Chapter II

Literature Search

New drug delivery systems are developed with the biomedical goal to enhance the site specificity, effectiveness and safety of medicinal agents, especially potent and complex drugs as well as enzymes, proteins, peptides and genetic materials. Knowledge of human physiology is helpful in bioengineering of different cells, such as carrier erythrocytes, nanoerythrosomes, bacterial ghosts, platelets, genetically engineered stem and dendrite cells that can be used to target the drugs at the specific receptors sites rather than tissues because of some functional properties which allow for easy surface recognition and receptor binding.

Background of Invention

2 Biological cellular Carrier systems

2.1 Bacterial Ghosts - The Bacterial Ghost (BG) platform technology is an innovative system for vaccine, drug or active substance delivery and for technical applications in white biotechnology (Hutchison, 1966). The bacterial ghost system is a novel vaccine delivery system endowed with intrinsic adjuvant properties (Ulrike et al., 2005). Bacterial ghosts are nonliving gram-negative bacteria cell envelopes devoid of cytoplasm contents, maintaining their cellular morphology and native surface antigenic structures including bio adhesive properties. They are produced by PhiX174 protein E-mediated lyses of gram-negative bacteria. The intrinsic adjuvant properties of bacterial ghost preparations enhance immune responses against envelope-bound antigens, including T-cell activation and mucosal immunity. Since native and foreign antigens can be expressed in the envelope complex of ghosts before E-mediated lyses, multiple antigens of various origins
can be presented to the immune system simultaneously. In addition, the extended bacterial ghost system represents a platform technology for specific targeting of DNA-encoded antigens to primary antigen-presenting cells. The potency, safety and relatively low production cost of bacterial ghosts offer a significant technical advantage, especially when used as combination vaccines.

BGs produced from *M. haemolytica* were used for the in vitro delivery of the moderate hydrophilic cytostatic drug doxorubicin (DOX) to human colorectal adenocarcinoma (Caco-2) cells (Langemann, 2010). One of the main advantages of BGs is that they are non-living. They retain all of the surface morphological, structural and antigenic components of their living counterparts. BGs also have vast loading capacity. The inner space of BG’s empty envelope can be loaded with a combination of protein peptides, drugs or DNA which gives us an opportunity to design new types of polyvalent vaccines.

### 2.1.1 Pharmaceutical Applications of Bacterial Ghost

#### 2.1.1.1 Bacterial ghost as drug delivery system

Bacterial ghost can be targeted toward dendritic cells and macrophages,(Felnerova, 2004) microvascular endothelial cells,(Gröger,2000) ocular surface diseases (Kudela,2011) as well as in gene transfer to melanoma cells(Kudela,2008).Bacterial ghosts are also studied for targeting potential toward human conjunctival epithelial cells using some in vitro models like Chang conjunctival epithelial cell lines and primary human conjunctiva-derived epithelial cells. These studies concluded efficient internalization of bacterial ghost in corneal cells lines with no cytotoxicity (Kudela et al., 2011). Use of anticancer agents is often related with a large number of side effects. Delivery of cytotoxic agents via bacterial ghost provides a novel platform for cancer targeting. Bacterial ghosts from *Mannheimia haemolytica* loaded with doxorubicin was studied against human colorectal
adenocarcinoma cells, whereby potent anticancer activity was observed with ghost loaded with doxorubicin as compared with plain drug in terms of higher antiproliferative effects. Use of bacterial ghost as an effective model to control fertility on animal model (*Trichosurus vulpecula*) has been documented. Ghosts encapsulated with possum zona pellucida protein-2 when subjected to be applied to the nostrils and eyes of female *Trichosurus vulpecula*, and its effects on fertility was determined by super ovulation and artificial insemination. Ghosts provoked humoral and cell-mediated immune leading to fertilization of only few eggs. Effects of immunisation on fertility were assessed, following super ovulation and artificial insemination. Both constructs evoked humoral [antibody] and cell-mediated immune responses in possums and significantly fewer eggs were fertilized in females immunized against zona pellucida protein-2C ghost (Walcher, 2008).

### 2.1.1.2 Bacterial ghost for protein and peptide delivery

*Escherichia coli* ghost carrying hepatitis B virus core 149 (HBcAg-149) proteins anchored in inner and the outer membrane of *E. coli* was compared. Both these strategies demonstrated an excellent means to deliver HBcAg-149 as antigen to female BALB/c mice (Jechlinger, 2005). Vaccine with adequate spectrum of activity is often desired for population. *V. cholera* vaccine as whole-cell cholera and toxoid vaccines has only offered transient protection. *V. cholera* ghost were administered to reversible intestinal tie adult rabbit diarrhoea model. Vaccination by *V. cholera* ghost resulted in increased levels of serum vibriocidal titers (Eko et al., 2003).

Bacterial ghost also offer a promising approach to immobilize plasmid DNA which offer a novel carrier along with intrinsic property of immunogenicity of gram-negative bacteria cell envelope (Mayrhofe et al.,2005). Tumour cells and antigen presenting cells when
transfected with bacterial ghost with plasmid DNA phagocytised them, bacterial ghost with antigens were capable of activating CD4+ and CD8+ T cells and thus they elicited immune response against antigens which are expressed against target cells (Trombetta et al., 2003).

2.1.1.3 Delivery of nucleic acid via bacterial ghost

Bacterial ghost technology could be an innovative approach in vaccine development due to their interaction and uptake by macrophages, monocytes, and dendrite cells. (Riedmann, 2007) Nucleic acids can easily be incorporated inside bacterial ghost system. Lyophilized bacterial ghost are suspended in DNA solution and then washed to remove excess of DNA. This system could be easily used for DNA and gene delivery. About 3000 minimum sized DNA plasmid copies could be encapsulated in ghost system. (Paukner, 2005)

2.1.1.4 Immunization by bacterial ghost

Effective immunization against gram-negative bacteria can be achieved using bacterial ghost technology. Vaccination of pigs with Actinobacillus pleuropneumoniae resulted in prevention of colonization of pathogen and effective immunization without any adverse effect (Huter et al., 2000). Bacterial ghost from P. multocida and M. haemolytica have been successfully evaluated for their immunogenic potential, where ghost effectively protected the animal models comparable to standard vaccines available in markets. (Marchart, 2003)

Preclinical testing on V. cholera ghost has been successfully completed, where mucosal administration of bacterial ghost has offered maximum protection due to humoral and cellular immune response (Riedmann et al., 2007; Walcher, 2004).
Immuno stimulating components like pathogen-associated molecular patterns constituting peptidoglycan, lipopolysaccharides, monophosphoryl lipid A, and so on are present on ghost surface, which, on interaction with cellular components, evoke immune response (Riedmann et al., 2007). Bacterial ghost also serve as an important means for enzymatic reactions. Although bacterial ghosts are produced when cytoplasmic material of bacteria is expelled out, but removal of cytoplasmic material do not confer total loss of enzymatic activity. Enzymes like membrane-bound β-galactosidase and chloramphenicol acetyl transferase are known to be present on bacterial surface (Buckley, 1986; Maratea, 1985).

2.1.2 Neutrophils- Neutrophils are an attractive carrier system for the transport of diagnostic or therapeutic agents to areas of acute inflammation. They are present in large numbers, can be highly purified, contain carrier proteins within their granules and are designed to accumulate in large numbers at area of pathology (Smith et al., 1994).

2.1.3 Lymphocytes- The concept of lymphocytes as a source of transfer macromolecules particularly nucleic acid is more defined for functioning in immune process. Thus it is concerned with the putative role of lymphocytes as a source of macromolecule particularly DNA for other cells (Harris, 1979). The discovery of several subsets of CD4+ T lymphocytes has contributed to refine and to challenge our understanding of the roles of CD4+ T cells in the pathogenesis of fibrotic lung diseases.

2.1.4 Fibroblasts- Fibroblasts are used as a source of lysosomal enzymes. The ability of skin fibroblasts to provide continuous source of lysosomal enzymes in-vitro was established by Dean et al., 1975. Fibroblasts are advantageous in replacement therapy because no surgery is needed for the recipient. Normal fibroblasts in –vitro produce all the enzymes necessary to correct each type of mucopolysaccharides and this obviates the need to isolate and purify or to encapsulate each enzyme.
2.1.5 Stem Cells—Stem cells are primal cells common to all multicellular organisms that retain the ability to renew them through cell division and can be differentiated into a wide range of specialized cell types. Modern therapeutics is having a lot of hope from stem cell research in the field of organ transplantation and replacement of lost tissue. The rigorous definition of a stem cell requires that it possesses two properties: Self renewal and unlimited potency. Self-renewal means the ability to go through numerous cycles of cell division while maintaining the undifferentiated state. Unlimited potency means the capacity to differentiate into any mature cell type (Rosenthal, 2003).

2.1.5.1 FUTURE PERSPECTIVES OF STEM CELL RESEARCH:

2.1.5.1.1 Low blood supply: Now the method to produce large numbers of Red blood cells has been developed. In this method precursor red blood cells, called hematopoietic stem cells are grown together with stromal cells, creating an environment that mimic the conditions of bone marrow, the natural site of red blood cell growth. Erythropoietin, a growth factor, is added coaxing the stem cells to complete terminal differentiation to red blood cells (Verfaillie et al., 2002). Further research into this technique will have potential benefits to gene therapy and blood transfusion.

