DELIVERY SYSTEMS FOR THE TREATMENT AND PREVENTION OF LEISHMANIASIS

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Current drugs for the treatment of leishmaniasis are far from satisfactory and no effective vaccine is available. In the recent years, the pharmaceutical technology has made a giant's causeway and is able to offer a plethora of delivery systems (DS) and nanomedicines that could improve the treatment and profilaxis of leishmaniasis. This article reviews the major class of DS evaluated as antileishmanial drugs carriers or as adjuvants in *Leishmania* vaccines. On regarding the published works we can conclude that pharmaceutical technology has shown potential to prepare DS of great efficacy for treating and preventing leishmaniasis, as exemplified in Ambisome® or in the adjuvant MPL-SE®. However, Ambisome® is not choice for many people because of its high cost. Thus, nowadays there are significant issues to resolve in order to get products to market based on these technologies.
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Leishmaniasis is a disease that ranges in severity from skin lesions to serious disfigurement and fatal systemic infection. WHO estimates that the disease results in 2 million new cases a year, threatens 350 million people in 88 countries and that there are 12 million people currently infected worldwide. Current treatment is based on chemotherapy, which relies on a handful of drugs with serious limitations such as high cost, toxicity, difficult route of administration and lack of efficacy in endemic areas. Pentavalent antimonials have been the mainstay of antileishmanial therapy for over 70 years with second line drugs, Amphotericin B (AmB) and Pentamidine, used in case of antimonial failure. Since the introduction of miltefosine at the beginning of this century, no new antileishmanial compounds have been approved for human treatment[1].

Leishmaniasis is considered one of a few parasitic diseases likely to be controllable by vaccination. However, to date no such vaccine is available despite substantial efforts by many laboratories. The development of a safe, effective and affordable antileishmanial vaccine is a critical global public-health priority[2].

Conceptually, many of the unlikely properties of conventional antileishmanial drugs or the poor immunogenicity of subcellular compounds of *Leishmania* could be improved through the use of Delivery Systems (DS) and nanodevices provided by the pharmaceutical technology. DS could improve the solubility of poorly water-soluble drugs (v.g. amphotericin B or atovaquone) or protect antigenic proteins, DNA or RNA from rapid degradation. Needle-free administration of antileishmanial drugs or vaccines (v.g. by oral or topical routes) would be also feasible[3]. Because of their particulate nature, DS should provide more selective targeting of drugs or Antigens (Ag) towards Monocyte-Phagocyte System (MPS). As *Leishmania* parasites are also mainly confined in macrophages, DS could improve the therapeutic index of antileishmanial drugs, decreasing the effective dose and the off-target toxic effects produced by un-adequate biodistribution[4]. As Antigen Presenting Cells belong to MPS system, DS should enhance the Ag uptake and contribute to increase the immunogenicity of subcellular vaccines[5].

This article introduces the concept of Delivery Technology and Nanomedicine and reviews the delivery strategies designed to improve the current options of treatment or vaccination against leishmaniasis. Results and possibilities are analyzed.

Delivery systems: concept, classification and biomedical applications

Drugs are hardly ever administered to a patient in an unformulated state. A drug dosage formulation consists of one or more active ingredients along with other molecules termed as excipients. It has been increasingly realized that the use of excipients is as important as the drug itself. Excipients facilitate the preparation and administration, enhance the consistent release of the drug and protect it from degradation. Thus, excipients are not longer considered to be inert substances because they can potentially influence the rate and extent or drug absorption and, thus, determine the bioavailability of the drug. The term of bioavailability refers to the amount drug available to the systemic circulation out of the total drug administered to a patient and it is an important consideration in pharmaceutical dosage forms because the presence of drug in systemic circulation is essential to reach its target site and exert its therapeutic effect. A drug needs to be formulated so as to extract the maximum therapeutic benefit out of it, and this is the underlying concept behind a Delivery System (DS).

The long-term objective of Pharmaceutical Technology is to prepare medicines or "magic bullets" for selectively targeting the drug to the site of action within the body without affecting healthy organs and tissues. This results in improved efficacy and reduced toxicity. Meanwhile, DS can solve any

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of the problems of drugs that limited their application. DS can protect a drug from degradation, or increase the stability of a wide variety of therapeutic agents as peptides, proteins or oligonucleotides, enhance drug adsorption even facilitating diffusion through epithelium, modify pharmacokinetic and drug tissue distribution profile and/or improve intracellular penetration and distribution. Also, DS can be used to improve the solubility of hydrophobic compounds in aqueous medium to render them suitable for parenteral administration (Box 1)[3, 6].

Box 1. The beneficial attributes of DS.

- Solubilization of poorly soluble compounds
- Protection of labile compounds like protein, DNA, RNA from premature degradation. Lower doses of bioactives are required
- Controlled release that could avoid unfavourable pharmacokinetic and reduce acute toxicity
- Trojan horse approach to cross biological barriers: needle-free administration routes
- Selective targeting to diseases tissues, avoiding side effects in non-target tissues

Historically, the field of DS has evolved from macroscopic devices as implants or topical patches with zero-order controlled release in the 1970s, microscopic devices with sustained release in the 1980s to the actual era of targeted nano-carriers. It is known that nano-sized carriers are a prerequisite for effective drug targeting. Carriers systems larger than 400 nm are rapidly captured by MPS and cannot circulate in the bloodstream for a long enough time to deliver efficient amounts to therapeutic targets. On the other hand, drugs with molecular weight smaller than ca. 40000 are excreted through the renal filtration system and they therefore cannot maintain stable circulation in the blood stream. Thus, it seems clear that the "magic bullet" will be a targeted nano-device produced by nanotechnology[7].

Following the evolution of DS, the first applications of DS took advantage of sustained release of drugs from them, so that the DS function in a manner similar to a drug infusion but with less patient inconvenience.

