes, macrophages, mannose, nanoparticles, vaccination

Expert Opin. Drug Deliv. (2008) 5(6):703-724

1. Introduction

The glycosylation of proteins and their interaction with carbohydrate-binding proteins (lectins) has been proven as an extremely important factor in a large variety of cellular recognition processes such as enzyme trafficking, cellular migration, cancer metastasis and immune functions. The high specificity of oligosaccharide–lectin interactions has already been used for the targeting of carbohydrate ligands to site-specific target receptors and many glycoconjugates have demonstrated the potential of 'glycotargeting' as a promising route to the 'magic bullet'. Within this general context, the effectiveness of mannosylated devices in vaccination or for drug delivery purposes can be ascribed to their ability to target mannose receptors, which are highly expressed in cells of the immune system [i.e., macrophages and dendritic cells (DCs)] [1-4].

Although initially mannosylated constructs were believed to target only the mannose receptor expressed on macrophages (MMR), other mannose receptor positive cells (i.e., DCs and endothelial cells) and many other lectins with mannose-binding activity have been identified [5]. However, mannose receptors vary in their immunological role as they differ in pattern, localization and level of expression in different cells. Similarly, although mannose receptors bind mannose-containing structures, different branching and spacing of these structures create unique sets of carbohydrate recognition profiles for each receptor and this aspect has not been clearly elucidated [6].

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2. Mannose receptors

2.1 Structures, multimerization and binding affinities

Lectins expressed in mice/humans that have shown mannose-binding activity include transmembrane proteins such as the classical MMR (group VI), Endo180, dendritic cell-specific intercellular adhesion molecule-3 grabbing non-integrin (DC-SIGN), DC-SIGN related (L-SIGN, group II, type 2 receptors) or their mouse homologues (SIGNR), Langerin or secreted/soluble proteins such as the collectins mannose binding protein (MBP), surfactant protein A and surfactant protein D (group III). Additionally, there is conflicting evidence for mannose type ligands for Dectin-2 [2,4]. All of these are C-type lectin receptors (CLRs) with the capacity to bind the carbohydrates via one or more carbohydrate recognition domains (CRDs) in a calcium-dependent manner [4-6]. The features of the main mannose rectors are summarized in **Table 1**.

The mannose specificity is determined by the amino acid sequence making up the CRD. Furthermore, the three-dimensional conformation and pattern of multimerization of each mannose receptor determine its ability to accommodate devices in a specific arrangement and interact with the mannose residues; all of them, however shared an increased affinity by a concomitant clustering of lectin binding site and carbohydrate recognition units in multivalent constructs (the glycoside cluster effect) [7].

The multimerization of CRDs can occur within one single molecule like in MMR. Thus, this receptor shows eight CRDs and three of them cooperate to achieve high-affinity binding to multivalent glycoconjugates [8-10]. In contrast, each molecule of DC-SIGN only contains a single CRD; however, it interacts with other DC-SIGN-forming tetramers on the surface of DCs [9,11,12]. The collectin mannose binding lectin (MBL) is also formed by a single CRD; although the basic unit is a trimer. Strong binding is only obtained with the formation of a trimeric complex and aggregation of up to six trimers that take the form of a bouquet of flowers [13]. This diversification of oligomeric states allows the accommodation and recognition of the distinct pattern of a carbohydrate, although the overlapping of ligands is frequent. In this context, the linear arrangement of the three CRDs in the single polypeptide of the mannose receptor would be suitable for the recognition of end-standing single mannose moieties or structures containing a dimannoside cluster, whereas the CRD of the tetrameric DC-SIGN has higher affinities for internal mannose branched structures with short spacing between the residues [14]. Finally, the binding of MBL is only significant with surfaces with appropriately spaced carbohydrates and very high density of terminal mannose, glucose or *N*-acetyl glucosamine [15].

2.2 Mannose receptor and antigen uptake

Transmembrane mannose receptors mediate endocytosis, function as antigen capture receptors and are involved in antigen capture and presentation [16-19]. MMR recognizes carbohydrate moieties of different pathogen ligands, including components from *Pneumocystis carinii*, *Candida albicans*, *Leishmania donovani*, *Trypanosome cruzi* and *Mycobacterium* species. DC-SIGN, however, binds to high-mannose N-linked glycans (branched trimannose structures) on the HIV-1 gp120 protein and on the surfaces of other viruses, including hepatitis C virus, human cytomegalovirus, Dengue or Ebola. It also recognizes the lipoarabinomannan from *Mycobacterium tuberculosis* or Lewis epitopes on *Helicobacter pilori* or *Schistosoma mansoni* [20].

After recognition, MMR delivers the antigen to the early endosomes and recycles to the surface [10]. The content of endosomes is subsequently targeted to lysosomes in which the degradation produces antigen fragments that, after presentation in MHC molecules, can stimulate the adaptive immune responses [16]. For DC-SIGN, this receptor delivers the bound components to late endosomes or lysosomes where they are degraded. The generated antigens are processed for MHC class II presentation to T cells [16,20]. Under specific conditions, both MMR and DC-SIGN appear to deliver the antigens in MHC class I molecules [21,22].

In contrast to the transmembrane mannose receptors, MBP plays a major role in innate immunity. MBP binds a large list of microorganism structures, including lipopolysaccharide (LPS) from *Escherichia coli*, lipoarabinomannan from *M. tuberculosis*, mannan from *C. albicans* and lipophosphoglycan from *L. donovani* [23]. Upon binding to microorganisms, MBL puts into action an effector mechanism characterized by the induction of agglutination to prevent the colonization followed by the activation of the complement by the lectin pathway, opsonization that enhances phagocytosis by macrophages and activation of their microbicidal action [23].

2.3 Mannose receptor and self-recognition

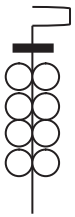
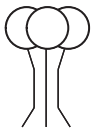
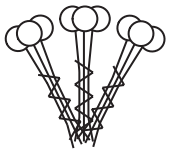
In addition to pathogens, mannose receptors also recognize self-glycoproteins with diverse functions [2,24]. Thus, the liver sinusoidal endothelial cells (that express MMR) participate in the clearance of sulfated glycoprotein hormones [25], collagen or gelatine [26]. Similarly, macrophages expressing MMR also play a role in the maintenance of tissue homeostasis and resolution of inflammation by eliminating self-glycoproteins as lysosomal hydrolases, tissue plasminogen activator or neutrophil myeloperoxidase.

The binding of DC-SIGN to endogenous ligands is principally associated with the migration of DCs [27] and DC-T interactions [28]. Finally, MBP binding appears to modulate inflammatory responses and apoptotic cell clearance [29].

2.4 Mannose receptor and immune activation: CLR and TLR cross talk

The role of MMR in endogenous or exogenous antigen uptake and presentation is clear. However, their participation in signal transduction pathways and modulation of cellular

Table 1. Main C-type lectins that recognize mannosylated ligands, from information in reviews [1-6].

	CLR	Pathogen (selected)	Endogenous ligands	Sugar and ligand specificity	Regulation	Expression	Function
MMR family 	MMR	*M. tuberculosis*, *C. albicans*, HIV, *C. neoformans*, *S. mansoni*, pneumocistis	Lysosomal hydrolases, thyroglobulin, L-selectin, lutropin, myeloperoxidase	Mannose, fucose, mannan, *N*-acetylglucoasamine via CRD, sulphated Le via CR, collagen via FNII	↑ PGE, IL-4, IL-10, IL-13; ↓ IFN-γ, LPS	DC subsets, macrophages, lymphatic and hepatic endothelium	Pathogen recognition, Ag presentation, regulation of circulating hormones, clearance of endogenous inflammatory molecules
	Endo 180	Unknown	Collagen, urokinase type plasminogen activator	Mannose, fucose, *N*-acetylglucosamine via CRD, collagen via FNII	Unknown	Fibroblasts, subsets of endothelial cells, macrophages	Extracellular matrix degradation, cell migration
Type II receptors 	DC-SIGN	Virus (HIV, HCV, CMV, Dengue), *M. tuberculosis*, *H. pylori*, *A. fumigatus*, leishmania, *S. mansoni*, *C. albicans*	ICAM-2 and ICAM-3	Mannan, Le, fucose, mannosyl lipoarabinomanan	↑ IL-13, ↓ LPS	DCs, alveolar, peritoneal, decidual macrophages	Pathogen recognition, Ag presentation, HIV transmission, cell migration, DC–T cell interactions
	DC-SIGNR (L-SIGN)	HIV, HCV, *S. mansoni*, *M. tuberculosis*	ICAM-3	Mannan, Le	Unknown	Hepatic and lymphatic endothelium, peritoneal and metallophilic macrophages	Pathogen recognition, HIV transmission
	Langerin	*M. leprae*	Unknown	Mannose, fucose, *N*-acetylglucosamine	↑ TGF-β, ↓ LPS	Langerhans cells and other subsets of DCs	Ag uptake
Collectins 	MBL	HIV, *S. aureus*, *S. pneumoniae*, *C. albicans*, *A. fumigatus* and many other bacteria, virus, fungi and protozoa	Dying and transformed cells, ischemic tissues, immunoglobulins, nucleic acids, phospholipids, metalloproteases	*N*-acetylglucosamine, fucose, glucose, *N*-acetylmannosamine		Serum	Agglutination, opsonization and enhanced phagocytosis, complement activation, regulation of inflammation

