Chapter 11

# Liposome-Mediated Immunosuppression Plays an Instrumental Role in the Development of "Humanized Mouse" to Study *Plasmodium falciparum*

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Additional information is available at the end of the chapter

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**Abstract**

The material world has been getting prone toward infectious diseases, and therefore novel strategies should be devised to treat chronic infectious disorders. The translational biomedical research scientists made early attempts to develop mouse-human chimera (humanized mouse) through the reconstitution of immunodeficient mouse with engraftment of human cells and tissues. Although the humanized mouse proved to be an effective tool in understanding various diseases such as human malaria and hepatitis, however, drug administration, retention capacity of the administered drug, toxicity, and ethical constraints are some of the major issues and need to be objectively addressed. The "humanization" of immunodeficient mouse needs pharmacological immunomodulatory reagents to control the excessively recruited cells of monocyte-macrophage lineage. Therefore, administration of liposome loaded with hydrophobic drug (clodronate) to induce selective apoptosis through "suicidal approach" in myeloid cells plays an instrumental role for controlling residual nonadaptive immune response of the host. Liposomes are spherical and hollow—structures consisting of lipid bilayer—and are used for the delivery of drug and vaccine candidates. The surface-engineered liposomes (ligand anchored) are used for targeted and controlled delivery. Clodronate-loaded liposomes play a pivotal role in developing humanized mouse. This mouse holds relevance to study pathophysiology and immunopathology of human malaria parasite, P. *falciparum*. The liposomal delivery of clodronate administered in immunodeficient mice to modulate their innate immune system is an amenable strategy with the minimal/acceptable range of systemic toxicity.

**Keywords:** humanized mouse, clodronate, liposomes, interleukin, immunity, innate response

## 1. Mouse-human chimera(s)

A "humanized mouse" is an immunocompromised mouse carrying identical functions of cell or tissue in origin as seen in humans. The depletion of adaptive immune system allowed sizeable grafting of human cells to understand the biology and pathology of various diseases to developing therapeutic interventions. The nude and severe combined immunodeficiency (SCID) mouse have been used for the humanization, but recently the immunodeficient background of NOG/NSG mouse has shown significant receptivity toward the significant engraftment and repopulation of human cells.

**The need of immunodeficient mouse**: An immunodeficient mouse is a laboratory mouse from a strain with a genetic mutation that causes a deteriorated or absent thymus, resulting in an inhibited immune system due to a greatly reduced number of T cells. The mouse is invaluable to translational research due to its susceptibility for different types of tissue and tumor grafts with less rejection episodes. These xenografts are commonly used in research to test new methods of imaging, treating tumors as well as understanding infectious diseases. The creation of a reproducible and straightforward animal model is inevitably required as it allows developing in-depth understanding on cellular and molecular mechanisms and pathophysiological manifestation responsible for the cause of systemic inflammatory diseases.

### 1.1. Human-hepatocyte transplantation

The initial attempt made toward developing a human liver chimeric mouse was the one that would accept human hepatocytes. SCID/bg mouse with the urokinase-type plasminogen activator (uPA) gene linked to an albumin promoter was the first one to be developed. The immunodeficient mice have subacute liver failure and are subjected to transplantation with fresh or cryopreserved human hepatocytes (huHep) via intrasplenic injection. Six to eight weeks after transplantation with human hepatocytes, large islands of human liver tissue are produced within the mouse liver, creating a mouse with a human/mouse chimeric liver [1]. The rate of successful engraftment in terms of huHep repopulation index (RI) is 60–70% as determined by calculating the human serum albumin levels.

The second mouse model that was developed had deficiency in the gene for the tyrosine catabolic enzyme fumarylacetoacetate hydrolase (Fah). These Fah−/− mice could engraft their hepatocytes only in the presence of 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC) and lost the engrafted Hepupon drug removal. This gene deficiency was bred into immunodeficient mice to create the FRGN mouse [2]. The mouse also supported the development of *Plasmodium falciparum* sporozoites into exoerythrocytic forms in the liver. Furthermore, when transplanted with human erythrocytes, they proved to be an effective model to study intraerythrocytic stages of *P. falciparum*.

### 1.2. TK-NOG mice for huHep transplantation

TK-NOG transgenic mouse in which mice express the herpes simplex virus thymidine kinase (HSVtk) transgenic construct containing the mouse albumin enhancer/promoter has drawn

significant attention. HSVtk mRNA is selectively expressed in the liver of NOG mice as a result of which they become prone to severe parenchymal liver damage after ganciclovir treatment

Of late, *reconstitution of TK-NOG mice with human hepatocytes led to orthotopic de novo engraftment and regeneration of huHep islands with controlled immunity and with broad repertoire*. The host is prepared by creating liver stroma with ganciclovir and further reducing their nonadaptive immune responses by clo-lip treatment and deploying immunosuppression strategies through tacrolimus laden polymeric hydrogels, huHep transplantation in TK-NOG mice.

*Prkdcscid* (protein kinase, DNA-activated, catalytic polypeptide; severe combined immunodeficient mouse *scid*): *Prkdc* plays a crucial role in repairing double-stranded DNA breaks and in recombining the variable (V), diversity (D), and joining (J) segments of immunoglobulin and T-cell receptor genes.

Homozygous mutants do not have mature T and B cells, are not capable to evoke cell-mediated and humoral immune responses, and are supportive to allogeneic and xenogeneic grafts. These models therefore are useful cancer research models. The *SCID* mutation renders NOD mice diabetes-free and thereby makes them useful for adoptive transfer of diabetes through T cell. This mutation in CB17 mice could allow engraftment of human peripheral blood mononuclear cells (PBMC's), fetal hematopoietic tissues, and hematopoietic stem cells (HSCs). The suboptimal engraftment efficiency and their inability to generate a potent and sizeable immune system are some of the striking limitations of this mouse model [3].

The above model proved to be unreliable because of the generation of mouse T and B cells, a phenomenon known as "leakiness," and generation of high levels of host NK cells. Besides, SCID mice resulted in a defective DNA repair system which results in an increased radiosensitivity.

### 1.3. RAG1 and RAG2 mutation (recombination activating gene 1 and gene 2)

Targeted mutations at gene Rag1 and Rag2 loci prevent mature T-cell and B-cell development in mouse but do not cause leakiness or radiosensitivity. Rag1 is essential for the V(D)J gene rearrangements that generate functional antigen receptors in T and B cells; homozygous $Rag1^{tm1Mom}$ mutants do not mature, functional T and B cells.