2.1.5.1.2 Baldness: Hair follicles also contain stem cells, and some researchers predict research on these follicles (Velu et al., 2004). Stem cell may lead to successes in treating baldness through "multihair-placation" known as "haircloning". This treatment is expected to work through taking stem cells from existing follicles, multiplying them in cultures, and implanting the new follicle cells which have shrunk during the ageing process, which in turn respond to these signals by regenerating and once again making healthy hair.

2.1.5.1.3 Missing teeth: The work on tooth generation has reached to a stage that it will be available to the general population in few decades. In the postulated theory, stem cells
taken from the patient could be coaxed in the lab into a tooth bud which, when implanted in the gums, will give rise to a new tooth, which would be expected to take two months to grow. It will fuse with jawbones and release chemicals that encourage nerve and blood vessels to connect with it.

2.1.5.1.4 Deafness: There has been success in re-growing cochlear hair cells with the use of Stem cells (Marshall et al., 2006).

2.1.5.1.5 Bone regenerations: Mesenchymal stem cells can be pumped and cutters expanded from animals and human and have been shown to regenerate functional tissue when delivered to the site of musculo-skeletal defects in experimental animals. Mesenchymal stem cells can regenerate bone in a clinically significant osseous defect and may therefore provide an alternative to autogenous bone grafts.

2.1.6 Resealed Erythrocytes: Erythrocytes are the most interesting carrier and posses' great potential in drug delivery due to their ability to circulate throughout the body, produce zero order release kinetics, reproducibility and ease of preparation. The primary aim for the development of this drug delivery system was to maximize therapeutic performance, reducing undesirable side effects of drug as well as to increase patient compliance. (Lewis.et al., 1984) The overall process is based on the response of these cells under osmotic conditions. Upon reinjection, the drug-loaded erythrocytes serve as slow circulating depots and target the drugs to disease tissue or organ.

2.1.6.1 Applications of Resealed Erythrocytes

Resealed erythrocytes have been proposed as delivery systems for a variety of applications in human and veterinary medicine. In vivo applications of the drug loaded erythrocytes are used either for prolonged drug release or for drug targeting to RES or non RES.
2.1.6.1.1 Targeting of bioactive agents to RES:

Damaged erythrocytes are rapidly cleared from circulation by phagocytic cells in liver and spleen. Targeting of the drug decreases its side effects and the dose to be administered as well as drug utilization (Balamuralidhara et al., 2011). Modifications of erythrocytes membranes accelerate their targeting to the liver as well as spleen. The treatment with the carrier erythrocytes containing certain substances gives rise to alterations in the properties of the loaded erythrocytes. These substances include antibodies, gluteraldehyde, sialic acid and sulfhydryl containing substances (Gupta et al., 2010).

Example of Modifying Agents:

- **Glutaraldehyde**: The treatment of loaded erythrocytes with glutaraldehyde enhances their properties as carrier systems. It has been observed that the erythrocytes treated in this way are more stable which increases their osmotic resistance, as well as their resistance to turbulences. It means that the output of the encapsulated substance from these erythrocytes into the circulatory flow is reduced (Talwar and Jain, 1992). Similarly, the treatment with glutaraldehyde increases the selectivity of the erythrocytes towards the RES and specifically, towards certain organs such as the liver and the spleen (Millan et al., 2004b).

- **Ascorbate and ferrous ions**: The chemical alteration of the erythrocyte membrane with substances such as ascorbate/Fe^{2+}, diamide or band 3-cross-linking reagents can induce increased uptake of modified red cells by macrophages (Millan et al., 2004a).

- **Biotin**: The surface modification of erythrocytes has also been addressed using phenylhydrazine and N-hydroxysuccinimide ester of biotin (NHS-biotin) which
increases the macrophage uptake of loaded erythrocytes in vivo (Mishra and Jain, 2002). Moreover, biotinylation of erythrocytes may also be a way of preparing immuno-erythrocytes attached to biotinylated antibodies that are stable in circulation.

- **Antibody:** Coating the loaded erythrocytes by anti-Rh or other types of antibodies is another method that makes the erythrocytes more recognizable by RES macrophages. In this technique targeting of erythrocytes is either spleen or liver. If the antibody is used as ligand in from of immunoglobin G, targeting to spleen is preferred; while if used in form of immunoglobin M type, the liver targeting is dominant (Erchler et al., 1986, Gupta et al., 2010)

- **Other means of modification:** Pre-exposing the carrier erythrocytes to thermal shock increase the up taking of loaded erythrocytes by RES (Ihler et al., 1973). Also oxidant compounds like azodicarboxilic acid bis (dimethylamide) increases the uptake of loaded erythrocytes by RES (Arias et al., 2010), they are reactive toward the sulfhydryl group-containing proteins of the cell membrane (Ihler et al., 1973). The enzyme neuraminidase as well as the proteolytic enzymes, also has been exploited to improve RES targeting of carrier erythrocytes with some degree of success (Millan et al., 2004b)

**2.1.6.1.2 Targeting to sites other than RES-rich Organs:**

Erythrocytes loaded with drugs have the ability to deliver a drug or enzyme to the macrophage-rich organs. Also, such cells have been used to target organs outside the RES. Co-encapsulation of paramagnetic particles, photosensitive agents in erythrocytes along with the drug to be targeted; application of ultrasound waves as well as site-specific antibody attachment to erythrocyte membrane have been tried (Hamidi et al., 2007b). The
magnetic erythrocytes, resulting from the co-encapsulation of the drugs with some ferrous fluids such as cobalt-ferrite and magnetite, have been reported to direct the encapsulated drug predominantly to the desired sites of the body by means of an external magnetic field. The magnetically guided erythrocytes have been tested successfully for targeting anti-inflammatory drugs to inflamed tissues (Markov et al., 2010; Ross, 2009). Photosensitized erythrocytes have been studied as a photo-triggered carrier and delivery system for methotrexate in cancer treatment. Moreover, carrier erythrocytes fused to the thermo-responsive liposomes and their localization using the external thermal source (Hamidi et al., 2007b).

2.1.6.1.3 Carrier erythrocytes as slow drug release system:

Slow release dosage forms are designed to obtain a prolonged therapeutic effect by continuously releasing medication over an extended period of time after administration of single dose (Hossain et al., 2004). Carrier erythrocytes have long life span in the circulation, so that they can be used as circulating depots for antitumor, antiparasitics, antibiotics as well as cardiovascular drugs. This happened only when the drug and the selected method for the drug loading don’t change the morphological and physiological parameters of erythrocytes (Gupta et al., 2010). Various bioactive agents encapsulated in erythrocytes are developed for the sustained release in circulation to allow effective treatment of diseases. Resealed erythrocytes serve as an ideal carrier for antineoplastic agents, antimicrobial drugs, vitamins and steroids (Gupta et al., 2010).
2.1.6.1.4 Erythrocytes as circulating bioreactors:

Erythrocytes have been realized as carriers for enzymes to serve as circulating bioreactors. Sometimes it is desirable to decrease the level of circulating metabolites that can enter erythrocytes. Erythrocytes have also been used as circulating bioreactors for the controlled delivery of antiviral drugs (Magnani and DeLoach, 1992).

2.1.6.2.1 Methods of Drug Loading Into Erythrocytes

Erythrocytes can be isolated from blood using a suitable anti coagulant (Hamidi et al., 2007b). Different sources such as human (Harisa et al., 2011), rats (Mishra and Jain, 2002), mice (Kravtzoff et al., 1990; Wang et al., 2010), rabbits (Hamidi et al., 2001a), dogs (Tonetti et al., 1991) are used as source for erythrocytes.

Figure 1: Schematic Illustration for methods of drug loading into erythrocytes

Figure 2: Scheme represents the details of hypotonic dialysis method.
Coupling of drug molecules to the surface of carrier RBC represents an alternative to encapsulation strategies that have been considered above. RBC membrane provides an extended surface area that may be used for anchoring multiple copies of protein or other therapeutic molecules. Several practical strategies for coupling therapeutics to carrier RBC surface have evolved and have been tested in vitro and in vivo in last two decades. These strategies can be divided into three wide categories,

i) Chemical coupling of agents to RBC surface (either covalent, or non-covalent);

ii) Coupling to RBC membrane of a receptor that binds a therapeutic agent (and, in some cases, augments its functions);

iii) Conjugation of therapeutics or their receptors with affinity ligands (e.g., antibodies or their fragments) that bind to RBC thereby anchoring cargoes on RBC (Figure 3). This is the latter strategy that permits loading drugs on RBC surface by injecting these conjugates into the bloodstream. Both these strategies provide coupling of antibodies, antigens, enzymes, cytokines and other biologically active substances to RBC and are being explored for vascular delivery of several classes of therapeutics including, more recently, model polymer nanocarriers anchored to RBC either non-specifically (Chambers et al., 2004, 2007) or via affinity peptides (Hall et al., 2007).

