



Optimisation and characterisation of Pheroid® entrapped radio tracer formulations

A van As



orcid.org/0000-0002-9566-5077

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Supervisor: Dr NI Barnard

Co-Supervisor: Prof JR Zeevaart

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Student number: 25752685

Erkenning

Here, U is my lewe, U sorg vir my. Wat ek ontvang kom alles van U af. 'n Pragtige deel is vir my afgemeet, ja, wat ek ontvang het is vir my mooi

Ps 16:5-6

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My Ma wat my aangemoedig het toe ek wou tou opgooi.

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ABSTRACT

Pheroid[®] is a lipid-based drug delivery system which has been shown to entrap, protect and deliver hydrophilic, hydrophobic and amphiphilic compounds across biological membranes. As a result Pheroid[®] has the advantage of increasing gastrointestinal absorption of drugs. Pheroid[®] could provide a means of oral delivery for drugs such as ^{99m}Tc-MDP (^{99m}Technetium methylene diphosphonate), an intravenously administered radiotracer used during bone scans. Oral administration of Pheroid[®] ^{99m}Tc-MDP was shown in rats. This suggested Pheroid[®] may enhance bioavailability of radiopharmaceuticals potentially allowing an alternative to intravenous administration. Thus increasing safety for medical staff as well as patient compliance. Due to instability and loss of activity, formulations containing radiotracers are prepared shortly before administration. For clinical applications an optimised formulation with ^{99m}Tc-MDP entrapment/association in Pheroid[®] within 2-6 hrs. is required.

Formulation parameters of Pheroid[®] were systematically altered and the effects studied using particle size distribution and ζ -potential as indication of stability. Formulations showing early stability were selected for further investigation. Light microscopy was used to confirm morphology while reflectance microscopy was used to qualitatively assess entrapment within or association of Sn-MDP with Pheroid[®]. Formulations displaying suitable entrapment were selected for quantitative determination of entrapment efficiencies utilizing a novel methodology. Entrapment efficiency was measured by incubating Pheroid-^{99m}Tc-MDP formulations on hydroxyapatite (HAP), as *in vitro* bone model. In the absence of metabolic function any amount of ^{99m}Tc-MDP unable to adsorb to the synthetic hydroxyapatite media may be considered to be associated with/entrapped within Pheroid[®] structures. Although stable formulations are necessary for pharmaceuticals, this study highlighted inadequacies of standard stability measurements as an indication of entrapment efficiency. Insight into the effect of certain formulation parameters on formulation stability as well as an alternative method for determining entrapment efficiencies for certain classes of compounds in Pheroid[®] formulations are highlighted. Two formulations were identified for further investigation for clinical application: Pheroid[®] vesicles with ^{99m}Tc-MDP added to aqueous phase (N₂O-H₂O) before preparation and Pre-prepared Pheroid[®] vesicles; ^{99m}Tc-MDP added 4 days after formulation. The latter having the advantage of ease of preparation within clinical settings. All experiments were conducted in triplicate and one way analysis of variance (ANOVA) was used to determine reproducibility, applying a significance level $\alpha = 0.05$.

Keywords: Pheroid[®], ^{99m}Technetium Methylene-diphosphonate (^{99m}Tc MDP), radiotracer, hydroxyapatite (HAP), entrapment efficiencies (EE)

UITTREKSEL

Pheroid[®] is 'n lipiedgebaseerde geneesmiddel-draersisteem waarvoor die volgende al bewys is: dit is in staat om hidrofiele, hidrofobiese en amfifiele verbindings oor biologiese membrane te dra, te beskerm en af te lewer. As gevolg hiervan bied Pheroid[®] die voordeel van verhoogde gastro-intestinale absorpsie van geneesmiddels. Pheroid[®] kan 'n manier bied om geneesmiddels soos ^{99m}Tc-MDP (^{99m}Tegnetium Metileen Difosfonaat), 'n intraveneus toegediende radioaktiewe merker molekule wat gebruik word tydens beenskanderings, mondelings toe te dien.

Mondelingse toediening van Pheroid[®] ^{99m}Tc-MDP is in rotte getoon. Dit het die vermoede geskep dat Pheroid[®] die biobeskikbaarheid van radio-farmaseutiese middels kan verbeter, wat 'n alternatief vir intraveneuse toediening moontlik maak. Dit sal die veiligheid vir mediese personeel sowel as pasiënt samewerking verbeter. As gevolg van onstabiliteit en verlies van aktiwiteit, word radioaktiewe formulerings kort voor die toediening voorberei. Vir kliniese toepassings sal 'n geoptimaliseerde formulasie benodig word waar die ^{99m}Tc-MDP binne 2-6 uur vasgevang word in/ge-assosieer word met die Pheroid[®].

Formuleringsparameters van Pheroid[®] is stelselmatig verander en die effekte is bestudeer met behulp van deeltjiegrootte analise en ζ-potensiaal as aanduiding van stabiliteit. Formulasies wat vroeë stabiliteit getoon het, is gekies vir verdere ondersoek. Ligmikroskopie is gebruik om morfologie te bevestig, terwyl refleksiemikroskopie gebruik is om kwalitatief te bepaal of die ^{99m}Tc-MDP vasgevang word in/ge-assosieer word met die Pheroid[®].

Formulasies wat belowende vasvangings getoon het, is gekies vir kwantitatiewe bepaling van vasvangdoeltreffendheid deur gebruik te maak van 'n nuwe analise metode.

Vasvangdoeltreffendheid is gemeet deur die inkubasie van Pheroid-^{99m}Tc-MDP formulasies op hidroksie-apatiet (HAP), as *in vitro* beenmodel. In die afwesigheid van metaboliese funksie kan enige hoeveelheid van ^{99m}Tc-MDP wat nie aan die sintetiese hidroksieapatietmedia adsorbeer nie, geag word as geassosieer met / binne die Pheroid[®]-strukture vasgevang te wees.

Alhoewel stabiele formulasies vir farmaseutiese middels nodig is, het hierdie studie aangetoon dat standaard stabiliteitsmetingsmetodes nie noodwendig 'n akkurate aanduiding is van vasvangdoeltreffendheid nie. Insig in die effek van sekere formuleringsparameters op formulasiestabiliteit sowel as 'n alternatiewe metode vir die bepaling van vasvangdoeltreffendhede vir sekere klasse verbindings in Pheroid[®] formulasies word uitgelig.

Twee formulasies is geïdentifiseer vir verdere ondersoek met die oog op kliniese toediening:

Pheroid[®] vesikels met ^{99m}Tc-MDP bygevoeg tot die waterige fase (N₂O-H₂O) voor voorbereiding en voorafbereide Pheroid[®] vesikels, ^{99m}Tc-MDP bygevoeg 4 dae na formulering. Laasgenoemde het die voordeel van gemak van voorbereiding binne 'n kliniese omgewing.

Alle eksperimente is in drievoud uitgevoer en een-weg analise van variansie (ANOVA) is gebruik om herhaalbaarheid te bepaal deur 'n betekenispeil $\alpha = 0.05$ toe te pas.

Sleutelwoorde: Pheroid[®], ^{99m}Tc Tegmentium Metileen-difosfonaat (^{99m}Tc MDP), radioaktiewe merker, hidroksie-apatiet (HAP), vavangdoeltreffendhed (EE).

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LIST OF ABBREVIATIONS

API	Active Pharmaceutical Ingredient
HAP	hydroxyapatite
keV	kiloelectron volt
LS	Liquid Scintillation
LSC	Liquid Scintillation Counting
mCi	millicurie
Necsa	South African Nuclear Energy Corporation
N₂O	Nitrous Oxide
NTeMBI	Nuclear Technologies in Medicine and the Biosciences
NWU	North West University
RL	Radiolabels
^{99m}Tc MDP	^{99m} Tc Methylene Diphosphonate
TLC	Thin layer chromatography

Chapter | 1

1.1. Background

1.1.1. Drug carrier prerequisites

Drug delivery is the process of administering a pharmaceutical compound to achieve a therapeutic effect in humans or animals (Wang *et al.*, 2005; Tiwari *et al.*, 2012). For a drug to be therapeutically active, it should be dissolved in either the dosage form or bodily fluids with high physical and chemical stability. To achieve this, drugs can be formulated within a drug carrier system. Drug delivery systems (DDS) are defined as formulations, substances or devices that enable the introduction of active pharmaceutical ingredients (API) in the body. DDS improve efficacy and safety by e.g. controlling the rate and targeting drug release (Jain, 2008). Considerations for a drug carrier in a liquid dosage form include: biocompatibility, should be biodegradable, of fine and uniform particle size, have good stability and be pharmaceutically acceptable (Müller *et al.*, 1998). For a drug carrier system to be pharmaceutically acceptable, it should be compatible with the physicochemical and chemical properties of the drug. The formulation containing the carrier and drug should be stable during preparation, storage and administration (Lasic & Papahadjopoulos, 1998).

1.1.2. The Pheroid[®] active ingredient delivery system

Pheroid[®] (hereafter referred to as Pheroid for simplicity) is a novel patented active pharmaceutical ingredient (API) delivery system consisting of essential and plant unsaturated fatty acids, which are emulsified in water saturated with nitrous oxide (N₂O) (Grobler & Kotze, 2006; Grobler *et al.*, 2008; Grobler, 2008). Three different types of Pheroid are currently recognised: Pheroid vesicles, Pheroid micro-sponges and pro-Pheroid[®] (Grobler *et al.*, 2008).

Pheroid technology has the capacity to entrap, protect and deliver API molecules across diverse biological membranes. Pheroid formulations can be manipulated for various therapeutic applications by successfully entrapping hydrophilic, hydrophobic and amphiphilic compounds (Du Plessis *et al.*, 2010b). Some applications of Pheroid technology include: anti-malarials, enhanced tuberculosis treatments, peptide delivery, topical drug delivery and improved antibiotic efficacy (Meyer, 1997; Grobler & Kotze, 2006; Grobler, 2008; Du Plessis *et al.*, 2010a; Du Plessis *et al.*, 2010b; Du Plessis *et al.*, 2014)

Lipid-based drug delivery systems are used mainly to improve bioavailability and reduce side effects (Gardner, 1987). As a lipid-based drug delivery system, Pheroid has the advantage of increasing gastrointestinal absorption of drugs (Steyn *et al.*, 2011). The Pheroid delivery system has been shown to improve the delivery of a range of complexes in terms of decreased onset of

action times, lower effective drug concentration doses and enhanced efficacy of drugs (Grobler *et al.*, 2008; Steyn *et al.*, 2011; Grobler *et al.*, 2014).

Significantly, Pheroid technology might provide a practical or alternative means of delivery for injectable currently orally unavailable drugs (Du Plessis *et al.*, 2014).

1.1.3. ^{99m}Tc -MDP as radiotracer

An appropriate definition of nuclear medicine was proposed in 1968: “Nuclear medicine is the scientific and clinical discipline in which free radionuclides or radionuclide compounds, redistributed *in vivo* or *in vitro* by physical or chemical mechanisms, are used for diagnostic, therapeutic or investigative purposes” (Cohn, 1968). Radionuclide imaging as a diagnostic tool provides a way to assess physiological changes that result from biochemical changes in the body. Different radionuclides, depending on the nature of the ligand, tend to concentrate in particular organs, systems or tissues (Maloth *et al.*, 2014).

^{99m}Tc (Technetium radioisotope 99) is a gamma emitting radionuclide. Technetium compounds have been used as imaging agents for some time (Wang *et al.*, 1979; Schwochau, 1994; M Rey, 2010; Barh *et al.*, 2014). ^{99m}Tc -MDP (technetium-99m methylene diphosphonate or medronic acid) in particular, accumulates in bone tissue and is used in skeletal imaging (Subramanian *et al.*, 1975; Kung *et al.*, 1978; Blake *et al.*, 2011). Bone scans provide images of the metabolic activity of the skeleton and are accomplished through imaging of the gamma rays emitted from the radioactive ^{99m}Tc isotope which accumulates particularly in areas with high/increased metabolic turnover (Budd *et al.*, 1989; Krasnow *et al.*, 1997; McCracken *et al.*, 2001).

^{99m}Tc MDP was first introduced in 1971 (Subramanian & McAfee, 1971), by 1980 ^{99m}Tc -MDP was characterised (Libson *et al.*, 1980) and by 1981 medical applications for this compound were already reported (Christensen & Krogsgaard, 1981). Today ^{99m}Tc -MDP is a well-known and understood radio-tracer that is used during routine bone-scans in most equipped hospitals (Iagaru *et al.*, 2012), including the Steve Biko Academic Hospital in Pretoria, South Africa.

MDP is available in commercial, ready-to-use kits. The kits contain methylene diphosphonic acid or a corresponding sodium salt, a reducing agent such as Sn(II) chloride or Sn(II) fluoride, and a stabilising agent such as 2,5 di-hydroxybenzoic acid (DHBA) or ascorbic acid (Lever & Lever, 2009).

Apart from acting as reducing agent, Sn(II) also forms Sn(II)diphosphonate complexes, hereafter referred to as Sn-MDP (Claessens & Kolar, 2000). Solutions of Sn(II) are easily oxidised, especially in solution, consequently ^{99m}Tc -MDP radio radiotracer doses have to be prepared shortly before administration (Lever & Lever, 2009).

^{99m}Tc -MDP is administered intravenously and gamma rays emitted during the decay of the radioisotope are used to produce images. A region where the isotope concentration is highest leads to higher gamma ray emission. This is a good indication of increased bone activity, which in turn points to new bone growth caused by e.g. healing fractures, bone growth around implants or tumour formation (McCracken *et al.*, 2001). When administered intravenously, the primary uptake route of ^{99m}Tc -MDP is through adsorption onto or into the crystalline structure of hydroxyapatite. After administration, 80% of the ^{99m}Tc -MDP is generally cleared from the body after 3hrs., while between 10 and 20% is found adsorbed to bone tissue.

An additional reason ^{99m}Tc -containing tracers have to be administered within a short time after preparation of the kits is due to the rate of decay ($^{99m}\text{Tc} \rightarrow ^{99}\text{Tc} + \gamma$), having a half-life of approximately 6 hrs. (McAfee *et al.*, 1964; Lever & Lever, 2009).

1.2. Rationale: Advantages of ^{99m}Tc -MDP entrapment within Pheroid

A study utilising ^{99m}Tc -MDP to observe the biodistribution of Pheroid has led to a patent for Pheroid-radiotracer formulations (Grobler & Zeevaart, 2015; Swanepoel, 2015). Apart from determining the biodistribution of Pheroid, this study showed that Pheroid has the potential to enhance the bioavailability of radiopharmaceuticals and potentially allow oral administration. This is noteworthy, as studies have confirmed that patients receiving radiotherapy prefer oral administration of drugs over the intravenous route. Main reasons for this preference include convenience and avoiding pain / discomfort associated with needles. (Liu *et al.*, 1997; Borner *et al.*, 2001). Oral administration also provides a safer alternative for medical staff, as needle related incidents pose potential injury and infection threats (Nsubuga & Jaakkola, 2005).

Swanepoel (2015) showed that Pheroid allows the oral administration of ^{99m}Tc -MDP in rats. If ^{99m}Tc -MDP, which is currently administered intravenously, can be formulated into an optimised orally administrable dosage form, it may improve patient compliance and comfort; and reduce needle related risks to medical staff.

Formulations containing radiotracers cannot be prepared a long time in advance to achieve the necessary entrapment into the carrier system. If the half-life of an administered radiotracer causes activity loss to such an extent that imaging is no longer possible, the advantages of oral administration are lost. When also taking into account the oxidation of Sn(II) after preparation of MDP kits it becomes clear that a readily prepared (in hospital), optimised formulation of Pheroid ^{99m}Tc -MDP is needed. For successful clinical implementation of such a diagnostic preparation, maximum entrapment in a dosage form ready for administration within 2-6 hrs of final formulation is required.

1.3. Aim of study

The aim of this study was to determine and study different factors that might affect the entrapment efficiencies (EE) of ^{99m}Tc -MDP / MDP in Pheroid and optimise these processes. The goal was to systematically change formulation parameters in order to study their effect on EE and thereby ultimately achieve a dosage form with optimal entrapment within 2 to 6 hrs of preparation. In order to achieve this, Pheroid[®]-Sn-MDP preparations with systematically varied formulation parameters was prepared and characterised in terms standardised methods, namely: particle size distribution, ζ -potential, pH and morphology.

Based on characterisation results, formulations show stabilisation within the shortest time were selected for further investigation. Entrapment efficiencies of various formulations was measured by incubating Pheroid[®]- ^{99m}Tc -MDP formulations on hydroxyapatite. The chemical nature of ^{99m}Tc -MDP leads to a very high affinity for hydroxyapatite. Any amount of ^{99m}Tc -MDP that is unable to adsorb to the synthetic hydroxyapatite (HAP) media may be considered to be associated with / entrapped within Pheroid[®] structures. As described by Jansen *et al.* (2009) HAP provides an *in vitro* model for measuring the affinity and adsorption capabilities of bone seeking radiotracers (Claessens & Kolar, 2000; Jansen *et al.*, 2009). This model does not seek to replicate *in vivo* conditions, but provides an intriguing means of investigating the entrapment of a bone seeking radiotracer in Pheroid, as the effects of metabolism which would be present in an *in vivo* model, may be excluded. The amount of radioactivity measured on HAP and within or associated with Pheroid components are influenced by the entrapment efficiency of the formulations rather than physiological factors.

1.4. Objectives of study

The objectives of this study can be summarised as follows:

1. Formulate Sn-MDP (non-radioactive) in combination with different types of Pheroid and with varying formulation parameters
2. Characterise and evaluate all Pheroid-MDP formulations in terms of particle size distribution, ζ - potential, pH and morphology over time.
3. Prepare ^{99m}Tc -MDP (radioactive) formulations based on promising results identified from objectives 1 and 2.
4. Determine the entrapment efficiencies of selected Pheroid-MDP formulations by means of unique quantitative radio adsorption measurements of hydroxyapatite adsorption as well as confocal reflectance microscopy.
5. Resolve optimal entrapment time requirements for Pheroid-MDP formulations.
6. Determine optimal Pheroid-MDP formulation parameters for future *in vivo* studies.

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Chapter | 2

2.1. Introduction: Drug carrier systems

Jain *et al.* (2014) provides a definition of a drug carrier system: “A drug delivery system is a formulation, ingredient or device enabling the introduction of a therapeutic substance in the body while improving its efficacy and safety by controlling different aspects of drug release in the body.” Drug delivery includes administration of active pharmaceutical ingredients (API), release of API's by the drug delivery system and the resulting transport of the API's across biological membranes / barriers to the desired site of action (Jain, 2008).

Reported problems posed by non-ideal drugs, as well as the therapeutic implications of their properties and the possible effect of drug delivery systems (DDS) are summarised by Allen and Cullis (2004) in table 2.1.

Table 2.1 The effect of DDS on drugs that present dosage form challenges (Allen & Cullis, 2004).

Properties of drugs	Implications	Effect of drug delivery systems
Poor solubility	Low amounts can be dissolved, and drugs precipitate very easily when introduced into an aqueous media i.e. blood plasma.	Lipid drug delivery systems contain both hydrophilic and hydrophobic components, presenting the possibility of both hydrophilic and hydrophobic dissolution.
Unwanted leakage into surrounding tissue	Leakage can cause tissue damage	DDS could possibly control release, reducing the possibility of leakage.
Easily metabolised	Drug loses activity before it can be effective.	DDS protect the drug, slowing down the rate at which drug is broken down.
Quick clearance from system	Requires higher dosages.	DDS can delay clearance, increase circulation time.
Non-selective bio-distribution	Drugs can unintentionally affect normal tissue.	DDS might be targeted to deliver the drug only to the affected site. DDS might also increase drug concentrations in diseased tissue.

Table 2.1. shows that the introduction of a drug delivery system can be advantageous. There are various types of drug delivery systems (Allen & Cullis, 2004). For the purposes of this study API delivery systems with characteristics (morphology, composition and delivery mode) in common to the Pheroid will be highlighted.

Lipid excipients display desirable physiological compatibility which has led to the development of various lipid-based drug carrier systems, for example liposomes, lipid-based nanoparticles, emulsions and lipid drug conjugates (Devalapally *et al.*, 2007; Bunjes, 2011).

Lipid-based drug delivery systems have the potential to address the issue of poor API solubility and are suitable for various administration routes (Westesen *et al.*, 1997; Gershkovich *et al.*, 2008; Porter *et al.*, 2008; Attama *et al.*, 2012; Li *et al.*, 2012).

Some common components of lipid-based drug delivery systems will be discussed. Colloidal emulsions and vesicular lipid-based drug delivery systems will also be reviewed in more detail. These systems are well suited for oral administration and share certain characteristics with Pheroid technology, the drug delivery technology investigated during this study.

2.2. General components of lipid based drug delivery systems

2.2.1. Excipients

Various lipid excipients are available. These excipients can influence the solubilisation, absorption and stability of drugs (Pouton, 2000; Porter *et al.*, 2008; Pouton & Porter, 2008; Kalepu *et al.*, 2013). Factors such as miscibility; solvent capacity; self-dispersion; digestibility and fate of digested products influence excipient choices. An increase in fatty acid chain length leads to an increase in melting point while unsaturation lowers melting points, an important formulation consideration (Jannin *et al.*, 2008). Regulatory factors include irritancy, toxicity, purity, chemical stability and cost (Pouton, 2000; Pouton & Porter, 2008).

2.2.1.1. Lipid excipients: triglycerides, mixed glycerides and polar oils

Triglycerides are lipid molecules containing three ester functional groups as well as three fatty acids (Fasman & Sober, 1977). Triglyceride vegetable oils are probably the most commonly used excipients in lipid-based drug delivery systems. Their main advantage is safety (fully digested and absorbed) (Porter *et al.*, 2008; Pouton & Porter, 2008). Triglycerides are classified based on chain length: long (LCT), medium (MCT) and short chain triglycerides (SCT).

Mixed glycerides are synthesised through partial hydrolysis of vegetable oils. The chemical composition of the mixed glycerides are determined by the starting materials (triglyceride) and the extent of hydrolysis (Strickley, 2007).

Oleic acid is an example of a mixed glyceride synthesised from the hydrolysis of olive oil, pecan oil or canola oil (DeBonte & Hitz, 1998; Strickley, 2004; Hauss, 2007; Strickley, 2007; Villarreal-Lozoya *et al.*, 2007; Grossi *et al.*, 2014).

2.2.1.2. *Lipid excipients: co-solvents*

Co-solvents are used to improve solubilisation in commercial drugs (Strickley, 2004; Strickley, 2007). Popular co-solvents include ethanol, glycerol, propylene and polyethylene glycol (Kalepu *et al.*, 2013). Co-solvents aid in increasing drug solvation capacity and dispersion of formulations containing high proportions of water soluble surfactants. Some limitations and disadvantages of co-solvents include increased precipitation of solubilised drugs after dilution as well as occasional immiscibility with oils used as lipid components in systems (Yalkowsky, 1999; Cole *et al.*, 2008).

2.2.2. Surfactants and their classification

A surfactant is a substance that adsorbs at the interface of a system containing distinguishable phases such as oil and water. The term **interface** indicates the border between two phases that are immiscible while **surface** refers to an interface where one phase is a gas. A surfactant acts to decrease interfacial free energy, stabilising the system (Attwood, 2012; Rosen & Kunjappu, 2012).

Surfactants have very specific chemical structures and contain two surface-active species: lyophobic groups (functional group with little attraction towards the solvent) and lyophilic groups (functional group strongly attracted to the solvent phase) (Rosen & Kunjappu, 2012).

Molecules containing both a lyophilic and lyophobic functional group are considered amphipathic. The amphipathic nature of surfactant molecules allows them to accumulate at interfaces. If water is the solvent, the lyophilic group will be hydrophilic and the lyophobic group hydrophobic. The polar / hydrophilic group is referred to as a head group and the nonpolar / hydrophobic as a tail group (Lawrence, 1994; Attwood, 2012; Rosen & Kunjappu, 2012). The head group may have a positive or negative charge, resulting in cationic or anionic surfactants, or it may consist of polyethylene oxide chains, resulting in non-ionic surfactants (Alexandridis & Hatton, 1995). The tail group is often a flexible hydrocarbon chain or aromatic hydrophobic group.

Surfactants can be classified based on charge as ionic (anionic, cationic or zwitterionic) or non-ionic (Salager, 2002). The characteristics as well as some advantages and disadvantages of abovementioned surfactants are summarised in table 2.2.

Table 2.2. Classification of surfactants based on charge.

Surfactant	Characteristics	Functional groups	Advantages	Disadvantages
Anionic	Surface-active functional group carrying a negative charge	Carboxylic acid salts, sulfonic acid salts, sulphuric acid ester salts, phosphoric and poly-phosphoric acid esters (Salager, 2002; Rosen & Kunjappu, 2012).		Not always compatible with non-ionic surfactants.
Cationic	Surface-active species with surface-active functional group carrying a positive charge	Long-chain amines and their salts, acylated diamines and polyamines and their salts, quaternary ammonium salts, polyoxyethylenated long-chain amines, quaternized polyoxyethylenated long-chain amines and amine oxides (Salager, 2002; Rosen & Kunjappu, 2012).	Compatible with non-ionic and zwitterionic surfactants. Adsorbs strongly onto most solid surfaces, can influence substrate characteristics. Allows deposition of active phases onto substrate.	Mostly incompatible with anionic surfactants, amine oxides are an exception. Generally more expensive. (Salager, 2002; Somasundaran, 2006; Rosen & Kunjappu, 2012)
Zwitterionic	Molecules with a surface-active group that can carry both a positive and negative charge. Divided into pH sensitive and pH insensitive.	Generic example: $RN^+H_2CH_2OO^-$, long chain amino acids (Rosen & Kunjappu, 2012) pH sensitive: amidoamines and amidobetaines, amine oxides, β -N-alkylaminopropionic acids, N-alkyl- β -iminodipropionic acids, imidazolinecarboxylates and N-alkylbetaines (Rosen & Kunjappu, 2012) pH insensitive: sulfobetaines and sultaines (Rosen & Kunjappu, 2012)	Compatible with all other types of surfactants, less irritating to skin and eyes than other types. Able to adsorb onto negatively or positively charged surfaces without formation of hydrophobic films (Salager, 2002; Somasundaran, 2006).	Often insoluble in organic solvents, even more so in polar solvents such as ethanol (Attwood, 2012)
Non-ionic	Carries no official ionic charge although might be polarised	Long carbon-chain alcohols. Fatty alcohols, derived from natural fats and oils, straight-chain primary alcohols		

Surfactants can also be classified according to HLB numbers. The hydrophilic-lipophilic balance (HLB) of a surfactant is an indication whether a molecule is more hydrophilic or lipophilic (Griffin, 1946; Griffin, 1955). HLB values are calculated theoretical values that can predict the behaviour of surfactants.

HLB values have a scale of 0 to 20. An HLB value of 0 implies absolute hydrophobic behaviour while a value of 20 implies absolute hydrophilic behaviour (Rosen & Kunjappu, 2012).

2.3. Colloidal emulsions

Colloidal systems are defined as homogenous dispersion systems of particles between 1 nm and 1 μm , throughout a continuous liquid phase (Bouchemal *et al.*, 2004; Fletcher *et al.*, 2013). Pashley and Karaman (2005) however note that the term is often more loosely applied to particles that can range from 10 μm to 0.1 nm as shown in figure 2.1.

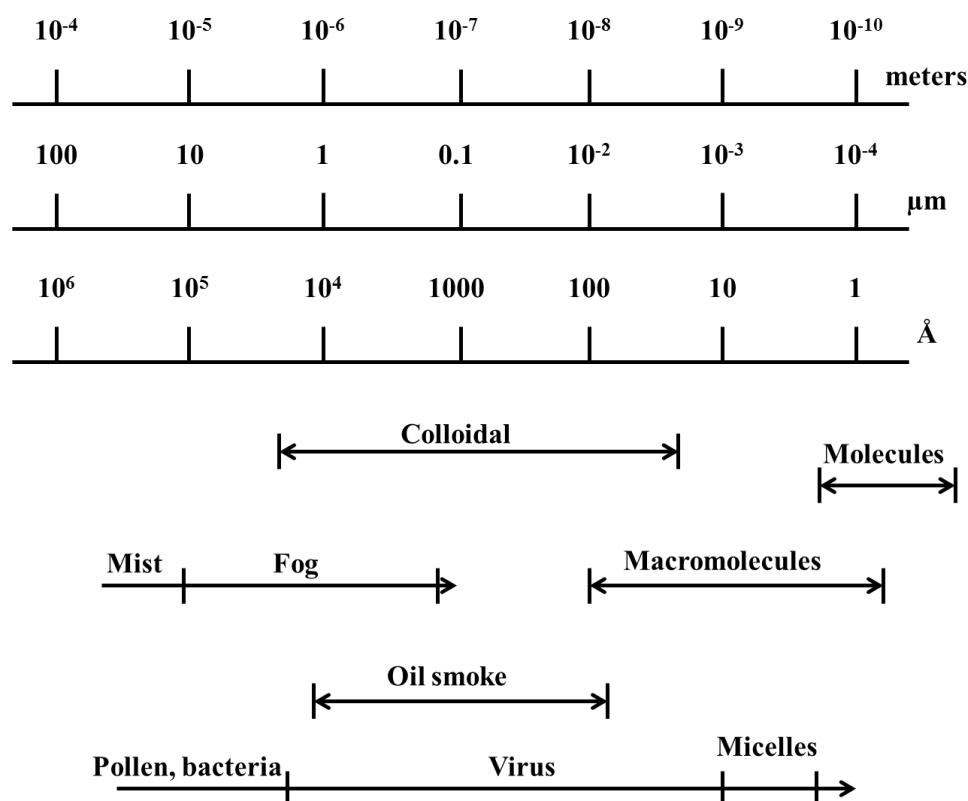


Figure 2.1. Scale of colloidal dispersed particles (Pashley & Karaman, 2005).

A colloidal classification is more dependent on particle behaviour than size. Colloidal particles are evenly dispersed in a dispersion medium and are in a constant metastable state where certain forces attract particles to each other while surface charges are repellent. This leads to dynamic stability where particles are not stationary but do not aggregate (Pashley & Karaman, 2005).

2.3.1. Colloidal emulsions: Micro- and Nanoemulsions

Two types of colloidal dispersions are widely used as drug delivery systems: oil-in-water (o/w) nanoemulsions and microemulsions. These two systems are similar with regard to composition, dimensions, structures and preparation methods (McClements, 2012), the primary difference being thermodynamic stability (Lovelyn & Attama, 2011). Thermodynamic stability is achieved when the product (in this case an emulsion) has a lower energy than the starting materials (McClements, 2015).

2.3.1.1. Microemulsions

Microemulsions are thermodynamically stable liquids with uniform physical properties and are formed by mixing oil, water, and surfactants (Jonsson, 1998; Fanun, 2008). The surfactant to oil ratio is higher for microemulsions than for nanoemulsions. The maximum oil to surfactant ratio depends on the following (Low & Antony, 2004; McClements, 2012):

- Characteristics of oil molecules: molar volume, polarity and geometry
- Characteristics of surfactant molecules: head and tail groups
- Environmental / variable formulation conditions: temperature, pH and ionic strength

Microemulsions form a wide range of systems depending on parameters such as composition and temperature during preparation. Microemulsions can contain a number of separate phases in equilibrium with each other (McClements, 2012). The structures within these phases can be spheroid, cylindrical, planar or sponge-like (Jonsson, 1998).

Theoretically, microemulsions can form spontaneously when oil, water and surfactant (s) are brought together at a specific temperature (Solans & Kunieda, 1996; Bouchemal *et al.*, 2004). However, mild external energy often needs to be applied in the form of heating or stirring to facilitate the formation of microstructures (McClements, 2012).

Microemulsions are thermodynamically stable under particular conditions (specific composition, storage temperatures) and if the initial conditions do not change, microemulsions should be stable indefinitely. Under less than ideal conditions, chemical changes in components, changes in initial environmental conditions, dilutions and changes in pH can lead to instability (Salager *et al.*, 2009; McClements, 2012).

Small surfactant molecules are best for stabilising interfacial tension between the oil phase and the aqueous phase, resulting in the thermodynamic stability that characterises microemulsions (McClements, 2012). The following are examples of applications of microemulsions in drug delivery:

- Microemulsions were shown to provide permeation enhancement for transdermal delivery through enhanced disruption of skin-lipid structures or by improving drug stability in formulations (Karande & Mitragotri, 2009).
- Microemulsions were investigated for topical delivery of anti-hypertensive drugs such as lacidipine (LCDP), a drug with poor water solubility and low bioavailability (Gannu *et al.*, 2010). Bioavailability studies in rabbits showed improved bioavailability of transdermal administration of a microemulsion gel compared to an oral suspension.
- Orally administered self emulsifying microemulsions containing vinpocetin and atorvastatin showed significant enhancement in bioavailability when compared to conventionally available tablets (Shen & Zhong, 2006).