**NOD-SCID mouse**: This model is advantageous over others as it reportedly supported higher levels of engraftment of human PBMCs and showed lower NK-cell activity with additional defects in innate immunity. However, the use of this model has been limited because of the development of thymic lymphomas over the period of time and shorter life span thereby. The residual activity of NK cells and other components of innate immunity are some of the drawbacks of this mouse model

**Mutation in (IL-2R) γ-chain**: The IL-2Rγ-chain allows signaling through high-affinity receptors such as IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21. The mutation in IL-2Rγ leads to developmental deficiencies in T cell, B cell, and NK cell. Also, this model supports engraftment of human tissues, HSCs and PBMCs. This model supported a remarkable decrease in the inflammation mediators (cytokines/chemokines), which, in turn, helped improve *P. falciparum*

survival. IL-2Rγ mutation on NOD-SCID genetic background conferred an advantage that supported greater and high rising parasitemia which remains stable for weeks with greater reproducibility. Additionally, NSG mice shown lesser effect of aging which was due to the minimalized effect by interposition impact rendered by the IL-2Rγ mutation, along with a small sample size exhibiting marginal benefit of aging in the said mouse strain. The NSG-IV murine model when transplanted with human cells showed a great complementation of IL-2Rγ mutation byclo-lip treatment in controlling inflammation mounted by the cell engraftment which reportedly showed the reduction in the erythrophagocytosis [1, 2, 4–7].

## 2. Liposomes: versatile carriers

Liposomes are small synthetic vesicles of spherical shape formulated from cholesterol and natural nontoxic phospholipids. The small size and hydrophobic and hydrophilic attributes of liposomes are some of the glaring features in addition to their biocompatibility and sustained release properties [8]. A liposome has an aqueous solution core surrounded by a hydrophobic membrane, in the form of a lipid bilayer, and therefore hydrophilic solutes dissolved in the core cannot readily pass through the bilayer [8].

The nature and attributes of liposomes vary depending upon the method employed for their formulation, lipid composition, and charge present on their surface. Moreover the choice of bilayer components determines the "rigidity" or "fluidity" and the charge of the bilayer [8]. For instance, unsaturated phosphatidylcholine species from natural sources (egg or soybean phosphatidylcholine) renders greater permeability with flexible stable bilayers, whereas the saturated phospholipids with long acyl chains (dipalmitoylphosphatidylcholine) form a rigid rather impermeable bilayer structure [8].

There has been experimental evidence on these phospholipids forming closed structures when mixed rigorously in aqueous phase [8]. These closed structures are hollow and therefore are used to deliver regardless of the nature of selected drug [8].

Hydrophobic chemicals are associated with the bilayer; lipid vesicles may be loaded with hydrophobic and/or hydrophilic molecules. The site-specific and controlled delivery of drugs/candidate vaccines is achieved by the fusion of lipid bilayer with other bilayers such as the cell membrane. However, delivery of entrapped content through liposomal formulations is a complex and non-spontaneous phenomenon [8].

## 3. Advent of liposomes as delivery vehicle

The origin of liposomal formulation liposomes goes back to the mid-1960s, and when Alec D. Bangham and his coworkers discovered that phospholipids in the presence of suitable solvents form bilayer membranes which beget hollow spheres to form unilamellar or multilamellar vesicles (MLVs) [9]. The background is studied in three phase: "Origin," "Medieval period," and "Modern era."

**Origin (1968–1975)**: The physiochemical characterization of liposomes had been carried out in this period. The approach of thin-film hydration was adopted for the development of multilamellar vesicles (MLVs). The closer resemblance to various biological membranes, liposome, had been a natural choice to study the nature and functions of biological membranes; Bangham had called his lipid structures "multilamellar smectic mesophases" or sometimes "banghasomes" [9]. A more common term liposome was later coined by Weissmann [8].

**Medieval period (1975–1985)**: Liposome's utility was improved following basic research that increased the understanding of their stability and interaction characteristic within the system. There were methodological advancements; so far the formulation of liposomes was concerned further; the in-depth understanding on physiochemical properties of liposomes, their behavior within the body, and their interaction with the cells led scientists to improve upon potential as drug carrier systems.

**Modern era (1985 onward)**: Liposomes have been widely used all across the scientific disciplines including material sciences, mathematics, physics, biophysics, biochemistry, colloid science, and nanobiology for their well-bestowed delivery potential [9]. Ambisome, a parenteral amphotericin-B-based liposomal product, was initially synthesized along with numerous products undergoing clinical trials or licensed for the market [9].

## 4. Classification of liposomes

The liposome size varies from very small (0.025 μm) to large (2.5 μm) vesicles comprising one or bilayer membranes. The circulation time of liposome is based on the size of the vesicle or in entrapment efficiency. The size and number of bilayers largely affect the amount of drug encapsulated in lipid vesicles, liposomes.

The bilayer behavior and size of liposomes provide an opportunity to categorize them as:

(a) **Multilamellar vesicles** (**MLVs**)

(b) **Unilamellar vesicles**: Unilamellar vesicles can also be classified into two categories: large unilamellar vesicles (LUVs) and [2] small unilamellar vesicles (SUVs). In unilamellar liposomes, the vesicle is surrounded by a single phospholipid bilayer sphere keeping the aqueous solution bound inside it. In multilamellar liposomes, vesicles have a structure similar to that of an onion. The combination of several unilamellar vesicles will give rise to a multilamellar structure of concentric phospholipid separated by water layers [10].

1. **Archeosomes**: Archeosomes are vesicles derived from archaebacteria lipids. These are very different from the eukaryotic and prokaryotic bacteria. They are less sensitive to oxidative stress, high temperature, and alkaline pH [11, 12].
2. **Cochleates**: Cochleates are derived from liposomes, suspended in an aqueous two-phase polymer solution and are subjected to phase separation which allows appropriate partitioning of polar molecule-based structures. When this solution is treated with cations like $Ca^{2+}$ or $Zn^{2}$, giving rise to cochleate precipitates less than 1 μm in dimension [13].