2.1.6.2.2 Polyethylene glycol conjugation: an approach to universal donor blood

Conjugation of highly hydrophilic polyethylene glycol (PEG) with the chain length in the range MW 3–10kDa has evolved since seventies as a universal “stealth” technology, prolonging circulation and masking from defense systems in the body of liposomes,
polymer nanocarriers, proteins and other drug carriers and drugs themselves. The goal of producing PEGylated “stealth” RBC, proposed by Scott and co-authors a decade ago, is to obtain a “universal” donor blood for haem transfusion by chemically camouflaging RBC antigen. (Scott et al., 1997, Scott, 2000). Indeed, PEG-coated RBC is less effectively opsonised, taken up by phagocytes and recognized by antibodies to RBC antigens (Armstrong, 1997, Murad, 1999, Bradley, 2001). Masking against immune reaction to mismatched ABO antigens is the most challenging goal; in this setting, PEG-coating offered no protection and rather aggravated haemolysis in the initial studies. PEG-coated RBC is less susceptible to parasite infection including malaria Plasmodium (Blackall, 2001) due to masking of their receptors (Sabolovic, 2000). In addition, PEG-modified RBC demonstrate reduced tendency to aggregation (Jeong, 1996) and improved rheological properties: reduced low shear flow viscosity (Armstrong, 1997) and enhanced thickness of membrane protective layer and plasticity necessary to endure hydrodynamic stress in circulation (Sabolovic, 2000; Chen, 2008; Jovtchev, 2008). In the last decade, PEG-RBC was an active area of research promising to alleviate acute problems of shortage of matched donor blood.

2.1.6.2.3 Elimination of circulating pathogens using RBC-coupled antibody heteropolymers

It is reported that RBC-coupled antibody complex when injected into body, can help in elimination of circulating pathogens. RBC transmembrane glycoprotein’s, binds C3b component of activated complement and immune complexes containing this protein and that macrophages in the RES safely detach such immune complexes including antibody-pathogen complexes from circulating RBC without damaging the cell (Jovtchev, 2008).
2.1.6.2.3 RBC as carrier for therapeutic agents which regulates the formation and dissolution of blood clots

In this theory, coupling of drugs to RBC surface may favourably alter their pharmacokinetics (i.e., prolong life-time in circulation) and optimize interaction with components of blood coagulation and fibrinolytic systems accessible from RBC surface. Attempts in this direction included conjugation of heparin to RBC to enhance potency of anticoagulant thrombo prophylaxis in patients predisposed to thrombosis and conjugation of pro-thrombotic RGD-containing peptide to RBC to design a substitute for platelet infusion in patients predisposed to hemorrhagic disorders. (Muzykantov, 1991)

2.1.7 Nanoerythrosomes- These nanovesicles offer a high degree of versatility for the encapsulation of biological or non biological compounds and for the binding of targeting agents. In particular, polyethylene glycols can be conjugated by a covalent link to the basic amino acid residues constitutive of the different proteins. The binding of polyethylene glycols to the nanoerythrosome membrane could be interesting for the therapeutic use as this system could overcome heterologous immunogenicity and reduce rapid clearance from circulation.

2.1.7.1 Method of preparation

2.1.7.1.1 Isolation of Erythrocytes, Preparation of Erythrocyte Ghosts and Nanoerythrocytes

2.1.7.1.1 Separation and Washing of Erythrocytes: Blood samples can be collected from animals by cardiac puncture (rats, mice etc.) / vein puncture (rabbit, human etc.) into a syringe containing heparin sodium (100 I.U. /ml in 0.9% saline) or vacutainers.
The freshly collected blood is centrifuged in a refrigerated centrifuge at 1000 rpm for 10 minutes at 4±1°C. The plasma and buffy coats are discarded and sediment erythrocytes are washed with washing buffer (pH 7.4). These washed cells are stored at 4±1°C.

2.1.7.1.1.2 Preparation of Erythrocyte ghosts: The hypotonic osmotic lysis method described by De Loach et al. (1980) can be used for the preparation of ghost suspension. The cells are lysed and washed several times with hypotonic saline solution. After each wash, the solution is centrifuged at 1000 rpm for 10 minutes at 4±1°C in a refrigerated centrifuge and the supernatants are aspirated and discarded. The ghost suspension is finally obtained when supernatant becomes colourless. Packed ghost cell suspension is diluted with 0.9% saline to obtain 50% hematocrit and stored at 4±1°C until used.

2.1.7.1.1.3 Preparation and Loading of Nanoerythrocytes: The nanoerythrocytes can be prepared using extrusion, sonication and electrical breakdown methods.

- In the extrusion method erythrocyte ghosts are passed through polycarbonate membrane filter, which causes them to break into smaller vesicles, nanoerythrocytes.
- In the sonication method erythrocyte ghosts are converted into small vesicles using a dismembator.
- The electrical breakdown method is used to convert ghosts into small vesicles under the influence of electrical potential.

Of the above described three methods, NEs prepared by extrusion method yield vesicles of more uniform size. It is a quicker and economic method in comparison to sonication and electrical breakdown. Furthermore, in the sonication and
LITERATURE SEARCH

electrical breakdown methods, heat generated during preparation if not controlled, may also modify the membrane.

- Drugs are conjugated to NEs with the help of a cross linker as the erythrocyte membrane contain 60% protein and 40% lipid by dry weight. The important protein of erythrocyte membrane is spectrin which is successfully used for binding of drug molecules on erythrocyte membrane.

Figure 3 Mechanism for formation of nanoerythrosomes using extrusion Method.
2.1.7.1.4 Chemistry of Cross linking: Chemically cross linking is the process of bond formation between two or more molecules. This technique is called bio conjugation when it involves the use with proteins and other bio molecules. Cross linking reagents (or cross linkers) are molecules that contain two or more reactive ends capable or chemically attaching to specific functional groups (primary amines, sulfhydryls, etc.) on proteins or other molecules. (www.piercenet.com)

2.1.7.1.5 Drug conjugation on nanoerythrosomes membrane

Despite the complexity of protein structure, including composition and sequence of 20 different amino acids, only a small number of protein functional groups comprise selectable targets for practical bio-conjugation methods. Just four protein chemical targets account for the vast majority of cross linking and chemical modification techniques on erythrocyte membrane protein.
Primary amines (−NH2): This group exists at the N-terminus of each polypeptide chain (called the alpha-amine) and in the side chain of lysine (Lys, K) residues (called the epsilon-amine). Because of its positive charge at physiologic conditions, primary amines are usually outward-facing (i.e., on the outer surface) of proteins; thus, they are usually accessible for conjugation without denaturing protein structure.

Carboxyls (−COOH): This group exists at the C-terminus of each polypeptide chain and in the side chains of aspartic acid (Asp, D) and glutamic acid (Glu, E). Like primary amines, carboxyls are usually present on the surface of protein structure.

Sulphydryls (−SH): This group exists in the side chain of cysteine (Cys, C). Often, as part of a protein's secondary or tertiary structure, cysteines are joined together between their side chains via disulfide bonds (−S−S−). These must be reduced to sulphydryls to make them available for cross linking by most types of reactive groups.

Carbonyls (−CHO): Ketone or aldehyde groups can be created in glycoproteins by oxidizing the polysaccharide post-translational modifications (glycosylation) with sodium meta-periodate.(www.piercenet.com)

2.1.7.1.6 Selection of Cross linkers:

Cross linkers are selected on the basis of their chemical reactivities (i.e., specificity for particular function groups) and other chemical properties that affect their behaviour in different applications:

Chemical specificity refers to the reactive target(s) of the crosslinker's reactive ends. A general consideration is whether the reagent has the same or different reactive groups at either end (termed homobifunctional and heterobifunctional, respectively;).
LITERATURE SEARCH

- **Spacer arm length** refers to the molecular span of a cross linker (i.e., the distance between conjugated molecules). A related consideration is whether the arm is cleavable (i.e., whether the linkage can be broken)

- **Water-solubility and cell membrane permeability** of a cross linker affect whether it can permeate into cells and/or crosslink hydrophobic proteins within membranes. These properties are determined by the composition of the spacer arm and/or reactive group.

- **Spontaneously reactive or photo reactive groups** in a cross linker affect whether it react as soon as it is added to a sample or can be activated at a specific time by exposure to UV light reversed or broken when desired. ([www.piercenet.com](http://www.piercenet.com))

2.1.7.1.1.7 Selection of Aldehydes as a cross linker: Aldehydes are preferred cross-linking agents. To cross-link the collagen/protein formaldehyde, glutaraldehyde, acetaldehyde, glyoxal pyruvic aldehyde, and dialdehyde starch may be used. Glutaraldehyde is particularly preferred for bio conjugation. To stop the cross linking reaction quenching agents are used. Quenching agents have functional groups that react with the functional groups of the cross-linking agent (e.g. aldehyde group) to form water soluble adducts which may be used to quench the cross-linking reaction. Quenching agents that have free amino groups are preferred like glycine, it is particularly preferred. The concentration of glutaraldehyde in the reaction mixture should be typically about 0.001% to about 0.05% by weight. The duration of the cross-linking reaction is usually in the range of one-half hour to about one week. The reaction is normally carried out at about 10°C to about 35°C. The quenching agent is added in at least stoichiometric proportions relative to the cross-linking agent. Excess quenching agent is preferred. ([www.piercenet.com](http://www.piercenet.com)). Glutaraldehyde has fairly small molecules, each with two aldehyde
groups, separated by a flexible chain of 3 methylene bridges. It is HCO-(CH$_2$)$_3$-CHO. The potential for cross-linking is obviously much greater than with formaldehyde because it can occur through both the -CHO groups and over variable distances. In aqueous solutions, glutaraldehyde is present largely as polymers of variable size (Monsan et al., 1975). There is a free aldehyde group sticking out of the side of each unit of the polymer molecule (Fig. 3), as well as one at each end. All these -CHO groups will combine with any protein nitrogens with which they come into contact, so there is enormous potential for cross-linking.

