Chapter 3

Physicochemical properties and cellular toxicity evaluation

Literature covering the determination of physicochemical properties, including factors such as size determination and entrapment efficacy specifically when accelerated stability studies are performed. This section also includes literature pertaining to toxicity studies such as reactive oxidative species determination and damage caused by lipid peroxidation

3.1. Introduction

Physicochemical properties refer to the chemical and physical properties that a system exhibit. In the case of liposomes this refers to the properties that the drug delivery systems present with, especially when going through stability testing. These properties need to be evaluated to ensure that the drug delivery system is safe for consumption (New, 1990). The effects that liposomes present when interacting with cells are also very important, especially any damage caused to the cells. This is referred to as toxicity. Each property has many different methods how they can be determined (Halliwell, 2007; Halliwell, 2006). The properties that are to be tested will be discussed in this chapter.

3.2. Stability studies

When the stability of a lipid or emulsion type product is tested, the consensus is that the product is stable if there is no change in the emulsion, such as coalescence, or creaming. This includes being frozen and thawed, or being exposed to high temperatures (temperatures between 40 ºC and 50 ºC). A stable lipid product can thus be described as one that does not change over time. There are four aspects to be taken into account when the stability of a lipid type product is tested. These four aspects are:

- The product must be exposed to conditions that are likely to be encountered in real-world situations, that is to say conditions during storage, shipping, handling as well as use.
- If at all possible, the product must be tested against a product with a known shelf-life and reactions to stability testing.
- Products cannot be tested immediately after manufacture, as the product may need time to reach an equilibrium state. This state may only be reached after 24 or even 48 hours, depending on the types of lipids used.
- The criteria against which the stability will be tested must be clearly defined beforehand, and the stability testing methods that are used have to be clearly developed and defined.

Stability in pharmaceutical products is not defined as the product not changing, but rather as changes that are acceptable, controlled and documented, thus not changing in a way that is unexpected. Therefore, stability testing must be defined in terms of when the change that occurs is unacceptable (Matthews, 1999).

3.2.1. Accelerated Stability studies

It is important to do stability studies on drugs and drug delivery systems, as to ensure the safety thereof for the consumer after the product was manufactured, handled and stored. It is difficult to be sure about the stability if the correct amount of testing was not done. This is a time consuming process that formulators and manufacturers dread to run. Accelerated stability testing methods could be an answer to this problem. Accelerated stability testing is a type of testing where the formulation being tested is exposed to high stress conditions for shorter amounts of time. The high stress conditions contribute to the fast breakdown of the components in these types of products. This gives large amounts of data in relatively short amounts of time. It may help the formulator to improve the formulation and to eliminate substandard formulations very quickly. It is important to be sure that the conclusions drawn from this type of study are extrapolated correctly. The point of doing stability testing on a product is, therefore, to ensure that the product will not undergo any unexpected changes, as well as being able to predict the shelf-life of the product, if the storage directions are followed (Pugh, 2002).

It is important to remember that accelerated stability storage is not a complete substitute for full stability testing; it is just an aid to identify better, more stable products in a shorter time. The accelerated stability testing is a precursor that may help to predict how a formulation will react over time (Pugh, 2002).

The temperatures normally used for accelerated stability studies are, 5 ^{o}C and 25 ^{o}C with a relative humidity of 60% respectively; and 40 ^{o}C with a relative humidity of 75%. The lowest temperature is kept constant by keeping formulations or drugs in a refrigerator and the higher temperatures are kept by the use of thermostatic cupboards (Pugh, 2002). One of the parameters often analysed during stability studies with lipid based formulations is the size of the particles.

3.2.2. Size determination

In stability studies of liposomes, the size determination is important. A variation in the size of liposomes, especially over time, can cause the internal volume of the liposome to either increase or decrease. This may affect the amount of entrapped drug or solute and cause a change in the efficacy of the drug delivery system (New, 1990).

Some of the methods used to determine the size of liposomes include (New, 1990; Vorauer-Uhl *et al.*, 2000; Childers *et al.*, 1989):

- Electron microscopy methods.
- Light scattering (also referred to as quasi-elastic laser light scattering).
- Gel filtration.
- High performance liquid chromatography (HPLC)
- Flow cytometry.

Some of the methods often damage or interfere with the actual size of the liposomes. Most of the methods have been known to need a high amount of expertise and are time consuming, before sufficient accurate data can be attained. A method that circumvents these problems is flow cytometry (Vorauer-Uhl *et al.*, 2000).