CLR: C-type lectin receptor; CMV: Cytomegalovirus; CR: Cystein rich domain; CRD: Carbohydrate recognition domain; DC: Dendritic cell; DC-SIGN: Dendritic cell-specific ICAM-3 grabbing non-integrin; DC-SIGNR: DC-SIGN related, also named L-SIGN; FNII: Fibronectin type II repeat; HCV: Hepatitis C virus; ICAM: Intercellular adhesion molecule; LPS: Lipopolysaccharide; MBL: Mannose binding lectin; MMR: Macrophage mannose receptor.

activation has not been clearly established. Pathogen-bearing mannose receptors produce different profiles of immune responses because they can cross react with several receptors, not only CLRs but also toll-like receptors (TLRs) [30].

In order to elucidate the real effect of mannose receptor activation, different studies with specific antibodies have been performed. For example, the pmel17 melanoma-associated antigen was linked to the heavy chain of anti-human MMR monoclonal antibody (B11) [31]. DCs treated with this conjugate (B11-pmel17) presented the antigen in the context of class I and class II molecules and generated CTL responses. In the same way, B11–ovalbumin fusion proteins elicited humoral immunity and efficient presentation of ovalbumin to CD4 and CD8 T cells in transgenic mice [22]. In all cases, the combination of MMR targeting with activation signals (CpG, poliI:C or resiquimod) enhanced antigen processing, presentation and tumour regression [22,32].

There is clear evidence that DC-SIGN is also a signalling receptor that can modulate TLR signals. Caparros and co-workers [33] demonstrated that triggering of DC-SIGN with an antibody resulted in extracellular signal regulated kinase activation, whereas Hodges *et al.* [34] reported activation of Rho-GTPase. The binding of pathogens to DC-SIGN can promote both T helper type 1 (T_H1) and T_H2-mediated responses [35]. Strikingly, there is also some evidence of a link between DC-SIGN and TLR by which some pathogen may evade the immune response. For example, the binding of ManLAM [a mannose capped glycolipid (ManLAM) localized in the cell wall of *M. tuberculosis*] to DC-SIGN delivers a signal that interferes with TLR4-mediated activation.

2.5 Pattern of mannose receptor expression

In steady-state conditions and in agreement with the role in the clearance of hormones and self-proteins, MMR appears to be mainly expressed in hepatic and lymphatic sinusoidal endothelial cells, intersticial cells of secretory organs, mucosal sites, tissue macrophages (Kupffer, lamina propia, dermal, peritoneal and alveolar), macrophages of the red pulp of the spleen and subcapsular sinus of lymph nodes [36]. The role of mannose receptors in the maintenance of tolerance also agrees well with the increase in their expression in macrophages in the presence of anti-inflammatory mediators such as IL-10, IL-4 or prostaglandin E. However, during an infection or upon stimulation with TLR agonists such as LPS, the pattern of MMR expression can change and it can be detected in lymph node DCs, located within B-cell follicles, and follicular DCs [36].

In contrast to MMR, the expression of DC-SIGN is restricted to DCs and tissue macrophages [37]. DC-SIGN is highly expressed by DCs in placenta at the interface of mother/child antigen transmission, a site of immune tolerance [38]. DC-SIGN is also highly expressed on ellipsoids in spleen at those sites where a direct contact between blood and tissue exists to enable antigen clearance from blood, without induction of an immune response. Finally, DC-SIGN is also localized in lymph nodes on DCs located in the T-cell area and on immature DCs located at the site close to the efferent sinus [39].

2.6 Immune response induced by mannosylated devices

The structure of mannosylated devices and mannose receptor expression profile dictates the quality of the immune response. At the body level, it will determine the antigen-presenting cells (APCs) involved in the recognition and the context in which this recognition will occur. In fact, after intravenous administration, mannosylated proteins will be predominantly cleared by MMR on liver sinusoidal endothelial cells (LSECs) and macrophages in the red pulp, with participation of DCs [40]. LSECs are a cell type of confusing identity implicated in the clearance of macromolecules and particles from the circulation that are smaller than 0.23 μm. The mannosylation of larger particles would increase their accumulation in Kupffer cells (cells expressing MMR). Similarly, agglutination induced by MBL binding would also favour their targeting to Kupffer cells [40]. The targeting of mannose devices to DCs probably would require high affinity of the ligand to DC-SIGN or highly specific MMR constructs and simultaneous administration of TLR agonists. In the absence of a danger signal, the proportion of DCs expressing MMR is very low [36].

At the cellular level, by interacting with a specific mannose receptor, different ligands can induce different routes of antigen processing and signalling pathways. Adding another level of complexity, most of the mannosylated constructs described in the literature and some of the pathogens bearing mannose moieties bind simultaneously to several mannose receptors [20]. The mannosylated ligands can also interact with a different set of receptors in different APCs [30]. As a consequence, the immunological outcome of mannosylated constructs becomes a priori unpredictable.

3. Mannose conjugates

Glycosylated antigen targeting to mannose receptors is a proposed method of developing new vaccines and treatments in which the immune system is involved. Thus, different strategies have been used to develop drug and delivery systems able to target the mannose receptors and related CLRs. Most of these attempts can be ascribed to the use of mannose conjugates based on the use of natural ligands of mannose receptors. Recently, synthetic ligands with high affinity and specificity for MMR or DC-SIGN have been developed and provide a novel approach for CLR-targeted systems [41,42]. In addition, some mannose ligands may also act as antigens; although, in such cases, the mannose derivative is usually conjugated

to a protein or peptide to modulate or potentiate the immune response.

Engering and co-workers [17,43] demonstrated that when the mannose receptor is involved in the uptake of antigens by DCs, it results in an approximately 100-fold more efficient presentation of the antigen to T cells compared with antigens internalized via a fluid phase. Carbohydrate ligands can be chemically added to the antigen resulting in mannosylated antigen or mannan–antigen conjugates.

Tan and co-workers [44] presented evidence that endocytosis of mannose receptor–antigen complexes by DCs takes place via small coated vesicles, while non-mannosylated antigens were mainly present in larger vesicles, yielding a superior presentation by DCs, which may be useful in vaccine design [44]. Thus, the mannosylation of synthetic peptides of mycobacterial HSP65 or mitochondrial antigen (imogen 55 – 70) resulted in a 200 – 10,000-fold enhanced potency to stimulate HLA class II-restricted peptide-specific T-cell clones compared with non-mannosylated peptides [44-46].

In another interesting work [45], the same group demonstrated that a 100 to 1000-fold lower concentration of mannosylated conjugates (HSP65 and imogen derivatives) was sufficient for complete blocking of the proliferative T-cell response against an agonist peptide compared with the non-mannosylated analogs. Moreover, mannosylated conjugates were similarly effective in the inhibition of the T-cell response against whole protein antigens; although, the enhanced presentation of mannosylated conjugates was blocked by mannan [44]. Furthermore, a strong increase in the efficiency of presentation of these conjugates was also observed with macrophages and peripheral blood mononuclear cells, which confirms that the mannosylation of peptides and proteins will result in preferential presentation by mannose receptors in professional APCs [44-47].