3. **Dendrosomes**: Dendrosomes are a version of liposomes that along with being nontoxic are biodegradable, self-assembled, hyperbranched, dendritic, spheroidal nanoparticles which are easy to prepare, relatively cheap, and highly stable as well as easy to handle. The dendrosomes have proven for their delivery potential and hold an edge over existing synthetic vehicles for gene delivery. Dried reconstituted vesicles (DRV): This technique allows preparing small, "empty" unilamellar vesicles, containing different lipids or mixtures. Once SUVs are dissolved in solubilized drug, the dehydration is performed. The rehydration then leads to the formation of large quantities of heterogeneous multilamellar vesicles followed by further processing to form liposomal vesicles loaded with drugs [14].
4. **Ethosomes**: The ethosomes (the engineered liposomes) as compared to conventional liposomes have proven efficient so far the delivery attribute is concerned. Also, these carriers have reportedly known to show better entrapment of drug(s) [15]. Ethosomal drug permeation through the skin was demonstrated in diffusion cell experiments. Ethosomal systems were composed of soy phosphatidylcholine, and about 30% of ethanol was shown to contain multilamellar vesicles by electron microscopy.
5. **Immunoliposomes**: Liposomes which are anchored with antibodies, Fabs, or peptide structures can be used in in vitro as well as in vivo applications [16, 17].
6. **Immunosomes**: The glycoprotein molecules attached onto the surface of preformulated liposomes are called "immunosomes." The immunosomes do not vary in their appearances with the prominent presence of spikes evenly distributed on their outer surface [18]. **Immunosomes** have structural and immunogenic characteristics closer to those of purified and inactivated viruses than any other forms of glycoprotein lipid arrangement.
7. **Immune stimulating complex** (**ISCOM**): ISCOMs are made up of saponin mixture Quil A, cholesterol, and phospholipids giving rise to spherical, micellar assemblies of about 40 nm in size. They are constituted of amphiphilic antigens such as membrane proteins. ISCOMs have an inbuilt adjuvant Quillaja saponin, isolated from *Quillaja* [19].
8. **Lipoplexes**: Cationic lipid-DNA complexes, called Lipoplexes, are efficient carriers for cell transfection, but the rendered toxicity limits their applications [20, 21]. These local and systemic toxicities may result from either cationic lipids or nucleic acids.
9. **LUVETs**: Large unilamellar vesicles prepared by extrusion techniques (LUVETs) are chiefly performed with high-pressure systems. These proved to be more stable and did not cause leakage on treatment with detergents [22, 23].
10. **Niosomes**: Niosomes are small unilamellar vesicles made from nonionic surfactants also called novasomes. Their chemical stability is comparable to that of archeosomes [24, 25].
11. **pH-sensitive liposomes**: This class of liposomes is characterized as follows:
    1. This class combines unsaturated phosphatidyl ethanolamine and acidic amphiphiles that render stability at neutral pH [26].
    2. The second class compiles liposomes composed of lipid derivatives which gives increased permeability to encapsulated solutes [26].

3. The third class of pH-sensitive liposomes operates at low pH to destabilize membranes. These are made of pH-sensitive peptides or fusion proteins [26].
4. The fourth class of liposome uses **pH-sensitive liposomes** and has pH-titrable polymers to stabilize membranes which are susceptible to change in shape at low pH [26] (**Figure 1**).

12. **Polymerized liposomes**: Polymerized phosphatidyl choline vesicles (35–140 nm) have been synthesized from lipids bearing one or two methacrylate groups per monomer. These vesicles showed improved stability and controllable time-release properties compared to non-polymeric analogs [27].
13. **Proliposomes**: Proliposomes (PLs) are defined as dry, free-flowing particles that immediately form a liposomal dispersion on contact with water. Proliposomes (PLs) are dry, free-flowing granular products composed of drug(s) and phospholipid(s) which, upon addition

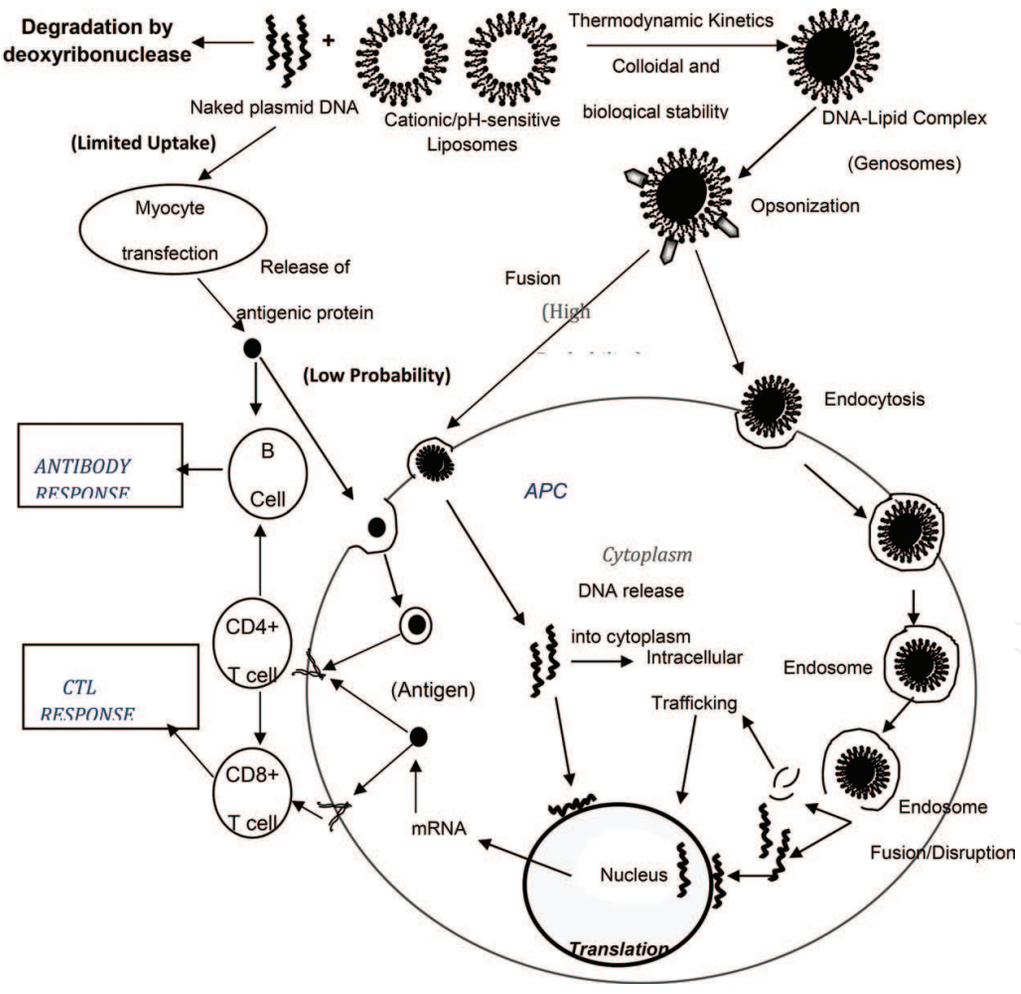


**Figure 1.** Schematic representation of proposed mechanism of DNA immunization via endocytic pathway. Naked DNA is taken up by a small number of myocytes after i.m. injection, which are then transfected episomally. The produced antigen is released from the cells to interact with APC and thus induce immunity. In contrast, liposomal DNA interacts with APC directly and induces better immune response. It also protects DNA from degradation by deoxyribonuclease attack.