3.2.3. Size determination – Fluorescence Activated Cell Sorter (FACS)

Flow cytometry gave scientists the opportunity to differentiate between different cells and particles according to size, shape as well as granularity (only applicable to cells). A Fluorescence Activated Cell Sorter (FACS) is able to do this by combining laser optics, fluidics, complex computer software and electronics. The FACS uses a system of mirrors and sensors in conjunction with a laser to determine specific properties of cells and particles (BD Biosciences, 2000).

The flow cytometer can measure the amount of light scattered by a particle when passing through the laser's field. The cytometer then measures the forward scatter (FSC), which is the intensity of the laser as a particle passes through. The intensity of the laser is proportionate to the size of the particle (Invitrogen, 2003; BD Biosciences, 2000). Calibration of the FACS is done with calibration kits that are available from the FACS manufacturer. The calibration kits are suspensions of polystyrene microspheres that are of a known diameter. These diameters are evaluated by transmission electron microscopy and are, therefore, an accurate method of calibration (Invitrogen, 2003). With known gates sizes, the size as well as the average size of cells, other particles or liposomes can be quickly and effectively determined with a high rate of accuracy (Childers *et al.*, 1989; Vorauer-Uhl *et al.*, 2000).

3.2.4. Entrapment efficacy

When any dosage form is being created, it is important to determine the dosage of the entrapped drug, as the effects of the drug are dose related. The amount of drug that remains inside the liposomes over a period of time is also important, as this affects the amount of drug available for treatment. The entrapment of a drug may change considerably over time, as the

entrapment of the drug is directly related to the stability of the liposomal membrane. A less stable membrane will lead to more of the drug escaping from the liposome interior (New, 1990).

When a liposome formulation is made, it is important to consider that the dose of the drug entrapped inside the liposome has to be reasonable, when compared to the amount of lipids beings used, as certain lipids given in large quantities can have toxic side effects. This is especially true for liposomes with a charge (Sharma & Sharma, 1997). The drug delivery system is specifically put into place to lessen toxic effects of the drug, not add more.

Many different methods of determining the amount of entrapment efficacy exist, with the method described by the following formula being used most (Maestrelli *et al.*, 2005):

$$EE\% = \left(\frac{Initial\ drug\ load - Unentrapped\ drug}{Initial\ drug\ load}\right) \times 100$$

This formula can be used in time studies to detect the leakage of any drug from the liposome.

3.3. *In vitro* evaluation of liposome toxicity

If the correct cell type is chosen beforehand for *in vitro* testing, problems with stability and toxicity can be discovered early and then sorted out. *In vitro* tests can be indicative of many possible toxicity problems early in the development of new delivery systems. Therefore, it is advised to do toxicity tests before moving on to more expensive and complicated *in vivo* tests (Blomme, 2008).

3.3.1. Reactive oxidative species (ROS) and lipid peroxidation

When oxidative stress is discussed, it is firstly important to understand the definitions and terminology associated with it. Firstly, an oxidant is defined as any and every compound that is capable of receiving an electron, and these molecules are also known as oxidising agents. The opposite is true for reductants. A reductant can lose an electron and is also known as a reducing agent. The process where electrons are lost is known as oxidation. When speaking in biological terms oxidants are often referred to as pro-oxidants (Kohen & Nyska, 2002).

These pro-oxidants or reactive oxidative species (ROS) can be split into radicals and non-radicals. The radicals are classified as such, because they have at least one unpaired electron. This group of radicals is especially reactive because of their high affinity for another electron to stabilise the structure. Examples of radicals are oxygen (O_2), superoxide ($O_2^{-\bullet}$), nitric oxide radical (NO^-), hydroxyl radical (OH^-), Alkoxyl (RO^-), peroxyl (ROO^-) and peroxynitrate ($ONOO^-$); with many others in biological systems. The formation of some of the main types of oxidants that form in biological systems can be seen in Figure 3.1. The non-radical species are often just as reactive, but are not necessarily radical by definition. The following are examples of non-radicals that are abundant or even created within a biological system: hydrogen peroxide (H_2O_2), hypochlorous acid (HClO) and ozone (O_3) (Kohen & Nyska, 2002; Halliwell & Cross, 1994). A superscripted dot is used in literature to denote a radical as in the case of Nitric oxide ($NO^\bullet$). Antioxidants on the other hand are most often described as agents that can stop or slow oxidation down. These types of reactions have to take place in cells to ensure the correct functioning of many vital cellular pathways and processes (Kohen & Nyska, 2002).