3.1 Mannosylated conjugates as vaccines against pathogens

3.1.1 Vaccines against group B streptococci

Group B streptococci (GBS) cause neonatal sepsis and meningitis, and invasive infections in non-pregnant women with underlying illnesses [48]. Although antibodies directed to the capsular polysaccharide antigens are protective, these antigens are variably immunogenic [49]. In order to potentiate the immunogenic response, the covalent coupling of these antigens to proteins has been commonly used [50-52]. However, generation of GBS oligosaccharides is a difficult task due to their instability in acid media. Recently, a variation of a classical carbohydrate degradation technique based on the sequential *N*-deacetylation and nitrous acid deamination [53] for the specific fragmentation of oligosaccharides was developed [54]. These oligosaccharides, which have a defined molecular weight, were first reduced at the resulting 2,5-anhydromannose terminal derivative. Then, sialic acid residues were modified by periodate oxidation to give a series of oligosaccharides, which were conjugated to tetanus toxoid [55]. The resulting conjugates stimulated the production in mice of high titres of type II and type III-specific antibodies, which induced opsonophagocytic killing of type II and III strains of group B streptococci and opsonic activity [52,55]. For type II conjugates, immunogenicity increased as oligosaccharide size decreased [56]. For the GBS type III glycoconjugates, however, the results were markedly different, with no real impact on opsonophagocytic activity by the modification of the polysaccharide size. These data are in good agreement with immunogenicity data obtained with conjugates of the pneumococcal type 14 PS fragments [54].

3.1.2 Vaccines against Mycobacterium tuberculosis

Tuberculosis has been considered a major worldwide cause of death for centuries. One-third of the world's population is infected with *M. tuberculosis*, which causes 2 million deaths per year. Although macrophages, and not DCs, are the primary targets for infection by mycobacteria, DCs are important for the cellular immune response and recent data demonstrate that DC function is modulated by *M. tuberculosis* [57], which may account for pathogen survival and persistence. DC-SIGN is the major receptor for *M. tuberculosis* on DCs [58]. DC-SIGN interacts with *M. tuberculosis* through its cell-wall component mannosylated lipoarabinomannan (ManLAM), blocking LPS-induced maturation and inducing the production of IL-10 [57].

Recently, identification of the specific carbohydrate structure recognized by DC-SIGN and its homologues has provided new strategies to combat *M. tuberculosis* interactions with these receptors [59]. These peptide-based mannosylated lipoarabinomannan (ManLAM) mimotopes were able to inhibit the binding of the monoclonal antibody CS40 to ManLAM in a concentration-dependent manner [60]. In addition, mice immunized with keyhole limpet haemocyanin-conjugated peptide developed antibodies that recognized ManLAM [60].

3.1.3 Vaccines against fungi

The major capsular polysaccharide of *Candida neoformans*, glucuronoxylomannan (GXM), conjugated to the tetanus toxoid, resulted in anti-GXM protective antibody responses [61,62] and monoclonal antibodies specific for GXM protection against experimental cryptococcosis [63]. However, the pleiotropic effects of GXM on host immunity [64], and the variable protective responses to GXM–carrier conjugates [65], prohibit the use of intact GXM in human vaccine development. Coupling a mannose heptasaccharide, which is thought to be the major GXM immunodeterminant, to a protein carrier induced antibodies against the heptasaccharide [65]. Similarly, the GXM peptide mimotope P13 conjugated to tetanus toxoid prolonged the survival of cryptococcal-infected transgenic mice owing to the production of human P13-specific IgG2 (but not IgG1) [66]. The effect of immunoglobulin isotypes

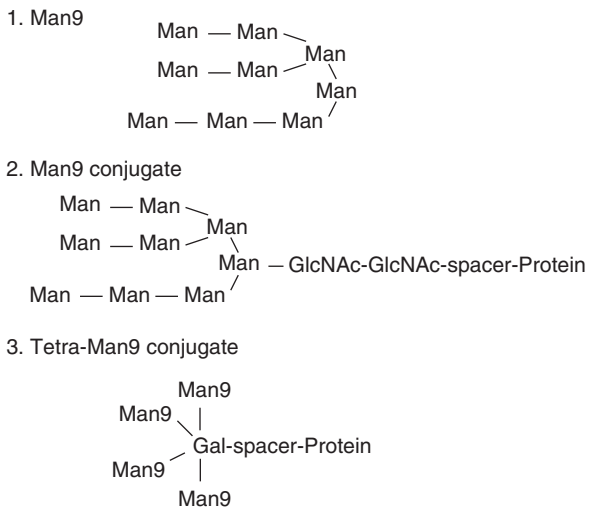


Figure 1. Schematic depiction of Man9, Man9 conjugate and Tetra-Man9 conjugate.
Adapted from [86].
Gal: Galactose; GlcNAc: *N*-acetylglucosamine; Man: Mannose.

correlates with the clinical observations, as IgG2 is commonly produced in response to bacterial capsular polysaccharides and by normal adults in response to GXM [67].

In *C. albicans*, short-chain β-1,2-linked oligomannosides are also recognized by antimannan antibodies that are protective against experimental candidiasis [68,69]. Antibodies against β-1,2-linked mannotriose or mannobiose protect mice against hematogenously disseminated candidiasis. *In vitro* synthesis of β-1,2-oligomannosides has led to the ability to mass produce this epitope and prototype vaccines consisting of synthetic β-trimannose coupled with protein carriers have been produced [70]. Normal rabbits produce high antibody titres to a trimannose–tetanus toxoid conjugate and, when rendered immunocompromised, they show enhanced resistance to disseminated candidiasis [71], which is consistent with antibody protection in neutropenic mice [72].

3.1.4 *HIV vaccines*

DC-SIGN plays a key role in the dissemination of HIV-1 by DCs through HIV-1 gp120 binding [73]. Resident mucosal DCs may capture HIV-1 through DC-SIGN. DC-SIGN does not mediate infection of DCs but protects the virus during migration to the lymphoid tissues, where DC-SIGN facilitates the transmission of HIV-1 to T cells [73].

As described by Trkola and co-workers [74], the human monoclonal antibody 2G12 is of particular interest for the development of a vaccine against AIDS. In fact, this antibody targets a unique carbohydrate antigenic structure on HIV-1 called gp120 [74]. The epitope of 2G12 consists of a cluster of oligomannose residues, on the 'silence face' of the HIV-1 envelope glycoprotein gp120 [75,76]. From different binding studies, it has been demonstrated that the terminal ManR1, 2Man unit was essential for 2G12 recognition but not sufficient for effective binding to 2G12 [77]. In addition, it was shown that the full-size Man9 displayed the highest affinity to 2G12 among several natural high-mannose oligosaccharides, and the synthetic mannose tetrasaccharide corresponding to the D1 arm of Man9 showed comparable affinity to the antibody as that of the Man9 moiety [78].

In this context, Ni and co-workers [79] designed and constructed synthetic oligomannose clusters and evaluated their ability to bind to 2G12 [77,80]. Thus, they observed that the galactose-based tetravalent Man9-cluster (Tetra-Man9) was 73-fold and 5000-fold more effective in binding to 2G12 than the monomeric Man9GlcNAc and Man6-GlcNAc, respectively [77]. This information was the starting point for designing a carbohydrate–protein conjugate for vaccination against AIDS, based on binding of the carbohydrate antigen (Man9 and the oligomannose clusters) to a strong T-helper epitope, such as the keyhole limpet hemocyanin and the universal T-helper epitope from tetanus toxoid (**Figure 1**) [79].

Preliminary immunization studies in rabbits suggested that moderate carbohydrate-specific antibodies were raised by the glycoconjugate immunogens; however, most antibody responses were directed to the linkers. In addition, the antisera were weakly cross reactive to HIV-1 gp120, but the carbohydrate-specific antibodies generated were not high enough to reach the level that could neutralize HIV-1 infection [79].

3.2 Mannosylated conjugates for the treatment of autoimmune diseases

3.2.1 *Inhibition of intracellular proteases*

Selective inhibition of enzymes involved in antigen processing in APCs, such as cathepsin E and cathepsin D, may provide alternatives for the regulation of autoimmune diseases [81]. Cathepsin E and cathepsin D are the major intracellular aspartic proteases in the endolysosomal pathway [82,83]. However, the aspartic protease inhibitors, including the highly potent pepstatin A [84], are inefficiently transported across the cell membrane [85].

In a recent study, mannose derivatives of pepstatin were used as cell-permeable aspartic protease inhibitors, and these inhibitors blocked ovalbumin processing in DCs. These conjugates showed higher solubility in water compared with pepstatin and were efficiently taken up by the cells via receptor-mediated uptake. These conjugates, however, displayed a low reduction in the inhibition of aspartic proteases due to the high stability of the link between pepstatin and the mannose derivative [81]. In order to overcome this problem, Free and co-workers [85] proposed a disulfide link between the mannose group (mannose–BSA neoglycoconjugate) and pepstatin to facilitate the release of the protease by disulfide reduction on endosomes [85,86].