of water, disperse to form a multilamellar liposomal suspension. These are economically feasible to formulate and be used up to a great extent on commercial scale. These hydrated membranes form vesicles upon contact with water. Moreover, the distribution, transfer, and storage become easy due to their availability in lyophilized form [28–30].

14. **Proteasomes**: Vesicles of bacterial origin were solubilized followed by ammonium sulfate precipitation and dialysis against detergent buffer. Proteins and peptides are non-covalently complexed to the membrane making them highly immunogenic [31].
15. **Reverse-phase evaporation vesicles** (**REVs**): Vesicles are formed by evaporation of oil in water emulsions resulting in large unilamellar liposomes. The main problem encountered in the usage of organic solvents is its trace content even after evaporation in the final solution which can be hazardous to human health and also may affect the stability of the vesicles. However, this issue may be addressed by the use of polycarbonate filters which allowed the separation based on their size and entrapment efficiency. Furthermore, as an alternative, diethyl ether can also be used as an organic solvent because of its lesser toxicity [32, 33].
16. **Stealth liposomes**: When liposomes are modified by coating them with polyethylene glycol (PEG), a synthetic hydrophilic polymer can greatly induce stability and their circulation half-life. These advantages have established glycolipids for surface anchoring in order to achieving targeted and sustained delivery. The engineering process of this class of liposome culminated with the observation that coating of liposomes with polyethylene glycol (PEG), a synthetic hydrophilic polymer, would improve their stability and lengthen their half-lives in circulation, rendering the use of glycolipids obsolete [34–38].

    The PEG coating stabilizing effect arises from high concentration of hydrated groups that inhibit both hydrophobic and electrostatic interactions of variety of blood components and thereby limits their recognition by the reticuloendothelial system (RES) [39, 40].
17. **Temperature-sensitive liposomes**: Temperature-sensitive liposomes permit easy gel to liquid crystallization phase transition above the physiological temperatures and are efficient in achieving target-specific drug delivery. This property is achieved by the usage of thermosensitive polymers [41], and therefore, content release, surface properties, and their cell-surface binding may be controlled by the temperature [42].
18. **Transfersomes**: Transfersomes formulated by phosphatidylcholine and cholate are highly deformable making them as preferred choice for transcutaneous delivery of drugs and candidate vaccines. This in contrast to conventional liposomes and niosomes offers needle-free delivery of vaccines and an increased concentration of antibody titer which may suffice the need of systemic and mucosal immunity by provoking humoral and cell-mediated branches of immune system. Moreover, these ultra-deformable carriers can easily overcome the skin barrier and efficiently deliver the antigenic payload [43, 44].
19. **Virosomes**: Virosomes are small unilamellar vesicles containing influenza hemagglutinin, by which they became fusogenic with endocytic membranes. The co-incorporation of other membrane antigens induces enhanced immune responses [10, 45].

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## 5. Engineered version of liposomes

A greater population has relied on the use of antibiotics. However, emergence of resistance against antibiotics has warranted a demand to identify a new class of antibiotics with an efficient mode of delivery.

The scientists engineered an artificial nanoparticles made of lipids, "liposomes," that closely resemble the membrane of host cell which target bacterial toxin [46]. Since bacteria are not targeted directly, the liposomes do not promote the development of bacterial resistance. In clinical medicine, liposomes are used widely as a vehicle to deliver specific medication into the body for achieving extended release [46]. The liposomal formulation, however, act as traps for bacterial toxins, sequesters, and neutralizes them instantly which would be subsequently eliminated by the host's own immune system.

## 6. Role of clodronate-loaded liposome in global immunosuppression

Clodronate (dichloromethylene bisphosphonate) is a nontoxic drug but impermeable to cell membrane. However, liposomes prepared by using phosphatidylcholine and cholesterol are not toxic and are engulfed by wandering macrophages. The hydrophobic drug clodronate when administered naked will be cleared from the circulation and gets absorbed by the digestive system and may face the leakiness issue. However, when administered through liposomes, it would not easily escape from the cell and is retained [47].

It is evident that clodronate may be delivered into phagocytic cells using liposomes as vehicles, therefore preventing it from being escaped from the cell [47].

The cell enzyme lysosomal phospholipases disrupt the phospholipid layer of liposome and induce release of drug clodronate which gets accumulated sizably within the cell. The free clodronate has an extremely short half-life in the circulation and is cleared from the circulation by the renal system. Therefore, specific entrapment of clo-lip formulation by macrophages induces selective apoptosis of macrophages. Therefore, a technique that involves the macrophage "suicide" approach, using the liposome-mediated intracellular delivery of dichloromethylene bisphosphonate (CI2MBP or clodronate) was deployed. The method is specific with respect to phagocytic cells of the mononuclear phagocyte system (MPS) [47].

## 7. Autoimmune hemolytic anemia (AIHA) liposome

Autoimmune hemolytic anemia is a disease in which autoantibodies against RBCs lead to their premature destruction. The autoantibodies of the IgG type lead primarily to the uptake and destruction of RBCs by splenic and hepatic macrophages. The current therapies such as corticosteroids and splenectomy are directed at interfering with this process. Clodronate-loaded liposomes (dichloromethylene diphosphonate) selectively deplete macrophages

within 24 h of administration by inducing apoptosis of macrophages. Therefore, liposomal clodronate would be a useful agent for treating sAIHA. This drug formulation was effective within hours by first blocking and then depleting phagocytic macrophages, and its action lasted for 3–4 days in vivo. Thus, in AIHA, liposomal clodronate therapy may act like a temporary, medicinal splenectomy. Therefore, clo-lip treatment may prove useful in situations where rapid response to therapy is critical or other medical therapies are inadequate.