2.3.1.2. Nanoemulsions

Nanoemulsions are defined as thermodynamically unstable colloidal dispersions. They consist of small spherical droplets of a liquid (dispersed phase) suspended within another immiscible liquid (dispersant) (Tadros, 2005; Lovelyn & Attama, 2011; McClements, 2012).

Nanoemulsions can be either oil-in-water or water-in-oil. Oil in water (o/w) nanoemulsions are defined as containing oil, water, a surfactant and occasionally a co-surfactant (McClements, 2012). O/w nanoemulsions are best suited for encapsulating, transporting and delivering lipophilic APIs in aqueous environments (Anton & Vandamme, 2009; Thiagarajan, 2011).

Because of their thermodynamic instability nanoemulsions do not form spontaneously (McClements, 2012). Energy is required to achieve dispersion. Emulsification is achieved by applying high-pressure homogenisers, high-shear stirring and/or ultrasound (Solans & Kunieda, 1996). Apparatus that supply energy in the shortest time and have the most homogeneous flow, produce the smallest particle sizes (Solans & Kunieda, 1996; McClements & Rao, 2011).

The following are examples of applications of nanoemulsions in drug delivery:

- Formulation of nanoemulsions containing risperidone for intra-nasal administration has been reported (Mistry *et al.*, 2009).
- Nanoemulsion formulations containing caffeine were developed for transdermal drug delivery. Comparison of *in vitro* skin permeability for respectively caffeine nanoemulsions and aqueous caffeine solutions displayed a significant increase in permeability for the caffeine nanoemulsion (Shakeel & Ramadan, 2010).
- Kelmann *et al.* (2007) developed a nanoemulsion formulation for Carbamazepine, an anticonvulsant drug with very poor water solubility.

2.4. Vesicular systems

Vesicular drug delivery systems are defined as highly ordered assemblies. They consist of one or more concentric bilayer formed due to self-assembly of amphiphilic molecules in the presence of water (Jain *et al.*, 2014).

Vesicular drug delivery systems are able to sustain drug action at a predetermined relatively constant rate because of zero order kinetics. Localisation of the drug action into the diseased tissue or organ can possibly be achieved through targeting of the drug delivery systems itself. This can be achieved through use of different carriers or chemical derivation and modification of components of the system (Jain, 2008).

The advantages of vesicular drug delivery systems can be summarised as follow (Jain *et al.*, 2014):

- Entrapment of both hydrophilic and hydrophobic drugs are possible
- Bioavailability of drugs can be improved.
- Metabolism of certain drugs can be delayed
- Circulation time of drugs in the body can be increased (a direct result of avoidance of metabolism)
- Targeted delivery of drugs can be achieved.

There are a wide variety of vesicular carriers, only a few which share common traits with systems investigated in the course of this study are highlighted in table 2.3.

Table 2.3. Examples of vesicular lipid drug delivery systems.

LIPOSOMES		
Characteristics and applications	Advantages	Disadvantages
- Colloidal, concentric bilayered vesicles with aqueous core entirely enclosed by bilayer membrane, mainly consisting of lipids. - Essential lipid components of liposomes include phospholipids and cholesterol. (Shashi <i>et al.</i>, 2012). - Applications include: Localised delivery of drugs (Agarwal <i>et al.</i>, 2001), topical treatment of fungal infections (Patel <i>et al.</i>, 2009), reduction of impact of anti-inflammatory medication on kidneys (Srinath <i>et al.</i>, 2000) and sustained release of diabetic medication (Divakar <i>et al.</i>, 2013).	- Liposomes can both deliver hydrophilic and lipophilic drugs. - Improved stability and protection of encapsulated drug from environment. - Reduced unnecessary exposure of tissues to drugs and their metabolites. - Liposomes can deliver both lower and high molecular weight drugs. - Improved pharmacokinetic properties due to reduced elimination and increased circulation life time (Himanshu <i>et al.</i>, 2011; Jadhav <i>et al.</i>, 2012; Akbarzadeh <i>et al.</i>, 2013).	- Liposomes are leaky by nature causing premature drug release. - Poor encapsulation efficiency for hydrophilic drug. - Expensive to prepare. - Possesses short half-life (Himanshu <i>et al.</i>, 2011; Akbarzadeh <i>et al.</i>, 2013).
TRANSFERSOMES		
Characteristics and applications	Advantages	Disadvantages
- Transfersomes are deformable, stress responsive vesicles containing an aqueous core surrounded by a bilayer of lipids. - Contains one natural amphiphilic lipid supplemented by a biocompatible surfactant. - Amphiphilic surfactants allow transfersomes to modify their membrane composition reversibly which provides their extreme flexibility. (Jain <i>et al.</i>, 2003; Gavali <i>et al.</i>, 2011; Prajapati <i>et al.</i>, 2011) - Applications include transdermal delivery of anti-inflammatory drugs (Duangjit <i>et al.</i>, 2010; Irfan <i>et al.</i>, 2012)	- Deformability allows penetration through the narrow pores of skin. - Both low and high molecular weight drugs are entrapped efficiently. - Protection of encapsulated drug from being metabolised. - Can be used for topical as well as systemic delivery of drugs (Jain <i>et al.</i>, 2003; Benson, 2006; Prajapati <i>et al.</i>, 2011).	- Chemical instability. - Preparation is very expensive (Prajapati <i>et al.</i>, 2011; Jain <i>et al.</i>, 2014).

COLLOIDOSOMES		
Characteristics and applications	Advantages	Disadvantages
• Used for delivery of proteins, vitamins, and food supplements. • Hollow shelled microcapsules consisting of coagulated particles at the interface of emulsion droplets. • Prepared by introducing colloidal particles into the continuous phase of w/o emulsions. Particles self-assemble at the interface and form a colloidal shell structure. • Applications include: encapsulation of enzymes, antimicrobial, antifungal and antiviral therapy as well as cosmetics and dermatology (Saraf <i>et al.</i>, 2011; Jain <i>et al.</i>, 2014).	• Size of colloidosomes easily controlled by controlling emulsion droplet size • Easy to prepare. • Good mechanical strength. • Preparation gentle enough to allow the encapsulation of materials such as biomolecules and cells without damage (Vyas & Khar, 2004; Saraf <i>et al.</i>, 2011).	• Yields are very poor • When transferred from organic to aqueous media, large amounts of colloidosomes are lost • Insufficient locking of shell leads to merging of colloidosomes (Vyas & Khar, 2004; Saraf <i>et al.</i>, 2011).

2.5. Pheroid technology

2.5.1. Introduction

Pheroid is a novel patented lipid-based drug delivery system (Grobler & Kotze, 2006; Grobler, 2008). It shares numerous characteristics with previously discussed lipid-based drug delivery systems: Pheroid is a colloidal system with a typical size distribution between 200 nm and 2 µm. The presence of Kolliphor EL, a non-ionic surfactant, causes self-assembly of Pheroid vesicles when the oil phase comes into contact with water, this is similar to the self-assembly of micelles. Pheroid vesicles consist of multiple bilayers, alternating the lipid and aqueous phases, a characteristic shared with vesicular systems.

Pheroid, however, may be considered more versatile than any of these individual systems as it can be formulated into a wider range of structures through manipulation of manufacturing parameters. It has the capacity to entrap, protect and deliver API's in biological systems. The formulations can be manipulated to accommodate hydrophilic, hydrophobic and amphiphilic molecules (Du Plessis *et al.*, 2010).

Three main types of Pheroid are identified, although variations are also possible (Grobler, 2009):

- Pro-Pheroid, a precursor to Pheroid vesicles, consisting of the fatty acid (oil), surfactant and gaseous components.
- Pheroid micro-sponges. The oil phase is manipulated by adding Incromega[®] E7010 (high in omega 3 fatty acid EPA) and Incromega[®] E3322 (high in ω-3 polyunsaturated fatty acids). This causes the formation of structures with crevices for API deposition
- Pheroid vesicles, depots or reservoirs that contain Pro-Pheroid as well as an aqueous phase.

2.5.2. Components and characteristics

Depending on the type of formulation Pheroid can consist of three components: an oil phase, aqueous phase and gaseous nitrous oxide (N₂O) phase. All types of Pheroid consist of at least two of the components: the oil phase and the gas phase.

2.5.2.1. The oil phase

Vitamin F ethyl ester is a combination of essential fatty acids that are esterified with ethanol. Fatty acid esters present are ethyl linoleate (25-50%), ethyl linolenate (25-50%) and ethyl oleate (10-25%). Estrification changes the form of the fatty acids to a *cis*-formation giving it the orientation of physiological fatty acids. According to the INCI (International Nomenclature Cosmetic

Ingredients) index it is alternatively identified as ethyl linoleate, ethyl lenolenate or ethyl oleat. In cosmetic formulations Vitamin F ethyl ester is used to treat dry skin and revitalise dull hair.

Vitamin F ethyl ester contains polyunsaturated essential free fatty acids, derived from plant oils with a high linoleic acid content. It is furthermore a lipid-soluble derivative of linoleic acid. Linoleic acid is an essential fatty acid and one of the most abundant polyunsaturated fatty acids in the western diet. Deficiencies in linoleic acid are linked to defective wound healing, growth retardation, and dermatitis (Collins *et al.*, 1971; Yamanaka *et al.*, 1980). F ethyl ester is used as oil phase in emulsions for intramuscular or subcutaneous administration (Johnson & Lloyd-Jones, 1987; Lovelyn & Attama, 2011).

DL- α -Tocopherol, also known as vitamin E, is another component of the oil phase. DL- α -Tocopherol is added as antioxidant to preparations that contain lipids to stabilize unsaturated oils against oxidation and extend shelf life (Burton & Ingold, 1981). In the cosmetic industry it is added as antioxidant to preparations containing lipids: creams, ointments, emulsions, body and face oils and cosmetics such as lipstick (Guaratini *et al.*, 2006). DL- α -Tocopherol also has applications in pharmaceutical preparations as an oil phase in emulsions for parenteral administration (Jeong *et al.*, 2006; Wasan, 2007).

Kolliphor[®] EL is a non-ionic surfactant that acts as solubiliser and emulsifier. It is useful for solubilising lipophilic substances such as vitamins A, D, E and K in aqueous solutions for oral administration (Reintjes, 2011). Kolliphor[®] EL is synthesized by reacting castor oil with ethylene oxide. Castor oil is a plant-derived oil which consists mainly of the glycerides of ricinoleic, isoricinoleic, dihydroxystearic and stearic acids in varying ratios. As a result the composition of Kolliphor EL can be widely varied depending on the castor oil origin (Uchegbu *et al.*, 2013).

Kolliphor EL is used for the solubilisation of many lipophilic drugs such as anaesthetics, sedatives and immunosuppressive agents (Berthelsen *et al.*, 2015). It can increase solubility up to a certain point, but when present in excess in preparations it retards or even prevents the formation of emulsion droplets. However, if the proportion of Kolliphor[®] EL is reduced to 5-10% of the water-insoluble substance, conditions exist for the formation of an emulsion (BASF, 2012). Kolliphor[®] EL can be added to either the aqueous or oil phase of emulsions and for Pheroid preparations it has been found effective when added to the oil phase (Uys, 2006).

2.5.2.2. The aqueous and N₂O phases

The aqueous phase of Pheroid usually consists of water, and can be altered through buffering and addition of salts that alter the ionic strength of the phase (Grobler, 2009). The aqueous phase makes up the bulk of Pheroid vesicles and micro sponges, and alterations in this phase can influence the

formation of the vesicles themselves. This can be used to manipulate Pheroid in terms of size and morphology for different applications (Grobler, 2009).

Nitrous oxide (N_2O) is a naturally occurring gas which is also commercially prepared through the decomposition of ammonium nitrate. N_2O has many applications: as oxidising agent in rocket fuels and internal combustion engines, as aerosol propellant in pressurised food products such as whipped cream as an inhalation anaesthetic and analgesic (Rosenberg *et al.*, 1979; Henderson, 1980; Zakirov *et al.*, 2001).

N_2O is also produced by cells in response to inflammation (MacMicking *et al.*, 1997; Mayer & Hemmens, 1997) and is therefore considered a biologically safe component of Pheroid already present in the body (Grobler, 2009).

2.5.3. Applications of Pheroid

The excellent properties of Pheroid technology as a drug delivery system has resulted in a number of applications of which a few are highlighted below.

2.5.3.1. Malaria treatment

Grobler (2014) and Grobler *et al.* (2014) investigated and evaluated the bioavailability of artemisone, an antimalarial drug, in combination with Pheroid in Vervet monkeys. Rapid absorption was observed and Pheroid technology increased the maximum artemisone serum concentration while significantly decreasing the time taken to reach said maximum.

Pheroid formulations containing mefloquine were also investigated. Results indicated that entrapping mefloquine in Pheroid might lead to decreased toxicity and improve its therapeutic index (Du Plessis *et al.*, 2014).

2.5.3.2. Treatment of tuberculosis

In vitro/in vivo studies involving Pheroid and rifampicin, an antibiotic used in the treatment of tuberculosis, were conducted (Grobler, 2009). Results showed an increase in drug plasma levels of rifampicin when combined with Pheroid. This was observed despite lower administration dosages. An increase in bioavailability; decreased time to reach the maximum serum concentration as well as larger therapeutic window were found. These results indicated that the combination of Pheroid with conventional tuberculosis treatment might make it possible to increase dosage intervals while decreasing administration dosages (Grobler, 2009).

2.5.3.3. Delivery of peptide drugs

Studies were conducted on various peptide drugs in combination with Pheroid:

- arginine vasopressin (antidiuretic) (Coetzee, 2007)
- insulin (De Bruyn, 2006; Oberholzer, 2009)
- calcitonin (blood calcium level regulating hormone) (Kotzé, 2005; Strauss, 2005; Du Plessis *et al.*, 2010)
- and recombinant human growth hormone (Steyn, 2006; Steyn *et al.*, 2010)

Administration routes of interest for these studies included peroral and intranasal routes. Topical applications were also investigated. A patent was registered for the delivery of peptide drugs using the peroral route (Grobler *et al.*, 2009).

2.5.3.4. Pheroid in conjunction with therapeutic or diagnostic radioactive compounds

Studies that used radiotracers to investigate the biodistribution of Pheroid brought attention to the potential of Pheroid as a delivery system for therapeutic or diagnostic radiopharmaceuticals (Swanepoel, 2015). ^{99m}Tc -MDP was used as radiotracer, and the Pheroid formulations were administered orally. Results showed that ^{99m}Tc -MDP, which is not absorbed through the gastrointestinal tract, was now being absorbed in sufficient quantities to make imaging possible (Swanepoel, 2015). A patent was registered for parenteral and oral administration of Pheroid containing radiopharmaceuticals (Grobler & Zeevaart, 2015). These results warranted further investigation, and are the base of this study.

2.6 Introduction: The use of radiotracers in medicine

A nuclide is an atom with a characteristic mass number (the total number of protons plus neutrons, symbol A), atomic number (number of protons in the nucleus, symbol Z) and nuclear energy state (Organization, 2006). All atoms from the same element have the Z, but when they have a different A they are considered isotopes (Ebbing & Gammon, 2010).

These isotopes can be either stable or unstable, depending on the relative numbers of protons and neutrons present. Nuclei with even numbers of protons or neutrons are generally more stable than those with odd numbers. An unstable atom nucleus may be caused by an excess of either neutrons or protons and is radioactive (Ebbing & Gammon, 2010; Zumdahl & DeCoste, 2012).

A radionuclide is a radioactive nuclide and similarly a radioisotope is a radioactive isotope. These terms (radionuclide and radioisotope) can be used interchangeably (Bain *et al.*, 2016)

A radiopharmaceutical consists of a radionuclide which is chemically attached to a chemical compound which acts as a targeting agent. Radiopharmaceuticals are drugs that are used in either diagnosis or therapy (Knapp & Dash, 2016). Radiopharmaceuticals used for imaging purposes contain small amounts of radionuclides, which are called radiotracers. (Cohn, 1968). Radio tracers are generally applied in the study of function, blood flow, metabolism and morphology of organs (Carlson, 1995).

George de Hevesy was the first scientist to recognise that radionuclides could be applied as tracers to follow the distribution of a compound containing an element in living organisms (Hevesy & Paneth, 1913). He observed the absorption and translocation of lead nitrate using ^{212}Pb as tracer (Hevesy, 1923). Radiolabeled tracers allow non-invasive measurement of distribution and function in a biological system. Radiopharmaceuticals are drugs that consist of two parts: a radionuclide that decays and a targeting molecule / organic ligand that determine the localization of the radiopharmaceutical.

Radiotracers can be used to obtain non-invasive imaging of diseases or as therapeutics to deliver treatment selectively to the site that requires it. Most of the development and optimization of radiopharmaceuticals revolve around designing or modifying the selectivity of a ligand for a radionuclide (Early, 1995; L'Annunziata, 2007; Maloth *et al.*, 2014).

Therapeutic radiopharmaceuticals contain radionuclides that decay to release α or β particles which can cause ionization and destroy tissue in e.g. a cancer site.

Diagnostic radiopharmaceuticals contain radionuclides that decay with enough energy to penetrate through body tissue and be detected externally while causing minimal undesired additional damage in tissue (Early, 1995).

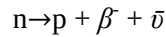
Traditionally, most radiopharmaceuticals were designed for either diagnostics or therapeutics. With the development of drugs that are target specific, the same targeting biomolecule or organic ligand can be used for both imaging and therapy. Such drugs are labelled “theranostics” (Early, 1995; L'Annunziata, 2007; M Rey, 2010).

2.7. $^{99\text{m}}\text{Tc}$ -MDP: a gamma emitting radio pharmaceutical / radiotracer

2.7.1. Concepts related to radioactivity

There are a total of 118 elements known today (Ts, 2005; Oganessian *et al.*, 2006; Ebbing & Gammon, 2010; Oganessian *et al.*, 2010). Of these $Z = 1$ to 92 (Z representing the atomic number) occur naturally, whereas the others are artificially produced (Ebbing & Gammon, 2010). In general, the elements with a lower Z tend to be stable compared to elements with higher Z which are radioactive (Welch & Redvanly, 2003). Structural arrangements and binding energies of nucleons

determine the stability of nuclides. As the number of particles inside an atom's nucleus increase, the forces keeping the particles together become less effective and increase the chances of particle emission (L'Annunziata, 2007; Saha, 2010). One criterion of stability is the neutron-to-proton ratio (N/Z) of the stable nuclides; radionuclides decay to achieve the N/Z of the nearest possible stable nuclide. When a nucleus has a higher N/Z ratio compared to the stable nucleus, it decays by β^- particle emission along with an antineutrino ($\bar{\nu}$), an almost mass less entity. Antineutrinos conserve energy during decay. In β^- decay, a neutron (n) decays into a proton (p) and β^- particle (Saha, 2010):



Radioactive decay by particle emission or electron capture changes the atomic number of the radionuclide, whereas decay by γ -ray emission does not. Many radioactive nuclides undergo successive transformations in which the original nuclide, called the parent, gives rise to a radioactive product nuclide, called the daughter (Krane, 1987; L'Annunziata, 2007; Saha, 2010).

The β^- particle can be emitted with energy varying from zero up to the decay energy (measured in electron volts (eV)). The decay energy is defined as the difference in energy between the parent and daughter nuclides. β^- decay may be followed by γ -ray emission, if the daughter nuclide is in an excited metastable state, the number of γ -rays emitted depends on the excitation energy (Krane, 1987; Saha, 2010).

An example of this behaviour is found in the decay of $^{99}_{42}\text{Mo}$ to $^{99m}_{43}\text{Tc}$, which in turn decays to Tc-99:

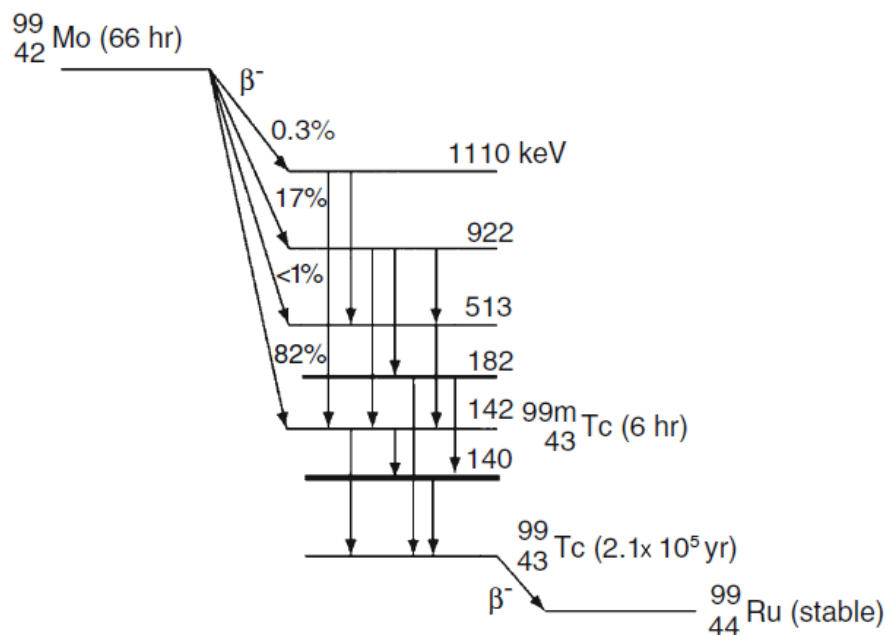
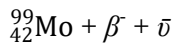


Figure 2.2. Schematic representation of the decay of ^{99}Mo to ^{99}Ru via ^{99m}Tc (Saha, 2010).

2.7.2. ^{99m}Tc in nuclear medicine

Imaging through the use of radionuclides is divided into two categories based on the type of decay of the nuclide as well as the resulting emission and therefore method of detection. Two recognised categories are positron emission tomography (PET) and single photon emission computed tomography (SPECT) (Brooks, 2005). SPECT is still the most common diagnostic method and utilises radionuclides emitting photons between 70 and 360 keV. Diagnostic imaging requires radiation that can penetrate the body and be detected by external instruments. The emitted energy needs to be higher than 50 keV to escape the body, but still low enough to not interact with or change tissue (Baum, 2014). Radionuclides that emit γ -rays or annihilation photons from positrons β^+ without additional α or β^- particle emissions are suitable. Radionuclides that decay with γ emission suitable for imaging include Gallium-67 (^{67}Ga), Iodine-123 (^{123}I), Indium-111 (^{111}In), Technetium-99m (^{99m}Tc) and Thallium-201 (^{201}Tl) (Mattos *et al.*, 2001; Brooks, 2005; Baum, 2014)

^{99m}Tc has favourable nuclear properties that make it an excellent radionuclide for diagnostics:

Single 140 keV photon emission, the absence of particle emission, a half-life of approximately 6 hrs and the availability via portable $^{99}\text{Mo}/^{99m}\text{Tc}$ generators (Baum, 2014). The use of ^{99m}Tc is further simplified through the availability of easy-to-use kits that allow for preparation at the hospital where it will be administered.

In 1960 early research demonstrated the use of ^{99m}Tc in medicine through means of tissue counting (Richards *et al.*, 1982). The uptake of pertechnetate into the thyroid was also reported. This led to the suggested use of ^{99m}Tc as a radiotracer (Richards, 1960; Richards *et al.*, 1982).

The advances in medical applications of ^{99m}Tc that followed these discoveries led to the development of ^{99m}Tc radiopharmaceuticals for various applications. Examples are summarised in table 2.4.

*Table 2.4 Radiopharmaceuticals as well as first registered trade names and applications. (Subramanian *et al.*, 1975; Schwachau, 1994; Banerjee *et al.*, 2001; Welch & Redvanly, 2003; Lever & Lever, 2009)*

Radiopharmaceutical	Short form	Clinical use
^{99m}Tc -Phytate	^{99m}Tc -Phy	Liver imaging
^{99m}Tc Sulphur Colloid	$^{99m}\text{TcS/C}$	Liver and spleen imaging
^{99m}Tc Mebrofenin	^{99m}Tc -mebro	Hepatobiliary function
^{99m}Tc Methylene diphosphonate	^{99m}Tc -MDP	Bone Scan
^{99m}Tc Red Blood Cells	^{99m}Tc -RBC	Cardiac Function and Blood Pool Scans

Radiopharmaceutical	Short form	Clinical use
^{99m}Tc Mibi OR ^{99m}Tc Sestamibi With Mibi or Sestamibi being an abbreviation for hexa (methoxyisobutylisonitrile)	^{99m}Tc -MIBI	Myocardial Perfusion (Heart Muscle Blood Flow)
^{99m}Tc Diethylene Triamine Penta Acetic Acid	^{99m}Tc -DTPA	Renal Function
^{99m}Tc Ethylene di cysteine	^{99m}Tc -EC	Renal tubular function
^{99m}Tc Glucoheptonate	^{99m}Tc -GHA	Kidney imaging
^{99m}Tc Ethyl cystienate dimer	^{99m}Tc -ECD	Brain Scan and Scans for Infection

^{99m}Tc -MDP is exclusively administered intravenously and γ - rays emitted during the decay of the radioisotope are used to capture images by means of planar γ imaging or SPECT/CT scans (single photon emission computerised tomography–computed tomography) (Basu & Alavi, 2016). A region of higher isotope concentration yields higher γ - ray emission. As the MDP complex has the highest affinity for bone tissue with increased metabolic activity, this is a good indication of increased bone activity, which in turn indicates new bone growth. Increased metabolic activity in skeletal tissue is indicative of healing fractures, bone growth around implants or bone growth due to tumour formation (McCracken *et al.*, 2001). Following intravenous administration ^{99m}Tc -MDP is deposited on bone primarily through adsorption onto or into the crystalline structure of hydroxyapatite. After administration, 80% of the ^{99m}Tc -MDP is generally cleared from the body after 3 hrs, whereas between 10 and 20% is found adsorbed to bone tissue (Blake *et al.*, 2009).

^{99m}Tc -containing tracers also need to be administered within a short time after preparation of kits due to the rate of decay of the system. $^{99m}\text{Tc} \rightarrow ^{99}\text{Tc} + \gamma$ has a half-life of approximately 6 hrs (McAfee *et al.*, 1964; Lever & Lever, 2009). Bone scans are also typically performed 3-4 h after administration. Too early imaging is not useful as the ^{99m}Tc -MDP must pass through soft tissue to reach the bone for optimal bone accumulation. Imaging too late will result in suboptimal images due to decay of the ^{99m}Tc .

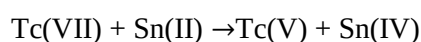
^{99m}Tc -MDP (Technetium-99m methylene diphosphonate or medronic acid) is a radiopharmaceutical with ^{99m}Tc as radionuclide. ^{99m}Tc -MDP is used for skeletal imaging as it mostly accumulates in bone with high growth rates (Subramanian *et al.*, 1975; Kung *et al.*, 1978; Blake *et al.*, 2011).

These bone scans are possible through imaging of γ - rays emitted from the radioactive ^{99}Tc isotope and provide images of the metabolic activity of the skeleton (Budd *et al.*, 1989; Krasnow *et al.*, 1997; McCracken *et al.*, 2001).

2.7.3. ^{99m}Tc -MDP characteristics and chemistry

Information regarding ^{99m}Tc MDP was first published in 1971 (Subramanian & McAfee, 1971). Currently ^{99m}Tc -MDP is used for routine bone-scans in operational hospitals globally. Conveniently pre-prepared MDP kits are commercially available. These contain methylene diphosphonic acid (MDP) or a corresponding sodium salt, a reducing agent such as Sn(II) chloride (stannous chloride dihydrate, $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$), and a stabilising agent such as 2,5 di-hydroxybenzoic acid (DHBA) or ascorbic acid (Lever & Lever, 2009). ^{99m}Tc is eluted from a $^{99}\text{Mo}/^{99m}\text{Tc}$ generator using saline (0.9% m/v NaCl), the eluate contains saline and pertechnetate ($^{99m}\text{TcO}_4^-$) (Steigman & Eckelman, 1992).

When the eluate is added to the commercial kits, Sn (II) reduces Tc(VII) to Tc(III) while being oxidised to Sn (IV):



SnCl_2 is very hard to purify, and commercial products contain at least 5% Sn(IV). Furthermore, Sn(II) is very easily oxidised to Sn(IV) which could be problematic as reduction of Tc(VII) is a prerequisite for the formation of pure, high yield ^{99m}Tc complexes (Lever & Lever, 2009). To ensure that enough Sn(II) is present to reduce the pertechnetate an excess of Sn(II) is present. Ratios of SnCl_2 to $^{99m}\text{TcO}_4^-$ are very high, in the order of 10^8 to 10^9 (Richards & Steigman, 1973). In order to preserve Sn(II) the kits are lyophilized and sealed under an oxygen-free atmosphere.

Apart from acting as reducing agent, Sn (II) also forms Sn(II)diphosphonate complexes, hereafter referred to as Sn-MDP (Claessens & Kolar, 2000). Typically, very little of the Sn(II) in kits is present as free ions in radiopharmaceutical solutions, most of it is complexed with a ligand or as colloidal tin aggregates (Steigman & Eckelman, 1992). An excess of coordinating ligand (MDP) is present in kits to ensure technetium complex formation and to minimize hydrolysis reactions (formation of tin hydroxides and technetium dioxide colloid). MDP does not only serve as chelating agent, but it also binds to Ca^{2+} in HAP, giving the radiopharmaceutical its bone targeting properties (Libson *et al.*, 1980; Ogawa & Saji, 2011)..

Thermodynamic stability of ^{99m}Tc complexes is important for safety and efficacy of radiopharmaceuticals (Cole *et al.*, 1987; Moi *et al.*, 1990; Banerjee *et al.*, 2001; Mazzi *et al.*, 2007). Thermodynamic stability refers to a system in its lowest possible energy state, implying that the most stable product has been formed (Ebbing & Gammon, 2010). The formation of ^{99m}Tc -MDP is thermodynamically favoured over the formation of Sn-MDP, which is why such a high percentage of ^{99m}Tc (in excess of 90%) is complexed with MDP (Lever & Lever, 2009). The exact structure(s) of the ^{99m}Tc -MDP complex has not been elucidated, although more than one complex is probably

formed, amongst which polymeric complexes. The reducing agent (Sn^{2+}) might also be incorporated into the complexes, complicating the structure even further (Jones & Thornback, 2007). Figure 2.3. shows one of the first proposed structures for $^{99\text{m}}\text{Tc}$ -MDP bound to Ca^{2+} found in HAP.

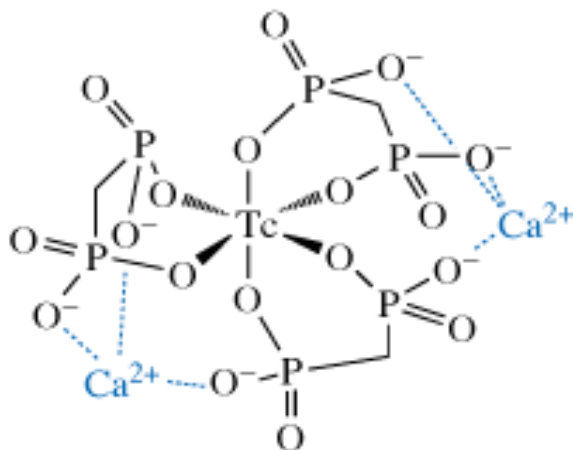


Figure 2.3. Chemical structure of the $^{99\text{m}}\text{Tc}$ -MDP complex (Subramanian & McAfee, 1971).

2.8 Radiotracers in lipid based drug delivery systems

Most solid tumours have a vascular pore cut-off size between 380 and 780 nm, although it is also tumour type, growth rate and microenvironment dependent (Hobbs *et al.*, 1998; Wu *et al.*, 1998). Therefore, the size range of a drug delivery system with encapsulated radiopharmaceuticals should ideally lie within or below that range.

For successful passive targeting, the delivery system needs to circulate in the blood for as long as possible, increasing the probability of the carrier passing the tumour site. This is achieved by having particle surfaces which are “invisible”, in order to avoid responses from the body. Hydrophilic polymers such as polyethylene glycol (PEG) are commonly used for this purpose because of a low degree of immunogenicity (ability to provoke an immune response) and antigenicity (capacity of a chemical structure to bind to antibodies) and chemical inertness of the polymer backbone (Tao & Zhang, 2005).

PEG-grafted liposomes, in the size range of 70 to 200 nm, displayed extended circulation half-lives of 15 to 24 hours in rodents and up to 45 hours in humans, whereas non-PEGylated liposomes had half-lives of only 2 hours (Moghimi *et al.*, 2001; Koo *et al.*, 2005).

Imaging agents associated with lipid delivery systems can also target sites that have compromised barrier function and increased permeability due to pathophysiology (conditions including but not limited to cancer) (Koo *et al.*, 2005; Attama *et al.*, 2012).