These mannose conjugates (MPC6) but not the mannose–BSA precursor, inhibited both enzymes with an IC_{50} of around 20 nM (cathepsin E) and 0.7 nM (cathepsin D). Critically, MPC6 was at least 100-fold more potent than pepstatin in inhibiting aspartic protease activity in DCs, presumably because of increased solubility and uptake. The inhibition of OVA and peptide presentation in wild type and cathepsin D-deficient mice clearly shown that the processing of OVA was inhibited in a dose-dependent manner by MPC6 using DCs derived from either cathepsin D-deficient or wild type mice. In contrast, MPC6 had little or no effect on presentation of the OVA peptide [87].

3.2.2 *Experimental autoimmune encephalomyelitis model*

Experimental autoimmune encephalomyelitis (EAE) serves as an experimental model for human multiple sclerosis, reproducing clinical aspects such as inflammation of CNS tissue [88,89]. It is depicted as a prototypic $CD4^+$ T_H1-mediated autoimmune disease [90] that depends on autoreactive T_H1 cells that traffic from the periphery into the CNS [91].

Kel and co-workers [92] demonstrated that the treatment with a soluble mannosylated epitope of proteolipid protein (M-PLP139-151) significantly inhibited disease mediated by autoreactive myelin-specific T cells. The conjugates were prepared by N-terminal elongation of an epitope of proteolipid protein (PLP139-151) with a lysine coupled to two tetra-acetyl-protected mannose groups. After reaction, the acetyl-protecting groups on the mannose moieties were removed using Tesser's base. Mice treated with the non-mannosylated peptide showed no significant effect on delayed-type hypersensitivity (DTH) responses in EAE models. On the contrary, significant reduction of EAE incidence and clinical symptoms was obtained when mannosylated peptide was administered before the onset of clinical symptoms. Treatment of established disease induced less pronounced effects, suggesting that treatment with the mannosylated conjugate is particularly effective during disease stadia involving (re)activation of autoreactive T cells [92].

3.3 Mannosylated conjugates for the development of cancer vaccines

The use of DCs for the development of therapeutic cancer vaccines is attractive because of their unique ability to present tumour epitopes via the MHC class I pathway to induce cytotoxic $CD8^+$ T-lymphocyte responses. However, the experimental conditions in which these conjugates are prepared may determine the specific response. In this context, mannan linked to the tumour-associated antigen MUC1 (a mucin-like protein) can induce strong T_H1 or T_H2-type immune responses, depending on the mode of conjugation [93]. Thus, as described by Karanikas and collaborators [93-95], MUC1 conjugated to mannan under reducing conditions induced strong T_H2-type immune responses and no protection in mice against a tumour challenge. By contrast, conjugation of MUC1 to mannan under oxidizing conditions yielded an immunogen capable of generating a T_H1-type response, as indicated by $CD8^+$ CTLs, a low level of IgG2a antibodies, and IL-12 and IFN-γ cytokine production. The binding of both conjugates to the mannose receptors was considered similar; however, the reduced mannan–MUC1 fusion protein would be preferentially presented by the MHC class II pathway, whereas the oxidized mannan–MUC1 fusion protein would be preferentially presented by the MHC class I pathway [94,95]. In fact, it appears that the presence of aldehydes in the oxidized mannan–MUC1 fusion protein would be crucial for endosomal escape of antigen into the cytoplasm [95].

An interesting approach was recently developed by Srinivas and co-workers [96]. In this case, they designed and synthesized conjugates containing a $CD8^+$ epitope of the Melan-A/Mart-1 melanoma antigen. These conjugates were obtained by coupling glycosynthons to small oligolysine-based peptides [97]. Glycosynthons [98,99] were prepared by an original two-step one-pot procedure involving the oligosaccharide reducing sugar and a peptide with a glutamyl residue in the N-terminal position. The glycosynthons were coupled to a peptide-oligo K, including the $CD8^+$ Melan-A epitope and an oligolysine tail. The yield of the process was about 55% and the predominant conjugate was the trivalent derivative: DiMan–Melan-A conjugate [96]. These mannose conjugates were efficiently taken up by DCs and concentrated in acidic vesicles. Furthermore, DiMan–Melan-A conjugates exhibited a high apparent affinity for DC-SIGN and MMR. The binding constant to MMR, however, was fourfold higher than that to DC-SIGN [14,100].

4. Mannosylated liposomes

Mannosylated liposomes have been considered as promising non-live vectors for targeted delivery purposes. In the past few decades several strategies have been developed to promote an adequate coating of liposomes, used as drug/antigen carrier, with the mannose derivative that specifically recognizes its receptor.

Baldeschweiler and co-workers [101] described the capacity of mannosylated liposomes to target cells of the monocyte–machrophage system using 6-aminomannose covalently linked to cholesterol. Later, Barratt and co-workers [102] demonstrated that fluorescently labelled mannosylated liposomes were more rapidly taken up by mouse peritoneal macrophages than by rat alveolar macrophages. This uptake rate was saturable at high liposome concentrations, although it was not inhibited by the presence of conventional liposomes. In addition, the rate of association was also related to the size of mannosylated liposomes. Thus, liposomes with a diameter of about 1.4 μm were taken up more quickly than those of 400 – 700 nm diameter.

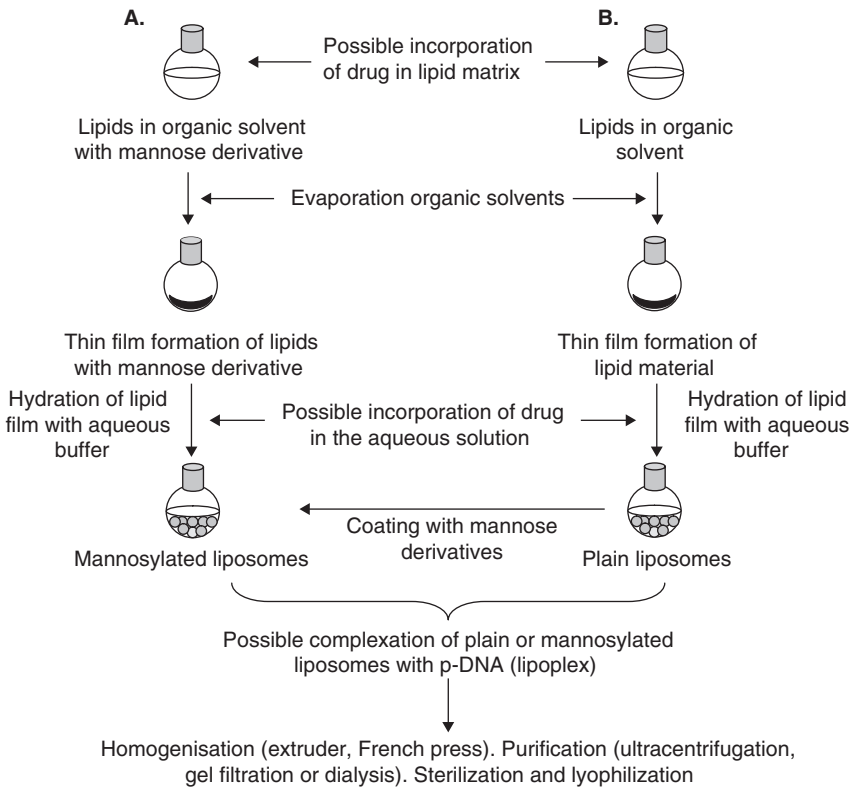


Figure 2. Schematic representation of possible strategies to obtain drug-loaded mannosylated liposomes (A) and plain liposomes (B).

In 1988, Garcon and co-workers [103] found evidence that the presence of a mannosylated ligand on the surface of tetanus toxoid-loaded liposomes led to more efficient binding of the vesicles to macrophages and enhanced adjuvanticy. These events were found to be related to the number of ligand molecules available on the surface of liposomes rather than the extent of ligand mannosylation.

4.1 Preparation of mannosylated liposomes

Generally, at least three different methods are widely used to attach any mannose derivative to plain liposomes: use of mannose–lipid conjugates as raw material for the preparation of liposomes; direct binding of mannose derivatives by chemical reaction to plain liposomes; and simple coating by adsorption onto the surface of liposomes. All of these methods can be associated with the most popular technique used to prepare liposomes, which is based on lipid film formation followed by hydration of lipids with an aqueous solution (**Figure 2**). For the first method, a mixture of lipids and mannose derivatives are dissolved in organic solvents. The solvents are evaporated under vacuum until dry mannose derivative/lipid film formation (**Figure 2A**). The residual thin film is then hydrated with an aqueous solution and, if necessary, the resulting liposomes are homogenized and/or purified. During film hydration and/or homogenization, mannose moieties (as hydrophilic branches of sugar-lipid derivatives) are self-reorganized displaying an extended conformation from the surface of the liposome to the aqueous medium [104-106]. The second method of mannosylation (**Figure 2B**) is based on the covalent attachment of mannose derivatives, such as *p*-aminophenyl-α-D-mannopyranoside, to phosphatidylethanolamine of the plain liposomes using glutaraldehyde [107]. Finally, direct coating of plain liposomes by simple incubation with mannan derivatives or oligomannose can also be used [108,109].