3.3.1.1. Damage caused by oxidative stress

An imbalance in the oxidant-antioxidant system can lead to cell damage or even cell death, as these ROS can cause DNA, mitochondrial and cell membrane damage through oxidative stress. This may initiate programmed cell death or apoptosis which leads to inflammation as well as a whole host of other symptoms and conditions, such as neuro-degeneration and aging (Halliwell, 2007; Halliwell, 2006; Halliwell & Whiteman, 2004).

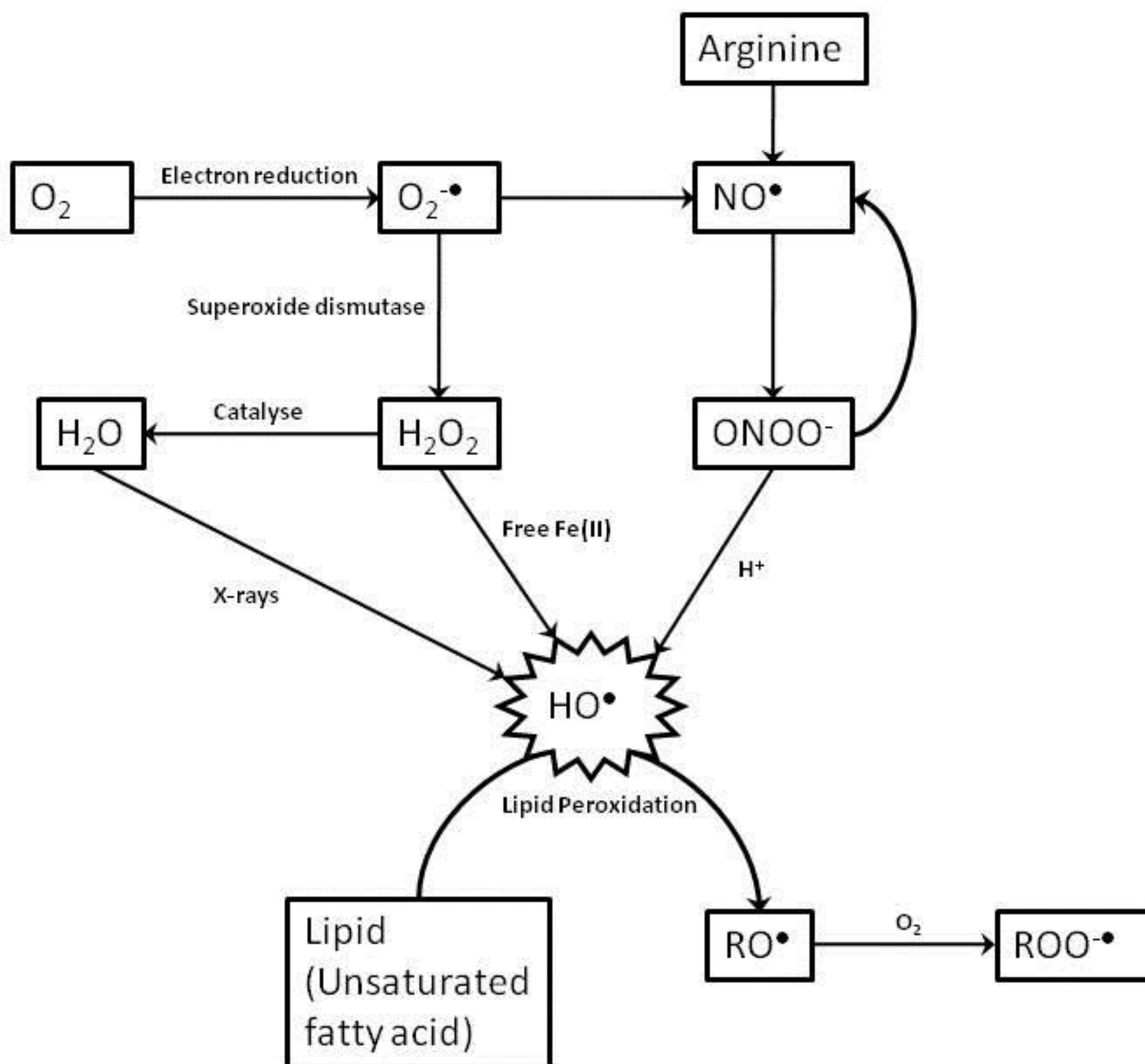

Figure 3.1: The formation of Reactive oxygen species, as adapted from (Stahl & Sies, 2002; Kohen & Nyska, 2002; Halliwell, 2006)