The next applications of DS profited from the natural tendency of particulate DS to localize to MPS (particularly to liver and spleen macrophages) for improving the efficacy of therapeutics and vaccination against infectious diseases[3, 8]. Therefore, DS with other targets and biomedical applications can be achieved by two general methods: passive and active targeting. The passive targeting of DS, determined by their physico-chemical properties (size and surface) and the EPR effect (enhanced permeability and retention) have improved the cancer and inflammation therapy. To achieve this goal, it is necessary to reduce the particle size or modify their surface characteristics (a common strategy is the "PEGylation") in order to avoid their rapid removal from the bloodstream by macrophages and prolong the circulation half-life. These particles have then chance to extravasate in tissues with increased permeability of vasculature as inflamed tissues and solid tumors. Moreover, because tumors have impaired lymphatic drainage, the carriers concentrate in the tumor and higher and more selective drug concentrations can be achieved relative to administration of free drug[9, 10].

Active targeting aimed to increase the delivery of drugs to a target through the use of specific interactions at target site. These interactions include antigen-antibody and ligand-receptor binding. Then, particles have been derivatized with ligands that bind to specific receptors expressed on target cells, like transferrin, folate, mannose or monoclonal antibodies.

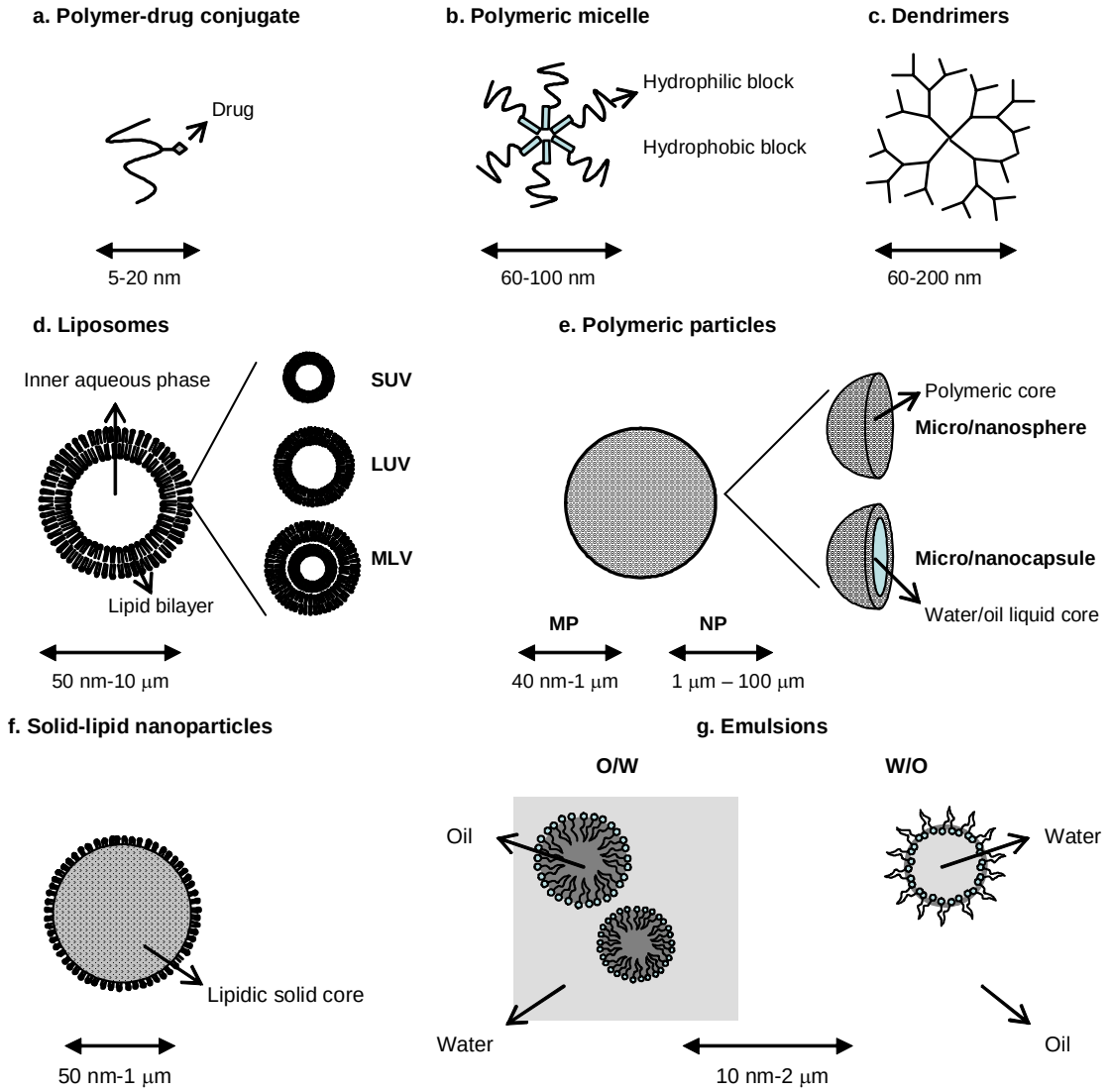
From the first liposomes proposed in 1974 by Gregoriadis and today, there was an explosion of devices suitable for drug delivery, materials and methods fro their preparation. A brief description of the most important ones appears below[11] (Figure 1).

Liposomes (50 nm-10 μm) consist of one or more phospholipid bilayers enclosing an aqueous phase. They can be classified as large multilamellar liposomes (MVL), small unilamellar vesicles (SUV) and large unilamellar vesicles (LUV), depending on their size and the number of lipid bilayers. Water soluble compounds can be included within the aqueous compartment and the lipophilic and amphiphilic compounds associated with the lipid bilayer. Liposomes are very attractive because they can be prepared with natural phospholipids which are well tolerated with minimal toxic effects. However, their application is limited by several disadvantages: poor encapsulation of hidrosoluble or high molecular weight drugs, low stability upon administration by interaction with plasma compounds, disruption after oral administration and uncontrolled drug release.