Koo *et al.* (2005) described how delivery systems can be functionalized to accommodate imaging agents. Alternatively the targeted system (imaging agent) can be encapsulated. This could enhance administration capacity with consequent release from the delivery system allowing the imaging agent to reach the targeted area. This however, is the subject of future *in vivo* investigations.

Studies conducted by Swanepoel (2015) encapsulating ^{99m}Tc -MDP into Pheroid showed imageable absorption levels could be achieved through entrapment in Pheroid and subsequent oral administration instead of intravenous injection. These results are the basis of this study: optimisation of the formulation parameters necessary to allow expedient oral administration of Pheroid entrapped ^{99m}Tc -MDP.

2.9. The use of hydroxyapatite as model for bone tracing models

2.9.1. Structure and medical applications of hydroxyapatite

Hydroxyapatite (hereafter referred to as HAP) is a calcium phosphate complex similar to human bone apatite in morphology and composition (Wei & Ma, 2004). HAP has a stoichiometric Ca/P ratio of 1.67, which is identical to bone apatite (Guo *et al.*, 2003; Kalita *et al.*, 2007; Teixeira *et al.*, 2009).

In comparison to other calcium phosphates, HAP is very stable. Thermodynamically, hydroxyapatite is the most stable calcium phosphate compound under physiological conditions such as temperature, pH and composition of body fluids (Kalita *et al.*, 2007).

In addition to its stability HAP is also very biocompatible, which has led to numerous medical applications (Chevalier & Gremillard, 2009; Huang *et al.*, 2010). HAP is effectively used to cover titanium and stainless steel implants, tricking the body and reducing implant rejection rates. HAP can also be used to treat bone defects through a process which places powders, blocks or beads of HAP in the affected areas of bone. This procedure provides an alternative to bone grafts and shortens the healing time (Ben-Nissan *et al.*, 2015).

2.9.2. Use of HAP to quantify adsorption of chemicals that exhibit affinity to bone

Jansen *et al.* (2009) described how HAP provides an *in vitro* model for measuring the affinity and adsorption capabilities of ^{117m}Sn -based bone-seeking radiotracers (Claessens & Kolar, 2000; Jansen *et al.*, 2009). The adsorption and desorption of Sn^{4+} onto HAP was studied by incubating Sn^{4+} -PEI-MP, a bone tracing radiopharmaceutical, on HAP. The supernatant was filtered after a specified time and the amount of Sn^{4+} on the HAP and in the supernatant was measured respectively. Since HAP is a main component of human bone, it follows that measurements of affinity of a chemical

towards HAP might be relevant to actual bone (Claessens & Kolar, 2000). Jansen *et al.* (2009) demonstrated that HAP can be used to quantify the chemisorptions of bone tracers.

For the purposes of this study the HAP model is used, uniquely, to determine the degree of ^{99m}Tc -MDP entrapment in Pheroid. This study aims to use the affinity of ^{99m}Tc -MDP for HAP to indirectly quantify the amount of ^{99m}Tc -MDP associated with or entrapped in Pheroid formulations. ^{99m}Tc -MDP in Pheroid formulations will be incubated on HAP, and the activity on both the HAP and in the supernatant was measured to determine % entrapment efficiencies.

In a system absent from biological influences, the free (or un-entrapped) ^{99m}Tc -MDP will be adsorbed onto HAP since ^{99m}Tc -MDP displays a high affinity for HAP. The remaining, unabsorbed portion may be considered successfully entrapped within the Pheroid active ingredient delivery system.

In the absence of metabolic activity it is postulated that ^{99m}Tc -MDP will exhibit such a high affinity for HAP that the portion of bone tracer not adsorbed onto HAP may be considered successfully entrapped within the Pheroid active ingredient delivery system.

It should be noted that strictly speaking the HAP adsorption model is a chemical model, rather than an *in vitro* model since *in vitro* assumes that biological conditions are mimicked (Vignais & Vignais, 2010). This model is simplified and omits biological parameters such as metabolism, interactions with components of blood and immunological responses.

2.10. Conclusions

The aim of this study was to prepare optimised Pheroid in terms of entrapment of ^{99m}Tc -MDP within the shortest time possible. This study however does not concern itself with the actual envisaged application (oral administration) which relies on biological systems for the release of ^{99m}Tc -MDP into the blood. This will require *in vivo* investigation and will be the subject of future studies.

2.11. References

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Chapter | 3

3.1. Introduction

The preparation and characterisation of Pheroid-MDP formulations are described in this chapter. Pheroid formulation parameters were varied to achieve optimal entrapment of MDP within Pheroid. The formulations were optimised with regard to time taken to entrap the MDP, the shortest possible time showing substantial entrapment being the most optimal. Formulation variations included different types of Pheroid, different ratios of Pheroidal components, addition of the MDP during different stages of formulation and buffering of the formulations at different pH. Characterisation methods for these formulations included particle size distribution measurements, ζ -potential and pH measurement, as well as confocal laser microscopy.

This study can be described as an eliminating process. A number of formulation possibilities were identified, and these formulations were then prepared. After preparation, formulations were evaluated in terms of particle size distribution and ζ -potential over a 24 hr. timeframe.

Based on results from this analysis the formulations that showed the most stable behaviour in the shortest time were selected for entrapment studies. Entrapment of the ^{99m}Tc -MDP in the Pheroid formulations was determined by incubating the formulations on HAP using methods described in literature (Claessens & Kolar, 2000).

Formulations that showed the best entrapment in the shortest time were then further investigated by means of confocal microscopy. The following aspects were investigated: morphology of vesicles, particle size distribution and association with / entrapment of Sn-MDP within the formulations.

This chapter will describe the instrumentation, methods and techniques used. The following topics will be addressed:

- The formulation of Pheroid vesicles and sponges
- The formulation of Pheroid vesicles and sponges containing Sn-MDP as well as Pheroid vesicles and sponges containing ^{99m}Tc -MDP
- Methods used to characterise Pheroid-MDP formulations
- Entrapment studies using HAP model
- Visualization of formulations by means of Confocal Laser Scanning Microscopy (CLSM)

3.2. Formulation of Pheroid

The first and third objectives of this study (as described in chapter 1) were to formulate Sn-MDP (non-radioactive) as well as ^{99m}Tc -MDP (radioactive) in combination with different types of Pheroid and with varying formulation parameters. Certain factors had to be taken into account when possible formulations were identified:

- MDP is a water-soluble compound, and should ideally be in a formulation that contains an aqueous component. Furthermore, ^{99m}Tc -MDP will always be presented in a saline solution as the Tc is eluted from the generator using medical saline.
- The size and morphology of Pheroid structures can be manipulated by altering the pH and ionic strength, and therefore the influence of pH and pH buffering on formulation characteristics need to be investigated.

Based on envisaged future use of this formulation in a hospital/clinical setting, the following aspects were considered:

- The formulation must be easily prepared by medical personnel with little or no formulation background.
- If the Pheroid should be prepared at the site of use, available resources need to be considered e.g. N_2O - H_2O is not freely available in a hospital. Current preparations of radio-tracer administrations utilize pre-prepared saline solutions widely available in this setting, therefore the possibility of formulating Pheroid with saline as aqueous phase was also investigated.
- MDP kits were received from NTP Radioisotopes SOC, and constituted (mixed) on site, the possibility of a pre-made appropriate Pheroid formulation which is then combined with the radio-active ^{99m}Tc -MDP solution was also considered.

The following section addresses the stated objectives of this study.

3.2.1. Materials

Vitamin F ethyl ester was purchased from Croda, South Africa, Kolliphor EL was purchased from BASF, South Africa, dL- α -tocopherol was purchased from Chempure, South Africa, Incromega[®] E7010 and Incromega[®] E3322 was purchased from Croda, South Africa and nitrous oxide was supplied by Afrox and used without further purification. MDP kits are manufactured by NTP Radioisotopes SOC and were gifted by Necsa.

The abbreviations provided in table 3.1 will be further used when describing different formulations.

Table 3.1. Abbreviations used when describing formulations

Abbreviation	Meaning	Abbreviation	Meaning
H_b	Before homogenising	V-A	Vesicles, H ₂ O aqueous phase
H_d	During homogenising	V-NS	Vesicles, Normal saline aqueous phase
H_a	After homogenising	V_i -MDP- H_b -A	Vesicles, H ₂ O-N ₂ O aqueous phase, MDP added before homogenising
VO_a	Vortexed after	V_i -MDP- H_b -NS	Vesicles, Normal saline aqueous phase, MDP added before homogenising
V	Vesicles	V_i -MDP- H_d -A	Vesicles, H ₂ O-N ₂ O aqueous phase, MDP added during homogenising
V_i	Vesicles, used immediately after manufacturing	V_i -MDP- H_d -NS	Vesicles, Normal saline aqueous phase, MDP added during homogenising
V_{4d}	Vesicles, used 4 days after manufacturing	V_i -MDP- H_a -A	Vesicles, H ₂ O-N ₂ O aqueous phase, MDP added after homogenising
V_{pH4}	Vesicles, buffered at pH = 4	V_i -MDP- H_a -NS	Vesicles, Normal saline aqueous phase, MDP added after homogenising
V_{pH7}	Vesicles, buffered at pH = 7	V_{pH4} -MDP- H_d -NS	Vesicles buffered at pH 4, Normal saline aqueous phase, MDP added during homogenising
V_{pH10}	Vesicles, buffered at pH = 10	V_{pH7} -MDP- H_d -NS	Vesicles buffered at pH 7, Normal saline aqueous phase, MDP added during homogenising
$V_{1:2.8(4d)}$	Vesicles, oil ratio of Vit. F to Kolliphor EL 1:2.8, 4 days after manufacturing.	V_{pH10} -MDP- H_d -NS	Vesicles buffered at pH 10, Normal saline aqueous phase, MDP added during homogenising
$V_{1:1(4d)}$	Vesicles, oil ratio of Vit. F to Kolliphor EL 1:1	$V_{1:2.8(4d)}$ -MDP- VO_a	Vesicles, oil ratio of Vit. F to Kolliphor EL 1:2.8, 4 days after manufacturing. Vortexed after addition of MDP.
$V_{2.5:1.3(4d)}$	Vesicles, oil ratio of Vit. F to Kolliphor EL 2.5:1.3	$V_{1:1(4d)}$ -MDP- VO_a	Vesicles, oil ratio of Vit. F to Kolliphor EL 1:1, 4 days after manufacturing. Vortexed after addition of MDP.
$V_{2.8:1(4d)}$	Vesicles, oil ratio of Vit. F to Kolliphor EL 2.8:1	$V_{2.5:1.3(4d)}$ -MDP- VO_a	Vesicles, oil ratio of Vit. F to Kolliphor EL 2.5:1.3, 4 days after manufacturing. Vortexed after addition of MDP.
S_{4d}	Sponges, used 4 days after manufacturing	$V_{2.8:1(4d)}$ -MDP- VO_a	Vesicles, oil ratio of Vit. F to Kolliphor EL 2.8:1, 4 days after manufacturing. Vortexed after addition of MDP.
NS	0.9% Normal saline	S_{4d} -MDP- H_a	Sponges, 4 days after manufacturing. Vortexed after addition of MDP.
A	H ₂ O-N ₂ O	V_{4d} -MDP- VO_a	Vesicles, 4 days after manufacturing. Vortexed after addition of MDP.

The following tables show components of the different Pheroid formulations.

Table 3.2 .Components of V-A and V-NS

Component	m/m%
Vitamin F ethyl ester	2.8 %
KolliphorEL	1.0 %
DL- α -Tocopherol	0.2 %
N ₂ O-H ₂ O OR N ₂ O-Saline (0.9% m/v)	96%

Table 3.3. Components of V_i-MDP-H_b-A, V_i-MDP-H_b-NS, V_i-MDP-H_d-A, V_i-MDP-H_d-NS, V_i-MDP-H_a-A and V_i-MDP-H_a-NS.

Component	m/m%
Vitamin F ethyl ester	2.8 %
KolliphorEL	1.0 %
DL- α -Tocopherol	0.2 %
N ₂ O-H ₂ O OR N ₂ O-Saline (0.9% m/v)	76%
Sn/ ^{99m} Tc-MDP in saline	20%

Table 3.4. Components of V_{pH4}-MDP-H_d-NS, V_{pH7}-MDP-H_d-NS and V_{pH10}-MDP-H_d-NS.

Component		m/m%
Vitamin F ethyl ester		2.8 %
KolliphorEL		1.0 %
DL- α -Tocopherol		0.2 %
N ₂ O-Saline (0.9% m/v)		66%
MDP in saline		20%
Buffer	pH 4 = Potassium Hydrogen Phthalate solution	10%
	pH 7 = Potassium dihydrogen phosphate, Sodium hydroxide	
	pH 10 = Sodium carbonate monohydrate, Sodium bicarbonate	

Table 3.5. Components of $V_{1:2.8(4d)}$ -MDP-VO_a, $V_{1:1(4d)}$ -MDP-VO_a, $V_{2.5:1.3(4d)}$ -MDP-VO_a and $V_{2.8:1.(4d)}$ -MDP-VO_a.

Component	m/m%			
Vitamin F ethyl ester	0.84 %	1.57%	2.07%	2.3 %
KolliphorEL	2.3 %	1.57%	1.07%	0.84 %
DL- α -Tocopherol	0.2 %			
N ₂ O-H ₂ O	76.32%			
MDP in saline	16.7%			

Table 3.6. Components of S_{4d} -MDP-H_a.

Component	m/m%
Vitamin F ethyl ester	2.3 %
KolliphorEL	0.84 %
DL- α -Tocopherol	0.17 %
Incromega E7010	0.21 %
Incromega E3322	0.21 %
N ₂ O-H ₂ O	79.57 %
MDP in saline	16.7%

Table 3.7. Formulations selected for entrapment efficiency study

	Type of formulation	Aqueous phase	Incorporation of MDP into formulation	Additional considerations
a.	V_i -MDP-H _b -NS	N ₂ O-Saline (0.9% m/v)	Added to aqueous phase before mixing with oil phase	None
b.	V_i -MDP-H _b -NS	N ₂ O-Saline (0.9% m/v)	Added to aqueous phase before mixing with oil phase	Agitated for 2 hrs. before incubation
c.	V_i -MDP-H _d -NS	N ₂ O-Saline (0.9% m/v)	Added to aqueous phase during mixing with oil phase	Agitated for 2 hrs. before incubation
d.	Pheroid- ^{99m} Tc-MDP vesicles	N ₂ O-H ₂ O	Added to aqueous phase before mixing with oil phase	None
e.	Pheroid- ^{99m} Tc-MDP vesicles	N ₂ O-H ₂ O	Added to formulation 4 days after manufacturing, vortexed for 1 minute to incorporate	None
f.	Pheroid- ^{99m} Tc-MDP vesicles: Vit. F ethyl ester: KolliphorEL ratio of 1:1	N ₂ O-H ₂ O	Added to formulation 4 days after manufacturing, vortexed for 1 minute to incorporate	None
g.	Pheroid- ^{99m} Tc-MDP micro sponges	N ₂ O-H ₂ O	Added to formulation 4 days after manufacturing, vortexed for 1 minute to incorporate	None
h.	Control	Saline	^{99m} Tc-MDP in saline	None

3.2.2. Methods

Unless stated otherwise all formulations were prepared using a Heidolph Diax 600 homogeniser (Labotec, South Africa) at 13500 rpm for at least four min or until the formulation temperature was below 40°C.

3.2.1.1. Preparation of MDP kits

Sn-MDP solutions were prepared by adding 6 mL 0.9% saline to a lyophilised vial of MDP. Under normal circumstances the volume of the saline does not matter, the quantity added correlates to the activity required. For the purposes of this study, and to simplify formulation, a constant volume of 6 mL was added to each vial. Standard procedures for preparation of MDP kits are as follows: add the saline to the vial and swirl for a minute. Thereafter the vial should stand for 10 min before being used.

3.2.1.2. Preparation of oil phase for V_i -MDP-H-A and V_i -MDP-H-NS

Vitamin F ethyl ester, Kolliphor EL and dL- α -tocopherol with ratios 2.8: 1: 0.2 were heated to 70 °C and mixed thoroughly. Known volumes (1 mL) of the mixture were weighed at room temperature (25 °C) to determine the density.

Table 3.8. Density of “standard oil phase”.

Volume	Mass	Density
1 mL	0.8717 g	0.8717 g/mL
1 mL	0.8909 g	0.8909 g/mL
1 mL	0.8912 g	0.8912 g/mL
AVERAGE	0.8846 g	0.8846 g/mL

The “standard oil phase” was therefore found to have an average density of 0.8846 g/mL. The purpose of determining the density was to increase efficiency of the preparation of small volume formulations. Weighing of individual components for small formulation volumes (10-50 ml) allows for continuous introduction of large errors. The systematic incorporation of human error was thus minimized by measuring and preparing, stock formulations of varying composition. These preparations were used within 1 month of formulation; where after a fresh batch was prepared. When used in formulations a single volume of the oil mixture was measured out and used. The oil component comprised 4% m/v of the Pheroid vesicle formulations, thus for 10 mL Pheroid the volume needed would be calculated as follows:

$$\begin{aligned}\text{Volume} &= \frac{\text{Mass}}{\text{Density}} \\ &= \frac{0.4\text{g}}{0.8846\text{g/mL}} \\ &= 0.45\text{ mL}\end{aligned}$$

3.2.1.3. V-A and V-NS

N₂O-H₂O or N₂O-Saline (9.6 mL) was heated to 70 °C. The pre-mixed oil phase (4% m/v, 0.45 mL) was also heated to 70 °C. The oil phase was then added to the aqueous phase and homogenised.

3.2.1.4. Preparation of *V_i-MDP-H_b-A*, *V_i-MDP-H_b-NS*, *V_i-MDP-H_d-A*, *V_i-MDP-H_d-NS*, *V_i-MDP-H_a-A* and *V_i-MDP-H_a-NS*.

N₂O-H₂O or N₂O-Saline (7.6 mL) was heated to 70 °C. The pre-mixed oil phase (4% m/v, 0.45 mL) was also heated to 70 °C. The oil phase was then added to the aqueous phase and agitated. Different methods of incorporating the MDP into the formulation were used:

1. Adding the MDP in saline (2 mL) to the aqueous phase before homogenising.
2. Adding the MDP in saline (2 mL) to the mixture while homogenising
3. Adding the MDP in saline (2 mL) to the mixture directly after homogenising.

3.2.1.5. Preparation of *V_{pH4}-MDP-H_d-NS*, *V_{pH7}-MDP-H_d-NS* and *V_{pH10}-MDP-H_d-NS*

N₂O-Saline (6.6 mL) and buffer solution (1 mL) were added together and heated to 70 °C. The pre-mixed oil phase (4% m/v, 0.45 mL) was also heated to 70 °C. The oil phase was then added to the aqueous phase and homogenised. MDP in saline (2 mL) was added to the mixture during the homogenisation process.

3.2.1.6. Preparation of *V_{1:2.8(4d)}-MDP-VO_a*, *V_{1:1(4d)}-MDP-VO_a*, *V_{2.5:1.3(4d)}-MDP-VO_a* and *V_{2.8:1(4d)}-MDP-VO_a*.

MDP was added to stable selected formulations four days after manufacturing. Uys (2006) found that formulations are fully stabilized only four days after manufacturing. Since this is indicated as the minimum time required to stabilize a formulation it was chosen for further experiments.

The different Vitamin F ethyl ester: KolliphorEL ratios were varied as follows:

1. 1 : 2.8 (1g : 2.8 g)
2. 1 : 1 (1.9 g: 1.9 g)
3. 2.5 : 1.3 (2.5 g : 1.3 g)
4. 2.8 : 1 (2.8 g : 1 g)

N₂O-H₂O (96 mL) was heated to 70°C. Vitamin F ethyl ester and Kolliphor EL was heated to 70°C. After removing from heat DL- α -Tocopherol (0.2 g) was added to the Vitamin F ethyl ester and KolliphorEL mixture. The antioxidant component, Tocopherol (Vit E) was kept constant throughout this experimental series. The oil phase was then added to the aqueous phase and homogenised. After homogenising the formulation was left under N₂O (200kPa) for four days. MDP in saline (2mL) was added to 10 mL of the prepared vesicles and vortexed for 1 minute.

3.2.1.7. Preparation of *S_{4d}*-MDP-*H_a*

N₂O-H₂O (95.5 mL) was heated to 70 °C. Vitamin F ethyl ester (2.8 g), Kolliphor EL (1 g), Incromega E7010 (0.25 g) and Incromega E3322 (0.25 g) was heated to 70 °C. After removing from heat DL- α -Tocopherol (0.2 g) was added to the oil mixture. The oil phase was then added to the aqueous phase and homogenised. The mixture was homogenised with a Heidolph Diax 600 homogeniser (Labotec, South Africa) at 13500 rpm for at least 4 min or until below 40°C. After homogenising the formulation was left under N₂O (200kPa) for four days. MDP in saline (2mL) was added to 10 mL of the prepared sponges and vortexed for 1 min.

3.3. Characterisation of formulations

The second objective of this study was to characterise all Pheroid-MDP formulations in terms of particle size distribution, ζ - potential, pH and microscopy.

ζ - Potential was a chosen parameter since it is a known indicator of stability in colloidal systems. (Hunter, 2013)

Grobler *et al.* (2008) found changing pH of the aqueous phase of Pheroid formulations could influence the structural and functional properties. pH is therefore a parameter that can possibly influence entrapment. pH was monitored to investigate whether the presence of MDP caused any changes which could be related to entrapment.

Microscopy offers the opportunity to investigate the morphology of formulations. The presence of MDP in the formulations could potentially change the morphology of the formulation, and microscopy was used to visualize any changes.

3.3.1. Particle size distribution measurements

Dynamic light scattering (DLS) is an important experimental technique in both research and industry because of its ease of use and inexpensive sample preparation capability (Bryant & Thomas, 1995). DLS has become a powerful technique for studying the properties of suspensions and solutions of colloids, biological solutions, macromolecules and polymers. It has the additional advantage of being non-invasive and non-destructive (Balog *et al.*, 2015).

Measurements are performed by applying a monochromatic light beam, such as a laser, into a suspension. The suspended particles are subject to Brownian motion, which in turn, causes a Doppler Shift when the incident beam hits a moving particle causing the light to scatter. This change is then related to the size of the particles. The velocity of the Brownian motion is defined by the translational diffusion coefficient in infinitely dilute solutions and is usually notated as D_0 . D_0 of a particle is calculated from measurements using an autocorrelation function (Berne & Pecora, 2000). Applying D_0 , it is possible to calculate the spherical size distributions and give a description of the particle motion within the solvating medium.

Results are presented as % volume of a sample that had a certain hydrodynamic diameter. Hydrodynamic diameter (R_H) refers to the radius of a particle that is diffusing within a fluid. The radius that is given is equivalent to the theoretical radius of a hard sphere that has the same translational diffusion coefficient as the particle. The nature of the surface of the particle as well as the ionic concentration of the solvating medium can affect the morphology, which in turn can affect the apparent size. The term “hydrodynamic” refers to the fact that the measurement reflects the apparent size of dynamic solvated particles (McClements, 2015).

Particle size distribution analysis was performed by laser diffraction with a Malvern Mastersizer Hydro SM instrument (Malvern Instruments Ltd., Malvern, Worcestershire, UK). This instrumental setup was chosen because it only requires a small sample volume, which was ideal as Pheroid-MDP was formulated in small quantities. Analysis was performed after suspending a small amount of sample in 50 – 120 mL distilled water and stirring at 1500 rpm. Enough sample was added to ensure an obscuration value between 10 and 15%. Particle size distribution was measured for formulations prepared in triplicate with 30 min increments for the first 12 hrs. after addition of MDP. After 12 hrs. the particle size distribution was measured every hour until 24 hrs. Measurements of Pheroid formulations that did not contain Sn-MDP were also measured after 48 and 72 hrs. to confirm stability of formulations.

Parameters of the particle size distribution that was noted included $d_{0.1}$, $d_{0.5}$ and $d_{0.9}$ values. These values indicate what percentage of the sample falls within a certain size range:

- $d_{0.1}$ indicates that 10% of the sample population have a diameter smaller than this value
- $d_{0.5}$ represents the median, which is the value where 50% of the population is bigger and the other half is smaller than the value.
- $d_{0.9}$ indicates that 90% of the sample population have a diameter smaller than this value.

The $d_{0.1}$, $d_{0.5}$ and $d_{0.9}$ values are used to calculate the span of the distribution which describes the width of the distribution. The weighted volume mean can also be calculated

$$\text{Span} = \frac{d_{0.9} - d_{0.1}}{d_{0.5}}$$

$$D[4,3] = \frac{\sum_i^n D_{vi}^4}{\sum_i^n D_{vi}^3}$$

The span provides a clearer indication of the width of the distribution as $d_{0.9}$ values might indicate outliers, which are present but do not really influence the formulation because of the small % present.

The weighted volume, notated as $V_{[4,3]}$, is an indication of the overall volume of the particles/vesicles in the formulation and is useful when comparing different formulations as a change in average volume is an indication of a shift in the distribution (Scientific, 2012).

3.3.2. Zeta (ζ)-Potential measurement

ζ -Potential is a scientific term for electro-kinetic potential in colloidal systems. This is an electric potential found at the interface between a particulate surface and a point in the bulk fluid away from the interface. ζ -Potential represents the potential difference between the dispersion medium and the stationary layer (slipping plane) of fluid attached to the dispersed particle (Kutscher *et al.*, 2010).

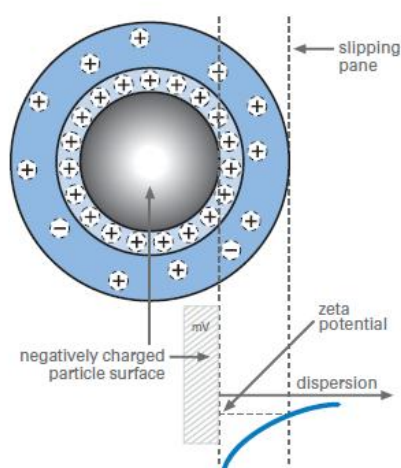


Figure 3.1 ζ -Potential is the measured at the slipping plane (Scientific, 2012)

Measurement of ζ -potential is a straightforward way to characterise the surface of charged colloids and deductions can be made from the analysis of its data regarding concentration, distribution, adsorption, ionisation, exposure or shielding of charged moieties. (Prokop *et al.*, 2002; Agnihotri *et al.*, 2004; Davis *et al.*, 2008; Huynh *et al.*, 2009; Singh & Lillard, 2009; Hotze *et al.*, 2014)

For ζ -potential measurement electrophoresis is mostly used because the measurements can be carried out relatively simply and quickly. Particle size is a limitation of this method as particles should ideally be between 10 nm and 10 μ m. Electrophoresis occurs when a fine dispersion in a dispersion medium is exposed to the effect of an electrical field. Single dispersed particles are

electrically charged according to the dispersion medium and an electrical double layer is formed around each of them.

If the liquid phase as a whole is prevented from flowing in one direction, only charged particles will be kept in motion within the electrical field, and they will travel, depending on their charge, towards the cathode or anode (Salopek *et al.*, 1992)

ζ -Potential analysis was performed by a Malvern Nano instrument (Malvern Instruments Ltd., Malvern, Worcestershire, UK). Samples were prepared by diluting Pheroid-MDP formulations 5000X using pure water. ζ -Potential was measured in triplicate formulations in 30 min increments for the first 12 hrs. after addition of MDP. After 12 hrs. the ζ -potential was measured every hour until 24 hrs.

ζ -Potential provides an indication of stability of the sample. When the absolute value of the ζ -potential approaches 0, it is an indication of a decline in stability. Possible causes for decreasing stability include aggregation or sedimentation of a formulation (Larsson *et al.*, 2012). In combination with the particle size distribution ζ provides a picture of the formulation's stability over time and confirms changes taking place within the matrix of the formulation.

3.3.3. pH measurement

Grobler *et al.* (2008) found that by changing the ionic strength and pH of the aqueous phase of Pheroid formulations the structural and functional properties can be manipulated. It is therefore important to monitor the pH of Pheroid formulations, as a parameter that can possibly influence entrapment.

For unbuffered formulations, pH was measured at 24 hrs. after the addition of MDP. For buffered formulations pH was measured 0, 2, 4, 6, 12 and 24 hrs. after the addition of MDP. Measurements were done using a Metler Toledo Seven2Go pro pH meter and calibrated directly before use.

3.3.4. Microscopy

The morphology of selected Pheroid formulations was assessed by confocal laser scanning microscopy (CLSM). Entrapment was confirmed by means of reflective laser scanning microscopy.

3.3.4.1. Confocal laser scanning microscopy (CLSM)

CLSM involves point by point scanning of a sample with a focused laser beam. Reflectance or fluorescence can be detected. A main advantage of CLSM is that it does not include out-of-focus information in the image. CLSM also allows simultaneous acquisition of images at different wavelengths, which is dependent on the instrument used. (Pawley & Masters, 1996)

An adaption of the method described by Du Plessis *et al.* (2010) was used:

The Confocal model was a Nikon C1 equipped with an Eclipse TE2000 inverted microscope, a DS-Ri1 digital camera with a confocal system including three lasers emitting at 408, 488 and 543 nm (peak emissions).

All Pheroid formulations were labelled with the flourophore Nile red (1 mg/ml), dissolved in DMSO. Nile red emits at wavelengths greater than 590 nm (Greenspan *et al.*, 1985). As a lipophilic flourophore, Nile red associates with the fatty acid components of the Pheroid vesicles/sponges and allows determination of their morphology.

Samples were prepared by adding 1 μ L Nile red to 50 μ L of a Pheroid formulation. Aliquots of the labelled formulation (5 μ L) was placed on a microscope slide and covered with a glass coverslip. Sealing of samples was not necessary as they were measured directly after preparation. Formulations as summarised in table 3.6 were prepared. Images were obtained from freshly prepared slides at time points 1, 2, 3, 4, 5, 6, 12 and 24 hrs. after addition of MDP.

3.3.4.2. Reflectance microscopy

Reflectance microscopy was used as a possible way to follow the incorporation of the MDP into the Pheroid formulations. MDP kits consist of different components, which are not necessarily chemically associated with the MDP itself:

- SnCl_2 (reducing agent)
- Methylene diphosphonate (MDP)
- 2,5 di-hydroxybenzoic acid (DHBA), stabilising agent

Individual analysis of components showed that DHBA emits an intense fluorescent signal when exposed to light over a range of wavelengths. Excitation wavelengths are pH dependent and are influenced by other compounds associated within the DHBA environment but are shorter than 500 nm. As DHBA will not necessarily be associated with MDP after dissolution it had to be excluded. This was achieved by using the 543 nm laser as a light source as this long wavelength was less likely to excite the DHBA than any other of the available light sources.

Analysis also showed while SnCl_2 did not emit any fluorescent signal, the tin in the molecule reflected between wavelengths of 520 and 680 nm. As Sn is associated with the MDP after preparation of the kits, this afforded a unique opportunity to observe possible entrapment or association of the Sn-MDP to Pheroid structures.

The Pheroid samples were analysed for reflectance without the addition of Nile Red. Light reflected by Sn-MDP was collected between 520 and 680 nm through a medium pin hole setting.

This was done to avoid fluorescence emitted by DHBA which fluoresces between approximately 380 and 520 nm. Photomultiplier tube sensitivity (PMT gain) which detects fluorescence was set at 165. Image darkness and saturation were set at 21 and 139 respectively.

While evenly dispersed throughout the formulation the Sn-MDP reflectance is also evenly dispersed. After some time the reflectance started to concentrate, in areas that corresponded with Pheroid vesicles, as confirmed by optical micrographs of the same samples. This concentration of the Sn-MDP is therefore a good indication of association with / entrapment within the Pheroid vesicles.

Table 3.9 Formulations analysed by microscopy

Formulations
Pheroid-MDP vesicles (N ₂ O-H ₂ O), MDP added to aqueous phase before mixing with oil phase
Pheroid-MDP vesicles (N ₂ O-H ₂ O), MDP added during mixing of aqueous and oil phase
Pheroid-MDP vesicles (N ₂ O-saline), MDP added to aqueous phase before mixing with oil phase
Pheroid-MDP vesicles (N ₂ O-H ₂ O), MDP added 4 days after manufacturing
Pheroid-MDP vesicles (N ₂ O-H ₂ O), Vit. F: Kolliphor EL (1:1), MDP added 4 days after manufacturing
Pheroid-MDP sponges (N ₂ O-H ₂ O), MDP added 4 days after manufacturing

Dr Matthew Glynn (PCDDP) is acknowledged for his contributions to the microscopy work.