**Figure 5:** Cross-linking of proteins with glutaraldehyde

2.2 Novel anti-malarial drug delivery systems

Development of anti-malarial drugs and novel delivery formulations is the major concern to improve the efficacy, specificity, tolerability and therapeutic index of existing drugs. Such novel formulations are likely to modify the traditional oral dosing schedule of drugs such as chloroquine(CQ) whose toxicity has been attributed to transiently high plasma concentrations following oral administration (Siqueira-Batista et al., 1998). Studies indicate successful controlled release of CQ from the synthetic polymer, Eudragit RS 100 (Ndesendo et al., 1996) as well as from pectin (polygalacturonic acid) (Musabayane et
Synthetic polymers are disadvantaged by the use of organic solvents and hence relatively harsh formulation conditions. These novel drug delivery systems are likely to optimise the therapeutic efficacy of anti-malarials. Novel drug delivery systems investigated to date include CQ-pectin formulations (Musabayane et al., 2003) lipid nanoemulsion or liposome-entrapping of primaquine (PQ) (Green et al., 2004). Biodegradable natural polymers (albumin, gelatin, alginate, collagen and chitosan) and synthetic polymers (lactide, glycolide, poly (lactide-co-glycolide) (PLGA) have been used as drug delivery systems although they have a relatively short duration of drug release (Rytting et al., 2008).

2.2.1 Liposomes

Liposomes are lipid vesicles used extensively for controlled delivery drug formulations. Liposome formulations inhibit rapid clearance by controlling the size, charge, and surface hydration of the drug. Some reports show that liposome when tried as carriers of antimalarial drugs (Singh and Vingkar, 2008) show increased bioavailability of artemisinin derivatives (Gabriels and Plaizier-Vercammen, 2003) to decrease malaria resistance in experimental animals (Chimanuka et al., 2002). Sustained and prolonged chloroquine release from liposome formulations (Owais et al., 1995) and primaquine (Dierling and Cui, 2005) has been reported. Antimalarial formulations targeting erythrocytes (Chandra, 2007) and the liver (Singh and Vingkar, 2008) have been developed. Many drugs and therapeutics including antitumor agents, antivirals, anti-fungals, antimicrobials, vaccines and gene can be delivered (Sharma and Sharma, 1997) with enhanced safety and efficacy.

2.2.2 Nanoparticles

Polymeric nanoparticle devices are biocompatible, slowly hydrolyzed polymer devices such as poly lactide, gelatin, albumin, polyacrylamide, poly isohexyl cyano acrylate and
poly diethyl-methylidene malonate that control delivery of drugs. Polymeric nanoparticle devices have been used to deliver anti-malarial drugs, halofantrine (Legrand et al., 2003), *Artemisia annua* derivatives (Wan et al., 1992) and chloroquine (Agrawal et al. 2007). An added advantage of nanoparticle delivery devices is that they can passively deliver drugs to lysosomes of phagocytes of the mononuclear phagocyte system, following intravenous administration (Rodrigues et al., 1994). The release of the drug at the site following lysosomal degradation enhances the efficacy of the active agent. Indeed, pharmacokinetic evaluation of the albumin-encapsulated primaquine (PQ) targeted to the mouse liver showed significantly higher concentrations of PQ in liver tissues relative to free drug (Green et al., 2004). Gelatin nanoparticles or galactose-coated drug dendrimers have also been studied for controlled PQ delivery (Agrawal et al., 2007). Reports indicate that PQ loaded-poly (d,l-lactide) nanoparticles were not only tolerated by healthy and Leishmania donovani-infected mice, but also reduced the 50% lethal dose when compared to the free PQ (Rodrigues et al., 1994).

### 2.2.3 Ceramic implants

Biodegradable Polymers like polylactic acid or gelatin or chitosan can be used as ceramic implants. Gelatin ceramic implants have been reported to sustain consistent therapeutic blood chloroquinine concentrations in animals (Saparia et al., 2001).

### 2.2.4 Microemulsions

In pharmaceutical and medical practice micro emulsion drug delivery systems containing water, oil and active agent have been used. Gum Arabic micro emulsions have been developed for chloroquine (Vaziri and Warburton, 1994) and primaquine (Nishi and Jayakrishnan, 2004) antimalarial drugs. The drug covalently couples via imine bond to aldehyde groups generated by oxidizing the polysaccharide with periodate and simultaneously fabricated into microspheres. Other studies indicate sustained controlled
chloroquine release from ethyl cellulose microsphere (Patel et al., 2006). A biodegradable polyphosphoester, poly [(cholesteryl oxo-carbonylamido ethyl) methyl bis(ethylene) ammonium iodide] ethyl phosphate] (PCEP) micelle has also been reported to efficiently deliver chloroquine in vitro (Wen et al., 2004).

2.2.5 Transdermal delivery

Transdermal delivery provides an alternative route of anti-malarial drugs. The advantages of transdermal route include avoidance of hepatic first-pass metabolism, easy administration and possibility of immediate withdrawal of the treatment. Although, transdermal delivery is limited by low skin permeability, methods such as active cationic liposomal delivery (Nair et al., 2009) and electroporation techniques (Sen et al., 2002) developed in the last decade’s enhanced permeation. Indeed, they reported physiological effects of Chloroquinine (CQ) following topical application of pectin-CQ hydrogel matrix patch on the rat skin using dimethyl sulphoxide as a penetration enhancer (Musabayane et al., 2003). The observations suggest that the anti-malarial can be delivered through the transdermal route. Sustained plasma concentration of PQ with concomitant inhibitory activity on asexual malaria parasites has also been reported in experimental animals following transdermal PQ administration (Mayorga et al., 1997). This is beneficial considering that the prophylactic and therapeutic applications of PQ are hampered by dose dependant adverse effects (Winstanley and Breckenridge, 1987).

2.2.6 Rectal delivery

The rectal drug delivery route has been used to deliver artemisinin formulations (Kuranajeewa et al., 2007) and quinine (QN) (Barenes et al., 1999) despite the observations that the route is associated with marked inter individual variability and bioavailability of the drugs (Kuranajeewa et al., 2007). The rate and extent of rectal drug
absorption are often low, possibly due to the relatively small surface area available for drug uptake. The composition of the rectal formulation appears to be an important factor determining the absorption process and pattern of drug release (Yong et al., 2003). Barennes et al., however, developed a rectally delivered Quinine cream formulation with improved parasitological efficacy and pharmacokinetics in children with *P. falciparum* malaria relative to intramuscular and intravenous injections. Rectal administration is a painless procedure that enables self-administration, and reduces the risk of infections from already used needles (Barennes et al., 1996).

2.2.7 Nasal Delivery

The nasal drug delivery route is easily accessible and offers a wide surface area for drug absorption (Illum, 2003). One particular advantage of the nasal route is the simplicity of administration, allowing easy treatment following the first sign of illness. The nasal cavity allows the drugs to be delivered directly to the brain via the nasal but limitations of this route include rapid removal of the drug from the site of deposition by mucociliary clearance, by enzymatic degradation, low permeability of the nasal epithelium and erratic bio-availability (Galloway and Chance, 1994). It is reported that nasally administered dihydroartemisinin formulations are effective for *Plasmodium* infection treatment in rodents (Touitou et al., 2006).