Polymeric particles can be divided into micro- (MP) or nanoparticles (NP) with size higher or lower than 1 μm respectively (50 nm-10 μm). There are long lists of polymers that can be used for their preparations: natural proteins (i.e. albumin) or natural polysaccharides (i.e. chitosan). The biodegradable and biocompatible synthetic aliphatic polyesters (poly(lactic acid) PLA, poly(glycolic acid) and their copolymers PLGA, or polycaprolactone) are the primary candidates for the development of MP-or NP-based DS, as they have been used for many years as suture material and as controlled-release delivery systems. They may consist of either a polymeric matrix (nano- or microspheres) or of a reservoir system (nano- or micro-capsules). They can be loaded with either hydrophilic or hydrophobic drugs or macromolecules like proteins. Depending on the preparation method the bioactive can be encapsulated inside the particle matrix or attached onto their surface.

Polymeric particles offer several advantages as compared with liposomes: high drug-loading capacity, long term stability and suitability for oral administration. Moreover, they can control the drug release. When prepared with biodegradable and biocompatible polymers, they are well-tolerated.

Figure 1. Structure of some types of DS. Bioactives can be encapsulated into the particles or attached onto their surface. Amphiphilic molecules can be also located in the lipid bilayer of liposomes or in the interface of the droplets in emulsions. SUV= Small Unilamellar Vesicles; LUV= Large Unilamellar Vesicles; MLV=Multi Lamellar Vesicles; MP= Microparticles; NP=Nanoparticles; O/W= Oil-in-water emulsion; A/W=Water-in-oil emulsion.



Solid lipid nanoparticles (SLN) were introduced at the beginning of the 90s. SLN are lipidic particles that combine the advantages of liposomes (biocompatibility) and polymeric nanoparticles (stability, high loading and controlled release). Additionally, they can be produced on a large scale with a general low cost.

Nanosuspensions contain particles consisting only of the drug and a minimum amount of surfactants, usually as an aqueous dispersion. It is the easiest and low-cost DS for improving the solubility and bioavailability of drugs poorly soluble in water.

Polymer-drug conjugates are prepared by chemical linkage between the bioactive and a water-soluble polymer or protein. The conjugation decreases the drug clearance, increase their plasma residence and alter its biodistribution. The linkage with poliethylenglicol (PEG) has increased the stability and activity of recombinant proteins, placing several products onto the market (v.g. PEG-α-interferon, PEG-G-CSF, PEG-asparaginase). Moreover, a growing list of polymer therapeutics is actually in clinical trials. The polymers that have been clinically used so far have a linear architecture. Nowadays, there are growing interest in hyperbranched dendrimers and dendritic polymer architectures.

Drawbacks of polymer therapeutics are the need of covalent linkage that can inactivate the bioactive. Moreover, chemical-linkers able to release the drug must be used. On the contrary, particulate carriers can entrap the drug in their loading space without the need of conjugation; offer a higher loading

capacity and better degree of protection than that afforded by polymer conjugates. However and this is a reason for their success, once bioactive-polymer conjugation is optimized, the fabrication of the product is easy to scale-up.

Self-assembling block copolymers (polymeric micelles) that have hydrophobic and hydrophilic domains are also employed to form micelles within and upon which the drug can be incorporated or attached. Micelles prepared with traditional surfactants have been used for many years to solubilise drugs. However they did not work as DS because they are not stable to dilution. Polymeric micelles, stable to dilution, offer the advantages of other nanosized DS.

Emulsions are dispersion or two or more immiscible liquids dispersed to give either oil-in-water (O/W) or water-in-oil (W/O) systems, stabilized with emulsifiers coating the droplets and preventing the coalescence. The size of the droplets can range from 10 nm to 2 μm. Low stability upon administration limited their capacity to improve drug biodistribution and targeting.

Finally, recently there is growing interest in non-biodegradable DS as metal colloids or carbon nanotubes. Metal colloids can be incorporated in other systems to confer additional specific properties such as magnetic, superparamagnetic and thermal properties. Carbon nanotubes belong to the family of fullerenes and consist of grafhite sheets rolled up into a tubular form where the bioactive can be attached. As long as they are not biodegradable, the risk of accumulation restricted their application mainly to diagnosis. Strategies applied to almost of the systems described above are the "PEGylation" in order to avoid their rapid clearance by MPS (named PEGylated, long-circulating or stealth particles) or the conjugation with monoclonal antibodies (immunoparticles) able to accumulate the carrier in the area within the body where the attached antibody recognize and bind its antigen. In spite that active targeted systems was expected to lead to more specific accumulation of the bioactive to the selected target, the conjugation of ligands or antibodies to particles was shown to increase their clearance by macrophages. Together with the known binding-site barrier effect, these complications have checked the expectative in immunoparticles for selective targeting[12].

Delivery of therapeutics against *Leishmania*

From their earliest days, DS found application for Visceral Leishmaniasis (VL) mainly due to the fact that *Leishmania* parasites colonize macrophages in liver and spleen, which are also responsible for clearance of DS[6]. The recognition of particles by macrophages is mediated by the opsonisation process or adsorption onto their surface of complement proteins, immunoglobulins and fibrinogen. The kinetic of clearance is mainly dependent on the physico-chemical characteristics of the particles, as their size and surface properties. Larger (200 nm and above) or hydrophobic particles are more effective at activating the complement system and are removed by the macrophages in minutes. Moreover, at the intracellular level *Leishmania* parasites survive within the phagolysosomes that is also the natural destination of conventional DS. As a matter of fact, the very first use of liposomal drugs in the treatment of infectious diseases was made in the case of *Leishmania*. Liposome-encapsulated antimonials were found to be 700-times more effective than unencapsulated drug in hamsters[13] After this seminal work, extensive literature reported that the activity of the most currently antileishmanial drugs was improved by encapsulation in DS. Readers are advised to review[14, 18]. In fact, liposomes, niosomes, emulsions, lipid or polymeric particles have been developed for current antileishmanial drugs as amphotericin B, mitelfosine, pentamidine, paramomicine, atovaquone or natural products with possible antileishmanial activity (Table 1).