3.4. Evaluation of Pheroid^{99m}Tc-MDP formulation entrapment efficiency using a HAP model

Literature has shown that HAP can be used as model to demonstrate how effectively a radiotracer adsorbs to bonelike compounds (Jansen *et al.*, 2009). ^{99m}Tc-MDP is a bone seeking radiotracer characterised by a high affinity for compounds present in bone tissue (Claessens & Kolar, 2000). It was hypothesised that if Pheroid-^{99m}Tc-MDP formulations were incubated on HAP and compared to a control of ^{99m}Tc-MDP only, the amounts of ^{99m}Tc-MDP adsorbed onto the HAP could be measured and compared. Previous studies conducted in this manner directly measured the affinity of the tracer to HAP which can then be extrapolated to the affinity of that compound to bone.

As discussed in Chapter 2 the HAP adsorption model is a chemical model, more than an *in vitro* model since *in vitro* assumes that biological conditions are mimicked (Vignais &

Vignais, 2010). The Pheroid- ^{99m}Tc -MDP on HAP model is simplified and omits all biological parameters such as metabolism and interactions with components of blood.

The fourth and fifth objectives of this study were to determine the entrapment efficiencies of selected Pheroid-MDP formulations and resolve optimal entrapment time requirements for Pheroid-MDP formulations. They are both addressed in the following sections.

3.4.1. Formulations assessed in entrapment study

Table 3.10 Formulations assessed in entrapment study

	Type of formulation	Aqueous phase	Incorporation of MDP into formulation	Additional considerations
a.	Pheroid- ^{99m}Tc -MDP vesicles	N ₂ O-Saline (0.9% m/v)	Added to aqueous phase before mixing with oil phase	None
b.	Pheroid- ^{99m}Tc -MDP vesicles	Saline (0.9% m/v)	Added to aqueous phase before mixing with oil phase	Agitated for 2 hrs. before incubation
c.	Pheroid- ^{99m}Tc -MDP vesicles	N ₂ O-Saline (0.9% m/v)	Added to aqueous phase during mixing with oil phase	Agitated for 2 hrs. before incubation
d.	Pheroid- ^{99m}Tc -MDP vesicles	N ₂ O-H ₂ O	Added to aqueous phase before mixing with oil phase	None
e.	Pheroid- ^{99m}Tc -MDP vesicles	N ₂ O-H ₂ O	Added to formulation 4 days after manufacturing, vortexed for 1 minute to incorporate	None
f.	Pheroid- ^{99m}Tc -MDP vesicles: Vit. F ethyl ester: KolliphorEL ratio of 1:1	N ₂ O-H ₂ O	Added to formulation 4 days after manufacturing, vortexed for 1 minute to incorporate	None
g.	Pheroid- ^{99m}Tc -MDP micro sponges	N ₂ O-H ₂ O	Added to formulation 4 days after manufacturing, vortexed for 1 minute to incorporate	None
h.	Control	Saline	^{99m}Tc -MDP in saline	None

Table 3.11 Results for comparison of techniques used to determine activity in supernatant and on HAP.

Samples	Total activity (uCi)	Activity in supernatant (uCi)	Activity on HAP (uCi)	% Activity in supernatant	% Activity on HAP
Filtered	2755	1734,2	1021	65.30%	34.70%
Centrifuged	2830	1797.7	1032	65.57%	34.43%

3.4.2. Measurement of entrapment

Triplicate samples containing 50 mg HAP and 3mL of each formulation were prepared to be incubated at 37°C. The total activities of all samples were counted in millicurie (mCi) before incubation. A Capintec dose calibrator was used to measure activity.

At time points 1, 2, 3, 6, 12 and 24 hrs. samples were removed from incubation and centrifuged at 5000 rpm for 5 min. The supernatant was decanted and the remaining HAP deposit was dried at 90°C for at least 12 hrs..

The dried HAP deposits were weighed and the radioactivity determined by counting using a Scintispec well counter. Prior to measurements the well counter was cross calibrated with the Capintec dose calibrator by making standard dilutions of the ^{99m}Tc eluate. The well counter gave readings as counts per µCi.

Some HAP was lost during decanting. To correct for this the amount of activity on the HAP was calculated using the following equation:

$$\text{Real amount of activity} = \frac{\text{Activity measured after centrifugation} \times \text{accurate mass before incubation}}{\text{Mass measured after centrifugation}}$$

Using the total sample count as well as the corrected count of the HAP measurements the % entrapment in Pheroid was calculated using the following formula:

$$\% \text{ Entrapment} = \frac{\text{Total activity in sample} - \text{Corrected activity on HAP}}{\text{Total activity in sample}} \times 100$$

Not all samples could be measured directly after collection, but as the half life of ^{99m}Tc is known, it possible to calculate the activities for the time of sample collection:

$$N(t) = N_0 e^{-\lambda t}$$

with $N(t)$ representing the activity at time t , the time when the HAP was measured after drying.

λ Represents the decay constant and can be rewritten as

$$\lambda = \frac{\ln 2}{t_{\frac{1}{2}}}$$

where $t_{\frac{1}{2}}$ represents the half life of the decaying isotope (6.0067 hrs for ^{99m}Tc) (Silva *et al.*, 2015).

After rearrangement of the equation, the activity of a HAP sample can be calculated for any given time since its preparation:

$$N_{(0)} = N(t) e^{\lambda t}$$

The portion of the total sample associated with the HAP is thus determined and represents the amount of MDP which is free to associate with the HAP i.e. not entrapped/associated with the Pheroid system.

3.5. Statistical measures of reproducibility

To statistically prove reproducibility of all measurements completed in triplicate, a single factor ANOVA (analysis of variance) was performed on all experimental results which were done in triplicate. Single factor ANOVA examines equality of population means for a quantitative outcome where there is only one variable involved. Single factor ANOVA analyses two or more sets of data and compares their means. A null hypothesis is proposed, and it states that the means are the same. If this hypothesis is proven true, then the data sets are identical and the experiment is reproducible (Salkind, 2006). It is however unlikely that any experiment can be repeated and yield identical results, and therefore the researcher should decide how much difference between the sets should be acceptable. A confidence level is selected, e.g. 90%, 95% or 99%. This means that the datasets should be 90%, 95% or 99% similar (Kiefer, 1977).

The α (significance level) value is associated with the confidence levels:

For a 90% confidence level, α is $1 - 0.90 = 0.10$.

For a 95% confidence level, α is $1 - 0.95 = 0.05$.

For a 99% confidence level α is $1 - 0.99 = 0.01$.

And in general, for a C% confidence level, α 's value is $1 - C/100$.

α Values provide the probability of a type I error. Type I errors occur when a null hypothesis is incorrectly rejected. The smaller the value of α , the less likely such an error is to occur (Kiefer, 1977; Hubbard, 2004). In literature a confidence level of 95% and $\alpha = 0.05$ is considered standard (Zar, 1984).

In order to apply ANOVA, the degrees of freedom (I), must be known and an F value must be calculated, as follows:

$$F = \frac{\text{Between-group variance}}{\text{Within-group variance}} \quad (\text{Winter, 2011}).$$

The different variances are easily calculated using formulae available in literature. The importance of the F values lies in the fact that it can be compared to values for a F probability distribution with the same degrees of freedom. Such values are also easily obtained from textbooks (Rohlf & Sokal,

1995). This value is referred to as F_{crit} and $F > F_{crit}$ leads to rejection of the null hypothesis, while F values $F < F_{crit}$ lead to acceptance of the null hypothesis (Maxwell & Delaney, 2004).

The p-value is also useful in deciding whether to accept a null hypothesis or not. P-values can be defined as the probability of the datasets in question actually being identical. P-values are often used in statistical hypothesis testing (Wasserstein & Lazar, 2016). This requires the selection of the null hypothesis, the confidence level and α value or significance level. A statistically significant difference between datasets will result in $p < \alpha$ while $p > \alpha$ indicates no significant difference (Hubbard, 2004).

For the purpose of validating the reproducibility of the experiments a null hypothesis was formulated that stated that there was no significant difference between means of data sets. The alternative hypothesis stated that there was a significant difference between means of data sets. Microsoft Excel was used to calculate an F and p value for triplicate data sets.

$\alpha = 0.05$ was used consistently and the summary of all ANOVA results is provided in Appendices 1 and 2. Unless stated otherwise all results were reproducible.

3.6. Statistical measures of differences between sample groups (formulations)

3.6.1 Single factor ANOVA

Single factor ANOVA was performed on all experimental results to compare different sample groups (formulations) with one another and determine whether differences were statistically significant. A null hypothesis was proposed, stating that the means were the same. If proven true, then differences between data sets were not statistically significant. In order for the null hypothesis to be true, $p > \alpha$ and $F < F_{crit}$ (Maxwell & Delaney, 2004).

A statistically significant difference between datasets resulted in $p < \alpha$ and $F > F_{crit}$. In cases where a statistically significant difference was detected, post hoc tests were performed to determine which groups significantly differed from one another.

$\alpha = 0.05$ was used consistently and the summary of all ANOVA results is provided in Appendices 1 and 2.

2.6.2 Tukey post hoc test

Tukey's HSD test is used to determine which sample groups differ from each other. HSD, "Honest Significant Difference", is a number which represents the distance between groups, and compares means with each other.

Tukey's post hoc test provides a simple method to perform pairwise comparison. Tukey's HSD post hoc test assumes equal size of all compared groups, while a modified Tukey-Kramer method exists which can be applied to comparisons of unequal-sized groups (Kim, 2015).

For the purpose of this study Tukey's test was performed whenever ANOVA revealed significant changes between groups. Tukey serves to identify which samples cause these differences. Unless mentioned in Chapters 4 and 5, all relevant Tukey data can be found in Appendices 1 and 2.

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Chapter | 4

Preparation and characterisation of Pheroid[®] formulations containing Sn(II)-Methylene diphosphonate

Adele van As^a, Nicola Isabel Barnard^a, Jan Rijn Zeevaart^b

^a DST/NWU Preclinical Drug Development Platform, North-West University, Potchefstroom Campus, 2520, South Africa.

^b Radiochemistry, South African Nuclear Energy Corporation SOC Limited (Necsa), Pretoria, 0001, South Africa.

Abstract

Pheroid[®] formulations containing Sn(II)-Methylenediphosphonate (MDP) used as nonradioactive analogy for ^{99m}Tc-MDP were prepared. The aim was to develop an optimal formulation in terms of formulation stability and entrapment with possible application as oral dosage form for ^{99m}Tc-MDP, an intravenously administered radiopharmaceutical. Various Pheroid[®] formulations containing Sn(II)-MDP were prepared and characterised. Particle size, stability, morphology and qualitative entrapment, using dynamic light scattering, ζ-potential, confocal laser scanning microscopy and reflectance confocal laser scanning microscopy techniques, were used respectively. Pre-prepared Pheroid[®] vesicles with Sn(II)-MDP rapidly introduced was found to be the most stable formulation. Stability of Pheroid[®] - Sn(II)-MDP formulations were negatively impacted by introduction of Sn(II)-MDP during the formulation of the carrier system, alteration of the surfactant ratio's and agitation of formulations after preparation. Pre-prepared Pheroid[®] vesicles, having had sufficient time to stabilise, showed the greatest stability in terms of particle size distribution, ζ-potential, and also showed rapid entrapment when Sn(II)-MDP was added.

Keywords: Pheroid[®], Sn(II)-MDP, formulation, dynamic light scattering, ζ-potential, confocal laser scanning microscopy, reflectance.

1. Introduction

Pheroid[®] technology is a novel patented drug delivery system consisting of essential and plant unsaturated fatty acids, which are emulsified in water saturated with nitrous oxide (N₂O) (Grobler & Kotze, 2006; Grobler *et al.*, 2008; Grobler, 2008). Three different types of Pheroid[®] are recognised: Pheroid vesicles, Pheroid micro-sponges and pro-Pheroid[®] (Grobler *et al.*, 2008).

Pheroid[®] technology has the capacity to entrap, protect and deliver active pharmaceutical ingredient (API) molecules across diverse biological membranes. Pheroid[®] formulations can be manipulated for various therapeutic applications by successfully entrapping hydrophilic, hydrophobic and amphiphilic compounds (Du Plessis *et al.*, 2010b). Some applications of Pheroid technology include: anti-malarials, enhanced tuberculosis treatments, peptide delivery, topical drug delivery and antibiotic improvement (Meyer, 1997; Grobler & Kotze, 2006; Grobler, 2008; Du Plessis *et al.*, 2010a; Du Plessis *et al.*, 2010b; Du Plessis *et al.*, 2014).

Lipid-based drug delivery systems are used mainly to improve bioavailability and reduce side effects (Gardner, 1987). As a lipid-based drug delivery system, Pheroid[®] has the advantage of increasing gastrointestinal absorption of drugs (Steyn *et al.*, 2011). The Pheroid[®] delivery system has been shown to improve the distribution of a range of complexes in terms of decreased onset of action times, lower effective drug concentration doses and enhanced efficacy (Grobler *et al.*, 2008; Steyn *et al.*, 2011; Grobler *et al.*, 2014). Significantly, Pheroid[®] technology might provide a practical means of delivery for previously orally unavailable drugs (Du Plessis *et al.*, 2014).

^{99m}Tc is a gamma emitting radionuclide, and has been used for imaging for some time (Wang *et al.*, 1979; Schwochau, 1994; M Rey, 2010; Barh *et al.*, 2014). ^{99m}Tc -MDP (technetium-99m methylene diphosphonate or medronic acid) in particular, accumulates in bone tissues and is used in skeletal imaging (Subramanian *et al.*, 1975; Kung *et al.*, 1978; Blake *et al.*, 2011). Bone scans provide images of the metabolic activity of the skeleton and are accomplished through imaging of the gamma rays emitted from the radioactive ^{99m}Tc isotope (Budd *et al.*, 1989; Krasnow *et al.*, 1997; McCracken *et al.*, 2001).

MDP is available in commercial ready-to use kits. These kits contain methylene diphosphonic acid or a corresponding sodium salt, a reducing agent such as Sn(II) chloride or Sn(II) fluoride, and a stabilising agent such as 2,5 di-hydroxybenzoic acid (DHBA) or ascorbic acid (Lever & Lever, 2009).

Apart from acting as reducing agent, Sn(II) also forms Sn(II)diphosphonate complexes, hereafter referred to as Sn-MDP. Chemically, Sn-MDP is very similar to ^{99m}Tc -MDP and can be used as a non-radioactive analogy (Claessens & Kolar, 2000).

^{99m}Tc -MDP is administered intravenously and gamma rays emitted during the decay of the radioisotope are used to produce images. When administered intravenously, the primary uptake route of ^{99m}Tc -MDP is through adsorption onto or into the crystalline structure of hydroxyapatite present in bone. After administration, 80% of the ^{99m}Tc -MDP is generally cleared from the body after 3 hours, while between 10 and 20% is found adsorbed to bone tissue.

A study utilising ^{99m}Tc -MDP to observe the biodistribution of Pheroid[®] has led to a patent for Pheroid[®]-radiotracer formulations (Grobler & Zeevaart, 2015; Swanepoel, 2015). Apart from determining the biodistribution of Pheroid[®], this study showed that Pheroid[®] has the potential to enhance the bioavailability of radiopharmaceuticals and allow oral administration. This is noteworthy, as studies have confirmed that patients receiving radiotherapy prefer oral administration of drugs over the intravenous route. Main reasons for this preference include convenience and avoiding pain / discomfort associated with needles. (Liu *et al.*, 1997; Borner *et al.*, 2001).

Swanepoel (2015) showed that Pheroid[®] allows the oral administration of ^{99m}Tc -MDP in rats. If ^{99m}Tc -MDP, which is currently administered intravenously, can be formulated into an optimised orally administered dosage form, it may improve patient compliance and comfort and reduce needle related risks to medical staff.

Formulations containing radiotracers cannot be prepared a long time in advance to achieve the necessary entrapment into the carrier system. If the half-life of an administered radiotracer causes activity loss to such an extent that imaging is no longer possible, the advantages of oral administration are lost. For successful clinical implementation of such a diagnostic preparation, maximum entrapment in a dosage form ready for administration within 2-6 hours of final formulation is required.

The aim of this study was preliminary identification of suitable Pheroid[®] formulation parameters for further investigation into their usefulness as carriers for ^{99m}Tc -MDP.

2. Materials and methods

2.1. Materials

Vitamin F ethyl ester was purchased from Croda, South Africa, Kolliphor EL was purchased from BASF, South Africa, dL- α -tocopherol was purchased from Chempure, South Africa, Incromega E7010 and Incromega E3322 was purchased from Croda, South Africa and nitrous oxide was supplied by Afrox and used without further purification. MDP kits are manufactured by NTP Radioisotopes SOC/Necsa and were gifted by Necsa.

2.2. Preparation of Sn-MDP solutions

Sn-MDP solutions were prepared by adding 6 mL 0.9% saline to a lyophilised vial of MDP. Under normal circumstances the volume of the saline does not matter, the quantity added correlates to the

activity required. For the purposes of this study, and to simplify formulation, a constant volume of 6 mL was added to each vial.

2.3. Preparation of Pheroid[®]-Sn-MDP formulations

2.3.1. General preparation of Pheroid[®] vesicle and sponge formulations

A general preparation method for Pheroid[®] vesicles is described by Du Plessis *et al.* (2010b). This method was slightly adjusted to suit the specific requirements of this study. The lipid components as well as surfactants (Vitamin F ethyl ester, KolliphorEL and DL- α -ocopherol) found in Pheroid[®] were heated to 70°C and combined until homogeneous. The mixture was cooled down and the density measured. The oil component, 4% (m/m), was heated to 70°C and added to 96% purified water saturated with N₂O(N₂O-H₂O) / Saline (0.9% m/v) (N₂O-Saline) and homogenised with a Heidolph Diax 600 homogeniser (Labotec, Johannesburg, South Africa) for 4 min and at not higher than 40°C. Vesicles were then allowed to cool to room temperature 25°C).

Pheroid[®] sponges were prepared in a similar way, with the addition of Incromega E7010 and Incromega E3322 to the oil phase.

2.3.2. Preparation of Pheroid[®] vesicles with Sn-MDP incorporated immediately

Vesicles were prepared with Sn-MDP incorporated at different stages during formulation:

- Sn-MDP added to aqueous phase before homogenising
- Sn-MDP added to formulation added during homogenising
- Sn-MDP added to formulation directly after formulation

These formulations were prepared with the aqueous phase being N₂O-H₂O **OR** N₂O-Saline.

Unless mentioned otherwise all formulations mentioned were prepared in triplicate.

2.3.3. Preparation of Pheroid[®] vesicles with Sn-MDP incorporated after formulation had been stabilised

Vesicles with varied lipid: surfactant ratio's were prepared and left under N₂O for four days to stabilise. Sn-MDP was introduced by vortexing the formulation for 1 min. Pheroid[®] formulations with the following lipid: surfactant ratios were prepared:

- Vit. F to Kolliphor EL 1:2.8
- Vit. F to Kolliphor EL 1:1
- Vit. F to Kolliphor EL 2.5:1.3
- Vit. F to Kolliphor EL 2.8:1 (standard ratio for Pheroid[®] vesicles)

These formulations were prepared with the aqueous phase being N₂O-H₂O.

2.3.4. Preparation of Pheroid[®] sponges with Sn-MDP incorporated after formulation had been stabilised

Pheroid[®] sponges were prepared and left under N₂O for four days to stabilise. Sn-MDP was then introduced by vortexing the formulation for one minute.

This formulation was prepared with the aqueous phase being N₂O-H₂O.

2.4. Characterisation of Pheroid[®]-Sn-MDP systems

2.4.1. Dynamic light scattering

Particle size distribution analysis was performed by laser diffraction with a Malvern Mastersizer Hydro SM instrument (Malvern Instruments Ltd., Malvern, Worcestershire, UK). This instrumental setup was chosen because it only requires a small sample volume, which was ideal as Pheroid[®]-Sn-

MDP was formulated in small quantities. Analysis was performed after suspending a small amount of sample in 50 – 120 mL distilled water and stirring at 1500 rpm. Enough sample was added to ensure an obscuration value between 10 and 15%. Particle size distribution was measured for formulations prepared in triplicate with 30 min increments for the first 12 hrs after addition of Sn-MDP. After 12 hrs the particle size distribution was measured every hour to 24 hrs.

Parameters of the particle size distribution that was noted included $d_{0.1}$, $d_{0.5}$ and $d_{0.9}$ values.

These values were used to calculate the span of the distribution which described the width of the distribution (eq. 1).

$$\text{Span} = \frac{d_{0.9} - d_{0.1}}{d_{0.5}} \quad (\text{Eq. 1})$$

2.4.2. Zeta (ζ)-Potential

ζ -Potential analysis was performed by a Malvern Nano instrument (Malvern Instruments Ltd. , Malvern, Worcestershire, UK). Samples were prepared by diluting Pheroid®-Sn-MDP formulations 5000X using pure water. ζ -Potential was measured for triplicate formulations in 30 min increments for the first 12 hrs after addition of Sn- MDP. After 12 hrs the ζ -potential was measured every hour to 24 hrs.

2.4.3. Microscopy

The following formulae were microscopically analysed:

- Pheroid®-Sn-MDP vesicles (N₂O-H₂O), MDP added to aqueous phase before mixing with oil phase
- Pheroid®-Sn-MDP vesicles (N₂O-Saline), MDP added to aqueous phase before mixing with oil phase
- Pheroid®-Sn-MDP vesicles (N₂O-H₂O), MDP added 4 days after manufacturing
- Pheroid®-Sn-MDP vesicles (N₂O-H₂O), Vit. F: Kolliphor EL (1:1), MDP added 4 days after manufacturing
- Pheroid®-Sn-MDP sponges (N₂O-H₂O), MDP added 4 days after manufacturing

2.4.3.1. Fluorescence and reflectance confocal laser scanning microscopy

A Nikon C1 Confocal model equipped with an Eclipse TE2000 inverted microscope and a DS-Ri1 digital camera with a confocal system including three lasers emitting at 408, 488 and 543 nm (peak emissions) was used.

For fluorescence measurement all Pheroid formulations were labelled with the flourophore Nile red (1 mg/ml), dissolved in DMSO. Nile red emits at wavelengths greater than 590 nm (Greenspan *et al.*, 1985). As a lipophilic flourophore, Nile red associates with the fatty acid components of the Pheroid vesicles / sponges and allows determination of their morphology.

Samples were prepared by adding 1 μ L Nile red to 50 μ L of a Pheroid formulation. 5 μ L of the labelled formulation was placed on a microscope slide and covered with a glass coverslip. Sealing of samples was not necessary as they were measured directly after preparation. Images were obtained from freshly prepared slides at time points 1, 2, 3, 6, 12 and 24 hours after addition of MDP.

An adaption of the method described by Du Plessis *et al.* (2010b) was used.

For reflectance measurement individual analysis was performed for each of the components of an MDP kit:

- SnCl_2 (tin(II)chloride - reducing agent)
- Methylene diphosphonate (MDP)
- 2,5 di-hydroxybenzoic acid (DHBA), stabilising agent

Each component was scanned over a wide wavelength range using the three lasers emitting at 408, 488 and 543 nm. The main aim of imaging was to track the movement of MDP to determine any association with Pheroid vesicles. Since it is known that Sn is associated with MDP after preparation of the MDP kit, the focus was only on wavelengths where SnCl_2 and MDP would emit light. Any fluorescence that did not correspond with SnCl_2 or MDP was excluded from any further imaging analysis.

SnCl_2 reflected between 520 nm and 680 nm while DHBA fluoresced at wavelengths shorter than 500 nm. Any DHBA interference was excluded from imaging by using the 543 nm laser as a light source.

The formulations mentioned in section 2.4.3. were analysed using the following parameters:

Pheroid[®]-Sn-MDP samples were analysed for reflectance, light reflected by Sn-MDP was collected between 520 and 680 nm through a medium pin hole setting. PMT gain was set at 165. Image darkness and saturation were set at 21 and 139 respectively. Samples were analysed at 1, 2, 3, 6, 12, and 24 hrs after addition of Sn-MDP to the formulations.

2.4.3.2. Light microscopy

A Nikon C1 Confocal model equipped with an Eclipse TE2000 inverted microscope and a DS-Ri1 digital camera fitted with a light source for light microscopy was used.

Samples were prepared by placing 5 μL of the various Pheroid[®]-Sn-MDP formulations on a microscope slide and covering with a glass coverslip. Sealing of samples was not necessary as they were measured directly after preparation. Light microscopy images were obtained from freshly prepared slides at time points 1, 2, 3, 6, 12 and 24 hrs after addition of MDP.

As with fluorescence and reflectance microscopy formulations mentioned in section 2.4.3. were analysed.

3. Results and discussions

3.1. Particle size analysis and ζ -potential measurements

All particle size analysis and ζ -potential measurements results will be evaluated in terms of the time taken for a formulation to achieve some measure of stability. Ideally formulations should stabilise within 2 to 6 hours after addition of Sn-MDP in order for them to be considered suitable for further exploration.

Dynamic light scattering (DLS) is an important experimental technique for studying the properties of suspensions and solutions of colloids, biological solutions, macromolecules and polymers. It has the additional advantage of being non-invasive and non-destructive (Balog *et al.*, 2015). It is a well established method for the analysis of Pheroid[®] formulations (Grobler *et al.*, 2008; Du Plessis *et al.*, 2010b; Steyn *et al.*, 2010; Steyn *et al.*, 2011; Du Plessis *et al.*, 2012; Du Plessis *et al.*, 2014).

Parameters of the particle size distribution that was noted included $d_{0.1}$, $d_{0.5}$ and $d_{0.9}$ values. These values indicate what percentage of the sample falls within a certain size range:

- $d_{0.1}$ indicates that 10% of the sample population have a diameter smaller than this value
- $d_{0.5}$ represents the median, which is the value where 50% of the population is bigger and the other half is smaller than the value.
- $d_{0.9}$ indicates that 90% of the sample population have a diameter smaller than this value.

Using equation 1, the span of the different formulations was calculated. The span gives a clearer indication of the width of the distribution as $d_{0.9}$ values might indicate outliers which are present but do not really influence the formulation because of the small percentage present.

ζ -Potential provides an indication of stability of the sample. When the absolute value of the ζ -potential approaches 0, it is an indication of a decline in formulation stability (Larsson *et al.*, 2012). In combination with the particle size distribution ζ provides a picture of the formulation's stability over time and confirms changes taking place within the matrix of the formulation.

The span as well as ζ -potential of Pheroid[®] formulations are given in Figure 1. **A** – standard Pheroid[®] N₂O-H₂O; **B** - Sn-MDP (N₂O-H₂O) added before homogenisation; **C** - Sn-MDP (N₂O-H₂O) added during homogenisation and **D** - Sn-MDP (N₂O-H₂O) added after homogenisation. From graph (**A**) it is clear that a standard Pheroid[®] (N₂O-H₂O) formulation does not truly stabilise within the first 24 hours after preparation. The span varies from 3.72 μ m to 34.49 μ m. This is confirmed by the ζ -potential which is widely varied with a maximum of -3.72 mV and a minimum of -29.83 mV.

The figure shows that the span of graph (**B**) doesn't exceed 10 μ m within the first 20 hrs after preparation which already shows improved stability compared to graph (**A**). The formulation appears most stable between 1.5 and 2.5 hrs, as well as between 9 and 16 hrs. In terms of possible application the stability between 1.5 and 2.5 hrs shows promise while the second stable window is less useful.

Between 1.5 and 2.5 hours the ζ -potential for graph (**B**) varied between -37.38 mV and -27.14 mV. Although this variation is quite large, both values are still within reasonable limits for Pheroid[®] formulations (Uys, 2006), making this a formulation suitable for further investigation. Uys (2006) noted that absolute ζ -potential above 30 mV indicated stabilised Pheroid[®] (N₂O-H₂O). By comparison this formulation stabilises more quickly than standard Pheroid[®] (N₂O-H₂O).

Graph (**C**)'s span stabilises after 2.5 hrs and remains stable for the next 10.5 hrs. The ζ -potential however is less stable, and the values are predominantly higher than -25 mV, indicating this formulation's stability is unsuitable, and was discarded for further studies.

Graph (**D**)'s span and ζ -potential remains unstable throughout the first 24 hrs after formulation, and therefore this formulation does not warrant any further attention.

Figure 2 gives the span as well as ζ -potential of Pheroid[®] (N₂O-saline) and three Pheroid[®]-Sn-MDP (N₂O-saline) formulations with MDP introduced at different stages of formulation.

Saline was introduced as aqueous phase since it is readily available in medical facilities. In the interest of simplifying the preparation method so that it can be easily handled by nursing staff, the possible use of saline was investigated.

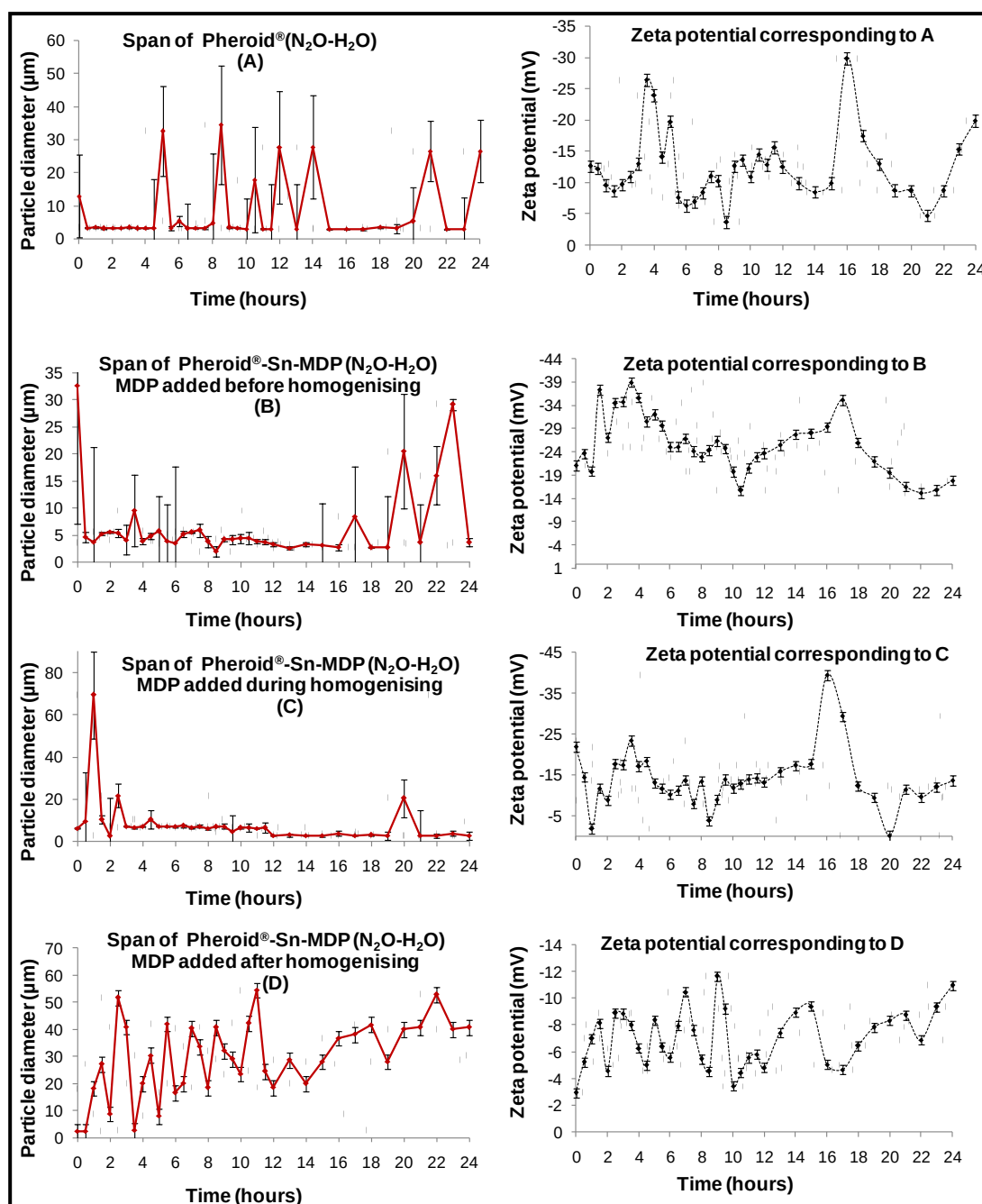


Figure 1. Curves depicting the change in span of Pheroid® (N_2O-H_2O) and three Pheroid®-Sn-MDP(N_2O-H_2O) formulations with MDP introduced at different stages of formulation.