In general, the most frequent approach is the use of mannose–lipid conjugates. In this context, Kawakami and collaborators [110] synthesized a novel mannosylated cholesterol derivative [cholesten-5-yloxy-*N*-(4-((1-imino-2-β-D-thiomannosyl-ethyl)amino) butyl) formamide] (Man-C4-Chol), which can be efficiently mixed with phospholipids to prepare liposomes. Other interesting mannose–lipid derivatives are Man4K3-DOG (a tetramannosyl head group connected, via a polyethylene glycol spacer, to a lipid moiety) [111], trimannose-dipalmitoylphosphatidylethanolamine (man3-DPPE) [106] and cholesteryl mannan (CHM) [108].

Mannosylated liposomes have been used to load different types of drugs, including dichloromethylene-bisphosphonate [111], proteins (i.e., ovalbumin) [106], antitumour drugs (i.e., doxorubicin) [107], quercetin [112],

nuclear factor κB [113], fluorescent markers [114], muramyldipeptide (MDP) as immunomodulator [115] and plasmid DNA [110,116].

4.2 Mannosylated liposomes for drug delivery

Table 2 summarizes some interesting studies associated with the use of mannosylated liposomes for the delivery of therapeutic agents. Efficient targeted drug delivery to APCs has been considered of high interest in the case of vaccination or for the treatment of many intracellular parasitic infections.

4.2.1 *Treatment of tuberculosis*

Many microorganisms infect and colonize alveolar APCs, in which they can survive and multiply [117,118]. In the case of *M. tuberculosis*, the interaction of this pathogen with host DCs (or other APCs) is thought to be critical for mounting a protective antimycobacterial immune response and for determining the outcome of infection [119,120]. In an interesting study, it was shown that the most effective particle size of mannosylated liposomes for ciprofloxacin (CPFX) targeting to alveolar APCs, following pulmonary administration is 1000 nm [121]. Uptake of liposomes by these cells increased by introducing surface mannose modification [122,123]. More recently, Chono and co-workers [124] confirmed that the efficient antibacterial effects of mannosylated CPFX liposomes against intracellular parasites in alveolar APCs may be exhibited at a lower dose than that used in clinical situations. This study, which was carried out by intratracheal administration of liposomes in rats, clearly indicated that the pulmonary administration of mannosylated CPFX liposomes could be an efficient drug delivery system for the treatment of respiratory intracellular parasitic infections, including *M. tuberculosis*, *C. pneumoniae*, *Listeria monocytogenes*, *Legionella pneumophila* and *Francisella tularensis* [124].

4.2.2 *Treatment of leishmaniasis*

Toxicity and drug resistance are major obstacles in the therapy of leishmaniasis induced by the presence and multiplication of the *Leishmania* parasite within macrophages. In this context, the antileishmanial property of a benzyl derivative of the antibiotic MT81 (Bz_2MT81) was tested in mannose-coated liposomes against visceral leishmaniasis in hamsters [125]. This formulation eliminated intracellular amastigotes of *L. donovani* within splenic macrophages more efficiently than control liposomes or free Bz_2MT81. At a dose equivalent to 7.5 μg/kg body weight of mannosylated liposomes subcutaneously injected for 15 days at an interval of three days, the splenic parasitic load decreased to the extent of 79% of the total parasite present in infected animals. For non-mannosylated liposomes or the free drug the splenic load parasitic decrease was calculated to be 55 and 50%, respectively. The activity of Bz_2MT81 mannosylated liposomes was higher than liposomal amphotericin B, which has been used in human clinical trials against visceral leishmaniasis. For amphotericin B-loaded liposomes, the effective dose of the drug used to reduce the splenic leishmania parasite by 80% in hamster was reported to be 31.36 μmol/kg [126], whereas Bz_2MT81-mannosylated liposomes were found effective with only 19.03 nmol/kg [125].

In a similar work, the leishmanicidal property of piperine loaded in mannose-coated liposomes was tested in experimental visceral leishmaniasis in hamsters. Mannose-coated liposomal-containing piperine eliminated intracellular amastigotes of *L. donovani* in splenic macrophages much more efficiently than did the uncoated liposomal piperine or the free drug. At a dose equivalent to 6 mg/kg body weight every 4 days (four doses in 12 days), the mannose-coated liposomal piperine was found to reduce the spleen parasite load to the extent of 90% in comparison to that achieved by uncoated liposomes (77%) or free piperine (29%) [127]. In a similar animal model, mannosylated liposomes containing andrographolide (a labdane diterpenoid isolated from *Andrographis paniculata*) were found to be more potent in reducing the parasitic burden in the spleen as well as in reducing the hepatic and renal toxicity [128].

In another related work [115], doxorubicin-loaded mannosylated liposomes combined with INF-γ were also considered effective for the treatment of leishmaniasis. Because leishmaniasis is accompanied by immunosuppression, immunostimulation by IFN-γ was found to act synergistically with mannosylated liposomal doxorubicin therapy. Doxorubicin, a highly cytotoxic and antineoplastic drug, had profound antileishmanial effects when incorporated into mannosylated liposomes. Thus, doxorubicin, when loaded in mannosylated liposomes, was almost 150 times more effective than a drug solution and about fivefold more potent than when incorporated in conventional liposomes. The results of this study suggest that the immunostimulatory effect of IFN-γ may enhance the efficacy of doxorubicin and reduce its toxicity. Furthermore, the combined treatment resulted in reduced levels of IL-4 but increased levels of IL-12 and inducible nitric oxide synthase, which indicates a plausible conversion of antiparasitic T-cell response from a T_H2 to a T_H1 pattern indicative of long-term resistance [115].

4.3 Mannosylated liposomes for vaccination purposes

Mannosylated liposomes have also been proposed for vaccination against some types of cancer and several pathogens including *Neisseria meningitis*, *Leishmania* spp. and HIV.

4.3.1 *Vaccination against N. meningitidis*

Meningitis caused by *N. meningitidis* is a serious threat to children and young adults. Vaccines based on capsular

Table 2. Examples describing some *in vivo* applications and beneficial therapeutic effects of mannosylated liposomes.

Therapeutic molecules	Aim/study	Achieved beneficial therapeutic effect	Ref.
Quercetin	Evaluation of the neuroprotective effect against cerebral ischemia–reperfusion evoked oxidative damage on Sprague Dawley young and aged rats	Quercetin-loaded Man-LIPO showed preservation of the antioxidant activity of enzymes and an inhibition of edema formation in neuronal cells of young and old rats	[177]
Hamycin	Evaluation of hamycin-loaded mannosylated liposomes in experimental aspergillosis in mice	Reduced toxicity	[178]
		Survival rates of 70% after 7 days	
Amp B	Biodistribution of Amp B-loaded in either *o*-palmitoyl mannan liposomes (OPM-lipo) and *p*-aminophenyl-mannopyranoside liposomes (PAM-lipo)	The rates and extent of accumulation in macrophage-rich organs were higher than for controls and the free drug	[179]
		PAM-lipo exhibited a high accumulation in the macrophages of the liver and spleen	
		OMP-lipo exhibited a tropism for the lungs (alveolar macrophages)	
Oligonucleotide-containing CpG sequences (CpG)	Antileishmania effect of mannosylated liposomes loaded with CpG (Man-CpG-LIPO), using Balb/c mice infected with *L. donovani*	Effective control of the infection in mice using Man-CpG-LIPO: suppression of parasite burden and revision of spleen size to normal	[153]
		Man-CpG-LIPO were found to be, at least, 10 times more effective than controls	
		Man-CpG-LIPO treatment resulted in reduced levels of IL-4, increased levels of IFN-γ and IL-12 in infected spleen cells	
Dox and IFN-γ	To evaluate the antileshmanial efficacy of Dox-loaded mannosylated liposomes (Dox-Man-LIPO) combined with INF in infected mice with *L. donovani*	Combination between IFN and Dox-Man-LIPO reduced parasite in spleen	[107]
		The combination induced conversion of antiparasitic T-cell response from a T_H2 to T_H1 pattern indicative of long-term resistance	
Nuclear factor κ-B decoy	Distribution of liposomes in mice	Man-LIPO was found to accumulate in parenchymal cells of the liver	[113]
	Evaluation of inflammatory cytokine production using murine LPS model	The production of TNF-α, IFN-γ, IL-1-β, ALT and AST were effectively reduced by Man-LIPO	

Amp B: Amphotericin B; APC: Antigen presenting cell; CTL: C-type lectin; DCDP: Dichloromethylene diphosphonate; Dox: Doxorubicin; LPS: Lipopolysaccharide; Man-LIPO: Mannosylated liposomes; T_H: T helper.