(2.3) The Disease and its management

2.3.1 Malaria (Martindale, 1993; Gilmam et al., IP 2010)

Malaria is an important cause of death and illness in children and adults, especially in tropical countries. The most critical problem currently limiting malaria treatment is the emergence and spread of parasite resistance to the majority of anti-marial drugs in use
(Woodrow et al, 2005). Improper or incomplete monotherapy of malaria has caused the development of resistance to the commonly used chloroquine (Mita et al., 2009, Rope et al., 2009) and mefloquine (Wisedpanichkij, 2009), and even toquinine, which has been a mainstay in the anti-malarial pharmacopeia for approximately two centuries (Nkrumah 2009). Malaria control requires an integrated approach, including prevention (primarily vector control) and prompt treatment with effective antimalarials. (WHO, 2010). The mortality, morbidity and economic burden demand that attention be focused on this area. According to the latest estimates, released in December 2013, there were about 207 million cases of malaria in 2012 (with an uncertainty range of 135 million to 287 million) and an estimated 627,000 deaths (with an uncertainty range of 473,000 to 789,000). Malaria mortality rates have fallen by 42% globally since 2000 and by 49% in the WHO African Region (WHO, 2013). Malaria is caused by Plasmodium parasites. The parasites are spread to people through the bites of infected Anopheles mosquitoes, called "malaria vectors", which bite mainly between dusk and dawn. Plasmodium falciparum is common in the tropics and causes the most serious form of the disease. Infections with this parasite can be fatal in the absence of prompt recognition of the disease and its complications and urgent, appropriate patient management. In recent years, some human cases of malaria have also occurred with Plasmodium knowlesi – a species that causes malaria among monkeys and occurs in certain forested areas of South-East Asia. (Collins et al., 2009) Resistance of parasites to antimalarial agents continues to be a threat to malaria control and elimination efforts globally. The emergence of resistance to artemisinin in worldwide is of particular concern.
Table 2: Malaria Parasite Species, Their Type

<table>
<thead>
<tr>
<th>S No</th>
<th>Species</th>
<th>Type</th>
<th>Nature</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Plasmodium falciparum</em></td>
<td>Malignant Tertian</td>
<td>Most Serious</td>
</tr>
<tr>
<td>2</td>
<td><em>Plasmodium vivax</em></td>
<td>Benign Tertian</td>
<td>Wide Spread, Rarely Fatal</td>
</tr>
<tr>
<td>3</td>
<td><em>Plasmodium malariae</em></td>
<td>Quatrian</td>
<td>Causes Fatal nephritis</td>
</tr>
<tr>
<td>4</td>
<td><em>Plasmodium ovale</em></td>
<td>Ovale Tertian</td>
<td>Rarely Fatal</td>
</tr>
</tbody>
</table>
2.3.2 Life cycle of malaria Parasite

Figure 6: Malaria Parasite life cycle

2.3.3 Transmission

Malaria is transmitted exclusively through the bites of Anopheles mosquitoes. The intensity of transmission depends on factors related to the parasite, the vector, the human host, and the environment. About 20 different Anopheles species are locally important around the world. All of the important vector species bite at night. Anopheles mosquitoes breed in water and each species has its own breeding preference; for example some prefer shallow collections of fresh water, such as puddles, rice fields, and hoof prints. Transmission is more intense in places where the mosquito lifespan is longer (so that the
parasite has time to complete its development inside the mosquito) and where it prefers to bite humans rather than other animals. For example, the long lifespan and strong human-biting habit of the African vector species is the main reason why about 90% of the world’s malaria deaths are in Africa (Kokwaro et al., 2009).

Transmission also depends on climatic conditions that may affect the number and survival of mosquitoes, such as rainfall patterns, temperature and humidity. In many places, transmission is seasonal, with the peak during and just after the rainy season. Malaria epidemics can occur when climate and other conditions suddenly favor transmission in areas where people have little or no immunity to malaria. They can also occur when people with low immunity move into areas with intense malaria transmission, for instance to find work, or as refugees.

Human immunity is another important factor, especially among adults in areas of moderate or intense transmission conditions. Partial immunity is developed over years of exposure, and while it never provides complete protection, it does reduce the risk that malaria infection will cause severe disease. For this reason, most malaria deaths in Africa occur in young children, whereas in areas with less transmission and low immunity, all age groups are at risk.

### 2.3.4 Symptoms

Malaria is an acute febrile illness. In a non-immune individual, symptoms appear seven days or more (usually 10–15 days) after the infective mosquito bite. The first symptoms – fever, headache, chills and vomiting – may be mild and difficult to recognize as malaria. If not treated within 24 hours, *P. falciparum* malaria can progress to severe illness often
leading to death. Children with severe malaria frequently develop one or more of the following symptoms: severe anaemia, respiratory distress in relation to metabolic acidosis, or cerebral malaria. In adults, multi-organ involvement is also frequent. In malaria endemic areas, persons may develop partial immunity, allowing asymptomatic infections to occur.

For both *P. vivax* and *P. ovale*, clinical relapses may occur weeks to months after the first infection, even if the patient has left the malaria prone area. These new episodes arise from dormant liver forms known as hypnozoites (absent in *P. falciparum* and *P. malariae*); special treatment – targeted at these liver stages – is required for a complete cure.

### 2.3.4.1 Clinical features of severe malaria

- Impaired consciousness (including unarousable coma)
- Prostration, i.e. generalized weakness so that the patient is unable to sit, stand or walk without assistance.
- Multiple convulsions: more than two episodes within 24h.
- Deep breathing and respiratory distress (acidotic breathing).
- Acute pulmonary oedema and acute respiratory distress syndrome.
- Circulatory collapse or shock, systolic blood pressure.
- Acute kidney injury.
- Clinical jaundice plus evidence of other vital organ dysfunction.
- Abnormal bleeding.
2.5 Disease Management- Anti malarial drugs

Multidrug resistance has been reported from most parts of the world and as a result, monotherapy or some of the available combination chemotherapies for malaria are either ineffective or less effective. New antimalarial regimens are, therefore, urgently needed and antimalarial combination chemotherapy is widely advocated. Antimalarial combinations can increase efficacy, shorten duration of treatment (and hence increase compliance), and decrease the risk of resistant parasites arising through mutation during therapy.

Combination therapy with antimalarial drugs is the simultaneous use of two or more blood schizontocidal drugs with independent modes of action and different biochemical targets in the parasite. The concept of combination therapy is based on the synergistic or additive potential of two or more drugs, to improve therapeutic efficacy and also delay the development of resistance to the individual components of the combination. In malaria drug combination therapy, the current trend is to co-formulate two or more agents into a single tablet, termed as multicomponent drug (e.g., Coartem®, lumefantrine-artemether) as opposed to the traditional cocktail therapy, so as to improve patient compliance (Morphy and Rankovic, 2005).
2.5.1 Classification of Anti malarial drugs

Anti malarial drugs can be classified according to anti malarial activity and structure.

2.5.1.1 According to anti malarial activity: (White, 1996)

2.5.1.1.1 Tissue schizonticides for causal prophylaxis: These drugs act on the primary tissue forms of the plasmodia which after growth within the liver, initiate the erythrocytic stage. By blocking this stage, further development of the infection can be theoretically prevented. Pyrimethamine and Primaquine have this activity. However since it is impossible to predict the infections before clinical symptoms begin, this mode of therapy is more theoretical than practical.

Tissue schizonticides for preventing relapse: These drugs act on the hypnozoites of P. vivax and P. ovale in the liver that cause relapse of symptoms on reactivation. Primaquine is the prototype drug; pyrimethamine also has such activity.

2.5.1.1.2 Blood schizonticides: These drugs act on the blood forms of the parasite and thereby terminate clinical attacks of malaria. These are the most important drugs in anti malarial chemotherapy. These include chloroquine, quinine, mefloquine, halofantrine, pyrimethamine, sulfadoxine, sulfones, tetracyclines etc.

2.5.1.1.3 Gametocytocides: These drugs destroy the sexual forms of the parasite in the blood and thereby prevent transmission of the infection to the mosquito. Chloroquine and quinine have gametocytocidal activity against P. vivax and P. malariae, but not against P. falciparum. Primaquine has gametocytocidal activity against all plasmodia, including P. falciparum.
2.5.1.4 Sporontocides: These drugs prevent the development of oocysts in the mosquito and thus ablate the transmission. Primaquine and chloroguanide have this action.

Thus in effect, treatment of malaria would include a blood schizonticide, a gametocytocide and a tissue schizonticide (in case of \textit{P. vivax} and \textit{P. ovale}).

2.5.1.2 According to structure:

2.5.1.2.1 Artemisinin derivatives (Capela et al., 2009; Araújo et al., 2009; Maude et al., 2010)

Derivatives of \textit{Artemisia annua L.} (Qinghao, Asteraceae) are considered the cornerstone of the treatment of falciparum malaria due to their potency and rapid action (WHO, 2006). Indeed, the introduction of artemisinin-based combination therapy (ACT) interventions decreased morbidity and mortality associated with malaria in several parts of the world (Ogbonna and Uneke, 2008). ACTs in common use include artemether-lumifantrine (Coartem), artesunate-mefloquine and artesunate-amodiaquine. Artemisinin based regimen possess gametocytocidal properties by inhibiting parasite transmission to probably reduce the development of anti-malarial resistance (Nosten and White, 2007). The WHO Expert Consultative Group recommended artemisin in combination therapy (ACT) to combat falciparum resistance based on these properties. Major limitations of ACTs have been ascribed to the imbalance between demand and supply, comparatively high cost, dosing complexity and the lack of clinical experience (Bloland, 2003). Reports of high failure rates associated with ACT therapy along the Thai–Cambodian border (Vijaykadga et al., 2006; Noedl et al., 2008) as well as in vitro drug-susceptibility data (Jambou et al., 2005) suggest the possibility of clinical artemisinin resistance. In addition, high doses of artemisinin derivatives have been reported to elicit dose, time and route
dependent central nervous system toxicity in laboratory animals (Petras et al., 2000). Interestingly, no serious side effects due to artemisinin derivatives have been reported in human studies to date. We suggest that the discrepancy between animal and human studies can partly be attributed to different routes of administrations (Hien et al. 1994)