In Figure 2 graphs (E) and (F) show that when Sn-MDP is incorporated before homogenising, it has little effect on the stability of the formulation, since the span for both graphs are very similar. The ζ -potential values of (F) is much lower than that of (E), indicating higher stability. This was also observed for (B), which might indicate that addition of Sn-MDP to the aqueous phase before homogenising might serve to stabilise the formulation. Graph (F) shows stability between 1.5 and 3.5 hrs, as well as between 9 hrs and 17 hrs. This formulation may warrant further investigation. Both Sn-MDP (N_2O -saline) added before homogenisation (G) and Sn-MDP (N_2O -saline) added during homogenisation (H) showed considerable instability in both their span and ζ -potential and will probably not be useful for further studies. The addition of saline did nothing to improve the stability of the formulations, in fact if graphs (G) and (H), are considered it might actually be concluded to have a detrimental effect on the stability of the drug carrier. The use of saline, other

than the small volume already present in Sn-MDP solutions, was therefore discontinued in further investigations.

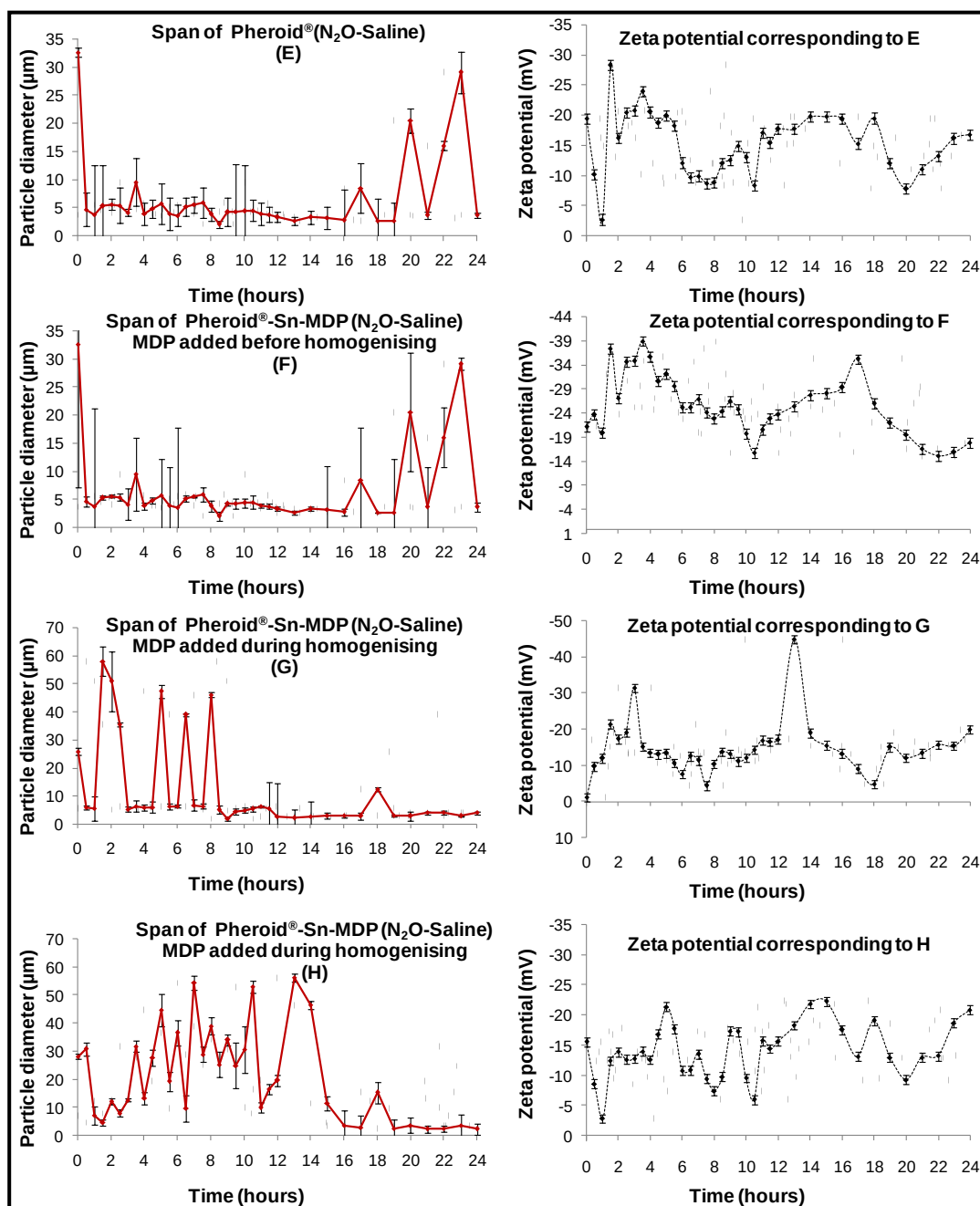


Figure 2. Curves depicting the change in span of Pheroid® (N₂O-saline) and three Pheroid®-Sn-MDP (N₂O-saline) formulations with MDP introduced at different stages of formulation.

Grobler (2009) and Uys (2006) mention particle size distribution and ζ -potential of Pheroid[®] formulations stabilise 4 days after preparation. In order to establish whether this stability might be useful in Pheroid[®]-Sn-MDP applications, Pheroid[®] (N₂O-H₂O) vesicles and Pheroid[®] (N₂O-H₂O) sponges were prepared and stored under N₂O for four days, after which Sn-MDP was incorporated into the formulation. Figure 3 gives the span and ζ -potential of Pheroid[®] (N₂O-H₂O) vesicles (I) and Pheroid[®] (N₂O-H₂O) sponges (K) as well as Pheroid[®]-Sn-MDP (N₂O-H₂O) vesicles (J) and Pheroid[®]-Sn-MDP (N₂O-H₂O) sponge (L) formulations.

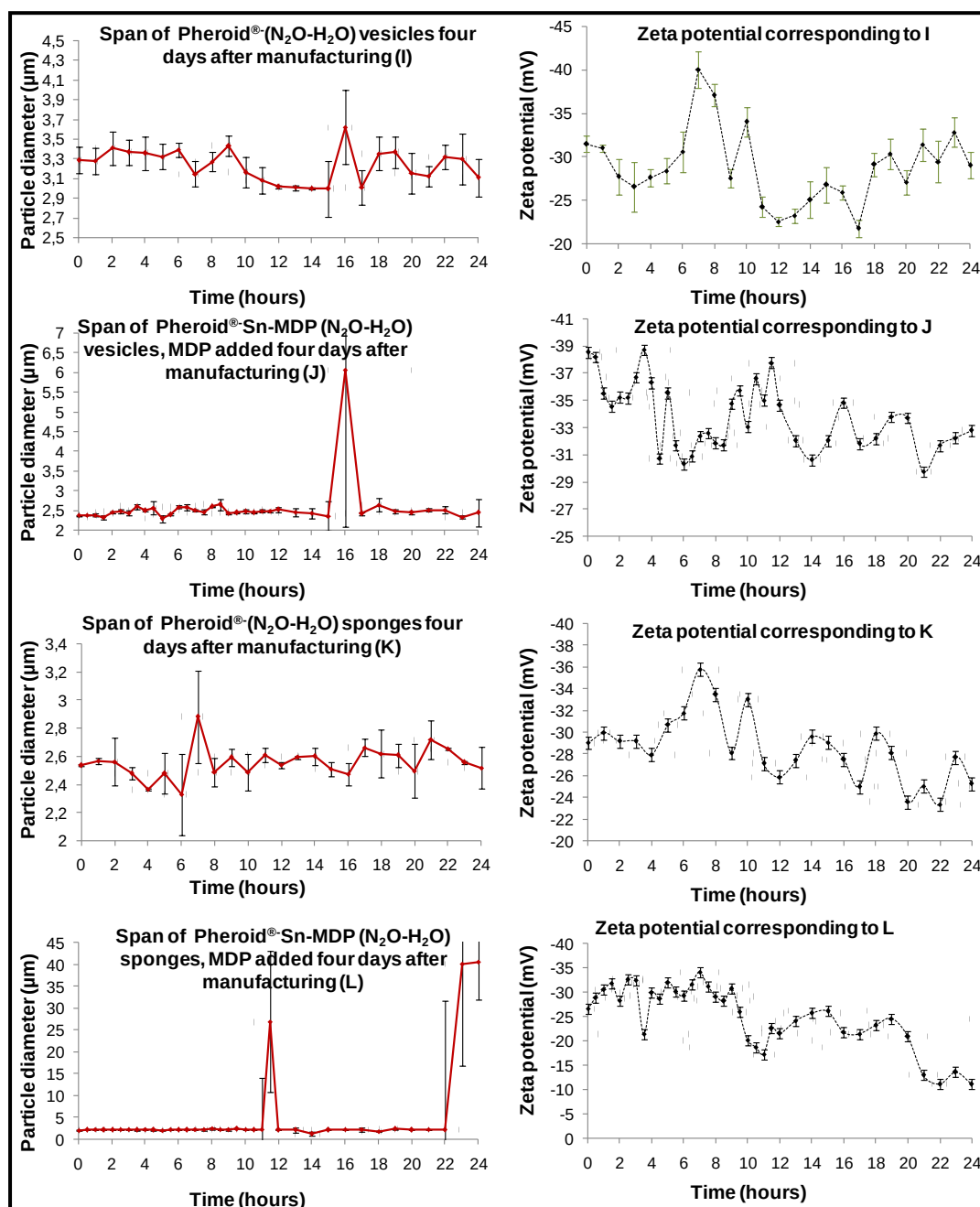


Figure 3. Curves depicting span and ζ -potential of Pheroid[®] (N₂O-H₂O) vesicles and Pheroid[®] (N₂O-H₂O) sponges as well as Pheroid[®]-Sn-MDP (N₂O-H₂O) vesicles and Pheroid[®]-Sn-MDP (N₂O-H₂O) sponge formulations with MDP introduced four days after preparation.

Graphs (I) and (K) show that the span of both Pheroid[®] (N₂O-H₂O) vesicles and Pheroid[®] (N₂O-H₂O) sponges stabilised after 4 days. Variations observed in the ζ -potential are within acceptable limits (Uys, 2006). The addition of Sn-MDP to the formulations does not impact the formulation stability within the first ten hours but an overall trend of decreasing stability can be observed in the

ζ -potential. The decrease in stability would however not impact the usability of the formulations for application as radiotracer carrier system, since they are being investigated for possible use within the first 2 to 6 hrs after preparation. When considering the stability of the formulations during the required time, these two formulations show the most promise.

The surfactant ratios in lipid based drug carriers can influence their stability as well ability to entrap API's (Pouton, 2000; Porter *et al.*, 2008; Pouton & Porter, 2008; Kalepu *et al.*, 2013). For this reason Pheroid[®] (N₂O-H₂O) vesicles with different lipid: surfactant ratios were also investigated. The results are given in Figure 4.

Figure 4 shows the span and ζ -potentials four days after preparation of Pheroid[®]-Sn-MDP (N₂O-H₂O) with different lipid: surfactant ratios. As an excipient, Kolliphor EL increases the miscibility of the Vitamin F ethyl ester in the aqueous phase. When too high a percentage of excipient is present, the lipid component becomes miscible to such an extent that the integrity of the drug carrier is reduced (Pouton, 2000; Porter *et al.*, 2008; Pouton & Porter, 2008; Kalepu *et al.*, 2013). Therefore the instability observed for both the span and ζ -potentials of graphs M and N is not completely unexpected. Equal ratios of lipid and excipient components for O and P yields an unstable formulation with an unacceptably broad span for Pheroid[®] vesicles (Uys, 2006). The larger span could be due to the increased excipient percentage resulting in increased miscibility with the aqueous phase forming less rigid vesicle structures. Since the stability of a formulation is not a definitive indicator of entrapment the observed changes in the span could actually indicate entrapment of Sn-MDP into the Pheroid[®] system. For this reason the Pheroid[®]-Sn-MDP (N₂O-H₂O) Vit F: Koll. EL 1: 1 formulation was included for further investigation.

Although some aforementioned formulations showed indications of stability, structural stability is not a conclusive indication of whether Sn-MDP has been entrapped within Pheroid[®] structures. The possibility should also be considered that Sn-MDP may be loosely associated with the structure, or that it is dissolved in the aqueous matrix and completely separate from the Pheroid[®] vesicles/sponges. If MDP is not suitably associated with Pheroid[®] the benefits of the carrier will not be transferred to allow oral administration of the bone imaging agent. Only when this aspect has been addressed by means of laser microscopy, section 3.2, can substantiated conclusions be drawn.

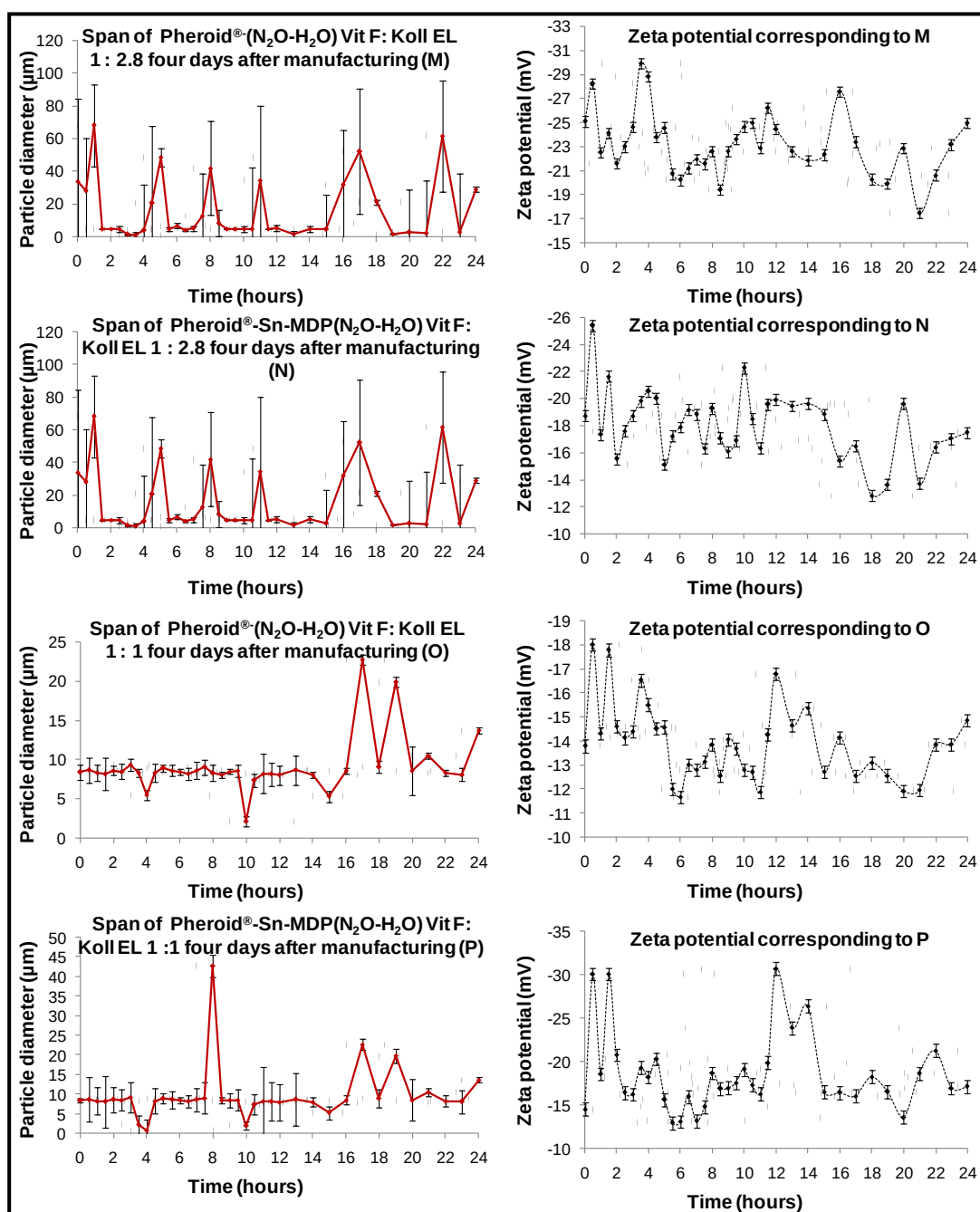


Figure 4. Curves depicting span (left) and ζ -potential (right) of Pheroid[®] (N₂O-H₂O) Vit F: Koll EL 1 : 2.8 vesicles and Pheroid[®] (N₂O-H₂O) Vit F: Koll. EL 1 : 1 vesicles as well as Pheroid[®]-Sn-MDP (N₂O-H₂O) Vit F: Koll. EL 1 : 2.8 vesicles and Pheroid[®]-Sn-MDP (N₂O-H₂O) Vit F: Koll. EL 1 : 1 vesicles with MDP introduced four days after preparation.

3.2. Reflectance microscopy studies

All components of the MDP kit was individually evaluated by scanning samples over a wide wavelength spectrum using a system that included three lasers emitting at 408, 488 and 543 nm.

It was found that MDP did not emit any light across this spectrum, which meant that it would not show up in any captured image. DHBA, which is present in MDP kits, emits an intense fluorescent

signal when exposed to light over a range of wavelengths shorter than 500 nm. It was determined that SnCl_2 reflected between wavelengths of 520 and 680 nm (as shown in Figure 5).

To exclude any interfering fluorescence from DHBA that could be misconstrued as entrapped Sn-MDP, a wavelength larger than 500 nm was selected since it was less likely to excite the DHBA. Taking into account the reflectance range of SnCl_2 a 543 nm laser was selected as light source.

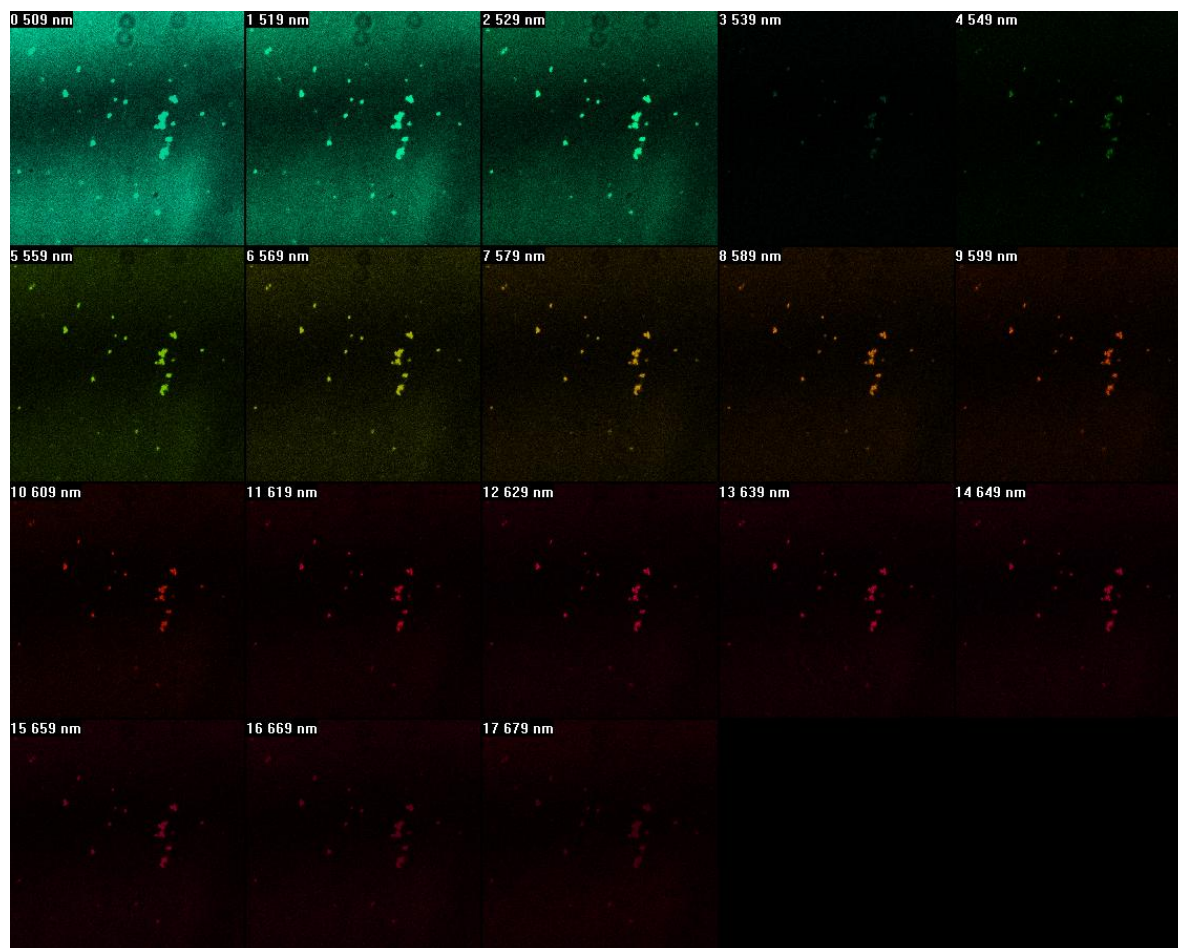


Figure 5. SnCl_2 solutions in sterile H_2O with undissolved SnCl_2 crystals present. Images captured at wavelengths varying from 509nm (image top left) to 679 nm (3rd image from bottom right).

Imaging techniques described in 2.4.3. were applied to selected formulations based on results from the particle span and ζ - potential stability assessments:

- Pheroid[®]-Sn-MDP vesicles ($\text{N}_2\text{O-H}_2\text{O}$), MDP added to aqueous phase before mixing with oil phase
- Pheroid[®]-Sn-MDP vesicles ($\text{N}_2\text{O-Saline}$), MDP added to aqueous phase before mixing with oil phase
- Pheroid[®]-Sn-MDP vesicles ($\text{N}_2\text{O-H}_2\text{O}$), MDP added 4 days after manufacturing
- Pheroid[®]-Sn-MDP sponges ($\text{N}_2\text{O-H}_2\text{O}$), MDP added 4 days after manufacturing
- Pheroid[®]-Sn-MDP vesicles ($\text{N}_2\text{O-H}_2\text{O}$), Vit. F: Kolliphor EL (1:1), MDP added 4 days after manufacturing

After preparation of MDP kits it is known that Sn complexes with MDP. Therefore following the movement of the Sn means that the movement of Sn-MDP is tracked. Using this principles the entrapment of Sn-MDP could be determined qualitatively.

3.2.1. Pheroid[®]-Sn-MDP vesicles (N_2O-H_2O), MDP added to aqueous phase before mixing with oil phase

Figure 6 shows that the amount of Sn-MDP within or associated the Pheroid[®] vesicles is not stable. The particle size analysis and ζ -potential measurements (Figure 1, formulation **B**) showed that the formulation was still undergoing changes, the microscopic images (Figures 6, 7 and 8) confirm the measured results. Of note is the high concentrations of Sn-MDP within or associated with the Pheroid[®] vesicles. For clinical applications this shows much promise, with the limitation that the formulation would have to be administered 1 hr after preparation. This small window for use poses problems in practical usage.

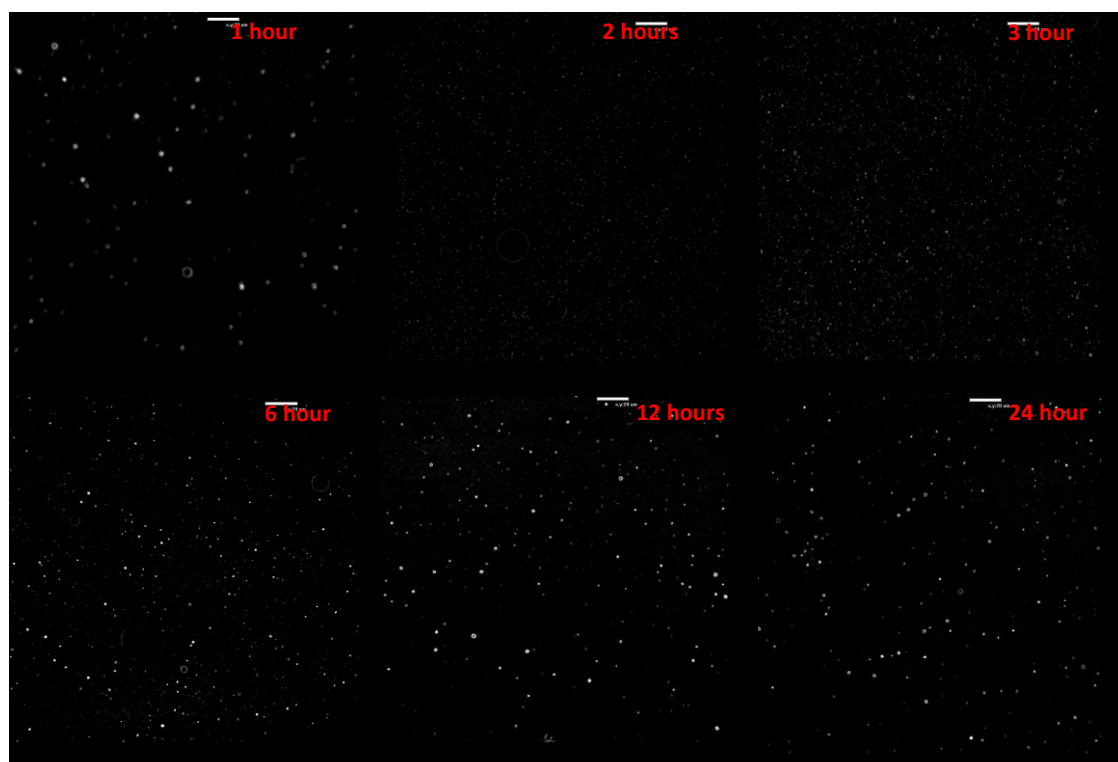


Figure 6. Reflectance micrograph of Pheroid[®]-Sn-MDP vesicles (N_2O-H_2O), MDP added to aqueous phase before mixing with oil phase after 1, 2, 3, 6, 12 and 24 hours. White areas indicate reflectance of a concentration of Sn-MDP.

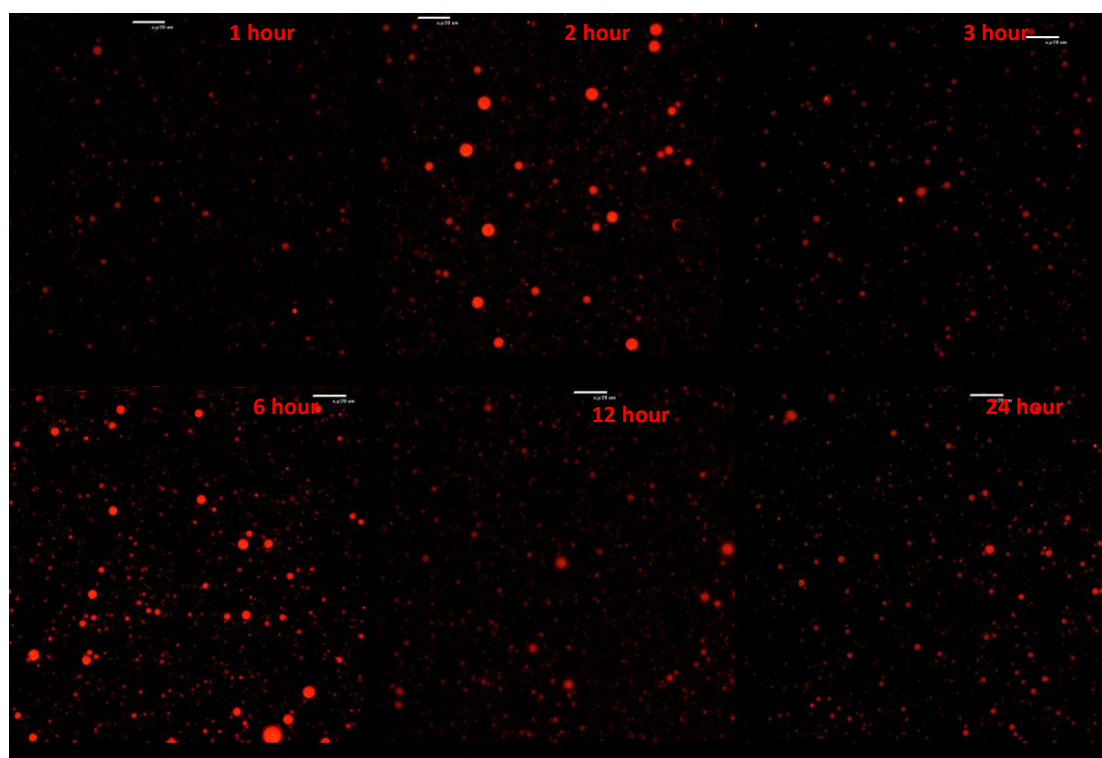


Figure7. Florescence micrograph of Pheroid[®]-Sn-MDP vesicles (N_2O - H_2O), MDP added to aqueous phase before mixing with oil phase.

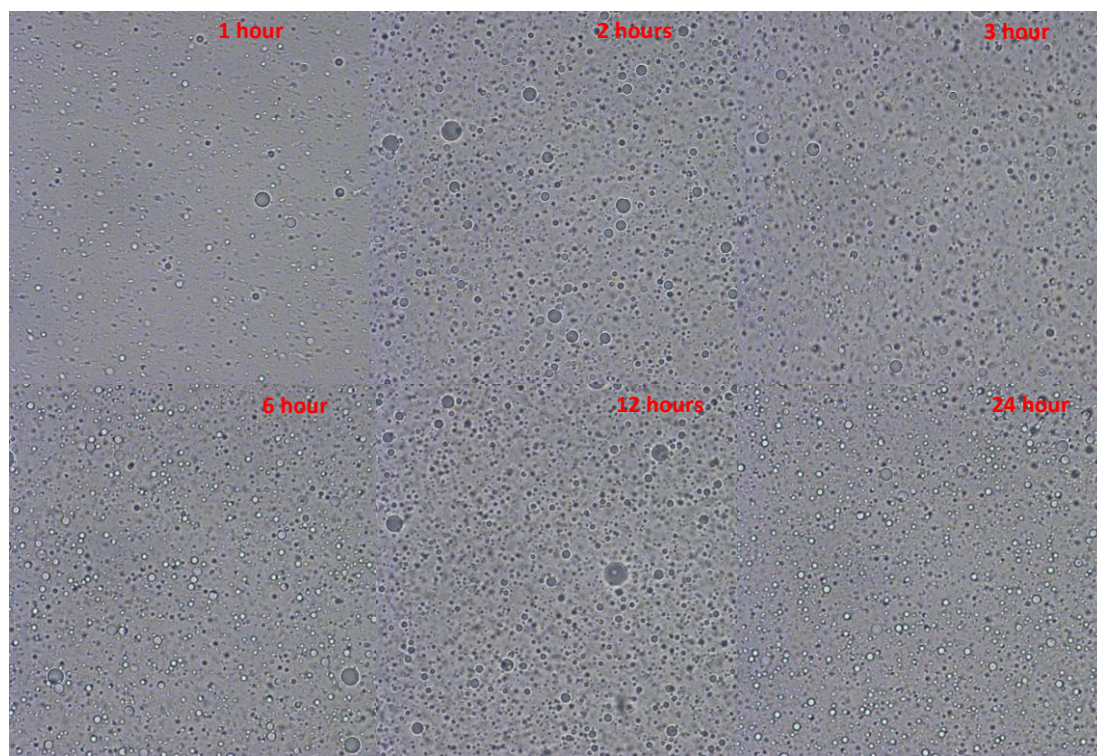


Figure 8. Light microscopy micrograph of Pheroid[®]-Sn-MDP vesicles (N_2O - H_2O), MDP added to aqueous phase before mixing with oil phase

Unless stated otherwise all microscopy images are provided in Appendix A.