3.3.1.2. DNA damage

DNA structures are relatively well protected and stable inside a cell, but DNA itself is not safe from ROS as these molecules can be found nearly everywhere. Direct interactions with some less reactive ROS molecules have little or no effect on DNA, but may lead to the formation of a more reactive radical that is able to do considerable damage. This is especially the case when $OH^{\bullet}$ or $ONOO^{-}$ is formed near DNA. Reactions of DNA with ROS can cause broken DNA chains, modification of DNA bases, damage to the deoxyribose sugars, splitting of the cross links between DNA proteins as well as damage to the DNA repairing systems (Halliwell & Whiteman, 2004). Damage and modification like this can have dire consequences, such as cancer and aging (Kohen & Nyska, 2002).

3.3.1.3. Protein damage

Proteins are another target of ROS as these molecules have many targets to attack on the protein structure. Proteins that are damaged by ROS can break up, change chemical form as well as undergo peroxidation (Halliwell & Whiteman, 2004). Proteins are everywhere in cells and control many cell functions. These functions include energy production, the creation of cell membrane potentials, transport across cell membranes, etc. Thus, the degradation of proteins also create major problems (Kohen & Nyska, 2002).

3.3.1.4. Lipid damage (Lipid peroxidation)

Cell membranes are a prime site for damage caused by ROS as cell membranes have a very high concentration of unsaturated fatty acids. The fatty acids with double bonds (unsaturated) in their structure are the most susceptible type. Fatty acids with only single bonds (saturated) are very stable. The double bond fatty acids or unsaturated fatty acids are open to attack from ROS. The ROS can easily take a hydrogen molecule from the fatty acid forming $RO^{\bullet}$. The ROS group is able to take a hydrogen group from methylene group, as the carbon in the methylene group has a double bond next to the hydrogen carbon bond, which weakens the carbon hydrogen bond. The fatty acid then rearranges itself in an attempt to become more stable. If the oxygen concentration in the surrounding area is high enough, the fatty acid will form a $ROO^{-\bullet}$ group. This $ROO^{-\bullet}$ is in itself capable of extracting a hydrogen atom from surrounding unsaturated fatty acids or other double bonds in the area, thus causing a chain effect if there are enough double bonds (Halliwell, 2006; Kohen & Nyska, 2002).

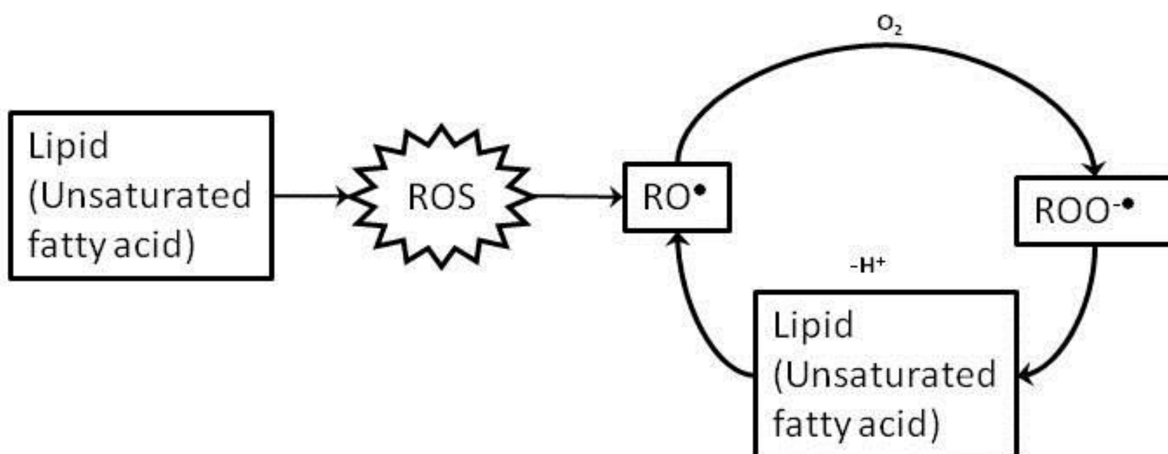

Figure 3.2: Lipid peroxidation as a cyclic process as adapted from (Kohen & Nyska, 2002; Stahl & Sies, 2002).