2.5.1.2.1 Artesunate

Current evidence indicates that artesunate is the drug of choice for the treatment of severe malaria. It is available in oral (in combination treatments), rectal and parenteral (injectable) formulations. When injected intramuscularly, artesunate is rapidly absorbed. Parasite clearance is faster than with quinine because artesunate kills young circulating ring-stage parasites. The drug is well tolerated, with no attributable local or systemic adverse effects. Rectal artesunate is the pre-referral treatment of choice for severe malaria, particularly in children; however, more studies are needed to clearly establish its effectiveness for pre-referral treatment in adults. Artemisinins can be used to treat pregnant women with severe malaria. While oral artemisinin-based monotherapy is not recommended for the treatment of uncomplicated malaria because of the risks for relapse and for promoting the spread of artemisinin resistance, use of parenteral artesunate alone is standard for initial treatment of severe malaria in order to achieve rapid plasma therapeutic levels, which are not achieved as rapidly after oral administration. Furthermore, patients are usually initially unable to tolerate oral medication. All cases of severe malaria should be treated with a full course of a locally effective artemisinin-based combination medication once they are able to take oral medication and after at least 24 hours of parenteral therapy have been completed.
2.5.1.2.1.2 Artemether

Artemether is available in oral (in combination treatment), rectal and intramuscular formulations. Its efficacy, side effects and availability are similar to those of artesunate, except that the parenteral formulation is oil-based and may be inadequately or erratically absorbed after intramuscular injection in severely ill patients.

2.5.1.2.1.3 Artemisinin-Based Combination Therapies

Artemisinin based combinations are known to improve cure rates, reduce the development of resistance and they might decrease transmission of drug-resistant parasites. The total effect of artemisinin combinations (which can be simultaneous or sequential) is to reduce the chance of parasite recrudescence, reduce the within-patient selection pressure, and prevent transmission. (Nelson, 2006)

2.5.1.2.2 Quinine (QN) related drugs

2.5.1.2.2.1 Quinine (QN)

Quinine (QN) derived from shrubs of various species of Rubia-ceous genera, Cinchona and Remijia was the first successful chemical against malaria. QN, a blood schizonticide is also active against the asexual erythrocytic forms of *P. falciparum*, *P. malariae*, *P. ovale* and *P. vivax*. In addition, QN is gametocytocidal for *P. malariae* and *P. vivax* but, has no direct activity against the gametocytes of *P. falciparum*. Although resistance to QN is rare, cases have been reported. QN salts may be given orally or intravenously (IV), intramuscularly (IM) or rectally (PR) (Barennes et al., 2006).

Intravenous quinine should always be given by rate controlled infusion and never as a bolus (‘push’) intravenous injection. It may also be given intra muscularly into the
anterior thigh (not the buttock) after dilution to 60–100mg/ml. While quinine commonly causes hypoglycaemia in pregnant women, it is safe for foetuses. Mild side-effects are common, notably cinchonism (tinnitus, hearing loss, dizziness, nausea, uneasiness, restlessness and blurring of vision); serious cardiovascular and neurological toxicity is rare. Hypoglycaemia is the most serious and frequent adverse side-effect. In suspected quinine poisoning, activated charcoal given orally or by nasogastric tube accelerates elimination.

2.5.1.2.2 Chloroquine (CQ)

CQ is a rapid acting schizontocide against P. falciparum with gametocytocidal activity against asexual erythrocytic forms of *P. malariae*, *P. ovale* and *P. vivax*. CQ remains the most frequently used drug option for falciparum malaria in sub-Saharan Africa countries (Winstanley et al., 2004) despite the emergence resistant parasites. Consequently, CQ can no longer be considered an adequately effective therapy of *P. falciparum* in the areas of Africa. Of note, several east African countries have replaced CQ with sulphadoxine–pyrimethamine (Fansidar, 1993). To rescue the therapeutic efficacy of CQ, approaches undertaken include the development of amodiaquine following structural modifications of the parent compound and linkage to a transition metal molecule to produce ferroquine (Kreidenweiss et al., 2006).

CQ is slowly eliminated from the body and hence accumulates in certain organs and tissues of the body such as the adrenal glands and alters physiological function of the organs. Studies indicate adverse CQ effects on kidney (Musabayane et al., 1996), cardiovascular system (Siqueira-Batista et al., 1998) and the retina (Ferreras et al., 2007).

2.5.1.2.3 Mefloquine

Mefloquine, a quinoline derivative structurally related to QN is a blood schizontocide which inhibits the asexual stages of *P. falciparum* and *P. vivax*, but has no effect on the
hepatic stage of malaria parasites. Resistance to mefloquine has been reported leading to the use of combinations of mefloquine with sulphadoxine/pyrimethamine (SP) or mefloquine/artemisinin derivatives/combinations. Neuropsychiatric adverse events associated with mefloquine prophylaxis have been reported (Winstanley, 1996). In view of the long half-life of the drug, its administration in areas with intensive malaria transmission is contra-indicated.

2.5.1.2.2.4 Primaquine (PQ)

The 8-aminoquinoline, PQ is a schizonticide used to eradicate the pre-erythrocytic liver latent tissue forms of *P. vivax* and *P. ovale* which cause malaria relapses. Whilst PQ remains the drug of choice to eradicate and control hypnozoites of *P. vivax* and *P. ovale*, the antimalarial may precipitate haemolytic anaemia in glucose-6-phosphate dehydrogenase (G-6-PD) deficient patients (Burgoine et al., 2010). Against this background its evidence suggests, however, that a course of 8 weekly doses may be a safe and effective alternative to the traditional 14 day course of the drug (Myat-Phone-Kyaw et al., 1994). In *P. falciparum* infection, a single dose of 0.75 mg/kg is given against the gametocytes (excluding patients <4 years and pregnant women) and is still superior to artemisinin derivatives. This dose can also be tolerated by G6PD deficient patients. PQ also eradicates the primary exoerythrocytic stage of Plasmodium species as well as gametocyticidal and sporontocidal phases. Primaquine is orally administered daily due to its short half life and high doses are associated with haematological disorders and gastrointestinal disturbances (Baird and Rieckmann, 2003). Transdermal Primaquine delivery studies may overcome these shortfalls (Mayorga et al., 1998).

2.5.1.2.3 Antifolates

The inhibition of falciparum folate metabolism remains an attractive target for malaria treatment. Anti-folate derivatives interfere with folate metabolism, a pathway essential
for survival of the parasite via inhibition of enzymes dihydrofolate reductase (DDFR) 
(Bzik et al., 1987) and dihydropteroate synthase (DHPS) (Lu et al., 2010). The 
combination of pyrimethamine, an inhibitor of DHFR, and sulphadoxine (SD) an 
inhibitor of DHPS, has been widely used for the treatment of malaria, but due to the 
development of resistance to DHPS and DFHR antagonists the activity has been lost in 
most parts of the world including tropical Africa.

2.5.1.2.3.1 Sulphadoxine/pyrimethamine (SP) combination

SP drug combination is the most widely used option for uncomplicated P. falciparum 
malaria in several African countries because it is affordable and practicable. SP 
combination has a long-life and is, therefore, prone to the rapid emergence of resistance 
falciparum parasites due to the slow elimination from the body (Nwanyanwu et al., 1996; 
Mugittu et al., 2005). Pyrimethamine is also formulated in fixed combination with 
sulpalene or dapsone.

2.5.1.2.3.2 Proguanil

Proguanil, a folate antagonist which destroys the malaria parasite by inhibiting DDFR 
is used for malaria prophylaxis in some countries (Mulenga et al., 2006). However, 
clinical failure rates have cast a shadow on the drug’s further development.

2.5.3 New anti-malarial drug combinations

Strategies in the discovery and development of new anti-malarial drugs have ranged 
from minor modifications and reformulation of existing drugs to designing agents that act 
against new targets. Currently, several treatment options have been developed based on 
existing anti-malarial drugs in contrast with the period when CQ monotherapy was the
standard treatment. The chemotherapeutic agents that have been developed to improve malaria treatment are described as following:

3.5.3.1 Artemether/lumefantrine combination

Current WHO guidelines recommend the ACT artemether/lumefantrine (AL) for the treatment of uncomplicated malaria caused by *Plasmodium falciparum* (WHO, 2008). Artemether is a methyl-ether derivative of artemisinin with blood schizontocidal and gametocytocidal activities, while lumefantrine is a racemic fluorine derivative with high blood schizontocidal activity (Wernsdorfer et al., 1998). Artemether with a half-life of 2–3 h is easily absorbed and rapidly eliminated from plasma, whereas lumefantrine with a half-life of three to six days is eliminated slowly and thus provides a high long-term cure rate. Therefore, the complementary pharmacokinetics and dissimilar modes of action of AL provides synergistic anti-malarial activity and hence rapid clearance of parasitaemia after a short treatment course. Studies have shown to be effective both in sub-Saharan Africa and in areas with multi-drug resistant *P. falciparum* in Southeast Asia (Hutagalung et al., 2005; Kokwaro et al., 2007; Achan et al., 2009). It should be noted that AL combination is active against the blood stages of *P. vivax*, but is not active against hypnozoites. Therefore, an 8-amino-quinoline derivative such as PQ should be given sequentially after the combination in cases of mixed infections of *P. falciparum* and *P. vivax* to achieve hypnozoites eradication. Several studies have confirmed the safety and tolerability of AL in a wide range of patient populations including children (van Vugt et al., 1999; Falade et al., 2005; Abdulla et al., 2008; Hatz et al., 2008).