Table 2. Examples describing some *in vivo* applications and beneficial therapeutic effects of mannosylated liposomes (continued).

Therapeutic molecules	Aim/study	Achieved beneficial therapeutic effect	Ref.
DCDP	Evaluation of the anti-inflammatory effect of Man-LIPO loaded with DCDP during pneumococcal meningitis	Monocyte depletion reduced the migration of white blood cells into the cerebrospinal fluid in experimental pneumococcal meningitis	[111]
Plasmid DNA expressing melanoma-associated antigen	Development of an antigen-presenting cell-targeted DNA vaccine against melanoma	Man-LIPO induced:	[116]
		Higher gene transfection into APCs than controls	
		CTL activity against melanoma	
Luciferase-encoding plasmid DNA	To design Man-LIPO containing plasmid DNA for selective targeting of antigen-presenting cells	Man-LIPO induced a higher gene expression in the liver, spleen, peritoneal exuded cells, and mesenteric lymph nodes than controls	[180-182]
		Man-LIPO enhanced gene expression in $F4/80^+$ and $CD11c^+$ cells in the spleen	

Amp B: Amphotericin B; APC: Antigen presenting cell; CTL: C-type lectin; DCDP: Dichloromethylene diphosphonate; Dox: Doxorubicin; LPS: Lipopolysaccharide; Man-LIPO: Mannosylated liposomes; T_H: T helper.

polysaccharides are available against serogroups A, C, and W135 [129]. However, a polysaccharide-based vaccine is not available for serogroup B meningococci, due to the low immunogenicity of their polysaccharides and the risk of induction of autoantibodies that cross react with glycosylated host antigens. In this context, class 1 porin protein (PorA), which is a major antigen and induces a strong bactericidal immune response [130], was loaded in mannosylated liposomes [131]. Mice were immunized subcutaneously to study the localization and immunogenicity of PorA liposomes. Thus, uptake of liposomes by DCs was significantly increased by mannosylation and resulted in the maturation of DCs. Furthermore, mannosylated liposomes displayed an increased localization in draining lymph nodes with respect to unmannosylated liposomes. Surprisingly, all types of liposomes induced similar high IgG titres comparable to those induced by outer membrane vesicles (containing lipopolysaccharide, OMVs) However, the number of responding mice per group increased from 50 to 60% with conventional PorA liposomes and OMVs to almost 100% of mice immunized with mannosylated PorA liposomes [131].

4.3.2 *Vaccination against leishmaniasis*

In an interesting study of vaccination against leismaniasis, two types of liposomes, including a soluble leishmanial antigen (SLA) and one coated with neoglycolipids containing oligomannose residues (mannopentaose, Man5-SLA, or mannotriose, Man3-SLA) were evaluated [132,133]. For mice treated with mannosylated liposome formulations, serum levels of SLA-specific IgG2a antibody titres were substantially higher than in controls, whereas the IgG1 antibody titres were considerably lower than in mice treated with SLA alone or SLA liposomes [133]. In addition, up to at least 6 weeks after a challenge with *L. major*, footpad swelling was suppressed in mice treated with mannosylated SLA liposomes, whereas mice receiving either SLA liposomes or SLA alone displayed typical progression of footpad swelling. More importantly, Shimizu and co-workers established that a threshold level of oligomannose in liposomes ranged between 0.005 and 0.01 would be more adequate to induce T_H1 immune response [132,133]. These findings are in agreement with the data reported by Sprott and co-workers [134] who studied the adjuvant activity of phosphatidylinositol mannoside from *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), which has a similar structure to the synthesized Man3-DPPE.

4.3.3 *Vaccination against HIV*

An antiviral vaccination strategy was studied using oligomannose-coated liposomes containing a neoglycolipid constructed with mannopentaose and dipalmitoylphosphatidylethanolamine [135,136], and encapsulating the epitope peptides of the HIV envelope glycoprotein gp120. These liposomes were proposed to induce an epitope-specific CTL response [109]. In BALB/c mice, subcutaneous immunization

with these mannosylated liposomes induced a MHC class I-restricted $CD8^+$ CTL response with a single immunization, whereas non-coated liposomes did not. These results are in agreement with previous data reporting a similar response for mannan-coated liposomes containing a hybrid protein of gag and env of human T-lymphotropic virus type 1 (HTLV-1) [137] and for DNA of HIV-1 incorporated into mannan-coated *N-t*-butyl-*N′*-tetradecyl-3 tetradecylaminopropionamidine and mannan-coated liposomes eliciting HIV-specific CTL activity [138,139].

4.3.4 *Vaccination against cancer*

Immunostimulants or immunomodulators such as CpG sequences or saponin Quil A can be associated with antigen-loaded mannosylated liposomes to achieve the desired immune response. In this context, White and co workers [140] proposed the construction of liposomes containing a lipid core peptide (SIINFEKL peptide) linked to a mannose residue [141,142] and the Quil A adjuvant. For determining the ability of these mannosylated liposomes containing the lipid core peptide and QuilA to act as prophylactic cancer vaccines and protect against tumour challenge, groups of C57Bl/6J mice were immunized subcutaneously and challenged with EG.7-OVA tumour cells. All naive mice challenged with tumour cells quickly developed measurable tumours. By contrast, mice immunized with mannosylated liposomes containing the lipid core peptide and Quil A were well protected, with 80% of animals remaining tumour free 4 weeks after the challenge [140].

Lu and co-workers [116] evaluated the hypothesis that intraperitoneal injection of a mannosylated liposome–pDNA complex (Man-lipoplex) may act as a DNA vaccine for melanoma by raising the cytotoxic immune response to a level leading to tumour rejection and regression. In this context, liposomes containing Man-C4-Chol and a plasmid coding for Gp100, which is abundantly expressed in both murine and human melanoma [143], covalently bound to ubiquitin (pUb-M gene) to enhance its degradation by proteasome and regulate the intracellular protein processing to be presented by MHC class I molecules [144,145]. These Man-lipoplex formulations induced significantly higher pUb-M gene transfection into APCs than unmannosylated liposomes and naked DNA. Thus, these mannosylated liposomes induced a strong CTL activity against melanoma, inhibiting its growth and prolonging the survival of mice after a lethal challenge with B16BL6 melanoma cells [116].

4.3.5 *DNA vaccination*

In gene therapy, DNA vaccination has great potential in the immunotherapy of cancer and infectious diseases [146]. On examining non-viral vectors, it appears that mannosylated cationic liposome would be one of the more appropriate systems for gene delivery *in vivo* [147]. In this context, pDNA-loaded liposomes prepared with Man-C4-Chol were found to provide higher transfection activity in primary cultured mouse peritoneal macrophages than that of conventional liposomes [110]. In mice, intravenous injection of these mannosylated liposomes exhibited high gene expression in the liver [110,148]. This mechanism was particularly observed in liver non-parechymal cells (NPCs) and was significantly reduced by predosing with mannosylated bovine serum albumin [110,149,150].

Animal studies have shown that DNA immunization induces not only an antibody response but also a potent cell-mediated immune response against the encoding antigen. In this context, the potency of mannosylated cationic liposomes (Man-liposomes) for DNA vaccination were studied by Hattori and co-workers [151] using OVA-encoding pDNA (pCMV-OVA) as a model antigen. After intravenous injection in mice, these mannosylated liposomes produced a stronger induction of IL-12, IFN-γ, and TNF-α release in the serum of animals than the unmodified liposome complex. In animals treated with naked pCMV-OVA, no cytokine release was detected. Similarly, the relatively copy number of mRNA of OVA extracted from $CD11c^+$ cells and the production of IFN-γ by spleen cells were found to be much more important in mice treated with Man-liposomes than with conventional vesicles. All of these findings were associated with the biasing of helper T cells towards differentiation to T_H1 cells when DNA vaccine was administered with Man-liposomes [151].