Actually there are three lipidic DS of AmB licensed for clinical use (Ambisome®, Amphocil® and Albecet®)[66, 67], although only one of them, Ambisome®, is recommended for treating patients with leishmaniasis who are unresponsive to antimonial. The three were found more effective than antimonials even with a single dose treatment. However, Ambisome® was better tolerated[68].

Ambisome® is the only true liposomal formulation of the three. It is composed of small, unilamelar vesicles (80 nm) that carry AmB very stable inserted into the bilayer. Amphotec® is composed of complexes between cholesteryl sulphate and AmB in equimolar proportions that have the form of thin discs of 120 nm diameter. Abelcet® is composed of complexes between two lipids and AmB that assemble in ribbons of 1-10 μm in length (Table 2)[66].

Their pharmacokinetic profile was very different. Ambisome® liposomes circulated for longer periods in blood and are slower uptake by MPS. On the contrary, Amphocil® and Albecet® deliver AmB rapidly to phagocytic cells because of their shape and size, respectively (Table 2).

Deeply analyzed, the higher efficacy of AmB-DS was due to higher doses that can be administered without sign of toxicity. The stable interaction of the drug with DS impaired AmB interaction with cholesterol membranes of mammalian cells and decreased the nephrotoxicity. The higher AmB doses that can be given allowed to persistent high drugs levels in infected macrophages and a better cure rate[69].

The benefits of DS for treating cutaneous leishmaniasis (CL) are less evident. In CL, *Leishmania* amastigotes reside deep in the dermis and also disseminate to the lymphatic system and mucosal membranes. CL is currently treated with the drugs given by intravenous route, because none topical treatments were effective. Two paramomycin ointments, that included permeation enhancers, are commercially available but their use is limited by their toxicity or lack of efficacy. Ambisome® was effective intravenously administered but not given by subcutaneous or intraperitoneal route, although in general the treatment of CL required higher doses than for VL. The efficacy of Ambisome® and Amphocil®, but not Albecet®, to treat CL was ascribed to the smaller size (100 nm) of the former that prolongs their circulation before to extravasate towards the skin lesions where the inflammation generated

Table 1. Examples of DS evaluated for treatment and vaccination against *Leishmania*.

Chemotherapy			
DS	**Drug**	**Parasite**	**References**
Liposomes	Antimonials	*L.donovani, L.major*	19,20
	Camptothecin	*L. donovani*	21
	Piperine	*L. donovani*	22
	Andrographolid	*L. donovani*	23
	Pentamidine	*L. donovani*	24
	Miltefosine	*L. donovani*	25
	Atovaquone	*L. donovani*	26
Niosomes	AmB	*L. donovani*	27
	Antimonial	*L. donovani*	28-31
Polymeric particles			
Starch-MP	Antimonial	*L. donovani*	32
PLGA-NP	AmB	*L. donovani in vitro*	33
PLA-NP	Pentamidine	*L. infantum*	34
	Primaquine	*L. donovani*	35
	DCM		36
Polymethylacrylate-NP	Pentamidine	*L. infantum*	37
Polyalkylcyanoacrylate NP	Primaquine	*L. donovani*	38
PolycaprolactoneNP	AmB	*L. donovani in vitro*	39
Albumin-MP	AmB	*L. infantum*	40,41
Nanosuspensions	AmB	*L. donovani oral*	42
	Aphidicolin	*L. donovani in vitro*	43
SLN	AmB	*L. donovani*	44
Emulsions	AmB	*L. donovani*	45-48
	Piperine	*L. donovani*	49
	Antimonial	*L. donovani*	50
	buparvaquone	*L. major* topical admon	51
	Sitamaquine	*L. major* topical admon	52
Drug conjugates	Antimonial	*L. donovani*	53
	AmB	*L. major*	54
	8-aminoquinoline	*L. donovani*	55
Vaccination			
DS	**Antigen**	**Parasite**	**References**
Emulsions			
Freund's adjuvant (IFA)	H1	*L. major*	56
	SLA	*L. donovani*	57
Montanide ISA720	H1	*L. major* in vervet monkeys	58
MPL-SE®	Leish-111f	CL and VL in human clinical trials	59
Liposomes	gp63	*L.donovani*	60
	Peptides	*L. donovani*	61
Liposomes + (non-coding pDNA)	SLA	*L. donovani*	62
Liposomes (+ CpG)	gp63	*L. major*	63,64
	LmSTI1	*L. major*	65

gp63=63-KDa glycoprotein of L. donovani promastigotes; H1=Ag Histone H1; LMSTI1=Leishmania major stress-inducible protein 1; MP=Microparticles; NP=Nanoparticles; SLA=Soluble Leishmania Ag.

by the parasite increased the vascular permeability. The large ribbons of Albecet® can not extravasate and resulted inactive[68]. Compared with Fungizone®, the three lipidic formulations had significant curative effect when topically applied to *L. major* lesions, in some cases with permeation enhancers (ethanol)[70].

The ideal treatment for leishmaniasis would be given by oral route. Oral therapy has a general lower cost (injectable formulations require the use of sterilized material and trained personal for administration) and better patient compliance. Among current options of treatment, only mitelfosine has shown activity against VL by oral route. The bioavailability

Table 2. Commercial formulations of AmB.