This process is known as lipid peroxidation. The fatty acid is broken down to lipid peroxide, which turns into cyclic endoperoxide, hydrocarbons, isoprotans or aldehyde. This chain reaction or lipid peroxide can continue until the entire cell membrane is destroyed. This process is illustrated in Figure 3.2. When tissues are stressed or damaged, increases of hyperperoxides are often observed (Spiteller, 1996). It is important to determine if lipid peroxidation is caused by adding any drugs or drug delivery systems, as the addition of these types of molecules can destabilise cells (Halliwell, 2007; Halliwell, 2006).

3.3.2. Defence against oxidative stress

As the atmosphere of our planet has an oxygen (O_2) concentration of nearly 21%, most life forms had to create mechanisms to protect themselves against oxidation. Just being exposed to higher concentrations of O_2 has side effects, such as coughing and chest soreness, which can later turn into lung damage (Halliwell & Cross, 1994). The major defence strategies against oxidation are efficient repair systems and antioxidants. Antioxidants are specifically used as a defence for oxidative stress, as they can directly remove oxidants or indirectly prevent oxidation (Halliwell & Whiteman, 2004; Kohen & Nyska, 2002). Broadly speaking, two types of antioxidants can be differentiated, namely antioxidant enzymes and low molecular weight antioxidants (LMWA) (Kohen & Nyska, 2002).

3.3.2.1. Antioxidant enzymes

Most life forms (except anaerobic life forms) on earth have superoxide dismutases (SODs) in one form or another, the SOD are mostly found in the mitochondria. These enzymes convert excessive amounts of $O_2^{-\bullet}$ to H_2O_2. The formed H_2O_2 can then be converted to H_2O by catalase from peroxisomes. Another mechanism to remove H_2O_2 is by glutathione peroxidase (GSHPX). This enzyme uses the H_2O_2 to oxidise reduced glutathione (GSH) to oxidised glutathione (GSSG). Glutathione reductase can convert GSSG back to GSH with reducing power provided by NADPH (Halliwell & Whiteman, 2004). Another enzyme that falls into this group is xanthine dehydrogenase (Kohen & Nyska, 2002).

3.3.2.2. Low molecular weight antioxidant (LWMA)

The antioxidants in this class are also known as scavengers. The first major advantage that the LMWA’s have over the enzymes is the ability to cross membranes quickly and easily, thus being

able to move to the specific site where they are needed. For the LWMA's to work effectively, a synergy must exist between the different types of LWMA's. The LWMA's have a distinctive mechanism of action, wherein they provide an electron to a reactive species and thus become a radical, although not a reactive radical. The scavengers can then be recycled by the antioxidant enzymes or other LWMAs (Kohen & Nyska, 2002). Examples of these so called scavengers include: GSH, uric acid, ascorbic acid and α-tocopherol; with α-tocopherol considered to be most important (Halliwell & Whiteman, 2004).

3.3.3. Oxidative stress in *P. falciparum*

When *P. falciparum* parasites infect erythrocytes, the parasites grow and multiply at a tremendous rate and this growth requires large amounts of energy and amino acids. The parasite gets these building blocks needed from the cytosol of the cell host. They metabolise haemoglobin (Hb) from the host cell. The haemoglobin is absorbed into the food vacuole of the parasite, where it is metabolised by haemoglobin proteolysis to release amino acids, with haem and $O_2^{-\bullet}$ as waste product (Francis *et al.*, 1997). Haem or ferriprotoporphyrin IX (FP) as it is also known, is very toxic and is capable of creating ROS which can cause oxidative stress. The parasite is capable of changing FP into less reactive haemozoin crystals, which is non-toxic, but this system is not completely effective, as some of the FP still escapes into the cytosol before being changed into haemozoin crystals (Becker *et al.*, 2004; Nogueira *et al.*, 2010; Francis *et al.*, 1997).

The parasite is capable of protecting itself against oxidative stress, as it carries its own supply of antioxidants to combat the problem of escaping FP. If a small amount of FP in the area of 0.5% does escape the transformation into crystal form, the oxidative stress it can cause is sufficient to damage the parasite and the host erythrocyte to cause lysis of the erythrocyte. The *Plasmodium* parasites have low molecular weight antioxidants (LMWA) and a few antioxidant enzymes to help deal with FP that escape. In the LMWA group, GSH is considered to be the most important (Becker *et al.*, 2004). A simplified illustration of the haemozoin formation as well as the GSH metabolism can be seen in Figure 3.3. The $O_2^{-\bullet}$ that is formed as a waste product of most of these processes is broken down by SODs. Malaria has two different SODs in its arsenal: the manganese and iron variations (Becker *et al.*, 2004).