2.5.3.2 Dihydroartemisinin and Piperaquine

Several studies indicate that the dihydroartemisinin (DHA) and piperaquine (PQP) combination has the potential to be an effective anti-malarial drug against multi-drug resistant falciparum malaria (Giao et al., 2004; Ashley et al., 2004, 2005; Zwang et al.,
The DHA/PQP combination rapidly reduces parasite biomass in the patient through the brief yet potent activity of DHA and the subsequent removal of uncleared parasites by the less active but more slowly eliminated PQP (Tarning et al., 2005). DHA, an artemisinin derivative, and PQP, a bisquinoline, have elimination half-lives of approximately 1 h (Newton et al., 2000) and approximately 2–3 weeks (Hung et al., 2003, 2004), respectively. The two components of DHA and PQP provide a combination that is relatively inexpensive and has been shown to be effective both in curing malaria and preventing re-infection (Zwang et al., 2009). DHA/PQP combination is well tolerated by all age groups. A major concern with the DHA/PQP combination is that the long half-life of PQP will facilitate the selection of drug resistant parasites.

2.5.3.3 Amodiaquine (AQ)/sulphadoxine/pyrimethamine (SP)

Amodiaquine (AQ) was widely used in various malaria endemic areas until WHO withdrew its endorsement for malaria control programme in 1990 as a result of reports of rare but severe toxic effects (Olliaro et al., 1996). However, there is renewed interest in AQ as a possible alternative to CQ, as it is effective even in areas of intense CQ resistance and has a side-effect profile similar to that of CQ and SP (Brasseur et al., 1999; Gorissen et al., 2000; Staedke et al., 2001). Several studies indicate that AQ is effective in treating CQ-resistant *P. falciparum* malaria parasites (Olliaro et al., 1996; Van Dillen et al., 1999), despite the reported hematological side effects (Phillips-Howard and West, 1990). Increasing *P. falciparum* resistance to CQ in sub-Saharan Africa necessitates use of alternative antimalarial agents. One alternative regimen, amodiaquine (AQ) plus SP, has shown surprisingly good efficacy in Uganda (Staedke et al., 2001; Dorsey et al., 2002; Gasasira et al., 2003). The SP/AQ antimalarial combination which has shown surprisingly good efficacy for treatment of uncomplicated malaria offers low-cost option for in Africa.
2.5.4 Optimization of therapy

A combination of new as well as old anti-malarial agents has been used as first-line therapy for malaria in Africa and other areas with widespread Plasmodium drug resistance. The options include amodiaquine/sulpadoxine/pyrimethamine (Ogungbamigbe et al., 2008), atreminisin (Kublin et al., 2002), chlorproguanil/dapsone (Fanello et al., 2008) and atovaquone/proguanil (Srivastava and Vaidya, 1999). Efforts to develop an effective malaria vaccine are yet to be successful and thus chemotherapy remains the mainstay of malaria control strategy. Plasmodium falciparum, the parasite that causes about 90% of all global malaria cases is increasingly becoming resistant to most antimalarial drugs in clinical use. This terrible situation is aggravated by reports from Southeast Asia, (Muregi et al., 2011) of the parasite becoming resistant to the “magic bullet” artemisinins, the last line of defence in malaria chemotherapy. Drug development is a laborious and time consuming process, and thus antimalarial drug discovery approaches currently being deployed largely include optimization of therapy with available drugs including combination therapy and developing analogues of the existing drugs. However, the latter strategy may be hampered by cross-resistance, since agents that are closely related chemically may share similar mechanisms of action and/or targets.

This may render new drugs ineffective even before they are brought to clinical use. Evaluation of drug-resistance reversers (chemosensitizers) against quinoline-based drugs such as chloroquine and mefloquine is another approach that is being explored.
2.5.5 Antimalarial drug resistance

Resistance to antimalarial medicines is a recurring problem. Resistance of *P. falciparum* to previous generations of medicines, such as chloroquine and sulfadoxine-pyrimethamine (SP), became widespread in the 1970s and 1980s, undermining malaria control efforts and reversing gains in child survival.

In recent years, parasite resistance to artemisinins has been detected in many countries. When treatment is an oral artemisinin-based monotherapy, patients may discontinue treatment prematurely following the rapid disappearance of malaria symptoms. This result in incomplete treatment and such patients still have persistent parasites in their blood. Without a second drug given as part of a combination (as is provided with an ACT), these resistant parasites survive and can be passed on to a mosquito and then another person. This causes resistance to develop against artemisinins and spreads to other large geographical areas, the public health consequences could be dire, as no alternative antimalarial medicines will be available for at least five years.

2.6 Malaria Elimination

Malaria elimination is defined as interrupting local mosquito-borne malaria transmission in a defined geographical area, i.e. zero incidences of locally contracted cases. Malaria eradication is defined as the permanent reduction to zero of the worldwide incidence of malaria infection caused by a specific agent; i.e. applies to a particular malaria parasite species.
2.7 Vaccines against malaria

There are currently no licensed vaccines against malaria or any other human parasite. One research vaccine against *P. falciparum*, known as RTS, S/AS01, is most advanced. This vaccine is currently being evaluated in a large clinical trial in 7 countries in Africa. A WHO recommendation for use will depend on the final results from the large clinical trial. These final results are expected in late 2014, and a recommendation as to whether or not this vaccine should be added to existing malaria control tools is expected in late 2015 (MVI 2006, Crompton et al.; 2010, Riley et al., 2013).

2.8 Relevant Reports

- Al-Achi A et al., (1990a) reported the interaction of doxorubicin with erythrocyte membrane to elucidate mechanism by which doxorubicin is taken up by the erythrocytes. The absorption isotherm revealed that the amount of doxorubicin absorbed per unit weight of erythrocyte ghost at a given drug concentration was similar to that of disrupted erythrocytes ghost.
- Ataullakhanov, (1994a) et al reported reversible binding of anthracycline antibiotics to erythrocytes treated with glutaraldehyde and suggested that...
glutaraldehyde-treated erythrocytes remain capable of reversible binding of anthracycline antibiotics.

- Lichtenberger et al. (1996) reported combination therapy of antisecretory agents and NSAIDs, chemically associated with phospholipids, has distinct advantages.

- Williams et al., (1994) conjugated methotrexate with phospholipids and studied the effect of this conjugate in suppression of joints inflammation and compared the effect with drug and free lipid preparation.


- Gaozynska et al., (1989) identified product substrate pattern for erythrocyte membrane self digestion using the original two dimensional electrophoretic technique.

- Manger et al., (1994) reported the synthesis of aara-lipid protein conjugates for protection against cytidinedeaminase catalyzed deactivation.

- Leo et al., (1989) studied the effect of ligustrazine on Ca++ uptake by inside out red cell membrane vesicle from renal hypertensive rats.


- Penniston and Green (1968) demonstrated a configuration change in erythrocyte ghost in the presence of ATP and concluded that this change in shape differs both in kind and in magnitude from the shape change caused by an osmotic gradient.

Schneeweise et al., (1997) reported a method for preparation of uniform haemoglobin free human erythrocytes ghosts using isotonic medium, electric field pulses of 16 KV/cm was utilized for 40 µ second duration.

Jain and Jain (1996) reported nanoerythrosomes based delivery of 6-meracaptopurine. The drug was covalently bound to nanoerythrosomes with the help of gluteraldehyde and the system was evaluated in-vitro and in-vivo depicting that nanoerythrosomes could be potentially used as drug carriers.

Jain and Jain (1997) covalently linked mitomycin –C to NEs and developed this system in lyophilized form, ready for reconstitution.

Jain and Jain (1999) have reported on engineered cellular nanostructure bearing methotrexate for tumor targeting and also evaluated the same in-vitro and in-vivo on cancerous cell line.

Muzykantov et al., (1996) reported Target-sensitive immunoerythrocytes and their interaction of biotinylated red blood cells with immobilized avidin which induced their lysis.

Perez et al., (1996) studied Heterogeneity of hypotonically loaded rat erythrocyte populations as detected by counter current distribution in aqueous polymer two-phase systems.

Moorjani et al., (1996) identified the mechanisms that make the nanoerthrocyes and daunorubicin (nEryt -DNR) complex more active than free DNR, using fluorescence microscopy and cellular-uptake; they observed that the nEryt-DNR complex cannot diffuse through the cell membrane.

DeLoach et al., (1980) reported use of an erythrocyte encapsulation dialyzer for preparing large quantities of erythrocyte ghosts and encapsulation of a pesticide in erythrocyte ghosts.
DeLoach et al., (1977) reported a dialysis procedure for loading erythrocytes with enzymes and lipids.


Kitao et al., (1978) studied agglutination of leukemic cells and daunomycin entrapped erythrocytes with lecithin in vitro and in vivo.


Hamidi et al. (2001), studied in vitro characterization of human intact erythrocytes loaded by enalaprilat.