AmB DS	Composition	Structure (Shape and size)	AUC (mg/L x h)	Toxicity LD50 (mg/kg)
Fungizone®	DOC-AmB (7:3)	Micelles aprox. 1 µm	34.25	4
AmBisome®	HSPC-Chol-DSPG-AmB (2:1:0.8:0.4)	SUV 50-100 nm	423.0	>175
Abelcet®	DMPC-DMPG-AmB (7:3:3)	Ribbon-like 2-5 µm	6.7	40
Amphocil®	CS-AmB (1:1)	Disks 122 nm por 4 nm	45.6	38

AUC= Area Under Concentration; LD50= Dose that produces 50% mortality. Results in mice are shown. DOC= sodium deoxicholate; AmB=Amphotericin B; HSPC=Hydrogenated soy phosphatidylcholine; DSPG=Distearoylphosphatidylglycerol; DMPC=Dimyristoylphosphatidylcholine; DMPG=Dimyristoylphosphatidylgycerol; CS= Cholesteryl sulphate.

of AmB is very low due to its low solubility and high molecular weight. Ambisome® was not effective after oral administration because liposomes are quickly destroyed by surfactants in the gastrointestinal tract. However, other DS have shown resistance against high concentrations of salts and degrading elements of the intestinal fluids and can solve the problems of drug that limit their oral bioavailability such as low solubility, mucosal permeability and first pass effect. A nanosuspension of AmB prepared by a high-pressure homogenization technique induced a significant reduction in the liver parasite load when orally administered against experimental VL (*L. donovani*)[42].

Nanoassemblies between antimonials (meglumine antimoniate) and b-cyclodextrins were prepared. Given by oral route they decreased the size of skin lesions on experimental CL (*L. amazonensis*) with effectiveness similar to that of the free drug given intraperitoneally at twofold-higher dose[71].

Although Ambisome® reduces the drug toxicity and the cost of hospitalization because a high single dose is demonstrated to be effective; this only partly offset the high purchase price of the drug. Hence, this high cost is unacceptable or prohibitive in the zones in which VL is endemic. In Europe where cost is not such a preponderant issue, Ambisome® has become the treatment of choice. For this reason, there are studies based on the development of low-cost methods of producing DS[72].

Heat-treatment of Fungizone® provokes the formation of 300nm superaggregates that are less toxic to mammalian cells, target the drug to macrophages and increased AmB activity against *L. donovani* in mice. It is a simple and cheap method of AmB improving therapeutic index[73].

Micelles and emulsions are also simple and low-cost systems that can be used to improve drug delivery. In fact, Fungizone®, the conventional AmB formulation is a micellar solubilisation of AmB with sodium deoxycholate. It solves the problem of AmB poor solubility but neither decreases the drug nephrotoxicity or improves the targeting to macrophages. A very recently emulsion of AmB showed higher accumulation in liver and spleen, more efficient elimination of the parasite and a reduction in AmB toxicity compared with Fungizone®[45, 48]. However, they remained far from benefits of Ambisome®. Fungizone®, directly infused with Intralipid (an emulsion of nutritional fluids administered intravenously) was tolerated much better showing reduced nephrotoxicity[74]. Similarly, sodium stibogluconate[50] and piperine[49] have been formulated in emulsions that increased its efficacy against *L. donovani*.

Niosomes, liposome-like vesicles consinting of mixtures of cholesterol and non-ionic surfactant, behave *in vivo* like liposomes. However, they are more attractive for industrial manufacturing because of lower cost of materials and easier fabrication conditions. A single dose of niosomes loaded with stibogluconate were found to be equally active® than Ambisome® in experimental VL, although did not protect against reinfection[28, 31]. AmB-loaded into niosomes presented lower activity than Ambisome® but higher than Albecet® and substantially higher than Fungizone® in experimental VL[27].

The possibilities of micro/nano polymeric particles have been also explored. AmB loaded in albumin MP were less toxic that heated Fungizone® and emulsions and more effective that Fungizone® to reduce the parasite number in spleen and liver at higher doses[40, 41]. Other polymeric particles such as poly(caprolactone) nanospheres were found three times more effective than free AmB in reducing parasite burden of infected macrophages in vitro[39].

Similarly, the encapsulation of pentamidine (second-line treatment)[34, 37] or primaquine[35, 38] in poly(D,L-lactide) (PLA) or polyalkylcyanoacrylate NP increased 3-6 fold their activity against *L. infantum* or *L. donovani* respectively.

DS technology has also been applied to anti-leishmanial agents of natural origin as dihydroxymethoxychalcone (DCM) or in order to explore the antileishmanial potential of various natural agents as quercetin[75], bacopasaponin[76] and arjunglucoside[77]. PLA NP encapsulated DCM was able to reduce 90% of parasite load at 5-lower concentrations than free drug that did not show any activity in experimental CL[36]. The antileishmanial activity of the natural products was improved in nanoparticulate form in the order: nanoparticles>niosomes>liposomes>microspheres, observing an inverse relationship between the efficacy and the size of the vesicles.

Several antileishmanial drugs were also chemically linked to polymers. AmB was conjugated by to a branched natural hydrosoluble polysaccharide, arabinogalactan, by reductive amination in aqueous medium at room temperature. The conjugate was soluble in water and more effective than Fungizone® and Ambisome® in reducing the number of mice with lesions and the lesion size in experimental CL. AmB-arabinogalactan was also 40 times less toxic than free AmB in mice[54].

The studies described above have been mainly taken advantage of the passive targeting of DS to macrophages to reach *Leishmania* within these cells. However, although the natural target of DS is the phagocytic cells, their uptake by macrophages can be greatly accelerated and increased by modifying their surface with ligands recognized by receptor expressed on these cells. Examples are sugar-bearing liposomes or immunoliposomes designed to target Macrophage mannose receptor (MMR) or Fc receptor respectively.