Clodronate-loaded liposomes completely halted the uptake of opsonized RBCs by the spleen in contrast to splenectomy which used corticosteroid treatment. However, this cannot replace the corticosteroid treatment but offers an advantage because the spleen is not removed, and its function is eventually restored by the natural replenishment of macrophages. Clodronate-loaded liposomes undertaken in this treatment are temporary but are spontaneous in treating AIHA as compared to corticosteroids.

## 8. Immunosuppression by liposome-mediated delivery of specific drugs

Normally, liposomes are now known for immunosuppression. However, liposome loaded with cytostatic drugs for cancer therapeutics is reported to cause more or less immune suppression [48]. These drug-loaded formulations opened new avenues in cancer immunotherapeutics.

The doxorubicin, an anticancer drug, commercially sold as doxil (a liposomal formulation) when administered in vivo, showed macrophage suppression. The immunosuppression was seen from the long-lived persistence of bacteria in the blood stream. We have tried immunomodulatory and pharmacological reagents/chemicals which were used to further suppress the residual innate immune response (**Table 1**) [47–50] of huRBC reconstituted immunodeficient mouse (huRBC-NSG) for the engraftment and survival of *P. falciparum*. Also, immunosuppression of nonadaptive residual immune responses of immunodeficient mouse was contained by clodronate-loaded liposomes in order to achieving significant huRBC grating in immunodeficient animals (NSG) (**Figure 2**).

### 8.1. Adjuvanticity of liposomes

When bestowed along with clodronate, EDTA, DTPA, or various calcium or metal ion complexes of these chelators have the potential to deplete macrophages in vivo. Liposomal formulations have reportedly enhanced the immune response both at humoral and cell mediated of a vaccine formulation [44, 51]. The adjuvant action of liposomes may be categorized as:

1. The marginal zone antigens of macrophages residing inside the spleen can be targeted through liposomes.
2. The liposomes are used to block or deplete the activity of suppressor alveolar macrophages.

Thus, liposomes offer the advantage of both drug administration and adjuvant [51, 52].

| Protocol tested | Dose | No. of mouse | % success (more than 2 days) | Parasitemia length average (days) | Best parasitemia (days) |
|---|---|---|---|---|---|
| DMSO | 5% | 18 | 88.8 | 7.18 | 12 |
| TGFβ | 100 ng/day | 17 | 23.5 | 7.25 | 8 |
| | 1 μg/day | 3 | 66.6 | 10 | 13 |
| Splenectomy | | 5 | 60 | 7 | 10 |
| Cyclophopshamide | 75 mg/kg | 7 | 100 | 7.6 | 9 |
| | 50 mg/kg | 12 | 41.6 | 6.8 | 9 |
| Coinfection *P. chabaudi P. falciparum* | | 7 | 71.42 | 11 | 24 |
| Coinfection *P. yoelii P. falciparum* | | 52 | 90.24 | 11.09 | 34 |
| NAC | 100 mg/kg | 25 | 56 | 11.07 | 19 |
| Vitamin E | 20 mg/kg | 13 | 77 | 8.25 | 34 |
| Trolox | 4 mg/kg | 5 | 60 | 3.85 | 6 |
| Anti-NK (TMβ-1) | 1 mg | 15 | 53.3 | 4.12 | 8 |
| Futhan | 20 μg/day | 4 | 50 | 2.75 | 4 |
| Bleeding | | 20 | 35 | 7.42 | 14 |
| *P. falciparum* various amount | 0.3% | 2 | 100 | 2 | 2 |
| | 1% | 2 | 100 | 4 | 4 |
| | 5% | 2 | 100 | 5.5 | 6 |
| | 7% | 2 | 100 | 4 | 4 |
| | 10% | 2 | 100 | 5.5 | 6 |
| pABA | 400 mg/kg | 4 | 100 | 4.5 | 5 |
| Folinic acid | 400 mg/kg | 4 | 100 | 4.5 | 5 |

**Table 1.** Coinfection of *Plasmodium chabaudi* and *Plasmodium yoelii*; NAC and vitamin E seem to have beneficial effect in *P. falciparum* survival; however, results are very heterogenous from one mouse to other and from one experiment to other [5].


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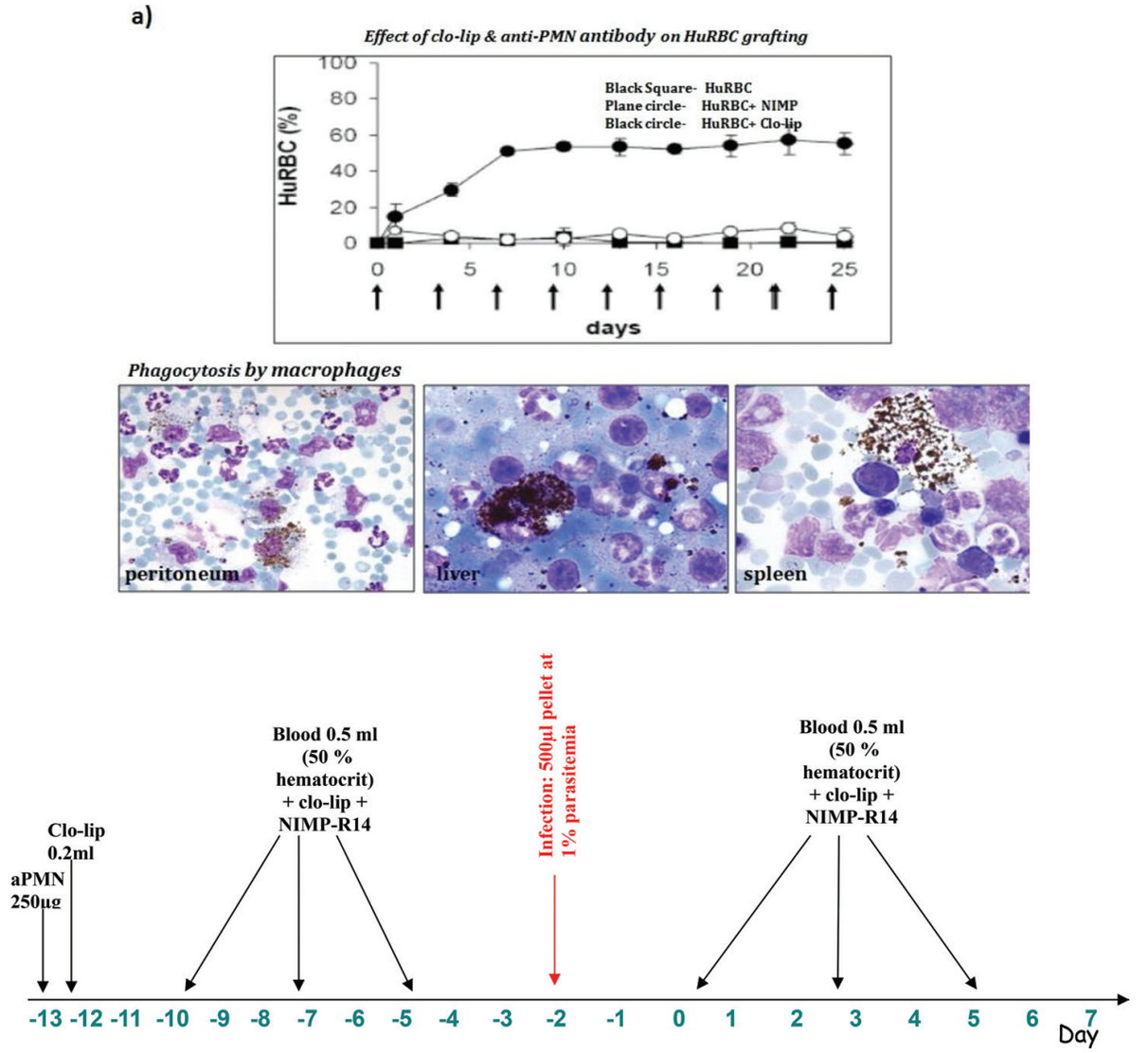