These systems are some of the main targets for antimalarial drugs. Many drugs can be made to interfere specifically with any of these enzymes and many more of the current drugs used today, already do. Drugs included here are the 4-amino-quinolines and artemisinins (Becker *et al.*, 2004).

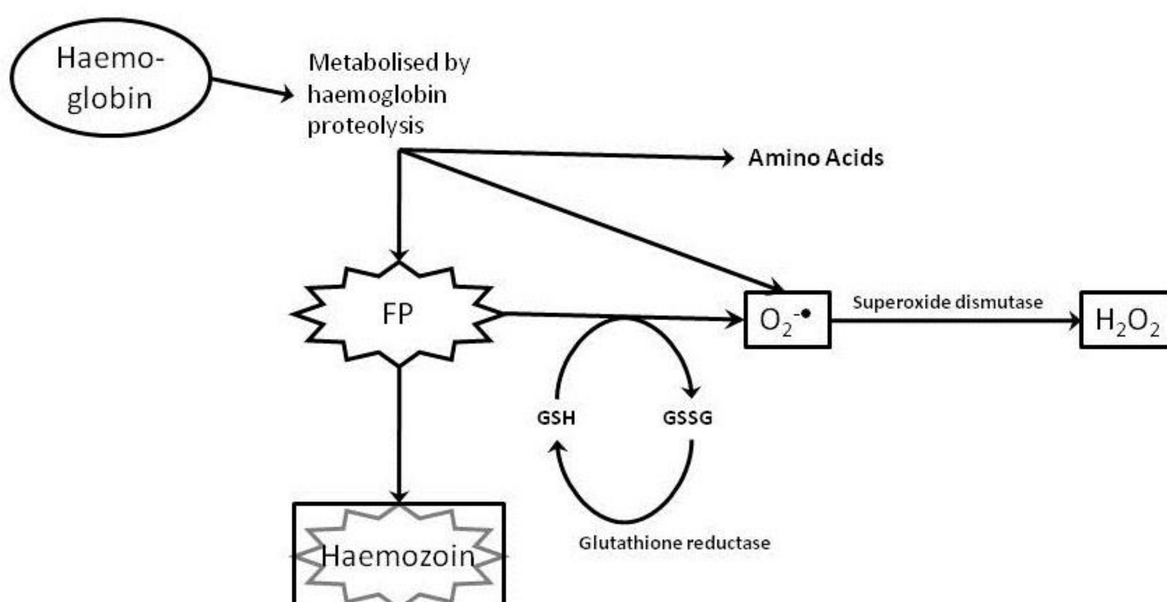

Figure 3.3: A schematic of the two main methods used by *P. falciparum* to detoxify haem. As adapted from (Becker *et al.*, 2004; Nogueira *et al.*, 2010)

It is on these systems that the 4-amino-quinoline antimalarials are thought to work. If it is with the ingestion of Hb, interfering with the polymerisation of heme, or interfering with the work of antioxidants in the parasite (Foley & Tilley, 1997; Sullivan *et al.*, 1998), most of the theories share a common mechanism, inducing oxidative stress (See section 1.9).

3.4. Conclusion

The physicochemical properties of any drug delivery system need to be tested, so as to ensure that the drug delivery system works as planned. During stability testing, many of the physicochemical properties change and are, therefore, an important criteria to test. Accelerated stability studies are some of the most preferred methods. Many different methods are used to determine the properties of a drug delivery system. Size determination can be done with the use of a fluorescence activated cell sorter. Entrapment efficacy can be determined by testing initial drug load added and testing the amount of drug that was not entrapped. *In vitro* studies are important to ensure that prospective formulations are not toxic or harmful. Testing for reactive oxidative species and lipid peroxidation can reveal the amount of damage that occurred. Erythrocytes are often used for these types of tests as they are abundant and prone to damage caused by ROS. ROS can damage DNA, proteins and lipids in cell membranes. Cells have built in defences against oxidation, as does *P. falciparum*. Cells protect themselves with antioxidants from two major classes: LMWA and antioxidant enzymes.