Thus, mannose-grafted liposomes loaded with hamycin[78] or pentamidine[24] showed improved activity over the inconjugated liposomes or the free drugs on experimental VL due to the increased macrophage uptake. After a single iv administration mannose-grafted lipid nanoparticles (SLN) loading AmB distributed in the liver and spleen faster than uncoupled SLN and showed enhanced antileishmanial activity (95% reduction in parasite burden)[44]. A 8-aminoquinoline analogue conjugated with the polymer (N-(2-hydroxypropyl)methacrylamine) bearing mannose was more active in experimental VL (*L-donovani*) than those without mannose moieties[55]. Empty IgG coupled liposomes were 3 times more active than free IgG and uncoupled liposomes in clearing *Leishmania* from macrophages *"in vitro"*[79]. *Leishmania* specific antibody-coupled liposomal doxorubicin was significant more active and much less toxic than free or liposomal doxorubicin in the treatment of VL[80].

Similarly, the peptide tufsin specifically binds to macrophages and increase their killer activity against pathogens. Tufsin-grafted liposomes loaded with sodium stibogluconate[81] and AmB[82] showed higher activity against experimental VL compared with plain liposomes or free drug. However, none of these strategies have been clinically tested yet. The difficulty of large-scale grafting contributes to the problems associated with scaling-up the production of DS.

Delivery systems as adjuvants for vaccinacion against *Leishmania*

The natural tendency of DS to localize to the MPS system found early application in vaccination. DS loading Ag were expected to increase Ag availability and uptake by Antigen Presenting cells (APC) and then, to work as "adjuvants". Evidences of the adjuvanticity of liposomes[83], emulsions, micro- or nanoparticles[84], and other DS can be extensively found in the literature[85]

The term adjuvant describes any substance, combination of substances or strategies that augment the specific immunity to an antigen as compared to that induced by the antigen or vaccine alone. The incorporation of any "adjuvant" is mandatory in the development of effective vaccines based on recombinant Ag (sub-cellular vaccines), which are poorly immunogenic *per se*.

Based upon their mechanism of action, the adjuvants can been classified in two categories [86]: Particulate adjuvants that target the Ag to APC and immunostimulants, usually Toll-like receptors (TLR) ligands. The three signals model from APC (specially dendritic cells, DC) required for the initiation of T-cell responses can help to understand the biological processes that contribute to adjuvant activity and the classification of adjuvants before said: signal 1 or increase of Ag uptake, processing and presentation and increased expression of MHC class I and class II molecules; signal 2 or enhanced expression of co-stimulatory molecules; signal 3 or production of cytokines as IL-12 or IL-18. The nature of signal 3 determines the type of specific immune response: Th1/CTL or Th2-humoral immune response.

Particulate adjuvants boost the signal 1: they protect Ag (usually protein, DNA or RNA) from rapid degradation and removal from the injection site. Therefore, they provide a depot that prolongs the exposure of APC to Ag (depot function). Ag loaded in small particles (< 2 μm) could be directly engulfed by APC (targeting function). Moreover, the uptake of particulate Ag by phagocytosis enhance the Ag presentation in the context of MHC class I molecules (termed as "cross-presentation").

Another group of adjuvants, TLR agonists enhance the signals 2 and 3[87]. TLR are pathogen recognition receptors (PRR) that recognize pathogen associated molecular patterns (PAMP) shared by microorganisms but not expressed in mammalian cells. There are at least 11 members of the TLR family that recognize specific components conserved from microorganisms. TLR4 detected lipopolysaccharide, TLR2 is able to recognise bacterial lipoproteins and lipoteichoic acids, TLR5 interacts with flagellin, TLR9 has affinity to unmethylated CpG DNA of bacteria and virus and TLR7 detected single-stranded viral RNA.

TLR engagement induced DC maturation and cytokines production that explain the adjuvanticity of TLR agonists. Moreover, TLR ligands not only trigger APC activation (signal 2 and 3) but also orientate the immune response towards a Th1 or Th2-cell response. In particular it is well established that LPS agonists, imidazoquinolines and CpG oligonucleotides induced Th1-cell responses after sensing by TLR4, 7/8 and 9 respectively.

Taking into account that DS seems to enhance signal 1 but not produce APC stimulation (signal 2 and 3), the use of DS as carriers for both ag and TLR agonist has been suggested as a perfect mix, that combine the benefits of both types of adjuvants[88]: DS to target both Ag and TLR to the same APC and TLR to induce APC activation and to direct the specific immune response. Moreover, when administered in close proximity, TLR seems to further enhance the "cross-presentation" of particulate Ag. Finally, the strategy could also avoid the spread of TLR through the bloodstream and their toxicity. In fact, TLR agonists are usually small molecules that readily enter the blood circulation after their administration. DS would be useful to prevent rapid elimination and encourage uptake by APC[89].

Nowadays, this scenario is changing. Besides Ag delivery functions, very recently it has been reported that some types

of DS can also activate innate immunity pathways *in vivo* (signal 2 and 3). Interestingly, it has been demonstrated that particles adjuvanticity is mediated by the protein complex NALP3/inflammosome in APC[90, 91]. The activation of NALP3 receptor produces a pro-inflammatory environment characterized by a rapid recruitment of leukocytes and APC at the injection site, that critically condition the ongoing specific immune response in a still undetermined way. Thus, a vaccine consisting on DS loading Ag plus TLR agonist perform also a combination between TLR and NLR signalling pathways. In this context, whereas the type of immune response induced by single TLR agonists is becoming clear, much more work must be done in order to define bias in the adjuvanticity of DS.

Actually, the adjuvant Alum is the only approved by the FDA over the world[92]. It is component of several licensed human vaccines (Table 3). It belongs to the category of particulate adjuvants and formulation is achieved through the adsorption of Ag onto aluminum particles. In Europe there are also two emulsions authorized in human vaccines. MF59® is a squalene-in-water emulsion with span® 85 and tween® 80 as emulsifiers. The size of the emulsion droplets is lower than 250 nm. It is used in Europe as an adjuvant in influence vaccines (Fluad®). Recently, AS03, a 10% oil-in-water emulsion was approved for use in influenza A Prepandrix®.