3.2.2. Pheroid[®]-Sn-MDP vesicles (N_2O -Saline), MDP added to aqueous phase before mixing with oil phase

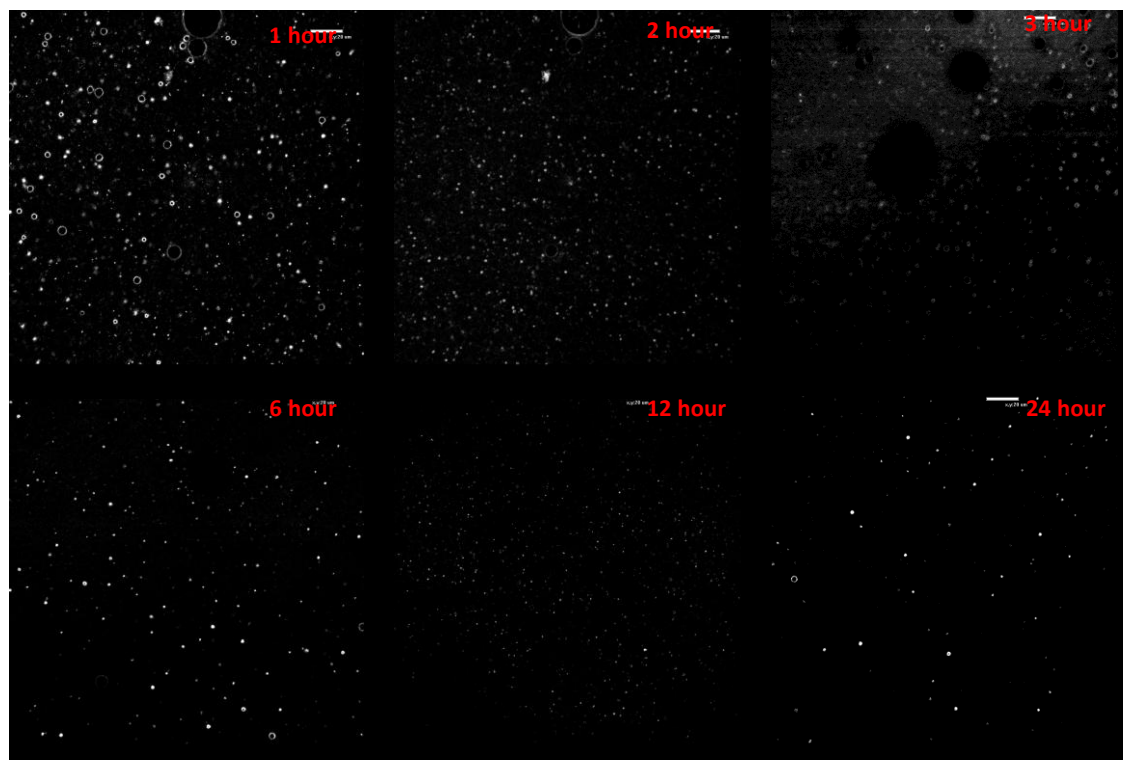


Figure 9. Reflectance micrograph of Pheroid[®]-Sn-MDP vesicles (N_2O -saline), MDP added to aqueous phase before mixing with oil phase.

Pheroid[®]-Sn-MDP vesicles (N_2O -Saline) formulation showed a large amount of entrapment within / association with Pheroid[®] vesicles within the first hr after formulation. At 1 hr the highest concentration of visible Sn-MDP is observed at the interface between the Pheroid[®] vesicles and the aqueous medium. This appears to indicate that Sn-MDP is associated with the aqueous bilayers which form part of the Pheroid[®] vesicles structure (Grobler, 2009). At 2 hours the visible Sn-MDP seems more concentrated in bright spots which might indicate that the Sn-MDP is now concentrated within the Pheroid[®] vesicles. This trend continues for the 3 and 6 hr images. For clinical applications this is very promising since it indicates that Sn-MDP is entrapped within / associated with the Pheroid[®] vesicles for the first 6 hrs after preparation, which falls well within the time frame for use.

This also highlights that stability of formulations as indicated by particle size analysis and ζ -potential measurements is not an indication of entrapment as this formulation showed considerable instability (Figure 2, formulation F).

3.2.3. Pheroid[®]-Sn-MDP vesicles (N_2O - H_2O), MDP added 4 days after manufacturing

Figure 10 shows that a certain amount of Sn-MDP is already concentrated within the Pheroid[®] vesicles at 1 hr after formulation. The amount does not change very much. As can be seen from the images obtained at 1, 2, 3, 6, 12 and 24 hrs there is little observable change in the concentration of Sn-MDP associated with the Pheroid[®] structures. Notably the concentration might be lower at 12 hrs, but at 24 hrs accumulation of the bone tracer molecules is again seen.

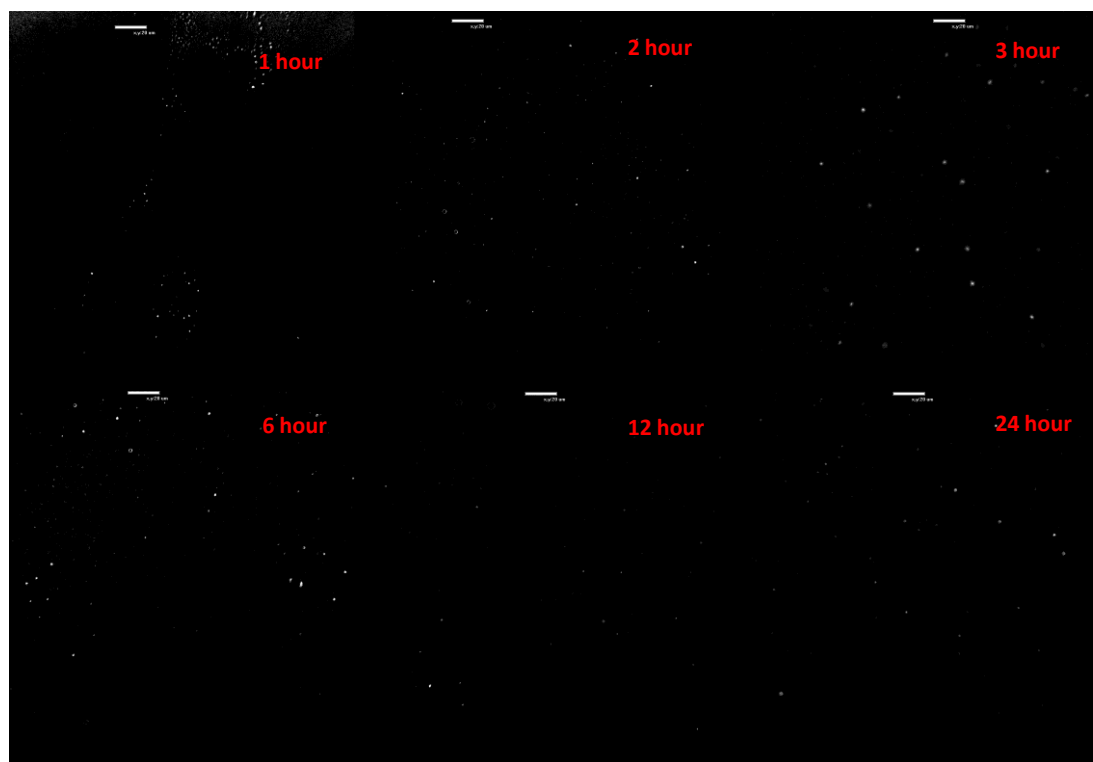


Figure 10. Reflectance micrograph of Pheroid®-Sn-MDP vesicles (N_2O - H_2O), MDP added 4 days after manufacturing.

The stability is also consistent with the particle size analysis and ζ -potential which indicated a stable formulation (Figure 3, formulations **I** and **J**). Association or entrapment took place within 1 hour after formulation and the formulation remained stable thereafter. Grobler (2009) does mention that a dynamic stability is established within the Pheroid® vesicles and this might account for the slight changes in Sn-MDP concentrations observed at different time points. This dynamic stability was observed in previous formulations which yielded good clinical results for a variety of active pharmaceutical ingredients (Grobler, 2009).

Although this formulation does not show very high concentrations of Sn-MDP associated with or entrapped within Pheroid® it offers a significant advantage for clinical application: It can be prepared in advance with just the standard Sn-MDP (or ^{99m}Tc -MDP) kit being added as required prior to administration to a patient. The stability of the formulation indicates it will be usable for at least the first 6 hrs after preparation, which does not place such a strict time constraint on medical staff.

3.2.4. Pheroid®-Sn-MDP sponges (N_2O - H_2O), MDP added 4 days after manufacturing

Figure 11 shows that very little association / entrapment of Sn-MDP occurred at any time point within the first 24 hrs after preparation. No large changes can be detected, and this is consistent with the stability indicated by the particle size distribution analysis and ζ -potential. There were however significant changes in the span at 12 hours and leading up to 24 hours (Figure 3, formulation **M**). This is not apparent from the reflectance microscopy as no changes in entrapment occurred, but it is evident from the fluorescence and light microscopy images, which are presented in Figures 12 and 13. This formulation didn't show very promising entrapment / association of Sn-MDP.

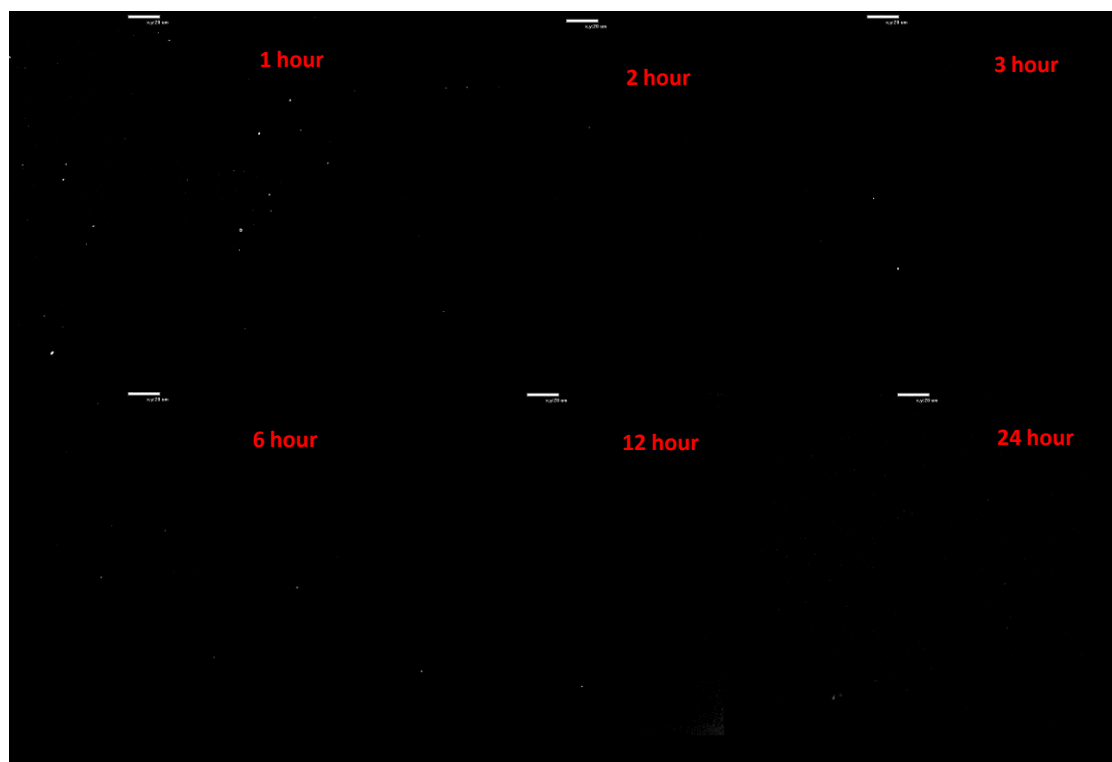


Figure 11. Reflectance micrograph of Pheroid[®]-Sn-MDP sponges (N₂O-H₂O), MDP added 4 days after manufacturing.

It is not completely unexpected that although the formulation shows marked stability, within the first 6 hours, it was determined to be unsuitable for the objectives of this study.

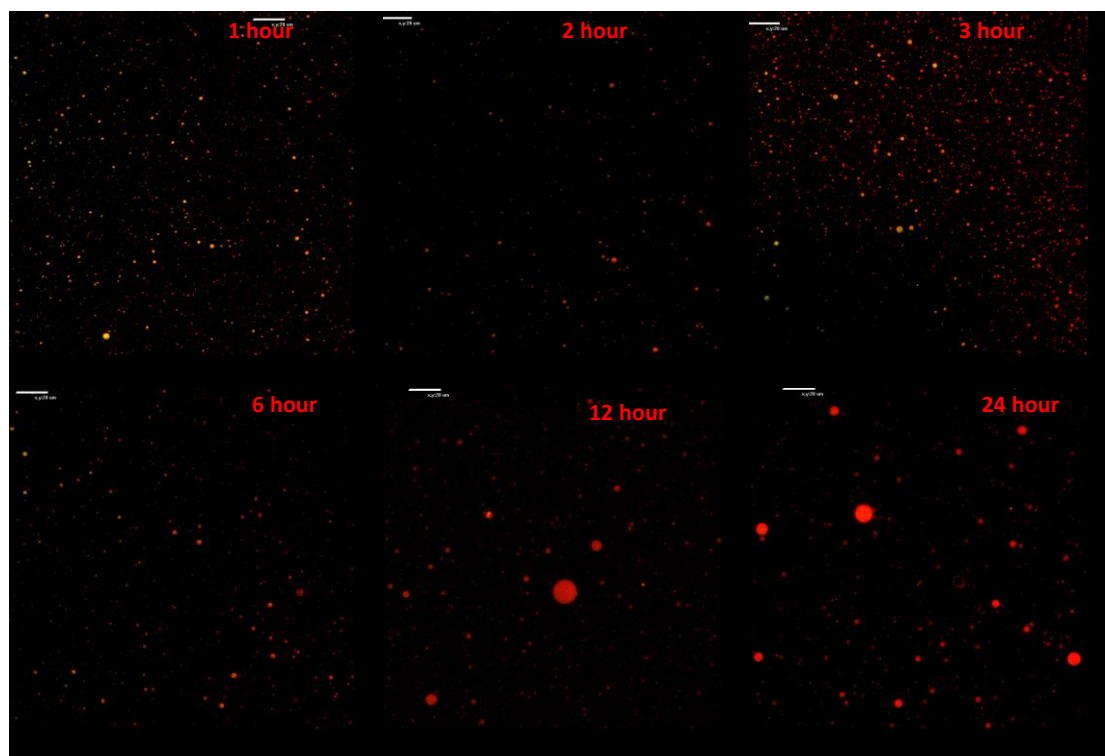


Figure 12. Fluorescence micrograph of Pheroid[®]-Sn-MDP sponges (N₂O-H₂O), MDP added 4 days after manufacturing.

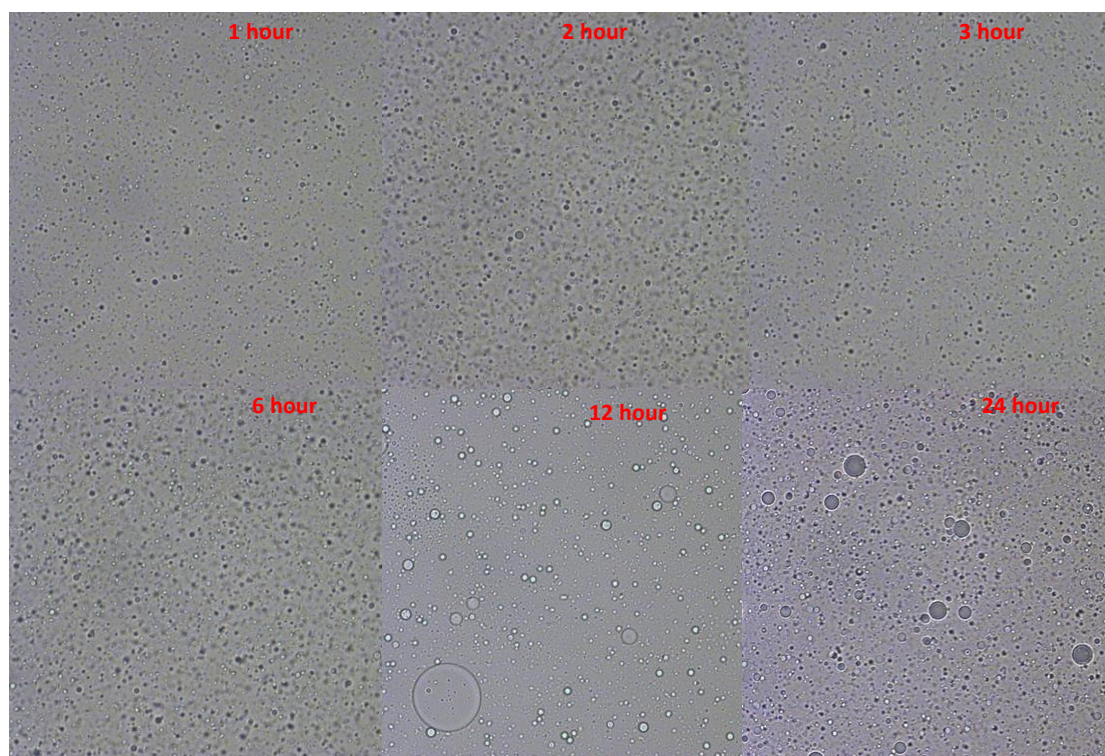


Figure 13. Light microscopy micrograph of Pheroid®-Sn-MDP sponges (N_2O-H_2O), MDP added 4 days after manufacturing.

3.2.5. Pheroid®-Sn-MDP vesicles (N_2O-H_2O), Vit. F: Kolliphor EL (1:1), MDP added 4 days after manufacturing

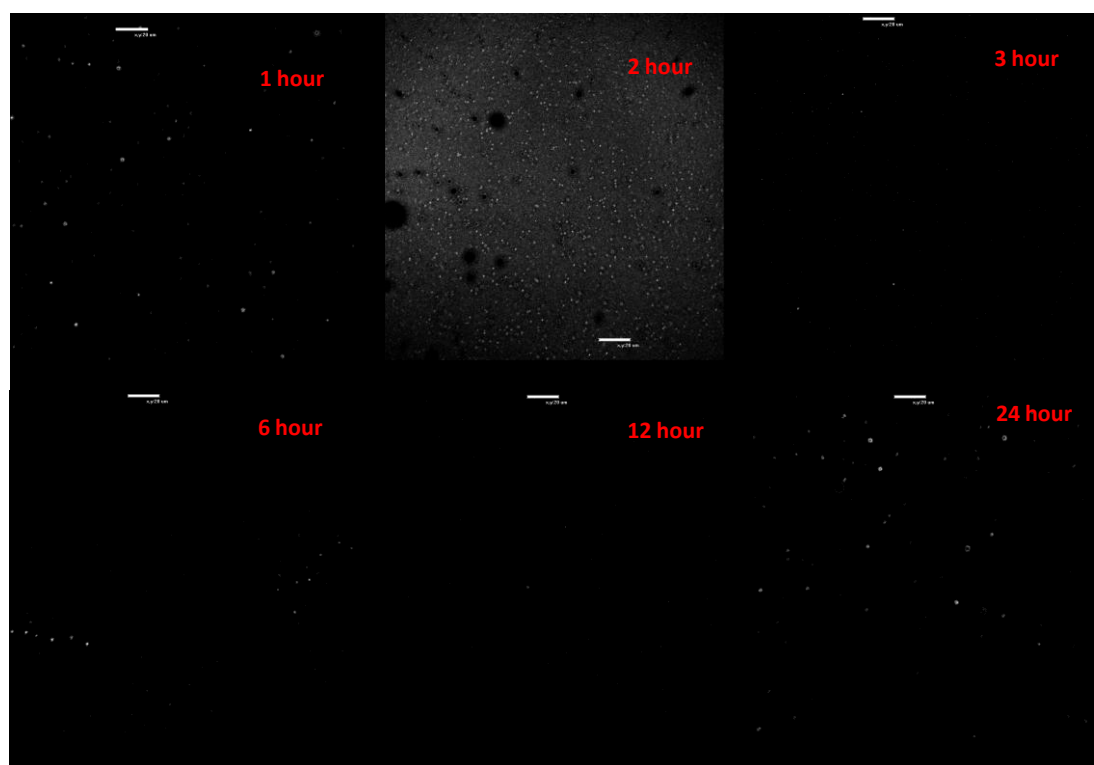


Figure 14. Reflectance micrograph of Pheroid®-Sn-MDP vesicles (N_2O-H_2O), Vit. F: Kolliphor EL ratio (1:1), MDP added 4 days after manufacturing.

Figure 14 shows that for the Pheroid[®]-Sn-MDP vesicles (N₂O-H₂O), Vit. F: Kolliphor EL (1:1) formulation the Sn-MDP was associated with the Pheroid[®] vesicles after which it clearly migrated away from the vesicles (at 2 hours). This easy migration might be due to the compromised structure of the vesicles, as a result of the high surfactant ratio, as discussed in section 3.1. Due to the instability of the formulation (Figure 4, formulation **O**) this formulation does not show any promise for the clinical application forming the basis of this study.

4. Conclusions

This study aimed to develop Pheroid[®]-Sn(II)-MDP formulations with possible future application as an active pharmaceutical delivery system which would allow oral administration of radio bone tracer MDP. Various formulation parameters were investigated. These included:

- Manipulation of the aqueous phase,
- addition of the radio tracer (Sn(II)-MDP) at different stages of formulation,
- surfactant ratios
- as well as pre-manufactured stable formulations with addition of Sn(II)-MDP preceding administration within a determinable timeframe.

In terms of stability as measured by means of particle size distribution analysis and ζ -potential, the formulations with Sn(II)-MDP added to the aqueous phase before mixing with the oil phase showed more stability than the formulations with Sn(II)-MDP added during mixing processes, or directly after mixing. Whilst the Pheroid[®]-Sn(II)-MDP vesicles (N₂O-H₂O) and Pheroid[®]-Sn(II)-MDP sponge did not show the greatest entrapment / association efficiency (N₂O-H₂O) formulations which were prepared and left for four days were shown to be stable, corresponding with previous observations (Uys, 2006; Grobler, 2009). The Pheroid[®]-Sn(II)-MDP vesicles (N₂O-H₂O), Vit. F: Kolliphor EL (1:1) formulation did not exhibit such stability, this is attributed to too high surfactant: lipid ratio resulting in leaky structures. The addition of Sn(II)-MDP did not have a large impact on the stability of the Pheroid[®]-Sn(II)-MDP vesicles (N₂O-H₂O) and Pheroid[®]-Sn(II)-MDP sponges (N₂O-H₂O) formulations over the first 12 hrs. The Pheroid[®]-Sn(II)-MDP vesicles (N₂O-H₂O), Vit. F: Kolliphor EL (1:1) formulation was shown to have undesirable stability from the onset, addition of Sn(II)-MDP did not improve stability, however applications requiring timed release may warrant further investigation.

Microscopy was used to confirm morphology and most importantly as a means to qualitatively assess entrapment of Sn(II)-MDP within the Pheroid[®] structures of the formulations. Microscopy showed that MDP undergoes dynamic association/entrapment with these pheroidal structures. Stability of the structures was also shown to be an inconclusive indication of entrapment. This was illustrated by Pheroid[®]-Sn(II)-MDP vesicles (N₂O-saline) with Sn(II)-MDP added to the aqueous phase before formulation which showed poor stability but excellent entrapment as discussed in section 3.2.2. Reflectance microscopy was found to be very useful in the process of identifying formulations that can be further investigated for possible clinical applications. The most promising formulations for orally administered radiopharmaceuticals were:

- Pheroid[®]-Sn(II)-MDP vesicles (N₂O-H₂O), MDP added to aqueous phase before mixing with oil phase
- Pheroid[®]-Sn(II)-MDP vesicles (N₂O-Saline), MDP added to aqueous phase before mixing with oil phase
- Pheroid[®]-Sn(II)-MDP vesicles (N₂O-H₂O), MDP added 4 days after manufacturing

Out of these three the Pheroid[®]-Sn(II)-MDP vesicles (N₂O-H₂O), MDP added 4 days after manufacturing (as summarised in Figure 15) is of most interest since it not only meets formulation

requirements, but it also offers an easy convenient way of preparing radiopharmaceutical formulations within a clinical setup.

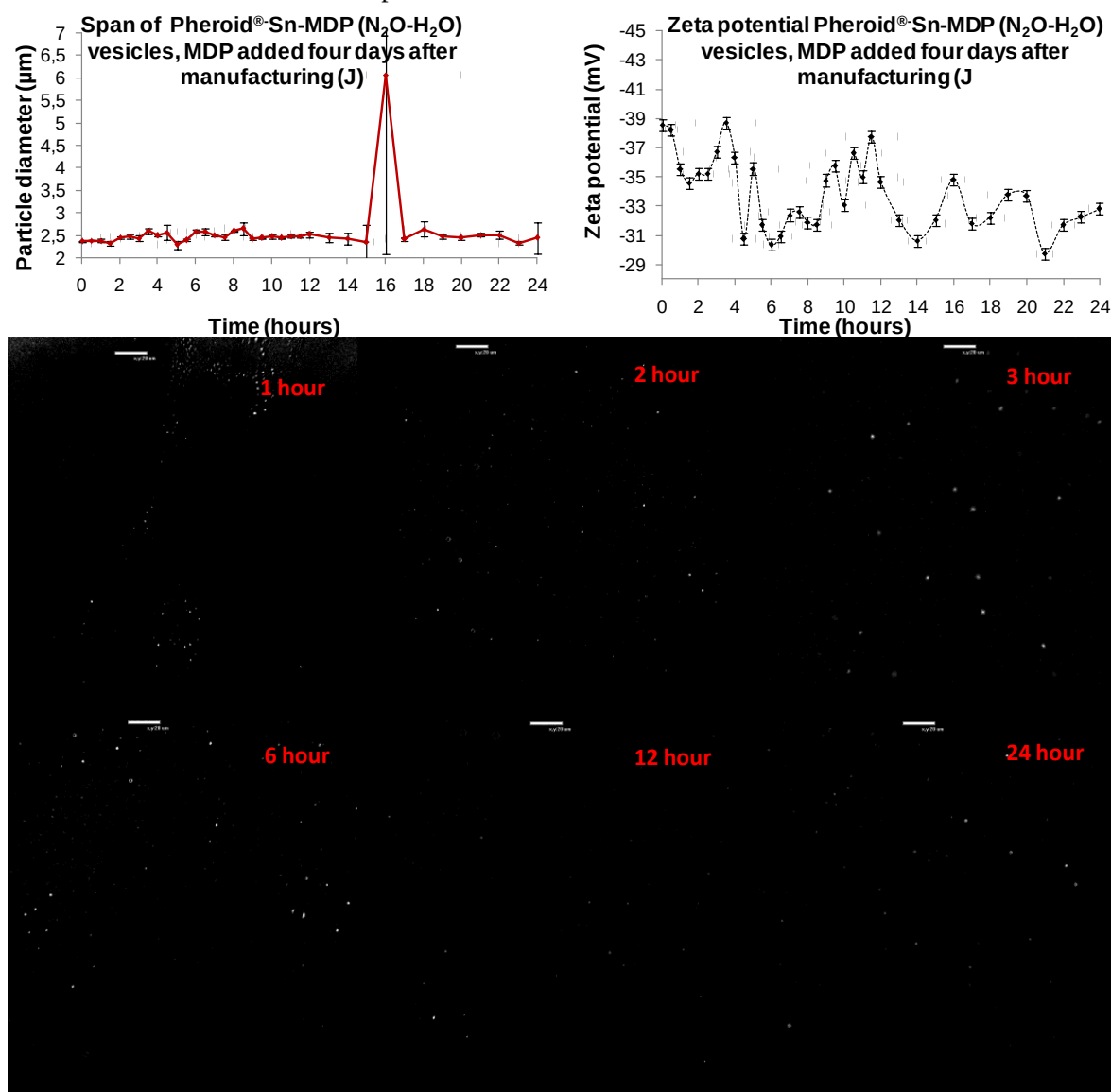


Figure 15. Pheroid®-Sn(II)-MDP vesicles (N_2O-H_2O), MDP added 4 days after manufacturing: Particle size span (top left) and ζ -potential (top right) as well as reflectance micrographs (bottom).

The quantitative measurement of entrapment as well as preclinical and clinical trials will be the focus of future studies.

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Appendix A

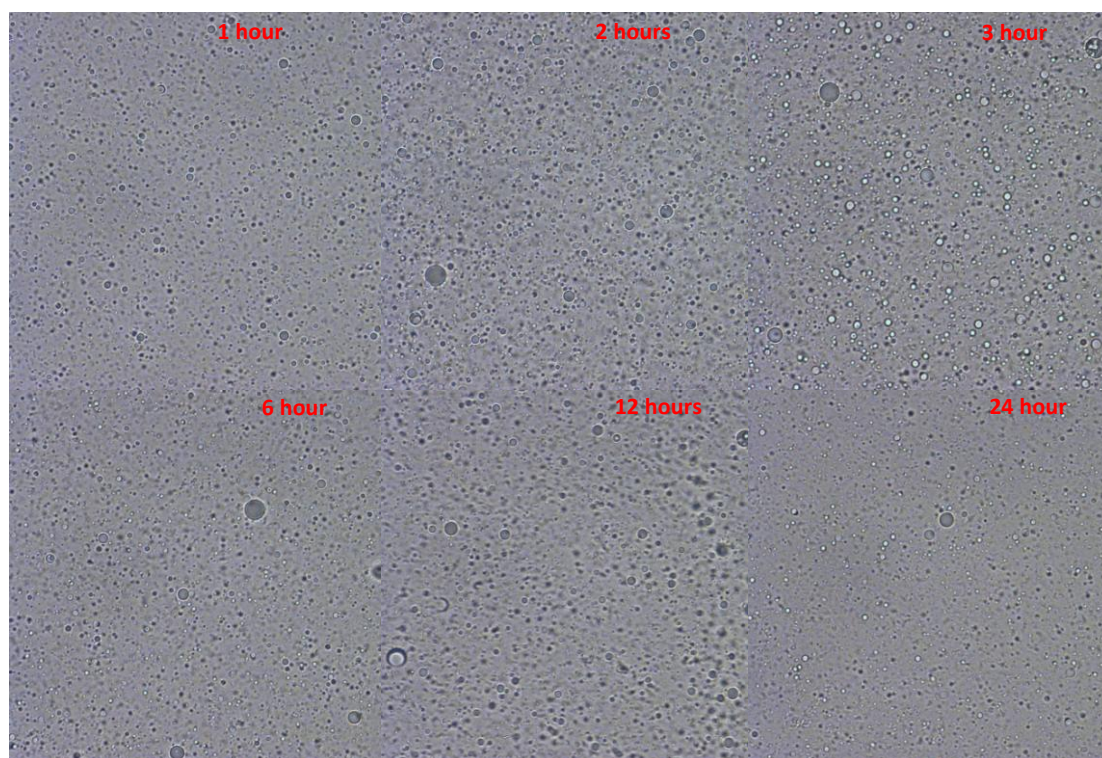


Figure I. Light microscopy micrograph of Pheroid®-Sn-MDP vesicles (N_2O - H_2 saline), MDP added to aqueous phase before mixing with oil phase.

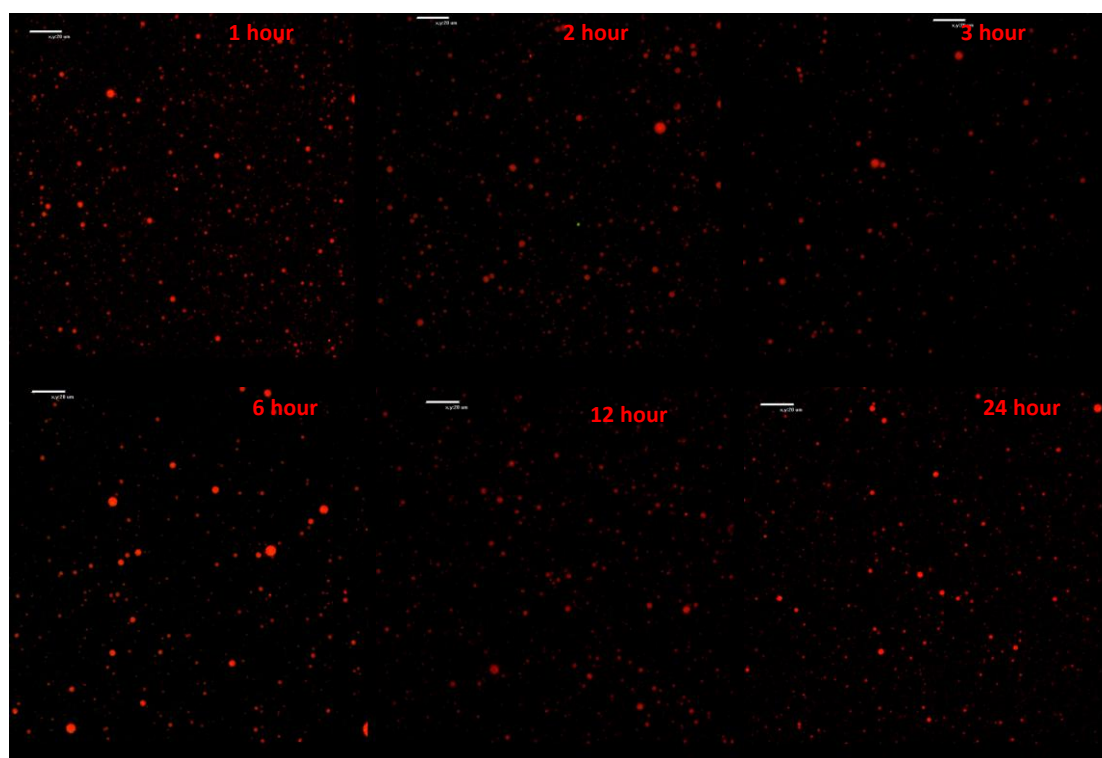


Figure II. Fluorescence micrograph of Pheroid®-Sn-MDP vesicles (N_2O -saline), MDP added to aqueous phase before mixing with oil phase.