**Figure 2.** Immunosuppression of nonadaptive residual immune responses of immunodeficient mice by the sustained delivery of (a) clodronate via liposomes and (b) chemical immunomodulation protocol to control residual innate immune responses for robust "humanization".

## 9. Clodronate-loaded liposomes play a crucial role in host's immunosuppression for sizeable *P. falciparum* grafting in a humanized mouse

To aid in human cell engraftment in recently developed transgenic/immunodeficient strain, TK-NOG [53] mouse is used. Clodronate-loaded liposome will ameliorate the residual nonadaptive immune response by depleting the sizeable number of cells from monocyte-macrophage lineage. The clo-lip (clodronate-loaded liposome) is scavenged by the cells of monocyte-macrophage lineage, triggering their apoptosis and creating stroma for huHep grafting. Recent surge in the usage of humanized mouse models owing to the results from earlier findings [4, 5] in which clodronate-loaded liposome treatment depletes

the macrophage level in an immunodeficient mouse (Pf-NSG-IV) to study of asexual blood stage infection of *P. falciparum* (**Figures 2** and **3**).

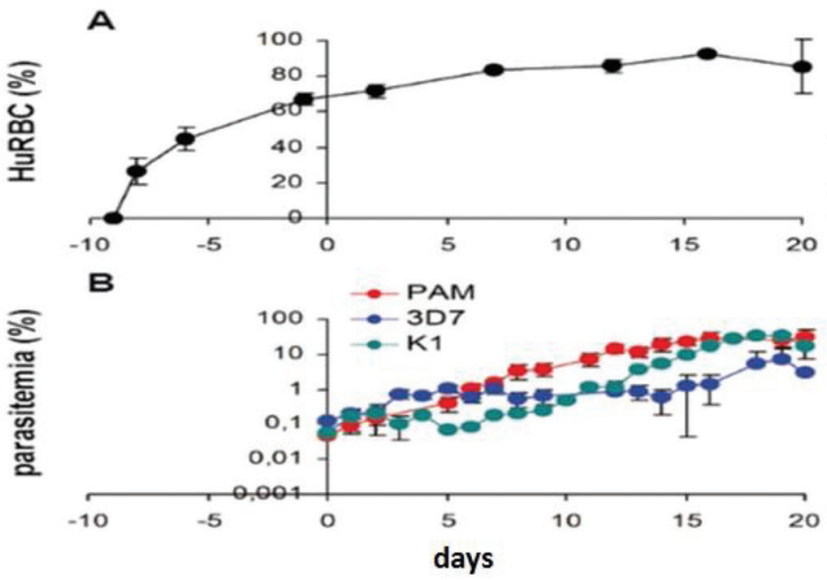


**Figure 3.** P. *falciparum* engraftment and development in an optimized humanized (PfhuRBC/NSG-IV) mouse [4, 5, 48]. A) Upper panel shows significant human blood chimerism in NSG mice over the period of more than 21 days, and B) lower panel illustrates the sizeable parasitemia using three (PAM, 3D7 and K1) P. falciparum strains in the huRBC reconstituted NSG mice.

## 10. Human malaria: a systemic inflammatory infectious disease

Malaria is one of the most deadly diseases in terms of mortality and morbidity affecting almost 2 million people worldwide. According to the WHO report, 2015, mortality due to malaria reached 438,000 deaths worldwide, majority of these occurring in African region (90%), followed by the Southeast Asia region (7%) and the Eastern Mediterranean region (2%).

However the malaria incidents dropped drastically by 37% across the globe and by 42% in Africa. Also there was a gradual decrease in the mortality rate by 60% globally and 66% in African region. The life cycle of malaria parasite is shown in **Figure 4**.

*P. falciparum* has gone to a wider trajectory to develop resistance against all the drugs and more complex patterns of multidrug resistance than anticipated. The suboptimal and uncontrolled use of drugs may lead to severe consequences of drug resistance in the field which could pose threat with unprecedented global health crisis. This scenario gets dangerous in the wake of unavailability of effective vaccines against *P. falciparum*.