There are other DS in clinical trials[93] (Table 3): HIV-1 DNA delivery using PLG MP has recently undergone Phase I clinical testing in the USA. Montanides (IS51 and ISA720), w/o emulsions containing mannide-mono-oleate as emulsifier, have been used in malaria, HIV and cancer vaccine trials. Montanides are similar to O/W emulsion IFA (uncomplete Freund's adjuvant), prepared with a biodegradable proprietary non-mineral oil. In fact, Montanides were developed in response to safety concern with IFA in animals. AS02 is an O/W emulsion containing MPL® and QS21®. MPL® is a non-toxic derivative of LPS and a potent stimulator of Th1 responses. QS21® is a purified component of the Saponin Quil-A with demonstrated adjuvant activity. AS02 is being evaluated in clinical trials for malaria, HPV, HBV, tuberculosis and HIV. AS01 is a liposomal formulation containing MPL® being actually evaluated in clinical trials for malaria. AS15 that incorporate CpG to the liposomal formulation AS01 is being tested in cancer vaccine trials. MPL®-SE an emulsion of squalene oil in water containing MPL® is currently being evaluated in several clinical trials for *Leishmania*.

The emulsions developed containing MPL® or QS21® illustrate the beneficial contribution of DS in the formulation of adjuvants. MPL® is unsoluble and prone to aggregation, which adversely affects its bioavailability. Although currently licensed as adjuvant in a new HBV vaccine, its efficacy and reliability is greatly enhanced when included in emulsions or liposomes. The saponin QS21® is a natural detergent which, when injected in a free form, produce hemolysis of red blood cells. The association of QS21® with the emulsion reduces its lytic activity.

Other DS with adjuvant activity are virosomes, virus-like particles (VLP) and immunostimulation complexes (ISCOMS). They are not used for the delivery of therapeutics and for this reason they were excluded from the general classification of DS.

Virosomes (50 nm-10 μm) are liposomes that carry viral fusion proteins in their membrane as for example Sendai fusion protein, influenza HA, E1 or E2 envelope glycoproteins of Rubella virus. A Virosome-based vaccine containing HA against Hepatitis A is registered in Europe.

Immunostimulating complexes (ISCOMs) are micellar assemblies of about 40 nm made of cholesterol, phospholipids and 70% of saponin QuilA that trapped the protein Ag through hydrophobic interactions. Clinical trials are in progress against infections including Influenza, HSV, HBV, HIV, malaria and cancer.

Virus-like particles (VLP) are capsid recombinant proteins that naturally self-assemble into non-infectious particles 20-100 nm in size. VLP vaccines against HBV and HPV are commercially available.

DS have been scarcely evaluated as adjuvants in the research of effective vaccines against *Leishmania*[94].

The administration of the recombinant produced Ag histone H1 adjuvanted with Montanide ISA720 promoted a reduced development of lesion size in vervet monkeys after challenge with *L.major*[58]. With IFA, it was shown that H1 elicited partial protection against *L.major* in mice[56].

A cocktail of *Leishmania* Ags (TSA, LmSTI1 and LeIF) were genetically linked in tandem to obtain the 111 KDa polypeptide Leish-111f. Leish-111f formulated with MPL®-SE conferred durable immunity to experimental Leishmaniasis for at least three months[59]. This vaccine formulation was also effective in inducing partial protection against visceral Leishmaniasis in animal models. However, it could not prevent disease development in a Phase III vaccine trial in dos. Therapeutic trial in mucosal leishmaniasis (Peru) and CL (Brasil) were both completed successfully in 2006. Now the vaccine trial is being performed in Sudan to evaluate the safety and immunogenicity in patients with post-kala azar dermal leishmaniasis. With these clinical trials against different species of *Leishmania* there is a hope for this adjuvant in human use against leishmaniasis.

Cationic liposomes loading Ag as the immunodominat 63-KDa glycoprotein (gp63) of *L.donovani* promastigotes[60, 64], or *Leishmania* major stress-inducible protein 1 (LmSTI1)[95] or soluble *Leishmania* Ag (SLA)[62], and very recently some polypeptides[61] were found to confer partial protection against experimental Leishmaniasis (both VL or CL, *L. donovani or L. major*) in BALB/c mice, estimated by the degree of reduction in parasite load in spleen and liver and/or by footpad swelling. As expected, the efficacy was further improved when CpG oligonucleotides[63, 65] or non-coding pDNA bearing CpG motifs[62] were co-encapsulated with these Ags in the liposomes. CpG is a TLR9 agonist and

Table 3. Adjuvants that belong to the category of DS incorporated in human vaccines.

	Structure and composition	Status
Aluminum salts	50-100 nm crystals of Aluminum hydroxide, Aluminum phosphate, Potassium aluminum sulfate (often called "Alum")	Currently incorporated in the vaccines: DPT, DT, HAV, HBV, HPV, *Streptococcus pneumonia*, meningococcal.
MF59®	O/W emulsion: 250 nm droplets composed with squalene, tween 80, Span 80 in citrate buffet	Influenza vaccine Fluad® Clinical trials: HBV; HSV, HIV
MPL-SE®	O/W emulsion of squalene oil in water containing MPL®	Leishmania
AS02	O/W emulsion containing MPL® and saponin QS-21®	HPV (Cervarix®). Clinical trials: HIV, Tuberculosis, Malaria, Herpes.
Montanides ISA51/ISA720	W/O emulsions prepared with a proprietary non mineral oil and a mannide monooleate surfactant	Clinical trials: HIV, Malaria, Cancer
ISCOMS	40 nm cage-like particles composed with cholesterol, phospholipids, protein antigen and the saponin Quil-A	Clinical trials: HIV, HSV, HPV, HCV, Cancer, Influenza, Malaria
Virus like particles (VLP)/Virosomes	40-60 nm self-assembled viral envelope proteins	HAV Epaxal®, HBV Recombivax®, Engerix-B ® HPV Gardasil®
AS01	Liposomes+MPL®	Clinical trials: HIV, Malaria, Cancer
AS15	Liposomes+MPL®+CpG	Clinical trials: Cancer
Microparticles	Poly(glycolic acid) MP	Clinical trials: DNA HIV-1