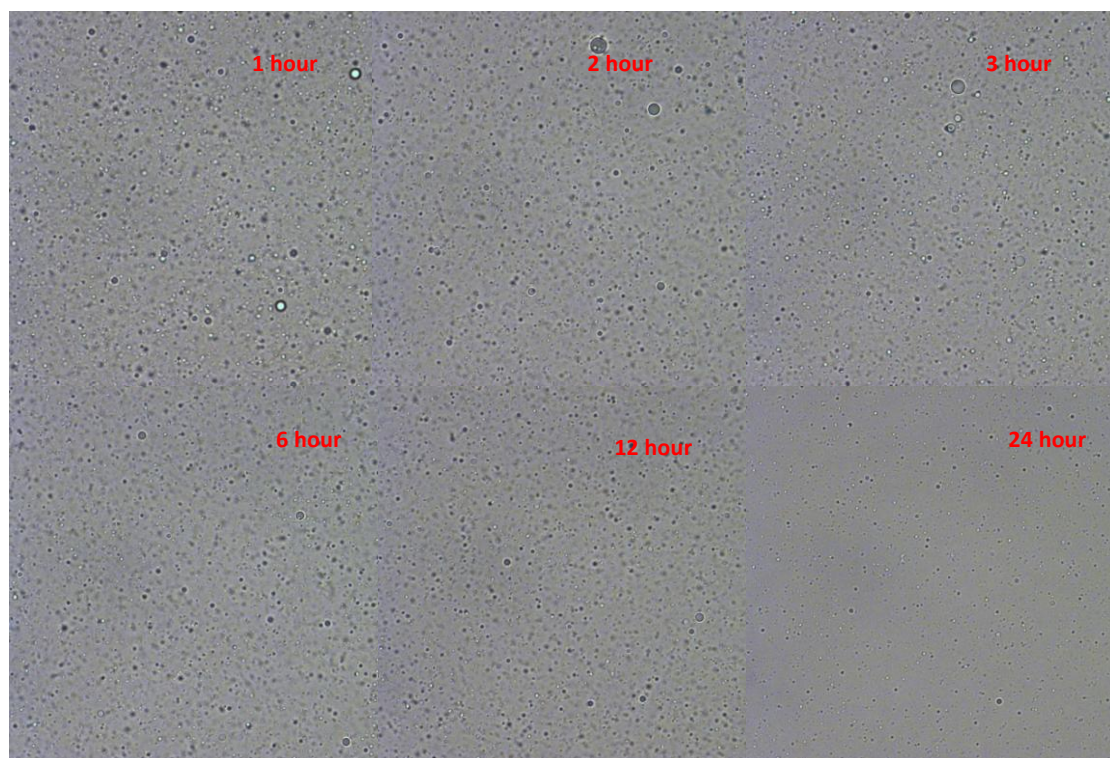


Figure III. Light microscopy micrograph of Pheroid[®]-Sn-MDP vesicles (N₂O-H₂O), MDP added 4 days after manufacturing.

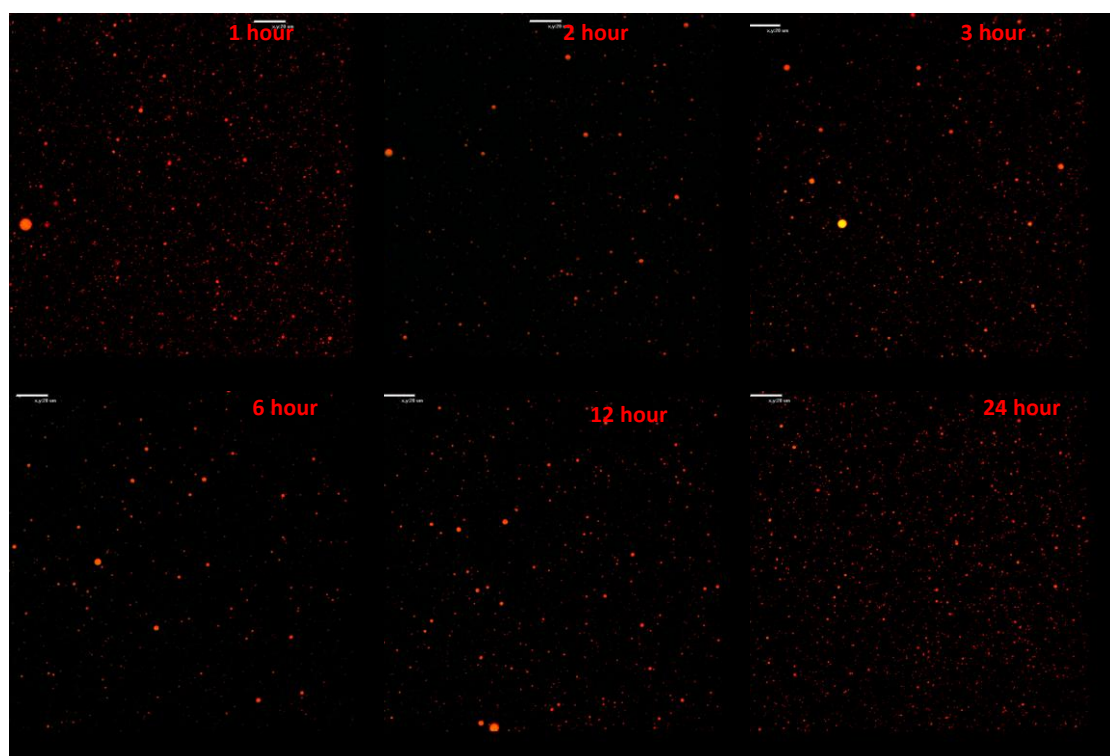


Figure IV. Fluorescence micrograph of Pheroid[®]-Sn-MDP vesicles (N₂O-H₂O), MDP added 4 days after manufacturing.

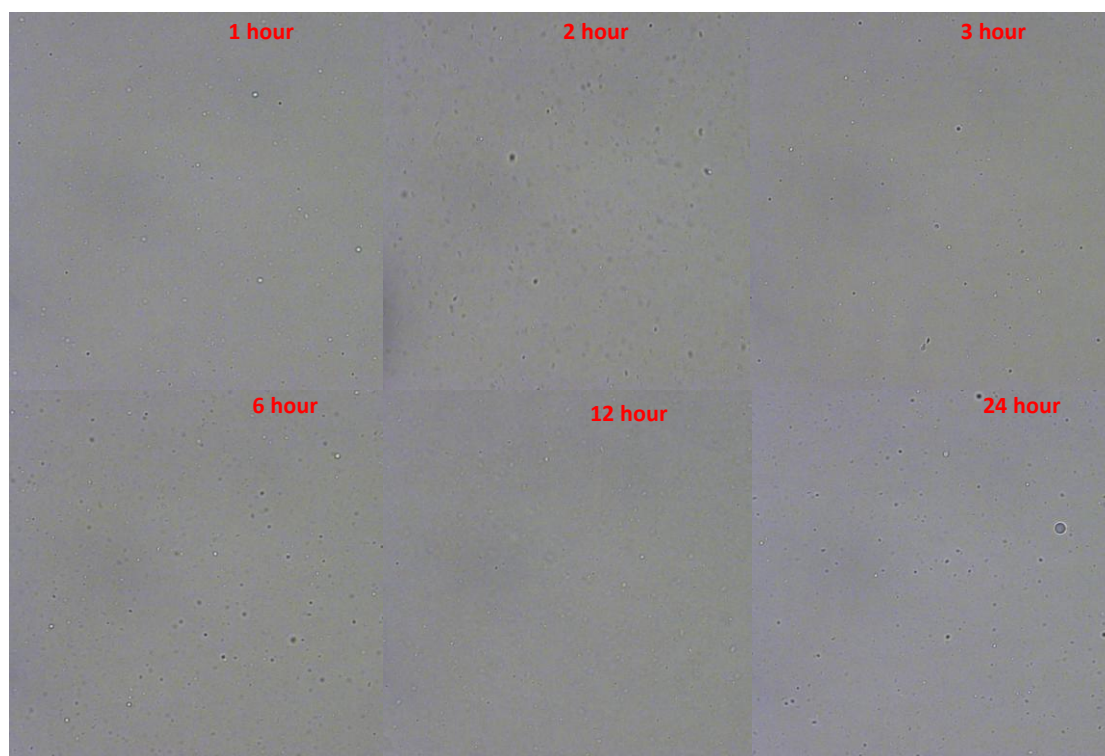


Figure V. Light microscopy micrograph of Pheroid[®]-Sn-MDP vesicles (N₂O-H₂O), Vit F: Koll. EL 1:1, MDP added 4 days after manufacturing.

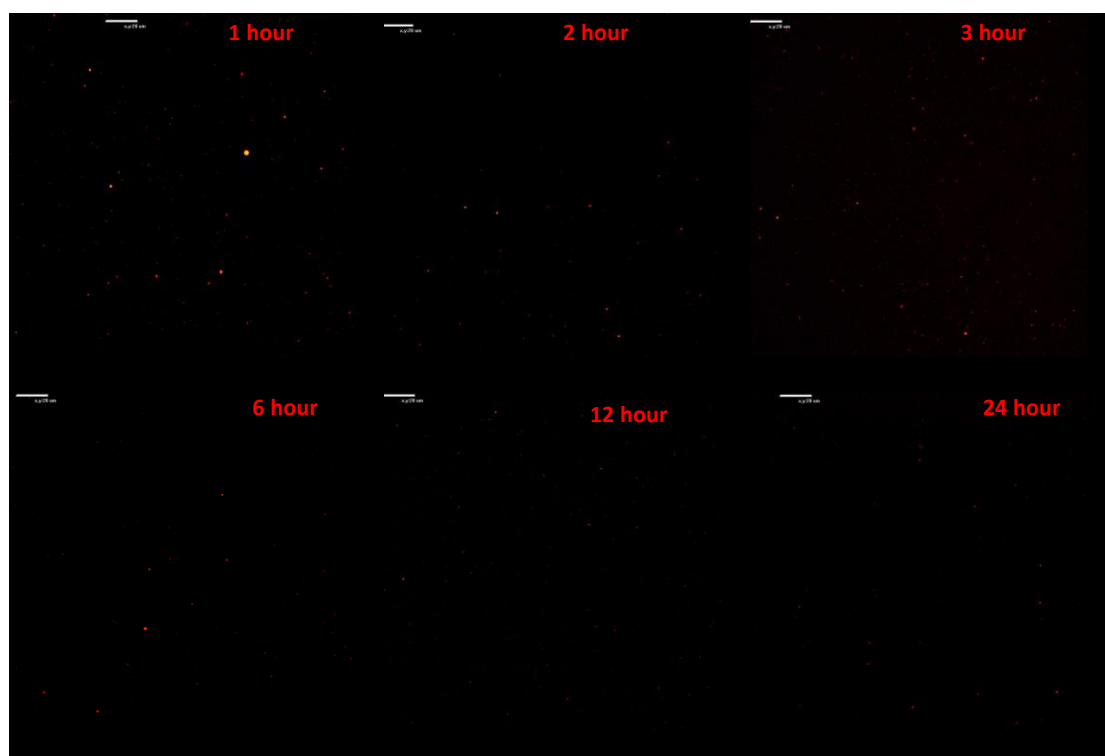


Figure VI. Fluorescence micrograph of Pheroid[®]-Sn-MDP vesicles (N₂O-H₂O), Vit F: Koll. EL 1:1, MDP added 4 days after manufacturing.

Chapter | 5

Method for quantitative measure of entrapment efficiencies for ^{99m}Tc -Methylene diphosphonate encapsulated in Pheroid[®]

Adele van As^I, Nicola Barnard-Marais^I, Jan Rijn Zeevaart^{II}

^I DST/NWU Preclinical Drug Development Platform, North-West University, Potchefstroom Campus, 2520, South Africa.

^{II} Radiochemistry, South African Nuclear Energy Corporation SOC Limited (Necsa), Pretoria, 0001, South Africa.

ABSTRACT

Selected Pheroid[®] formulations containing ^{99m}Tc -Methylene diphosphonate (MDP) were prepared. With the aim to develop a method to quantify the entrapment efficiencies of ^{99m}Tc -MDP encapsulated in the Pheroid[®] drug delivery system. In order to achieve this, the affinity of ^{99m}Tc -MDP for hydroxyapatite (HAP), a component of bone and used as an in vitro model for bone in this study, was used to indirectly quantify the amount of ^{99m}Tc -MDP associated with or entrapped in Pheroid formulations. In the absence of any metabolic activity it was found that ^{99m}Tc -MDP not associated with HAP is associated with Pheroid[®]. The amount of ^{99m}Tc -MDP associated with HAP was measured by means of γ emission detection. Entrapment efficiencies varied, depending on the type of Pheroid[®] formulations. Addition of ^{99m}Tc -MDP to the aqueous phase of Pheroid[®] vesicles before preparation yielded $98.69 \pm 1.004\%$ entrapment (after 2 hours) while addition of ^{99m}Tc -MDP pre-prepared Pheroid[®] vesicles yielded $98.26 \pm 0.07\%$ entrapment (after 12 hours).

Highlights:

- Indirect method to determine entrapment efficiencies in sensitive systems
- Statistically reproducible

Keywords: Pheroid[®], Sn-MDP, entrapment efficiencies, hydroxyapatite

1. Introduction

^{99m}Tc -MDP is an established radiopharmaceutical with bone seeking characteristics while Pheroid[®] technology is a novel patented drug delivery system consisting of unsaturated fatty acids, emulsified in water saturated with nitrous oxide (N_2O) (Grobler & Kotze, 2006; Grobler *et al.*, 2008; Grobler, 2008).

Pheroid[®] is a dynamic system with the capacity to entrap, protect and deliver hydrophilic, hydrophobic and amphiphilic active pharmaceutical ingredients (API) molecules across biological membranes (Du Plessis *et al.*, 2010). Due to its dynamic nature Pheroid[®] is usually characterised using techniques such as light microscopy, confocal laser scanning microscopy (CLSM) and dynamic light scattering (DLS) particle size determination, which have the advantage of being non-invasive and non-destructive (Balog *et al.*, 2015). DLS has the disadvantage that it only provides quantitative information about the whole formulation without being able to elucidate the entrapment efficiencies of APIs. CLSM on the other hand could provide information about entrapment, but because it is mostly qualitative its usefulness is also limited.

Technetium compounds have been used as imaging agents for some time (Wang *et al.*, 1979; Schwochau, 1994; M Rey, 2010; Barh *et al.*, 2014). ^{99m}Tc-MDP, the technetium radiopharmaceutical investigated in this study, accumulates in bone and is useful in skeletal imaging (Subramanian *et al.*, 1975; Kung *et al.*, 1978; Blake *et al.*, 2011).

MDP is commercially available in kits containing methylene diphosphonic acid or a corresponding sodium salt, a reducing agent such as Sn(II) chloride or Sn(II) fluoride, and a stabilising agent such as ascorbic acid or 2,5 di-hydroxybenzoic acid (DHBA)(Lever & Lever, 2009).

^{99m}Tc-MDP is administered intravenously and gamma rays emitted during the decay of the radioisotope are used to produce images. When administered intravenously, the primary uptake route of ^{99m}Tc-MDP is through adsorption onto or into the crystalline structure of hydroxyapatite. After administration, 80% of the ^{99m}Tc-MDP is generally cleared from the body after 3 hrs, while between 10 and 20% is found adsorbed to bone tissue.

A preceding study utilising ^{99m}Tc-MDP to observe the biodistribution of Pheroid[®] resulted in a patent for Pheroid[®]-radiotracer formulations (Grobler & Zeevaart, 2015; Swanepoel, 2015). Swanepoel (2015) showed that Pheroid[®] allows the oral administration of ^{99m}Tc-MDP in rats. If ^{99m}Tc-MDP can be formulated into an optimised orally administered dosage form, it may provide an alternative to intravenous administration and improve patient compliance and comfort.

Medical applications require that the formulations must be characterised and the entrapment efficiencies of the ^{99m}Tc-MDP in Pheroid[®] determined. As mentioned before the dynamic nature of Pheroid[®], creates problems if the formulation is not fluorescent, reflective or labelled with a fluorophore. Furthermore it is problematic to analyse radioactive formulations in standard laboratories and equipment since there is a very real risk of contamination.

Jansen *et al.* (2009) demonstrated that hydroxyapatite (HAP), a component of bone, can be used to quantify the chemisorptions of bone tracers. The adsorption and desorption of Sn⁴⁺ onto HAP was studied by incubating Sn⁴⁺-PEI-MP, a bone tracing radiopharmaceutical, on HAP. The supernatant

was filtered after a specified time and the amount of Sn^{4+} on the HAP and in the supernatant was measured respectively.

This study aimed to use the affinity of $^{99\text{m}}\text{Tc}$ -MDP for HAP to indirectly quantify the amount of $^{99\text{m}}\text{Tc}$ -MDP associated with or entrapped in Pheroid[®] formulations. The adsorption of $^{99\text{m}}\text{Tc}$ -MDP onto HAP is influenced by pH as well as the presence of other ions in solution

In the absence of metabolic activity it was postulated that $^{99\text{m}}\text{Tc}$ -MDP would exhibit such a high affinity for HAP that the portion of bone tracer not adsorbed onto HAP is strongly enough associated with or entrapped within the structures of Pheroid[®].

2. Materials and methods

2.1 Materials

Vitamin F ethyl ester, Incromega E7010 and Incromega E3322 were purchased from Croda, South Africa, dL- α -tocopherol was purchased from Chempure, South Africa. Kolliphor EL was purchased from BASF. Nitrous oxide was acquired from Afrox. All were used without further purification. MDP kits are manufactured by NTP Radioisotopes SOC/Necsa and were gifted by Necsa.

2.2 Preparation of $^{99\text{m}}\text{Tc}$ -MDP solutions

Radiolabelling was achieved by adding $^{99\text{m}}\text{Tc}$ in 10 mL saline to a lyophilised vial containing 5 mg MDP. Thin layer chromatography (TLC) was used to confirm the degree of labelling, mobile phase being acetone.

R_f , the retardation factor, is the fraction of a compound that is found in the mobile phase of a chromatographic system (Decristoforo *et al.*, 2007). R_f values can be measured for radioactivity from TLC plates in a similar fashion, the activity at the point of origin and at the solvent front can be measured and expressed as percentage MDP that is radiolabelled:

$$\% \text{ labelled} = \frac{^{99\text{m}}\text{Tc-MDP (at origin)}}{^{99\text{m}}\text{Tc-MDP (at origin)} + ^{99\text{m}}\text{Tc-MDP (unbound, at solvent front)}} \times 100 \quad (\text{Eq. 1})$$

2.3 Preparation of $^{99\text{m}}\text{Tc}$ -MDP-Pheroid[®] formulations

2.3.1 General preparation of Pheroid[®] vesicle and sponge formulations

Du Plessis *et al.* (2010) described a general preparation method for Pheroid[®] vesicles. This method is flexible and was adjusted to meet the specific requirements of this study. Table 1 summarises the formulations that were analysed for entrapment efficiency.

Table 1. Various ^{99m}Tc -MDP and Pheroid[®] formulations prepared for entrapment efficiency studies

	Description
Formulation 1	Pheroid [®] vesicles with ^{99m}Tc -MDP added to aqueous phase (N_2O - H_2O) before preparation
Formulation 2	Pheroid [®] vesicles with ^{99m}Tc -MDP added to aqueous phase (N_2O -saline) before preparation
Formulation 3	Pre-prepared Pheroid [®] vesicles with Vitamin F: Kolliphor EL 1:1 ratio; ^{99m}Tc -MDP added 4 days after formulation
Formulation 4	Pre-prepared Pheroid [®] vesicles; ^{99m}Tc -MDP added 4 days after formulation
Formulation 5	Pre-prepared Pheroid [®] sponges; ^{99m}Tc -MDP added 4 days after formulation

2.3.1.1 Preparation of **Formulation 1**

N_2O - H_2O (7.6 mL) was heated to 70°C. The pre-mixed oil phase (4% m/m, 0.45 mL) was also heated to 70°C. Just before combining the two phases, ^{99m}Tc -MDP in saline (2 mL) was added to the aqueous phase. The oil phase was then added to the aqueous phase and homogenised with a Heidolph Diax 600 homogeniser (Labotec, Johannesburg, South Africa) at 8000 rpm for 4 min. The formulation was prepared in triplicate.

2.3.1.2 Preparation of **Formulation 2**

This formulation was investigated due to the presence of a small amount of saline in the MDP kit as well as the availability of saline solutions in potential clinical setting. N_2O -saline (7.6 mL, 0.9 g/L) was heated to 70°C. The pre-mixed oil phase (4% m/m, 0.45 mL) was also heated to 70°C. Just before combining the two phases, ^{99m}Tc -MDP in saline (2 mL) was added to the aqueous phase. The oil phase was then added to the aqueous phase and homogenised at 8000 rpm for 4 minutes. The formulation was prepared in triplicate.

2.3.1.3 Preparation of **Formulation 3**

N_2O - H_2O (96 mL) was heated to 70°C. Vitamin F ethyl ester (1.9 g) and Kolliphor EL (1.9 g) was heated to 70°C. After removing from heat DL- α -tocopherol (0.2 g) was added to the Vitamin F ethyl ester and Kolliphor EL mixture. The oil phase was then added to the aqueous phase and homogenised. After homogenising the formulation was left under N_2O (200kPa) for four days.

Preliminary investigations showed that standard Pheroid preparation involving slow agitation over 12 hrs yielded unstable poorly entrapped MDP-Pheroid formulations and therefore this practise was abandoned.

Instead ^{99m}Tc -MDP in saline (2mL) was added to 10 mL of the pre-prepared vesicles and vortexed for 1 min. The formulation was prepared in triplicate.

2.3.1.4 Preparation of **Formulation 4**

N_2O - H_2O (96 mL) was heated to 70°C . Vitamin F ethyl ester (2.8 g) and Kolliphor EL (1 g) was heated to 70°C . After removing from heat DL- α -tocopherol (0.2 g) was added to the Vitamin F ethyl ester and Kolliphor EL mixture. The oil phase was then added to the aqueous phase and homogenised. After homogenising the formulation was left under N_2O (200kPa) for four days. ^{99m}Tc -MDP in saline (2 mL) was added to 10 mL of the prepared vesicles and vortexed for 1 minute. The formulation was prepared in triplicate.

2.3.1.5 Preparation of **Formulation 5**

N_2O - H_2O (95.5 mL) was heated to 70°C . Vitamin F ethyl ester (2.8 g), KolliphorEL (1g), Incromega E7010 (0.25g) and Incromega E3322 (0.25 g) was heated to 70°C . After removing from heat DL- α -tocopherol (0.2 g) was added to the oil mixture. The oil phase was added to the aqueous phase and homogenised. The mixture was homogenised with a Heidolph Diax 600 homogeniser (Labotec, South Africa) at 13500 rpm for at least 4 min or until below 40°C . After homogenising the formulation was left under N_2O (200kPa) for four days. ^{99m}Tc -MDP in saline (2mL) was added to 10 mL of the prepared sponges and vortexed for 1 min.

All formulations were used immediately after preparation.

2.4 Validation of sampling method

In literature hydroxyapatite adsorption is used to determine the affinity of prospective bone-seeking radiopharmaceuticals (Jansen *et al.*, 2009). In described experimental procedures, radiotracer solutions are incubated on hydroxyapatite and then filtered through $0.22\ \mu\text{m}$ Millipore filters. The assumption is made that the supernatant is homogeneous and that an aliquot represents the whole sample (Jansen *et al.*, 2009).

After incubation of Pheroid[®] on hydroxyapatite, forceful separation of Pheroid[®] and hydroxyapatite (such as filtering through a microfilter) might influence the integrity of Pheroid[®] structures, resulting in a non-homogenous supernatant. Since the hydroxyapatite would not be compromised when separated from the supernatant, it was decided not to analyse the supernatant but rather the radioactivity present on the hydroxyapatite itself after temperate separation.

To confirm the validity of this analysis method, identical ^{99m}Tc -MDP saline samples were incubated on 50 mg hydroxyapatite for 15 min. One sample was filtered, as per literature and the amount of activity in the supernatant and on the hydroxyapatite was calculated. The other sample was

centrifuged, the supernatant decanted and dried, the amount of activity on the hydroxyapatite was measured and used to calculate the activity in the supernatant.

2.5 Measurement of entrapment efficiencies of ^{99m}Tc -MDP-Pheroid[®] systems

2.5.1 Preparation of HAP and ^{99m}Tc -MDP-Pheroid[®] samples

Triplicate samples containing 50 mg hydroxyapatite and 3mL of each formulation were prepared to be incubated at 37°C. The total activities of all samples were counted before incubation.

At time points 0, 1, 2, 3, 6, 12 and 24 hrs (after preparation) samples were removed from incubation and centrifuged at 5000 rpm for 5 minutes. The supernatant, containing Pheroid[®] as well as associated/entrapped ^{99m}Tc -MDP was decanted and the remaining hydroxyapatite deposit was oven dried at 90°C for at least 12 hrs. The deposited material was used for further analysis due to equipment requirements.

2.5.2 Measurement of activity on hydroxyapatite after drying

The total activities of all samples were counted in millicurie (mCi) before incubation. A Capintec dose calibrator was used to measure activity.

At time points 1, 2, 3, 6, 12 and 24 hrs samples were removed from incubation and centrifuged at 5000 rpm for 5 min. The supernatant was decanted and the remaining HAP deposit was dried at 90°C for at least 12 hrs.

The dried hydroxyapatite deposits were weighed and the radioactivity determined by counting using a Scintispec well counter. Prior to measurements the well counter was cross calibrated with the Capintec dose calibrator by making standard dilutions of the ^{99m}Tc eluate. The well counter gave readings as counts per μCi . All activity determinations were decay corrected.

A fraction of hydroxyapatite was lost during decanting. To correct for this, the amount of activity on the hydroxyapatite was calculated using:

$$\text{Activity on original HAP} = \frac{\text{Activity measured after centrifugation} \times \text{accurate mass before incubation}}{\text{Mass measured after centrifugation}} \quad (\text{Eq. 2})$$

The portion of the total sample associated with the hydroxyapatite is thus determined and represents the amount of MDP which is free to associate with the hydroxyapatite i.e. not entrapped/associated with the Pheroid[®] system.

2.5.3 Calculation of amount of ^{99m}Tc -MDP associated with / entrapped in Pheroid[®]

Using the total sample count as well as the corrected count of the hydroxyapatite measurements the % entrapment in Pheroid[®] was calculated using the following formula:

$$\% \text{ Entrapment} = \frac{\text{Total activity in sample} - \text{Corrected activity on hydroxyapatite}}{\text{Total activity in sample}} \times 100 \quad (\text{Eq. 3})$$

This model assumes that all ^{99m}Tc -MDP not adsorbed to hydroxyapatite is in some way strongly enough associated with Pheroid[®] to overcome its affinity for hydroxyapatite.

3. Results and discussions

3.1 Validation of sampling method

The activity of each sample was measured before separation, and for the filtered sample the supernatant activity was measured while the activity of the hydroxyapatite was measured when the sample was centrifuged and decanted.

$$\text{Total activity} = \text{Hydroxyapatite activity} + \text{Supernatant activity}, \quad (\text{Eq. 4})$$

hence the activity on the hydroxyapatite and in the supernatant could be calculated for both samples.

For the filtered sample:

- **Total activity** = measured
- **Supernatant activity** = measured
- **Hydroxyapatite activity** = **Total activity** – **Supernatant activity** (Eq. 5)

For the centrifuged sample:

- **Total activity** = measured
- **HAP activity** = measured
- **Supernatant activity** = **Total activity** – **HAP activity** (Eq. 6)

It was confirmed that filtering or centrifuging a radiopharmaceutical solution incubated on HAP yielded the same activity measurement results. This validated the second method which was then further used in this study. Results for this part of the study are presented in Table 2, all activity measurements have been time corrected to account for decay.

Table 2. Results for comparison of sampling method used to determine activity in supernatant and on HAP.

Samples	Total activity (uCi)	Activity in supernatant(uCi)	Activity on HAP (uCi)	% Activity in supernatant	% Activity on HAP
Filtered	2755	1734,2	1021	65.30%	34.70%
Centrifuged	2830	1797.7	1032	65.57%	34.43%

3.2 Statistical analysis of results

3.2.1 Reproducibility of results

One way analysis of variance (ANOVA) was used to determine reproducibility of recorded measurements. A null (H_0) hypothesis stated that means of the different data sets for each formulation would be equal, while the alternative hypothesis (H_a) stated that means of the different data sets for each formulation would be different: ($H_0: \mu_1 = \mu_2$ vs. $H_a: \mu_1 \neq \mu_2$). A significance level $\alpha = 0.05$ was applied.

The F-statistic value refers to variation within data sets and is compared to a F_{crit} ratio of explained to unexplained variation, given a true null hypothesis. F_{crit} values are available in tables for different degrees of freedom.

Reproducibility could only be claimed (H_0 was accepted) if the following held true: $p > \alpha$ and if $F \leq F_{crit}$.

Values of importance are found in Table 3, while all relevant data can be found in Annexure A.

Table 3. Statistical determination of reproducibility of measurements

	ANOVA <i>p</i> - value	ANOVA F Statistic	ANOVA Inference
Formulation 1	0.17	2.01	Reproducible
Formulation 2	0.33	1.18	Reproducible
Formulation 3	0.89	0.12	Reproducible
Formulation 4	0.52	0.69	Reproducible
Formulation 5	0.73	0.32	Reproducible

3.2.2 Comparison of formulations to determine statistically significant differences

Single factor ANOVA ($\alpha = 0.05$) was performed on all experimental results to compare different sample groups (formulations) with one another and determine whether differences were statistically significant. Tukey's HSD (Honest Significant Difference) post hoc test was used to determine which sample groups differ from each other. For Tukey's test an $\alpha = 0.01$ value was used to ensure that no false significant differences were detected.

A one-way between subjects ANOVA was conducted to compare the effect of different formulation parameters on the entrapment efficiency of ^{99m}Tc -MDP in Pheroid. Formulation parameters had a significant effect on entrapment efficiencies [$F(4, 25) = 26.81$, $p = 1.08 \times 10^{-8}$].

Post hoc comparisons using the Tukey HSD test indicated that Formulations 1 and 2, 1 and 3, 2 and 4, 3 and 4 as well as 4 and 5 differed significantly. The aqueous phase composition, time given for

formulation stabilisation as well as oil phase composition and surfactant ratios proved important parameters in these formulations, causing detectable differences between groups.

Table 4. Tukey post hoc formulation comparison

	Tukey HSD <i>p</i> - value	Tukey HSD Q Statistic	Tukey HSD Inference
Formulation 1vs. Formulation 2	0.001	8.489	$p < 0.01$, significant difference
Formulation 1vs. Formulation 3	0.001	10.830	$p < 0.01$, significant difference
Formulation 1vs. Formulation 4	0.899	0.768	Insignificant differences
Formulation 1vs. Formulation 5	0.001	9.787	$p < 0.01$, significant difference
Formulation 2 vs. Formulation 3	0.479	2.341	Insignificant differences
Formulation 2 vs. Formulation 4	0.001	7.712	$p < 0.01$, significant difference
Formulation 2 vs. Formulation 5	0.881	1.297	Insignificant differences
Formulation 3 vs. Formulation 4	0.001	10.062	$p < 0.01$, significant difference
Formulation 3 vs. Formulation 5	0.899	1.044	Insignificant differences
Formulation 4 vs. Formulation 5	0.001	9.019	$p < 0.01$, significant difference

3.3 Quantification and analysis of entrapment efficiencies

All results were evaluated in terms of the minimum and maximum entrapment efficiencies, time taken to achieve maximum entrapment as well as stability of entrapment.

3.3.1. Formulation 1

^{99m}Tc -MDP has a high affinity for hydroxyapatite, and any ^{99m}Tc -MDP not associated with hydroxyapatite will be accepted as entrapped / associated with the Pheroid® to such an extent that it can't adsorb onto the hydroxyapatite. When ^{99m}Tc -MDP was added to the aqueous phase (N_2O - H_2O) before preparation of Pheroid® vesicles very little ^{99m}Tc -MDP was associated with the hydroxyapatite and after 1 hr the entrapment was $98.26 \pm 0.197\%$. Maximum entrapment ($98.69 \pm 1.004\%$) was achieved after 2 hrs and minimum entrapment occurred after 3 hrs (97.67%). As shown in Figure 1 the stability of entrapment established after 1 hr, with the largest difference between values being 1.06%. Clinical applications require formulations to stabilise within at least the first 6 hrs, this formulation warrants further investigation. Despite differences between entrapment % at different times, ANOVA analysis suggests that entrapment % does not differ statistically, [$F(5, 13) = 1.3434$, $p = 0.3113$].