4.4 Mannosylated liposomes for non-specific immunostimulation

The association of mannosylated liposomes and adjuvants has been proposed for the non-specific stimulation of the immune system. This immunostimulation may be of interest for the treatment of intracellular pathogens and to combat cancer. Thus, the association of Man-liposomes and CpG sequences was proposed for the elimination of intracellular pathogens. These formulations were prepared by covalent coupling of p-aminophenyl-α-D-mannopyranoside to CpG-loaded liposomes (Man-lip-CpG) [152]. When susceptible BALB/c mice infected with *L. donovani* were treated with Man-lip-CpG, the experimental infection was completely controlled. This fact was confirmed by the complete suppression of spleen parasite burden and reversion of spleen size to nearly normal levels. Thus, Man-lip-CpG was found to be 150 times and 10 times more efficient than free CpG and lip-CpG, respectively, by 100% removal of spleen parasites. The microbicidal activity of these mannosylated liposomes was correlated with their ability to enhance the generation of nitric oxide by macrophages [153].

In a recent study, Kuramoto and collaborators [154] evaluated the immunostimulatory effect of mannosylated liposomes containing CpG sequences (Man/CpG lipoplex) to combat refractory peritoneal dissemination of tumour cells in mice. The number of tumour cells in the greater omentum and the mesentery of Man/CpG lipoplex-treated mice was about 1.3 and 0.12%, respectively, of that in the

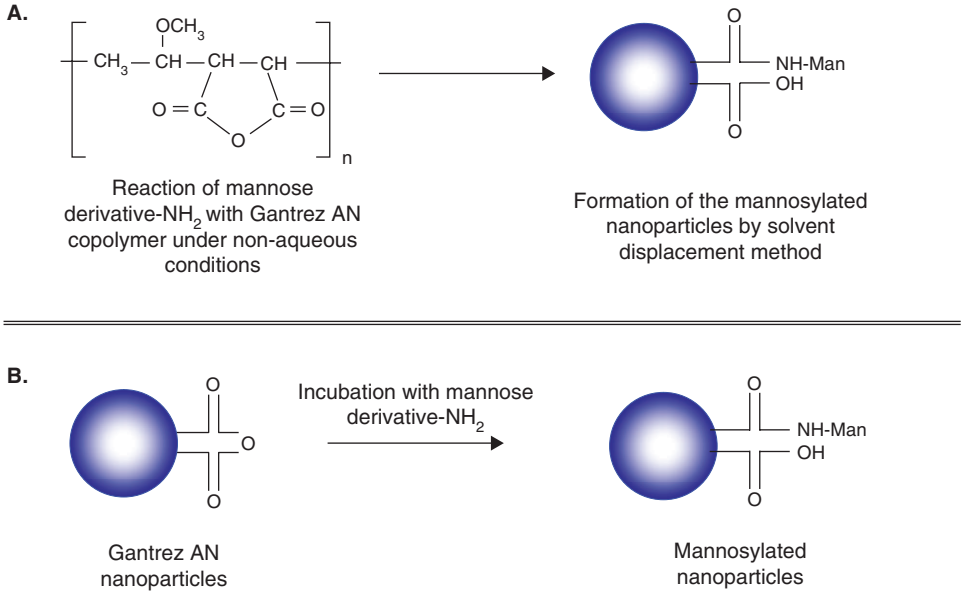


Figure 3. Fabrication of mannosylated polyanhydride (Gantrez AN) nanoparticles by the reaction of mannose derivative with the copolymer before the nanoparticles' formation (A), or direct coating of the just formed nanoparticles (B).

control. Furthermore, the survival time of mice was prolonged with manosylated CpG-loaded liposomes compared with the conventional CpG-lipoplex. In fact, approximately 40% of the mice treated with the mannosylated vesicles survived more than 30 days whereas all the mice in the unmannosylated control group died in < 20 days [154].

In another interesting work [115], mannosylated liposomes containing MDP were prepared by incorporation of Man-C4-Chol into small unilamellar liposomes consisting of cholesterol and distearoyl phosphatidylcholine (Man-lip-MDP). These liposomes were evaluated in an experimental liver metastasis model. In contrast to free MDP or lip-MDP treatments, which showed little effect on the inhibition of metastasis, Man-lip-MDP significantly reduced the number of metastatic colonies in the liver and increased the survival of the tumour-bearing mice. This finding was related to the ability of these mannosylated liposomes to target the liver non-parenchymal cells via mannose receptors [115].

5. Other mannosylated carriers

In the past few years, different types of mannosylated carriers have been proposed for specific antigen/drug delivery, including micelles, polymer nanoparticles, metal colloids, niosomes and nanoemulsions. Some of these carriers offer interesting advantages over soluble conjugates or liposomes, mainly related to the possibility of antigen/drug delivery by mucosal or topical routes and better properties for the controlled release of the loaded molecule. To date, however, few research groups are involved in the development of mannosylated particulate carriers. Thus, experimental data are very scarce.

5.1 Preparation of particulate carriers

In general, mannosylated particulate carriers have been prepared by desolvation [155], precipitation [156], emulsification [157] or micellization [158] techniques. For poly(anhydride) nanoparticles, mannosylation take place before or after nanoparticle formation by desolvation (**Figure 3**) [155]. By contrast, iron oxide nanoparticles were prepared by precipitation of Fe(II) and Fe(III) salts with ammonium hydroxide according to two methods. Mannosylation was carried out by either precipitation of iron salts in the presence of D-mannose solution or oxidation of magnetite nanoparticles with sodium hypochlorite followed by addition of D-mannose solution [156]. Another method of mannosylation consists of the attachment of a mannose derivative to a chitosan polymer by chemical reaction with mannopyranosyl-phenylisothiocyanate prior to nanoparticle formation [159,160]. In another study [158], the mannose unit was conjugated to the hydrophilic chain terminus of mixed micelles composed of poly(acrylic acid-b-methyl acrylate) and mannosylated poly(acrylic acid-b-methyl acrylate) by transfer radical polymerization. For niosomes, these carriers were prepared with sorbitan stearate (Span 60), cholesterol, and stearylamine by the reverse-phase evaporation method. Then, these carriers were coated with a modified polysaccharide *o*-palmitoyl mannan [161,162]. Finally, mannosylated nanoemulsions (composed of soybean oil, egg phosphatidylcholine and Man-C4-Chol in a ratio of 70:25:5) were prepared by dissolution in chloroform, vacuum desiccation and resuspension in PBS, and sonication for 1 h [157].

5.2 Applications of mannosylated particulate carriers

5.2.1 *Parenteral administration*

Mannosylated nanoemulsions (Man-emul), when intravenously administered to mice, were rapidly eliminated from the blood circulation and preferentially recovered in the liver. By contrast, control emulsions were retained more in the blood circulation. The hepatic uptake clearances of Man-emul were 3.3 times greater than those of controls. Furthermore, as described for mannosylated liposomes [110,150], Man-emul were concentrated in the non-parenchymal cells, whereas control nanoemulsions appear to target the parenchymal cells [157]. In a more recent work [163], the efficient targeting of these nanoemulsions was shown to be largely controlled by the effect of mannose density on Man-emul.

In another interesting study [160], mannosylated chitosan nanoparticle-based cytokine gene therapy suppressed cancer growth in mice. In Balb/c mice bearing CT-26 carcinoma cells, intratumoural injection of mannosylated chitosan nanoparticles/plasmid encoding murine IL-12 complex suppressed tumour growth and angiogenesis.

5.2.2 *Oral administration*

Mannosylated polyanhydride nanoparticles have demonstrated a high capacity to develop specific bioadhesive interactions with the mucosa of the gastrointestinal tract of rats [155]. This specific bioadhesive interaction was dependent on mannose density on the surface of the nanoparticles. Furthermore, fluorescence microscopy experiments confirmed that mannosylated nanoparticles were able to establish interactions with components of the enterocytes and cells of the Peyer's patches. These mannosylated nanoparticles, loaded with OVA as model antigen, were orally administered to mice (single dose of 100 μg). This formulation induced a strong and more balanced humoral immune response of IgG2a (T_H1 response) and IgG1 (T_H2 response) compared with OVA-non-mannosylated nanoparticles or a solution of the protein. Furthermore, mannosylated nanoparticles were able to elicit a significant intestinal secretory IgA response, at least for 6 weeks. All of these results correlate with the bioadhesive properties and effective lymphoid uptake of mannosylated nanoparticles [164].

Another example of oral-targeted delivery was described for mannosylated niosomes loaded with tetanus toxoid and their application in oral mucosal immunization [161]. In fact, niosomes were coated with a modified polysaccharide *o*-palmitoyl mannan (OMP) to protect them from bile salts causing dissolution and enzymatic degradation in the gastrointestinal tract and to enhance targeting of APCs of Peyer's patches. These niosomes elicited a combined serum IgG2a/IgG1 response in albino rats, suggesting that they could elicit both humoral and cellular responses. In addition, a significant mucosal immune response (sIgA levels in mucosal secretions) was also observed [161].