### 10.1. The malaria vaccine development: a challenging task

Malaria is actually caused by the parasite called *Plasmodia* spp. which is highly evolved and a complex organism. The mercurial behavior of parasite because of the secretion of tens of thousands of proteins

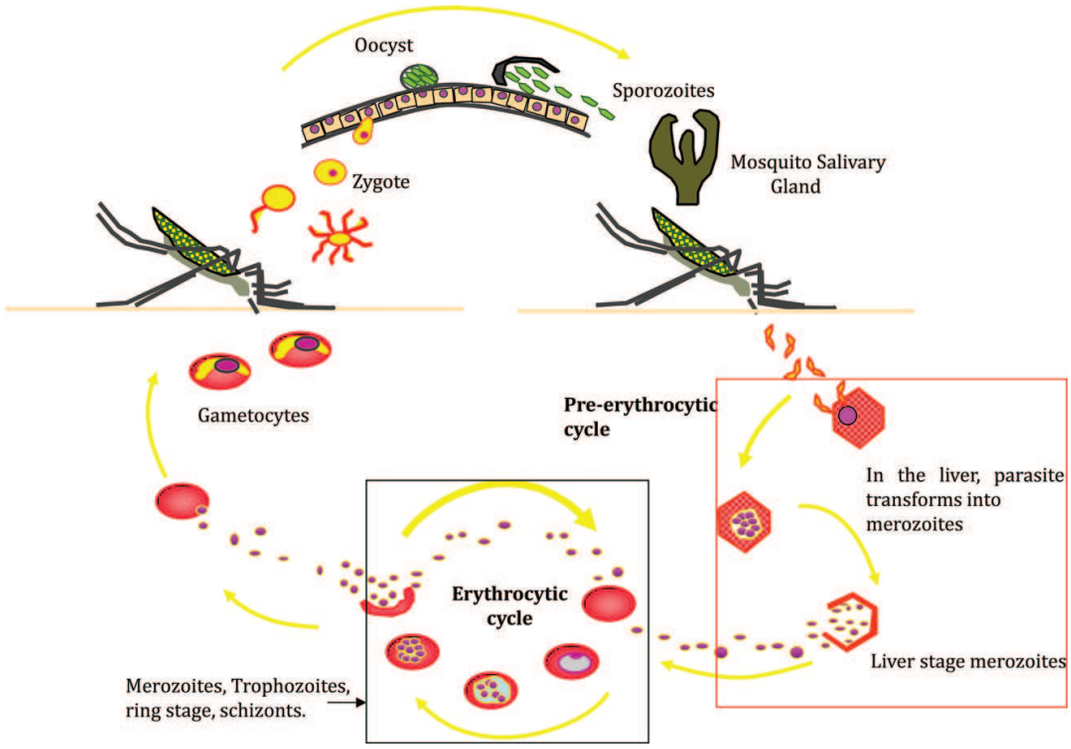


**Figure 4.** Life cycle of malaria parasite.

at each and every stage establishes it as one of the biggest challenges of humanity. The classical way of making a vaccine is to take the whole organism, the whole bacterium, or the virus and to inactivate it and to inject that as vaccine. That can be done for malaria, but it has been very hard to deploy that into a product—it is actually even harder to cultivate parasite especially liver stage in the lab as the conditions are not commensurate for its replication. The induction of potent and sterile immune responses is very difficult against LS of *P. falciparum*.

Parasite undergoes liver-stage development culminating in the formation and release of tens of thousands of first-generation parasites going undetected as this stage is asymptomatic. The asexual parasite stage can be cultivated in vitro, but the sporogonic stages require a workable humanized mouse model.

The *P. falciparum* consists of about 23 megabase nuclear genomes which has 14 chromosomes and encodes about 5300 genes. For example, two genes mainly Pfmdr1 and Pfmdr2 (*Plasmodium falciparum* multidrug resistance), *Plasmodium falciparum* multidrug resistance-associated protein (Pfmrp), are linked with the amount of drug that accumulates inside the digestive vacuole. Efficacy of drugs is dependent on these transporters as they shuffle them from intravacuolar to extravacuolar and vice versa. This observation suggests the common element of multigenic mechanism associated with mefloquine, halofantrine, and dihydroartemisinin. As the same gene has different effects depending on the type of gene mutation, it becomes difficult to identify any one functional gene emanating resistance. Therefore, vaccine development is a challenging and a herculean task to achieve [54].

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## 11. Liposome as a vaccine candidate

Liposomes are tools that can be used in tumor-targeting, gene-silencing, antisense therapy; immunomodulation; and genetic vaccination [48]. Liposomes (pH-sensitive liposomes) are majorly used for targeted and cytosolic delivery of vaccine candidate for achieving perdurable immune responses as well as delivery of drugs. They can be used as an efficient tool in vaccine development. The mycobacterial lipids when used to formulate liposomes have shown immense potential for mounting upon the sizeable cellular response which are considered as Th1 adjuvant.

The 19 kDa fragment (carboxy-terminal) of merozoite surface protein-1 of *Plasmodium falciparum* (PfMSP-119) is delivered directly into the cytosol with the help of liposomes to enhance immunogenicity. Engineered liposomes are used for sustained release of entrapped content and to increase immunogenicity. The liposomal vesicles have entrapped core of polymer that provides mechanical strength to them.

Gel core liposomes (engineered liposomes) were potentially tested for their utility in intramuscular delivery of transmission blocking antigen Pfs25 (recombinant protein antigen). Further, by using these engineered liposomes, the study evaluated the effect of coadministration of vaccine adjuvants CpG-ODN on the immune system of Pfs25. Liposome formulations of caryostatics, antibiotics, photosensitizers, enzymes, hormones, cytokines, and nucleic acids are being used to achieving some very promising results.

For targeting the DNA vaccine uptake and expression, APC are a preferred alternative to muscle cells. Antigen-coding plasmid DNA when administered via liposomes could bypass the need of muscle involvement and facilitates its uptake by APC, for instance, those infiltrating the site of injection or in the lymphatic, at the same time protecting DNA from nuclease attack [48, 55]. Engineered liposomes show advantage because of their evasion ability to escape the invasive route of administration making it an efficient carrier for the delivery of entrapped contents. Transfection of APC with liposome-entrapped DNA could be rendered by selecting an appropriate vesicle surface charge and lipid composition or by the co-entrapment of other adjuvants together with the plasmid DNA [48, 55–58] (**Figure 5**).

The proposed concepts of antimalarial vaccine based on liposomal construct of various types (cocktail formulation):

**a.** Recombinant protein-containing liposomes target specific, however, non-pH sensitive to deliver rDNA protein from (CSP 22), SPF 66 form of sporozoites and merozoites, so that proteins are specifically processed through endosomal pathway and presented by APCs through MHC II.

**b.** The second population of liposomes will contain dendritic cells targeted pH-sensitive liposomes bearing rDNA lipid complexes (target specific and pH sensitive) to deliver them to the cytosol of specific cells, that is, dendritic cells for subsequent expression of liver-stage and erythrocytic stage antigenic cellular expression and MHC I restricted presentation for Th-1 cellular CTL responses.