DPT=Diphtheria-pertussi-tetanus, DT=Diphtheria-tetanus, HAV=Hepatitis A virus, HBV=Hepatitis B virus, HCV=Hepatitis C virus; HPV=Human papilloma virus, HSV=Herpes simplex virus.

well-known Th1 inductor. In all these studies the degree of protection was correlated with a Th1 bias (IFN-γ/IL-4) and higher IgG2a/IgG1 ratio.

The efficacy of liposomes was highly affected by their physico-chemical characteristics. The influence of their composition and charge were evaluated. *L. donovani* promastigotes membrane Ag (LAg) loaded in liposomes formulated with lipids having liquid crystalline transition were more rigid, stable, elicited higher parasite $CD8^+$ and $CD4^+$ T cell immune response and better protection in BALB/c mice and hamster against infection by *L.donovani* [96]. What charge is concerned, cationic liposomes loading SLA were superior as adjuvants as they induced greater extent of protection against *L. donovani* compared with neutral and anionic liposomes[97, 99]. In fact, cationic liposomes enhanced the uptake of Ag by APC which subsequently leads to enhanced Ag presentation in the context of the major histocompatibility complex class I and class II pathways.

Actually, there is still a big lack in the performance of studies trying to find correlations between the physicochemical properties of DS and their adjuvant effect. Parameters as v.g. size could have great influence. The size determines whether particles are uptake by APC at the injection site or after migration towards the draining lymph nodes. Moreover, different subsets of DC could be implied in the uptake. At the cellular level, the mechanism of uptake and their intracellular trafficking could also be different, affecting the Ag processing and presentation. In this context, bigger particles are longer retained into the phagosome before to be transported to lysosomal compartments, resulting in a higher efficiency to cross-presentation pathway than NP. After administration in mice, 2-8 μm PLGA particles produced higher antibodies titers than smaller or bigger particles. Therefore, it was found that small particles (200-400 nm) favour Th1 immune response compared with 2 μm MP that promote IL-4 secretion[100]. Many other characteristics of DS that could have influence in their adjuvant activity have not yet been evaluated. In this context, improved data on mechanisms and adjuvant properties would open the possibility to predict the type of immune response that will be optimally induced by a particular adjuvant or combination of adjuvants, and for the first time raise the opportunity for rational adjuvant design, as opposed to past trial-and-error methods for adjuvant selection.

It is well-known that Alum tends to induce Th2 and B-cell responses, whereas TLR ligands tend to favour more Th1 and CTL responses[90]. Effective protection against *Leishmania* needs the development of Th1 and CTL immune responses. The bias induced by MF59® and other DS have not been clearly established. However, it has been hypothesized that the activation of NALP3 inflammasome induced by Alum (also observed with MF59® and PLGA particles) could be implied in the Th2 polarization[86].

Thus, we can anticipate that an effective vaccine against *Leishmania* will need a TLR agonist in their composition. However, in spite of the multitude of TLR agonists with evident efficacy actually in preclinical and clinical testing, there remains a surprising reliance on aluminium based compounds as the dominant adjuvants in human vaccines. In fact for FDA approval, new adjuvants must convincingly demonstrate efficacy and particularly safety, because the vaccination is given to a predominately

healthy population. The toxicity of TLR would be produced by an excessive innate immune system activation and inflammatory cytokines production. Their selective targeting to APC by DS could be an important strategy to ensure that TLR-expressing bystander cells and tissues do not become involved (avoiding off-target effects) and to decrease the dose to be administered for efficacy, keeping on minimal the adjuvant reactogenicity. Moreover, a lot of works evidenced that the hypothetical Th2 bias induced by DS-mediated activation of NALP3 inflammasome is surpassed in presence of TLR agonists that determine the immune response[101, 102].

Heat-stability, mono-dose presentation and needle-free administration are other desirable characteristics of an ideal vaccine, especially important for the accomplishment of vaccination programs in developing countries. DS, especially polymeric particles, could help to achieve a *Leishmania* vaccine with these attributes.

We finally highlight that there are other hurdles in the development of an effective vaccine against *Leishmania* besides an optimal choice of the adjuvant. In fact, the identification of Ags which induce protective immunity in all species of *Leishmania spp*. is a fundamental requirement to achieve this goal[103].

Conclusion

Far from providing a "magic potion", DS technology has shown potential to prepare systems of great efficacy for treating and preventing Leishmaniasis. However, a system that has great efficacy with poor commercial feasibility is merely futile. The commercial feasibility of any DS is governed by the cost of the material and the ease of manufacturing and scale-up[11, 104]. Nowadays, there is a big gap between the realms of scientists and the realities of pharmaceutical development. Most of DS products are extremely complex to manufacture at industrial level. There is a huge cost and development time penalty to pay in the development of processes required to provide well-defined DS products. Once the manufacturing and quality control processes are ultimately in place, the process of approval of such a product is problematic. Ambisome® was the first nanomedicine put into the market. However, Ambisome® is not a choice for most people. The pathway to commercialization for nanomedicine will require a substantial improvement in therapeutic outcome for the patient at the same or lower cost than conventional treatment. Thus, nowadays DS have still significant issues to resolve in order to get products to market based on these technologies.

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