Reddy K.R., (2000) reviewed recent developments in novel injectable drug delivery mechanisms and outlined the advantages and disadvantages of each. He suggested injectable continuous-release systems which deliver drugs in a controlled, predetermined fashion and are particularly appropriate when it is important to avoid large fluctuations in plasma drug concentrations.

Lasie (1996) encapsulated Doxorubicin in sterically stabilized liposomes and found improved liposome stability and drug retention significantly increased the anticancer activity of encapsulated doxorubicin (Doxil), enhancing the effectiveness of chemotherapy and potentially reducing its toxicity.

Asher et al., (1977) studied effects of temperature and molecular interactions on the vibrational infrared spectra of phospholipid vesicles.

Muga et al., (1991) reported membrane binding induces destabilization of cytochrome C structure. A lipid-induced conformational perturbation of ferricytochrome C is also indicated by a marked decrease in the thermodynamic stability of the membrane bound protein.

Woodle et al., (1991) suggested the surface PEG may produce a steric barrier for colloids. Reduced in vivo uptake may result from inhibition of plasma-protein adsorption, or opsonization, by the steric coating.

Woodle (1998) studied the incorporation of polymer-lipid conjugates, initially using PEG and subsequently other selected flexible, hydrophilic polymers, into lipid bilayers which gives rise to sterically stabilized liposomes that exhibit reduced blood clearance and concomitant changes in tissue distribution largely because of reduced, but not eliminated, phagocytic uptake.


Franco et al., (1987) studied the nature and kinetics of red cell membrane changes during the osmotic pulse method of incorporating xenobiotics into viable red cells.

Alvarez et al., (1998) studied behaviour of isolated rat and human red blood cells upon hypotonic-dialysis encapsulation of carbonic anhydrase and dextran. Both
markers are incorporated to slightly greater extents by human than by rat RBCs by hypotonic treatment. Cell recovery of rat and human RBCs loaded with either carbonic anhydrase or fluorescent dextran accounted for 49% and 80% respectively.

- Sanz et al., (1999) encapsulated Glutamate dehydrogenase (GDH) into mouse erythrocytes by a hypotonic dialysis/isotonic resealing method and studied GDH entrapment yield. The osmotic fragility curves (OFC) indicated that dialyzed/resealed-RBCs are more resistant to hypotonic haemolysis than native-RBCs.

- Zola et al., (1991) reported kinetic studies of protein encapsulation by moderate hypotonic dialysis, which allows entrapment of molecules with MW less than 50,000 Da with negligible stress of the erythrocyte membrane. Furthermore data reveal that the resealing procedure commonly used is insufficient to completely seal pores of loaded erythrocytes, allowing entrapped proteins with MW less than 12-14,000 Da to escape.

- Richieri et al., (1985) studied temperature effects on osmotic fragility, and the erythrocyte membrane and reported higher temperatures enabling a substantial transient increase in surface area of intact cells (up to at least 14% of 40 degrees C), with a corresponding increase in the cell's hemolytic volume (up to 21%). The hemolytic volume apparently increases linearly with temperature, since steady-state ghost volumes are found to increase linearly with the temperature at which the ghosts were produced.

- Kasahara et al., (1985) showed that a membrane protein fraction solubilized with 0.5% Triton X-100 or 30 mM octylglucoside catalyzed D-glucose uptake when incorporated into liposomes made from soybean phospholipids.
Rossi et al., (2001) reported erythrocyte-mediated delivery of a new homodinucleotide active against human immunodeficiency virus and herpes simplex virus. Loaded erythrocytes were modified to increase their recognition and phagocytosis by human macrophages. By administering Bis-PMEA-loaded erythrocytes to macrophages, 47% of Bis-PMEA and 28% of PMEA was still present 10 days after phagocytosis; in contrast, only 12% of PMEA was found in macrophages receiving PMEA-loaded erythrocytes. Bis-PMEA-loaded erythrocytes were then added to macrophages infected with HIV-1 and HSV-1 and their antiviral activity.

Alvarez et al., (1996) reported in vivo survival and organ uptake of loaded carrier rat erythrocytes.

Millan et al., (2005) reported encapsulation and in vitro evaluation of amikacin-loaded erythrocytes.


Ribeiro IR and Olliaro P studied the safety of artemisinin and its derivatives and published a review on clinical trials.
LITERATURE SEARCH


➢ Woodrowet et al., (2005) studied artemisinins and reported that these compounds combine potent, rapid antimalarial activity with a wide therapeutic index and an absence of clinically important resistance. Artemisinin containing regimens meet the urgent need to find effective treatments for multidrug resistant malaria and have recently been advocated for widespread deployment.

➢ Wisedpanichkijet et al., (2009) studied in vitro antimalarial interactions between mefloquine and cytochrome P450 inhibitors. They investigated antimalarial activity of inhibitors of cytochrome P450 (CYP) enzyme including their interactions with the antimalarial mefloquine against chloroquine-resistant (K1) and chloroquine-sensitive (3D7) *P. falciparum* clones in vitro.

➢ Cunha-Rodriguez et al., (2006), Antimalarial drugs - host targets (re)visited and emphasized the potential role of host genes and molecules as novel targets for newly developed drugs.

➢ Arya et al., (1986) studied Clinical Manifestations of Complicated Malaria.

➢ Chotivanich et al., (1998) studied rosetting characteristics of uninfected erythrocytes from healthy individuals and malaria patients. In a series of studies to examine the characteristics of the uninfected RBC which contribute to rosetting. The ability of RBC from healthy donors to form rosettes was found to be greater in the cells of group A and B than in those of group O (P = 0.05), and it decreased during storage under blood-blank conditions. Normal RBC exposed for ≥30
minutes to quinine, artemisinin or artemether (each at 0.25 microgram/ml) in vitro showed significantly decreased rosetting.

- Sherman et al., (2003) reported a critical summary of recent advances in the characterization of the molecules of the infected red blood cell involved in adhesion, i.e. parasite-encoded molecules (PfEMP1, MESA, rifins, stevor, clag 9, histidine-rich protein), a modified host membrane protein (band 3) and exofacial exposure of phosphatidylserine, as well as receptors on the endothelium, i.e. thrombospondin, CD36, ICAM-1 (intercellular adhesion molecule), and chondroitin sulfate.

- Barnwell et al., (1989) studied the tissue cell receptor for a ligand on the surface of the infected erythrocytes is Mr 88,000 glycoprotein (GP88) recognized by the MAb OKM5, which also blocks cytoadherence of IE.

- Roberts et al., (1989) studied Plasmodium falciparum infected erythrocytes containing mature trophozoites and schizonts that sequester along venular endothelium. This sequestration may protect the parasite from splenic destruction and may play a role in the pathogenesis of cerebral malaria.

- Meryman et al., (1972) reported a method for freezing and washing red blood cells using a high glycerol concentration.

- Pirmin Schmid et al., (2011) reported RBCs preserved with glycerol and thawed with a widely used protocol showed a recovery of 41 ± 16 % (mean ± standard deviation) while those thawed with a modified glycerol protocol showed a recovery of 76±8%. RBCs preserved by droplet freezing with sucrose/dextrose (S+D) showed a recovery of 56±11% while those preserved by droplet freezing with PVP showed a recovery of 85±6%. Recovery values were similar with
ethylenediaminetetraacetic acid (EDTA) or heparin anticoagulants, differing freezing rates, and varying droplet volumes.

- Henkelman et al., (2010) tested RBCs for aggregability (aggregation index [AI]), deformability (elongation index [EI]), and various hematologic variables. The AI of thawed RBCs was reduced, compared to fresh and liquid-stored RBCs (p<0.05). The AI of stored RBCs was significantly enhanced over a shear stress range of 2.0 to 50Pa compared to fresh RBCs (p<0.05). No significant differences in EI between thawed and 21- or 35-day liquid-stored RBCs were observed.

- Clark (2009) reported Embryo toxicity of the artemisinin anti-malarial and potential consequences for use in women in the first trimester.

- Stepniewska et al., (2009), studied population pharmacokinetics of artesunate and amodiaquine in African children.


- Das et al., (2013) reported malaria treatment failure with novel mutation in the Plasmodium falciparum dihydrofolate reductase (pfhfr) gene in Kolkata, West Bengal, India.


- Garg et al. (2012) studied novel mutations in the antifolate drug resistance marker genes among Plasmodium vivax isolates, exhibiting severe manifestations.

- Mishra et al., (2011) studied prescription practices and availability of artemisinin monotherapy in India.
Mullick et al., (2011) studied efficacy of chloroquine and sulphadoxine-pyrimethamine either alone or in combination before introduction of ACT as first-line therapy in uncomplicated *Plasmodium falciparum* malaria in Jalpaiguri District, West Bengal, India.

Pareek et al., (2008) reported a comparative study of efficacy and safety of hydroxychloroquine and chloroquine in polymorphic light eruption in a randomized, double-blind, multicentric study.


Finbloom et al., (1985) reported comparison of hydroxychloroquine and chloroquine use and the development of retinal toxicity.

Breckenridge et al., (1989) reported risks and benefits of prophylactic antimalarial drugs.