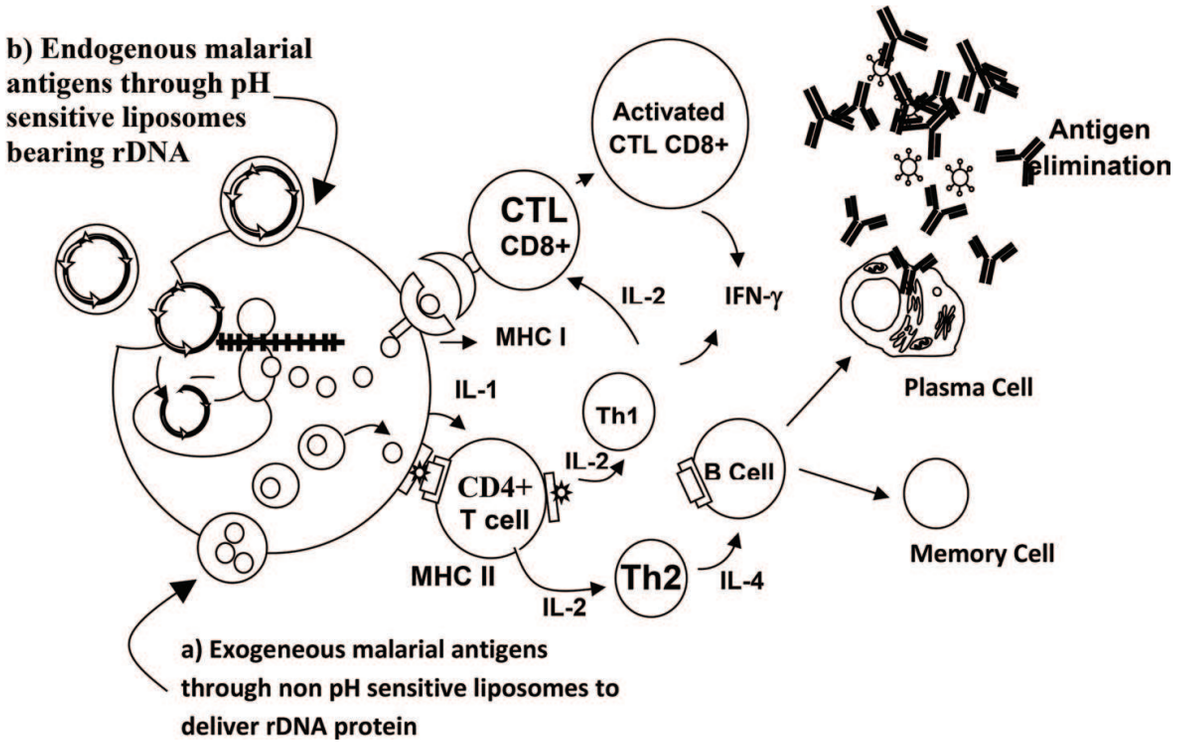


**Figure 5.** Schematics illustrating the proposed concepts of antimalarial vaccine based on liposomal construct of various types (cocktail formulation).

## 12. Cytotoxicity rendered by liposomes

1. **Dermal toxicity**: Conventional doxorubicin is used actively in various malignant tumors giving rise to a number of side effects like cardiotoxicity and myelosuppression. A different approach of this chemotherapeutic agent enclosed in PEGylated liposomes, in which liposome encapsulation prevents doxorubicin from penetration to compartments with tight endothelial cells junctions and facilitates its distribution to tissues with abnormal blood vessels [59]. This results in higher drug accumulation within the tumor than normal tissues. Consequently, a decreased incidence of cardiac and hematological toxicity is observed. PEGylated liposomal doxorubicin (PLD) has the ability to deposit itself within the skin and to induce specific mucocutaneous reactions. There are six types of PLD-related dermal disorders, and the most common is palmar-plantar erythrodysesthesia (PPE) [60]. Other less frequent manifestations are intertrigo-like dermatitis, a diffuse follicular rash, a maculopapular rash, melanotic macules, or a recall phenomenon. Dermal toxicity is the most common adverse reaction limiting PLD therapy. Skin lesions usually appear in regions prone to trauma such as the palms and soles. This was reported in a patient suffering from ovarian cancer showing partial response to chemotherapy [61].
2. **PEGylated liposomal doxorubicin (PLD)** was administered in patients. These PEGylated liposomes show a lower rate of cardiotoxicity and myelosuppression but show some obvious adverse effects including palmar-plantar erythrodysesthesia (PPE and some dermal manifestations such as intertrigo-like dermatitis, diffuse follicular rash, melanotic macules,

maculopapular rash [61]. Some studies have advocated that dimethyl sulfoxide or corticosteroids may be beneficial in the treatment of PLD-induced dermal complications as they accelerate skin recovery. The only well-established preventive management includes dose intensity modification or complete chemotherapy discontinuation.

## 13. Future challenges

For some drugs like DaunoXome, AmBisome, Doxil, Epaxel, etc., liposomes have proved to be a reliable delivery vehicle with some major challenges:

1. **Uptake by reticuloendothelial system**: Liposomes may be formulated as aerosol and as semisolid form such as cream, gel, or dry powder and are administered. They will then be readily taken up by the mononuclear phagocyte system (MPS) such as Kupffer cells of the liver and spleen. This is the natural route for the uptake of liposomes; however, they lack the ability to target site-specific receptors expressed on the surface of diseased cells and hence are inefficient for site-specific delivery. Therefore, liposomes which may evade rapid uptake by MPS need to be developed and further explored. PEG-coated or sterically stabilized liposomes are a few glaring examples.
2. **Large-scale production**: Production of liposomes from small scale (laboratories) to a large scale is a challenging task. The regulatory norms of the use of chloroform and methanol are not recommended more than the permissible limits. Preparation of liposomes also involves various steps like evaporation of solvent system under reduced pressure, preparation of thin lipid film, sonication, etc. These procedural hurdles pose a challenge across the researchers to develop these vehicles on a large scale.
3. **In process stability**: The oxidation and/or hydrolysis of phospholipids used in liposomal preparation does not allow the long-term storage and therefore less shelf-life. The physical and chemical instability of prepared liposomes is something to be explored further. However, fewer formulations are used in a lyophilized form which is to be reconstituted in a suitable buffer before use. Also, liposomes cannot withstand the degradation from proteins and enzymes in an animal model due to electrostatic stabilization.

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