In a related study [162], the same group used mannosylated niosomes as vaccine carriers for the oral delivery of a plasmid-expressing sequence coding for the small proteins of the hepatitis B virus. In Balb/c mice, all animals orally immunized with these niosomal formulations were seropositive in 2 weeks and the antibody levels were sufficient to provide seroprotection against hepatitis B. Furthermore, mannosylated niosomes elicited significantly higher mucosal immune responses than controls [162].

6. Clinical studies

Clinical studies with the conjugate mannan–MUC1 were started 13 years ago. The product, known as mannan fusion protein (MFP) has been registered as Prima's proprietary Cvac™ technology (Prima BioMed Ltd). More specifically, it was demonstrated that the mannan–MUC1 conjugate was non-toxic; in general, however, antibody responses predominated over cellular responses. More than 250 patients with advanced carcinoma of the breast, colon or other adenocarcinomas who overexpressed the MUC1 antigen were treated with the conjugates and moderate cellular immune responses and substantial antibodies responses were reported. Clinical responses, however, were not apparent in these patients [93,94]. The T_H2 type response in these patients was explained by the fact that natural antibodies recognized mannan–MUC1 which subsequently bound to Fc receptors and generated predominantly B-cell rather than T-cell responses [165]. To overcome this drawback, a number of variations were tried, including the use of cyclophosphamide (to switch off presumed 'suppressor cells'), although this solution had no effect on the response [95]. More recently, the same group [166] performed another clinical study to collect PBMC for culture *in vitro* with IL-4 and GM-CSF, making DCs which were pulsed with mannan–MUC1 before injection. The patients received three injections over 10 weeks and T-cell and antibody responses were measured. The results reported for 10 patients are encouraging and have demonstrated that the treatment was well tolerated by all patients. All patients produced strong cellular immune responses, DTH reactions occurred at the injection sites, and three of 10 made weak IgG or IgM antibodies. In this context, another Phase I trial [167] in patients with advanced adenocarcinoma who received Mannan–MUC1 pulsed DCs demonstrated that immunization with DCs manipulated and treated *ex vivo* with mannan–MUC1 produced T-cell responses in all patients and even stabilization of the tumour in some.

Promising results were reported in a pilot Phase III clinical trial using oxidized mannan–MUC1 immunotherapy against early breast cancer [168]. In this study, 31 patients with stage II breast cancer and with no evidence of disease received subcutaneous injections of either placebo or oxidized mannan–MUC1 to immunize against MUC1 and prevent cancer recurrence/metastases. The recurrence rate in patients receiving the placebo was 27% whereas those receiving immunotherapy had no recurrences and measurable antibodies and T-cell responses.

More recently, Mayer and collaborators [169] reported a Phase I study with a mannosylated antibody-enzyme fusion protein (MFECP-1 or the conjugate between mannosylated anti-carcinoembryonic antigen [CEA] single-chain Fv antibody and the bacterial enzyme carboxypeptidase G2) for selective targeting of tumour cells expressing the oncofetal antigen (CEA), in which the enzyme has to convert a prodrug into a toxic drug. In this study, patients with non-resectable, locally recurrent, or metastatic histologically proven colorectal or other CEA-expressing cancer were eligible for the study. MFECP1 was found to be safe, well tolerated and localized in tumour cells. More particularly, the best response was a 10% reduction of tumour diameter in a patient with peritoneal cancer. Eleven of 28 patients had stable disease after 8 weeks and 17 had progressive disease.

Finally, DermaVir (Genetic Immunity) is a novel DNA immunization method designed to improve antigen presentation and induce cytotoxic T-cell responses for the treatment of HIV/AIDS. In DermaVir the DNA is formulated with a cationic polymer (PEIm) in glucose. The cationic polymer forms a complex with the DNA, resulting in a small mannosylated particle, and the glucose stabilizes the complex by inhibiting aggregation prior to the vaccine application. DermaVir has to be applied directly to the epidermis, above the basal keratinocytes, to penetrate the skin surface and reach the network of sentinels that serve to initiate immune responses against pathogens [170-174]. From preclinical studies it was confirmed that this mannosylated nanomedicine was trapped by epidermal Langerhans cells and transported to draining lymph nodes. While in transit, Langerhans cells mature into DCs, which can efficiently present the DNA-encoded antigens to naive T cells for the induction of cellular immunity [174-176]. Clinical testing of DermaVir in HIV-1-infected individuals has demonstrated the safety, tolerability and induction of long-lasting, high-magnitude and broad HIV-specific T-cell responses of DermaVir [174-176].

7. Expert opinion

The preferential expression of mannose receptors in the cells of the immune system explains the documented benefits of mannose receptor-targeted delivery for chemotherapeutic treatments against pathogens that survey and multiply within the macrophages. Therefore, with the identification of mannose receptor expression in DCs, the most important APCs, mannosylation has been successfully applied in vaccination and, in fact, several constructs are currently undergoing clinical trials. For these purposes new and innovative methods of delivery of therapeutic agents have been proposed in the past few years. All of these methods can be considered as nanomedicines and include mannose conjugates and mannosylated carriers or particulates (i.e., liposomes, nanoparticles and niosomes).

Mannose conjugates formed by chemical reactions between a mannose derivative and a protein or a therapeutic agent (usually an antigen) are, in general, easy to produce. In addition, the link between the therapeutic and the mannose derivative may determine the stability of the conjugate within the body and the release of the antigen, and thus, the efficacy of the system. These conjugates can be administered by a parenteral route and they are quite efficient at reaching and targeting the mannose receptors which are abundantly expressed in lymphatic and hepatic endothelial cells and APCs. For vaccination against pathogens, the mannose derivative can be used for both targeting the mannose receptors and inducing the immune response (as antigen). However, as polysaccharides are poorly immunogenic, the mannose derivative is then bound to a protein or peptide, which is able to induce strong immune responses.

Mannosylated particulates are formed by the association of the mannose derivative to a carrier (liposome, nanoparticle, niosome etc.). The mannose derivative acts as a targeting agent whereas the particulate or carrier acts as a reservoir for the therapeutic agent. Compared with mannosylated conjugates, the drug or antigen has not risk of activity or immunogenic alteration produced by chemical modifications. Furthermore, because of their particulate nature, particles tend to be engulfed by phagocytic APCs and mannosylation will further improve their phagocytosis mediated by specific receptors. Comparing liposomes with other carrier delivery systems, liposomes offer multiple advantages for drug delivery, including their biocompatibility, biodegradability and safety. However, they show poor stability in biological fluids, especially in the gastrointestinal tract, and, in many cases, the preparative processes are difficult to scale up for industrial production.

It is clear that there is a positive influence of mannosylation on targeting antigens to APCs. However, diverse studies about their effect in APC activation, the contribution of mannose receptors to the maintenance of homeostasis and the implication of carbohydrates in the escape of pathogens support the strategy of combining mannosylated devices with adjuvants (such as TLR agonists) to elicit effective immune responses. Immune cells express several receptors that share mannose-binding activity but show various immunological roles. The three-dimensional configuration of mannosylated devices seems to dictate the specific recognition for a particular mannose receptor, although all of them share increased affinity for structures with multiple mannose moieties, in accordance with the cluster effect.

Overall, most published reports undoubtedly confirm the effectiveness of mannosylation strategies. The construction of mannosylated devices with many sugar units (that have shown increased avidity by mannose receptors) and co-delivery of danger signals (that upregulate the expression of mannose receptors in immune cells) have been shown as

ways of improving the efficacy of glycotargeting. The optimization and full exploitation of mannose-targeted drug delivery systems, however, require a deeper understanding of structure–activity relationships and a knowledge of their immunological functions.

It is important to note that nanotechnology is changing the way of designing new and safer drug/antigen delivery systems. This new technology is facilitating the use of information and discoveries obtained from basic research to yield new therapeutic strategies and devices for clinical use. In this context, mannosylated delivery systems (conjugates, liposomes, nanoparticles, etc.) are good examples of these new nanomedicines which, in the near future, may improve the treatment of a number of diseases (including cancer) and improve the quality of life of patients.

Declaration of interest

The work of this group is supported by grants from the 'Ministerio de Educación y Ciencia' (Projects SAF2004-07150 and AGL2004-07088-CO3-02/GAN), Instituto de Salud Carlos III (Project PI070326), regional Government of Navarra (Dep. de Salud; Res. 2118/2007), Foundation 'Universitaria de Navarra' and 'Asociación de Amigos Universidad de Navarra' in Spain.

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