Formulation 1		
Time	Average (% entrapment)	Stdev
1 hr	98.26 (N=3)	0.197
2 hr	98.69 (N=3)	1.004
3 hr	97.63 (N=3)	0.262
6 hr	98.00 (N=3)	0.509
12hr	98.28 (N=3)	0.380
24 hr	98.28 (N=3)	0.381

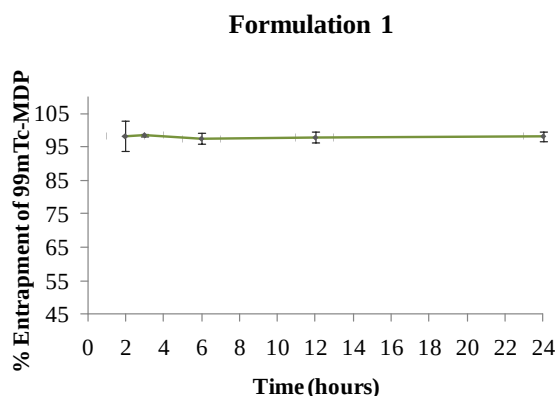


Figure 1. Summary of % entrapment efficiencies measured over 24 hrs for **Formulation 1**

3.3.2 Formulation 2

When ^{99m}Tc -MDP was added to a saline aqueous phase (N_2O -saline) before preparation of Pheroid[®] vesicles the formulation showed clear signs of entrapment, however the entrapment / association was unstable. The difference between formulations 1 and 2 was the amount of saline present in the aqueous phase. Although a small amount of saline will always be present in Pheroid[®]- ^{99m}Tc -MDP formulations, it is clear that an additional amount of saline is detrimental to entrapment efficiency of the drug delivery system. Maximum entrapment ($73.75 \pm 0.553\%$) was achieved after 12 hrs and minimum entrapment occurred after 3 hrs ($66.78 \pm 0.572\%$). As shown in Figure 2 entrapment does not stabilise during the first 24 hrs, with the largest difference between values being $6.97 \pm 3.654\%$. Since clinical applications require formulations to stabilise within at least the first 6 hrs, this formulation would not be suitable. Despite differences between entrapment % at different times, ANOVA analysis suggests that entrapment % does not differ statistically, [$F(5, 13) = 1.0108$, $p = 0.4499$]. Therefore, this formulation would exhibit statistically similar entrapment % at all time points measured. It is however important to note that despite the measurements at different time points not being significantly different, the formulation itself differs from the other formulations discussed.

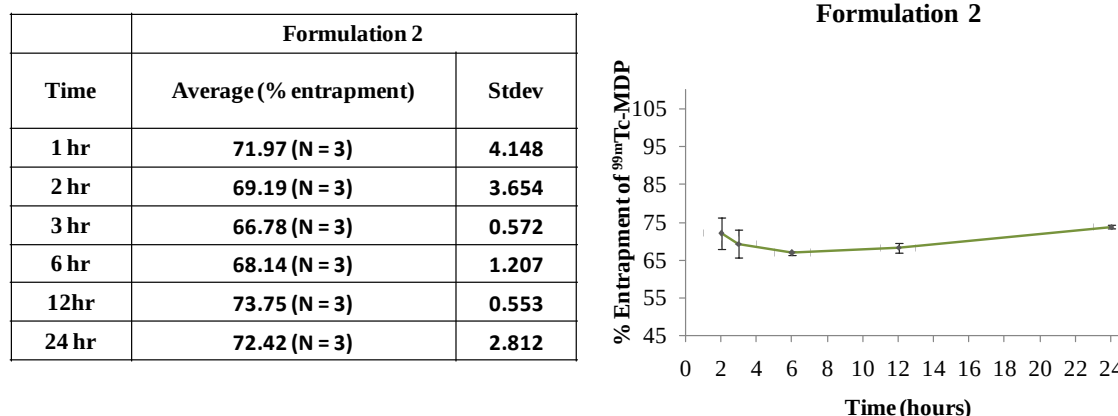


Figure 2. Summary of % entrapment efficiencies measured over 24 hours for **Formulation 2**

3.3.3 Formulation 3

^{99m}Tc -MDP was added to pre-prepared Pheroid[®] vesicles with Vitamin F: Kolliphor EL 1:1 ratio. The changed surfactant ratio influences the integrity and rigidity of the vesicles and can have an impact on stability. This parameter caused compromised stability for this formulation and unstable entrapment efficiencies were observed.

After 1 hr the entrapment was 65.33 ± 4.54 %, and the entrapment decreased over the next two hrs: 55.20% after 2 hrs and 50.84% (minimum entrapment) after 3 hrs. Only after 6 hrs did the entrapment % start to increase again, reaching maximum entrapment (78.99 ± 1.47 %) after 24 hrs. Figure 3 clearly shows that the entrapment % for this formulation does not stabilise within the first 24 hrs. This was confirmed by ANOVA analysis [$F(5, 13) = 10.5937$, $p = 0.0004$] which implies that the measurements taken for each time slot significantly differs from one another.

Because of its compromised integrity due to the surfactant : oil ratios, the vesicles could have dissembled when vortexed. This would explain the initial decrease in entrapment %. The high surfactant : oil ratio of the altered Pheroid[®] formulation is characteristic of microemulsions, which are known to self assemble (Solans & Kunieda, 1996; Bouchemal *et al.*, 2004). This means that the Pheroid[®] formulation would be able to reassemble and recover from the impact of vortexing. The entrapment % increase after 6 hrs could well be the result of Pheroid[®] vesicles reforming.

Due to its unstable entrapment % this formulation would not be suited for clinical application.

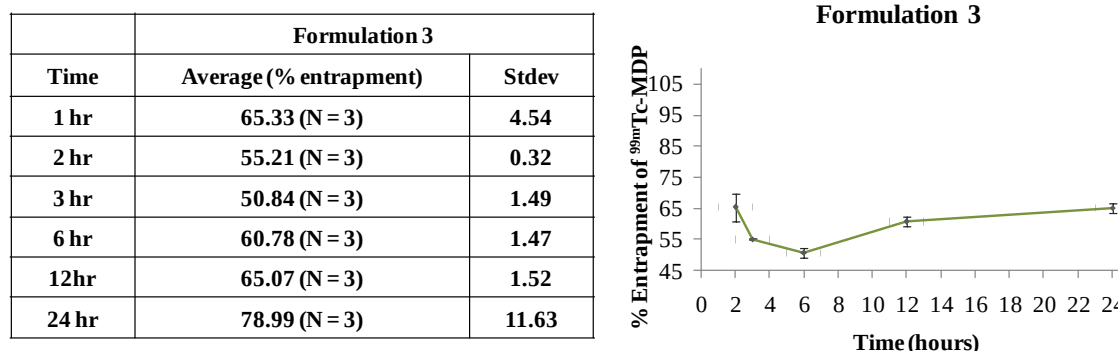


Figure 3. Summary of % entrapment efficiencies measured over 24 hours for **Formulation 3**

3.3.4. Formulation 4

^{99m}Tc -MDP was added to pre-prepared Pheroid[®] vesicles 4 days after formulation. Despite the incorporation of ^{99m}Tc -MDP into the formulation by means of vortexing, the formulation shows exceptional stability. The only formulation parameter difference between formulations 3 and 4 was the different surfactant ratios. Although a small amount of saline will always be present in Pheroid[®] - ^{99m}Tc -MDP formulations, it is clear that a higher Kolliphor EL ratio is detrimental to entrapment. After 1 hr the entrapment was $97.44 \pm 0.80\%$. For the first 6 hrs the entrapment % remains stable with the largest difference between values being 0.53%. Maximum entrapment ($98.26 \pm 0.07\%$) was achieved after 12 hrs and minimum entrapment occurred after 24 hrs ($86.51 \pm 10.22\%$). Graphical evidence suggest that this is a very stable formulation, and it is confirmed by ANOVA, [$F(5, 13) = 2.3479$, $p = 0.3590$]. Therefore this formulation would exhibit statistically similar entrapment % at all time points measured.

Its stable entrapment % makes this formulation suitable for possible clinical applications. It will also be very easily prepared by medical staff, which would be advantageous in a clinical setting.

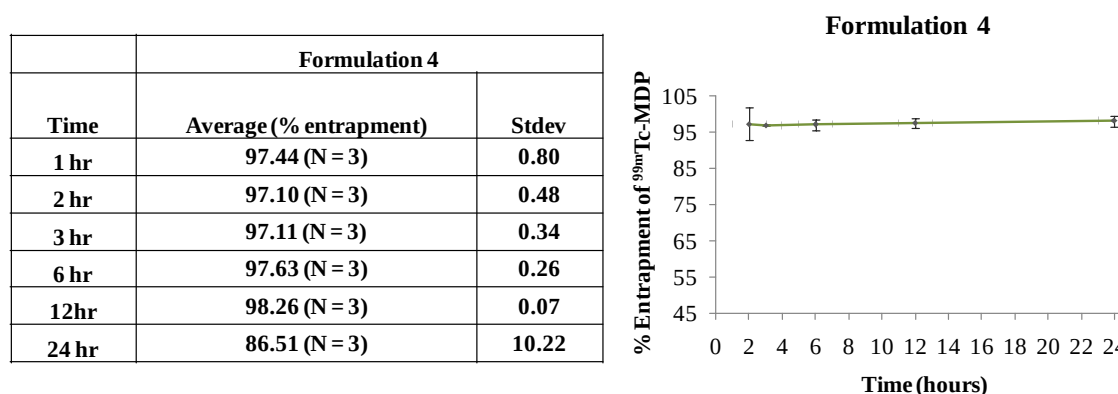


Figure 4. Summary of % entrapment efficiencies measured over 24 hours for **Formulation 4**

3.3.5 Formulation 5

^{99m}Tc -MDP was added to pre-prepared Pheroid[®] sponges 4 days after formulation. According to Uys (2006) and Grobler (2009) sponges are little lipid reservoirs which are best suited to entrap lipophilic active ingredients and previous studies showed significant enhancement in delivery of these compounds (Grobler, 2009). Pheroid[®] microsponges are able to cross most biological barriers, and its main advantage lies in its ability to cross biological barriers such as the digestive tract or skin (Grobler, 2009). This formulation was included in the study because of its barrier crossing abilities and to ensure all avenues were pursued to find an optimal preparation. Since sponges prefer lipophilic API, Tc^{99m} -MDP in aqueous solution was identified as a possible barrier to proper entrapment.

After 1 hr the entrapment was measured at $74.15 \pm 24.73\%$, thereafter entrapment decreased over the next two hrs: $52.21 \pm 5.63\%$ after 2 hrs and $48.71 \pm 17.37\%$ (minimum entrapment) after 3 hrs. Only after 6 hrs did the entrapment % start to increase again, reaching maximum entrapment ($81.79 \pm 6.65\%$) after 24 hrs. Figure 5 clearly shows that the entrapment % for this formulation does not stabilise within the first 24 hrs. This is confirmed by ANOVA which indicates that there are significant differences between measurements taken at different times, $[F(5, 13) = 3.1766, p = 0.0468]$

Due to varying stability as well as comparatively poor entrapment this formulation would not be suited for clinical application.

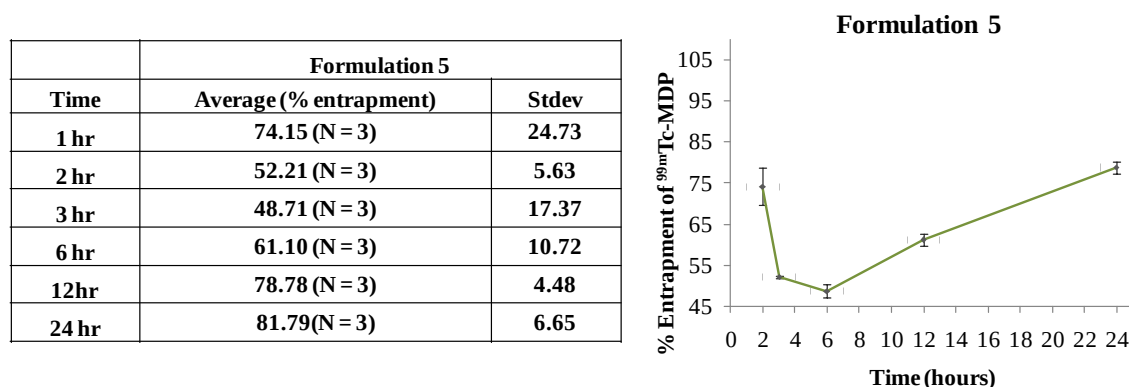


Figure 5. Summary of % entrapment efficiencies measured over 24 hours for **Formulation 5**.

4. Conclusions

The aim of this study was to develop a method for quantitative measurement of the entrapment efficiencies of the Pheroid[®] active pharmaceutical ingredient delivery system for the bone tracing radiopharmaceutical ^{99m}Tc -MDP.

Five potentially useful Pheroid® - ^{99m}Tc -MDP formulations were selected for investigation. All experiments were conducted in triplicate, 3 formulations were prepared separately for each formulation and subsequent entrapment studies were conducted on each preparation. ANOVA was used to determine reproducibility, applying a significance level $\alpha = 0.05$. Reproducibility was confirmed for all five formulations.

Formulations 2, 3 and 5 did not display high entrapment % and immediate entrapment stability; various possibly contributing formulation factors were considered and discussed in sections 3.3.2; 3.3.3 and 3.3.5.

- **Formulation 2** (maximum entrapment 73.75% after 12 hrs, no entrapment stability during first 24 hrs)
- **Formulation 3** (maximum entrapment 78.99% after 24 hrs, no entrapment stability during first 24 hrs)
- **Formulation 5** (maximum entrapment 81.79% after 24 hrs, no entrapment stability during first 24 hrs)

Formulations 1 and 4 displayed high entrapment % and rapid entrapment stability. The quantifying method confirmed both entrapment efficiency and stability as of 1 hr after preparation:

- **Formulation 1** (maximum entrapment 98.69% after 2 hrs, entrapment stability established after 1 hr and maintained for 24 hrs)
- **Formulation 4, Pre-prepared Pheroid® vesicles; ^{99m}Tc -MDP added 4 days after formulation.**
Maximum entrapment 98.26% after 12 hrs, although entrapment stability established after 1 hr and maintained for first 12 hrs. Its stable entrapment % makes this formulation suitable for possible clinical applications.

This quantitative measurement technique could prove useful when measuring entrapment of other compounds with a high affinity for HAP. The possibility also exists to apply the method to other compounds of interest which have radioactive isotopes surfaces materials with a high affinity for them. This study provides good indication of which Pheroid® - ^{99m}Tc -MDP formulations might have potential for further investigation and ultimately clinical application.

5. Acknowledgements

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Appendix A: Reproducibility of measurements

Formulation 1

Formulation 1: Entrapment efficiencies						
	Time=1 hr	Time=2	Time=3 hr	Time=6 h	Time=12 hr	Time=24 h
Set 1	98,43384725	99,817	97,57623656	98,0147	98,3958214	97,86138
Set 2	98,30583011	98,375	97,92021497	98,5012	98,59656206	98,60189
Set 3	98,04699465	97,887	97,40684811	97,4833	97,86138253	98,37774
Average	98,262224	98,693	97,63443321	97,9997	98,28458866	98,28034
STD	0,197078291	1,0035	0,261584634	0,50911	0,380002337	0,379742

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Row 1	6	590.0992	98.34987	0.622327
Row 2	6	590.3003	98.38339	0.065537
Row 3	6	587.0637	97.84396	0.129898

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	1.096114	2	0.548057	2.010575	0.168426	3.68232
Within Groups	4.088807	15	0.272587			
Total	5.184921	17				

Formulation 2

Formulation 2: Entrapment efficiencies						
	Time=1 hr	Time=2	Time=3 hr	Time=6 h	Time=12 hr	Time=24 h
Set 1	68,6384573	69,05	67,34162005	69,3234	74,33563766	75,47542
Set 2	76,61382126	72,911	66,79884972	68,1709	73,68216411	71,84196
Set 3	70,64805842	65,608	66,19891564	66,9098	73,2366251	69,94182
Average	71,96677899	69,19	66,77979514	68,1347	73,75147563	72,41973
STD	4,147996569	3,6536	0,571590457	1,20721	0,552775014	2,811677

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Row 1	6	424.1648	70.69413	11.23352
Row 2	6	430.0185	71.66976	13.20621
Row 3	6	412.5429	68.75715	8.984222

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	26.37404	2	13.18702	1.183614	0.3332	3.68232
Within Groups	167.1198	15	11.14132			
Total	193.4938	17				

Formulation 3

Formulation 3: Entrapment efficiencies						
	Time=1 hr	Time=2	Time=3 hr	Time=6 h	Time=12 hr	Time=24 h
Set 1	67,0794571	55,28	49,15039891	62,4154	65,92481081	65,97182
Set 2	68,73194644	54,852	52,01123892	59,5519	63,31701988	82,67306
Set 3	60,17358485	55,482	51,34683389	60,3826	65,97181653	88,33919
Average	65,32832946	55,205	50,83615724	60,7833	65,07121574	78,99469
STD	4,539958622	0,322	1,497228896	1,47324	1,519359968	11,62853

Anova: Single Factor**SUMMARY**

<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
Row 1	6	365.822	60.97034	52.21221
Row 2	6	381.1367	63.52278	123.477
Row 3	6	381.6961	63.61602	171.1569

ANOVA

<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Between Groups	27.04659	2	13.5233	0.116968	0.890417	3.68232
Within Groups	1734.23	15	115.6153			
Total	1761.277	17				

Formulation 4

Formulation 4: Entrapment efficiencies						
	Time=1 hr	Time=2	Time=3 hr	Time=6 h	Time=12 hr	Time=24 h
Set 1	98,25095765	97,51	97,48595103	97,3577	98,29723712	98,30616
Set 2	97,40566342	97,216	97,02112632	97,6519	98,17216065	80,24459
Set 3	96,65419565	96,579	96,83178821	97,885	98,30615861	80,97418
Average	97,4369389	97,102	97,11295519	97,6315	98,25851879	86,50831
STD	0,798840308	0,4757	0,336610536	0,26421	0,074921259	10,22375

Anova: Single Factor**SUMMARY**

<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
Row 1	6	587.2081	97.86801	0.211474
Row 2	6	567.711	94.6185	49.74499
Row 3	6	567.2306	94.53843	44.65703

ANOVA

<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Between Groups	43.30352	2	21.65176	0.686533	0.518453	3.68232
Within Groups	473.0675	15	31.53783			
Total	516.371	17				

Formulation 5

Formulation 5: Entrapment efficiencies						
	Time=1 hr	Time=2	Time=3 hr	Time=6 h	Time=12 hr	Time=24 h
Set 1	94,74456346	45,902	58,0303214	68,6709	83,43339816	74,50487
Set 2	46,71911768	56,734	59,43738163	48,825	78,40349962	87,5222
Set 3	80,98181308	53,99	28,66536941	65,7907	74,50487493	83,34644
Average	74,14849807	52,208	48,71102415	61,0955	78,7805909	81,79117
STD	24,73118489	5,6314	17,37429596	10,7237	4,47619036	6,646565

Anova: Single Factor

SUMMARY

<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
Row 1	6	425.2858	70.88096	306.8309
Row 2	6	377.6407	62.94012	271.3088
Row 3	6	387.2789	64.54649	424.6348

ANOVA

<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Between Groups	211.5259	2	105.7629	0.316411	0.733507	3.68232
Within Groups	5013.872	15	334.2582			
Total	5225.398	17				

Chapter | 6

6.1. Summary

Pheroid is a novel patented delivery system consisting of essential and plant unsaturated fatty acids, which are emulsified in water saturated with nitrous oxide (N₂O) (Grobler & Kotze, 2006; Grobler *et al.*, 2008; Grobler, 2008). Pheroid can entrap, protect and deliver hydrophilic, hydrophobic and amphiphilic compounds across diverse biological membranes (Du Plessis *et al.*, 2010b). Some established applications of Pheroid include: anti-malarials, enhanced tuberculosis treatments, peptide delivery, topical drug delivery and improved antibiotic efficacy (Meyer, 1997; Grobler & Kotze, 2006; Grobler, 2008; Du Plessis *et al.*, 2010a; Du Plessis *et al.*, 2010b; Du Plessis *et al.*, 2014). As a lipid-based drug delivery system, Pheroid has the advantage of increasing gastrointestinal absorption of drugs (Grobler *et al.*, 2008; Steyn *et al.*, 2011; Grobler *et al.*, 2014).

Significantly, Pheroid technology could provide a practical alternative means of delivery for injectable currently orally unavailable drugs (Du Plessis *et al.*, 2014). An example of such a drug is ^{99m}Tc-MDP, an intravenously administered radiotracer used during bone scans.

A study utilising ^{99m}Tc-MDP to observe the biodistribution of Pheroid led to a patent for Pheroid-radiotracer formulations (Grobler & Zeewaart, 2015; Swanepoel, 2015). Swanepoel (2015) showed that Pheroid allows the oral administration of ^{99m}Tc-MDP in rats and suggested that Pheroid might enhance the bioavailability of radiopharmaceuticals and potentially allow oral administration. This is noteworthy, as studies have confirmed that patients receiving radiotherapy prefer oral administration of drugs to an intravenous route. (Liu *et al.*, 1997; Borner *et al.*, 2001; Nsubuga & Jaakkola, 2005).

Due to half-lives of the radioisotopes as well as other considerations revolving around radio-pharmaceuticals, formulations containing radiotracers need to be prepared on site in designated areas by medical personal shortly before administration. To ensure that entrapment in the drug carrier does not take too long (after which imaging might not be possible) an optimised formulation of Pheroid^{99m}Tc-MDP is needed. For successful clinical implementation, maximum entrapment of ^{99m}Tc-MDP in the Pheroid delivery system within 2-6 hrs of final formulation is necessary.

In order to achieve optimal entrapment in the shortest time span this study aimed to determine and study different factors that might affect the entrapment efficiencies (EE) and stability of ^{99m}Tc-MDP / MDP in Pheroid and to optimise these processes.

The objectives of this study can be summarised as follow:

7. Formulate Sn-MDP (non-radioactive) in combination with different types of Pheroid and with varying formulation parameters
8. Characterise and evaluate all Pheroid-MDP formulations in terms of particle size distribution, ζ- potential, and pH and morphology over time.
9. Prepare ^{99m}Tc-MDP (radioactive) formulations based on promising results identified from objectives 1 and 2.
10. Confocal reflectance microscopic imaging of selected formulae.
11. Develop a method for and determine the entrapment efficiencies of selected Pheroid-MDP formulations by means of unique quantitative radio adsorption measurements of hydroxyapatite adsorption.
12. Resolve optimal entrapment time requirements for Pheroid-MDP formulations.
13. Determine optimal Pheroid-MDP formulation parameters for future *in vivo* studies.

6.1.1. Chapter 4 (Preparation and characterisation of Pheroid[®] formulations containing Sn-Methylene diphosphonate)

Objectives 1, 2 and 4 were achieved and reported in Chapter 4.

To achieve these goals formulation parameters were systematically changed in order to study their effect on morphology and stability of the Pheroid-MDP system. Parameters that were changed included: manipulating the aqueous phase, adding Sn-MDP at different stages of formulation, changing surfactant: lipid ratios and allowing a stabilisation period for the Pheroid component before addition of Sn-MDP. The effects of pH variance (as well as/in) buffered systems was also evaluated.

The Pheroid-Sn-MDP formulations with systematically varied formulation parameters were prepared and characterised using particle size distribution, ζ -potential, pH and morphology. Particle size distribution analysis and ζ -potential was used to measure formulation stability. All formulations were prepared and analysed in triplicate and statistically verified for reproducibility.

- Formulations with Sn-MDP added to aqueous phase before mixing with the oil phase showed more stability than formulations with Sn-MDP added during mixing, or directly after the mixing processes.
- Pheroid-Sn-MDP vesicles (N₂O-H₂O) and Pheroid-Sn-MDP sponges (N₂O-H₂O) formulations which were prepared and left for four days were shown to be the most stable.
- The Pheroid-Sn-MDP vesicles (N₂O-H₂O), Vit. F: Kolliphor EL (1:1) formulation did not exhibit such stability, probably be due to a too high surfactant: lipid ratio. Similarly, formulations with a saline aqueous phase showed a marked decrease in stability.

Microscopy was used to confirm morphology and most importantly as a means to qualitatively assess entrapment within or association of Sn-MDP with Pheroid formulations. Reflectance microscopy showed that stability as determined by particle size distribution analysis and ζ -potential is not necessarily an indication of entrapment. A good example of this is the Pheroid-Sn-MDP vesicles (N₂O-saline) with Sn-MDP added to the aqueous phase before formulation, which showed poor stability but good entrapment.

Reflectance microscopy was useful in the process of identifying formulations that warrant further investigations for possible clinical applications. These formulations were:

- Pheroid-Sn-MDP vesicles (N₂O-H₂O), MDP added to aqueous phase before mixing with oil phase
- Pheroid-Sn-MDP vesicles (N₂O-Saline), MDP added to aqueous phase before mixing with oil phase
- Pheroid-Sn-MDP vesicles (N₂O-H₂O), MDP added 4 days after manufacturing

6.1.2. Chapter 5 (Method for quantitative measure of entrapment efficiencies for ^{99m}Tc-Methylene diphosphonate encapsulated in Pheroid)

The results of objectives 3, 4 (entrapment efficiency determination), 5 and 6 were reported in Chapter 5.

Based on characterisation results, formulations that showed stabilisation within the shortest time as well as the best entrapment according to confocal microscopy, five formulations were selected for further investigation.

All experiments were conducted in triplicate and one way analysis of variance (ANOVA) was used to determine reproducibility. Applying a significance level $\alpha = 0.05$, reproducibility was confirmed for all five formulations.

Entrapment efficiencies of various formulations was measured by incubating Pheroid- ^{99m}Tc -MDP formulations on hydroxyapatite. The reasoning behind these measurements was that, in the absence of metabolic activity, any amount of ^{99m}Tc -MDP that is unable to adsorb to the synthetic hydroxyapatite (HAP) media may be considered to be associated with / entrapped within Pheroid[®] structures.

Due to formulation factors, the following formulations did not display high entrapment % and/or stability within the necessary time constraints (within 6 hrs of preparation).

- Pheroid vesicles with ^{99m}Tc -MDP added to aqueous phase (N_2O -saline) before preparation, (maximum entrapment 73.75% after 12 hrs., no entrapment stability during first 24 hrs.)
- Pre-prepared Pheroid vesicles with Vitamin F: Kolliphor EL 1:1 ratio ; ^{99m}Tc -MDP added 4 days after formulation (maximum entrapment 78.99% after 24 hrs., no entrapment stability during first 24 hrs.) and
- Pre-prepared Pheroid sponges; ^{99m}Tc -MDP added 4 days after formulation (maximum entrapment 81.79% after 24 hrs., no entrapment stability during first 24 hrs.).

The following formulations displayed high entrapment % and entrapment stability within 6 hrs:

- Pheroid vesicles with ^{99m}Tc -MDP added to aqueous phase (N_2O - H_2O) before preparation (maximum entrapment 98.69% after 2 hrs., entrapment stability established after 1 hour and maintained for 24 hrs.) and
- Pre-prepared Pheroid vesicles; ^{99m}Tc -MDP added 4 days after formulation (maximum entrapment 98.26% after 12 hrs., although entrapment stability was established after 1 hour and maintained for the first 12 hrs.).

6.1.3. Optimised parameters for Pheroid-MDP formulations with potential clinical application

Comparison of the results obtained from objectives 1 – 6 allows identification of optimised formulations for further study, in line with the final objective 7. Pheroid vesicles with ^{99m}Tc -MDP added to aqueous phase (N_2O - H_2O) before preparation and Pre-prepared Pheroid vesicles; ^{99m}Tc -MDP added 4 days after formulation were identified as the most likely candidates for further investigation. Results pertaining to these two formulations are summarised in figures 6.1 and 6.2.

Figure 6.1. shows unstable and less than ideal particle size span distribution (larger than values deemed acceptable in literature) (Uys, 2006) as well as unstable zeta potential values. In Figure 6.1. the reflectance microscopy shows clear entrapment / association of Sn-MDP with Pheroid and this is confirmed by the % entrapment efficiency results. Despite the formulation showing less desirable stability based on characterisation methods used, the formulation displayed suitable % entrapment of ^{99m}Tc -MDP in Pheroid, making this formulation an advantageous candidate for further investigation.

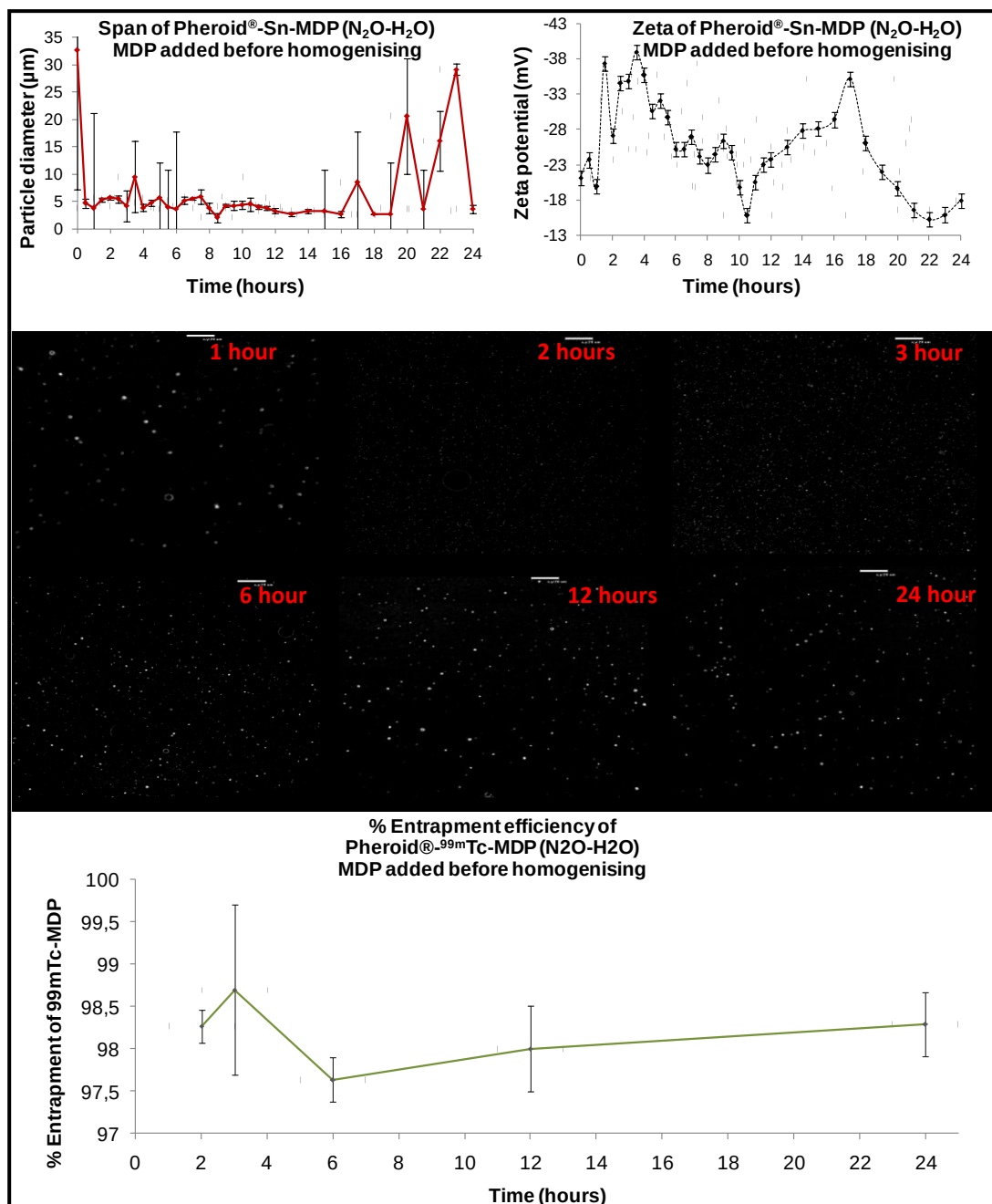


Figure 6.1. Particle size distribution span measured (top left), zeta potential measured, reflectance spectroscopy measured (middle images) and % entrapment efficiency measured over 24 hrs. for Sn-MDP / ^{99m}Tc-MDP added to aqueous phase (N₂O-H₂O).

Figure 6.2. shows stable and acceptable particle size span distribution (values from literature) (Uys, 2006) as well as relatively stable zeta potential values. In Figure 6.2. the reflectance microscopy shows signs of entrapment / association of Sn-MDP with Pheroid and this was confirmed by the % entrapment efficiency results. Not only did this formulation display stability, it also showed suitable % entrapment of ^{99m}Tc-MDP in Pheroid. Therefore this formulation is an ideal candidate for further investigation.

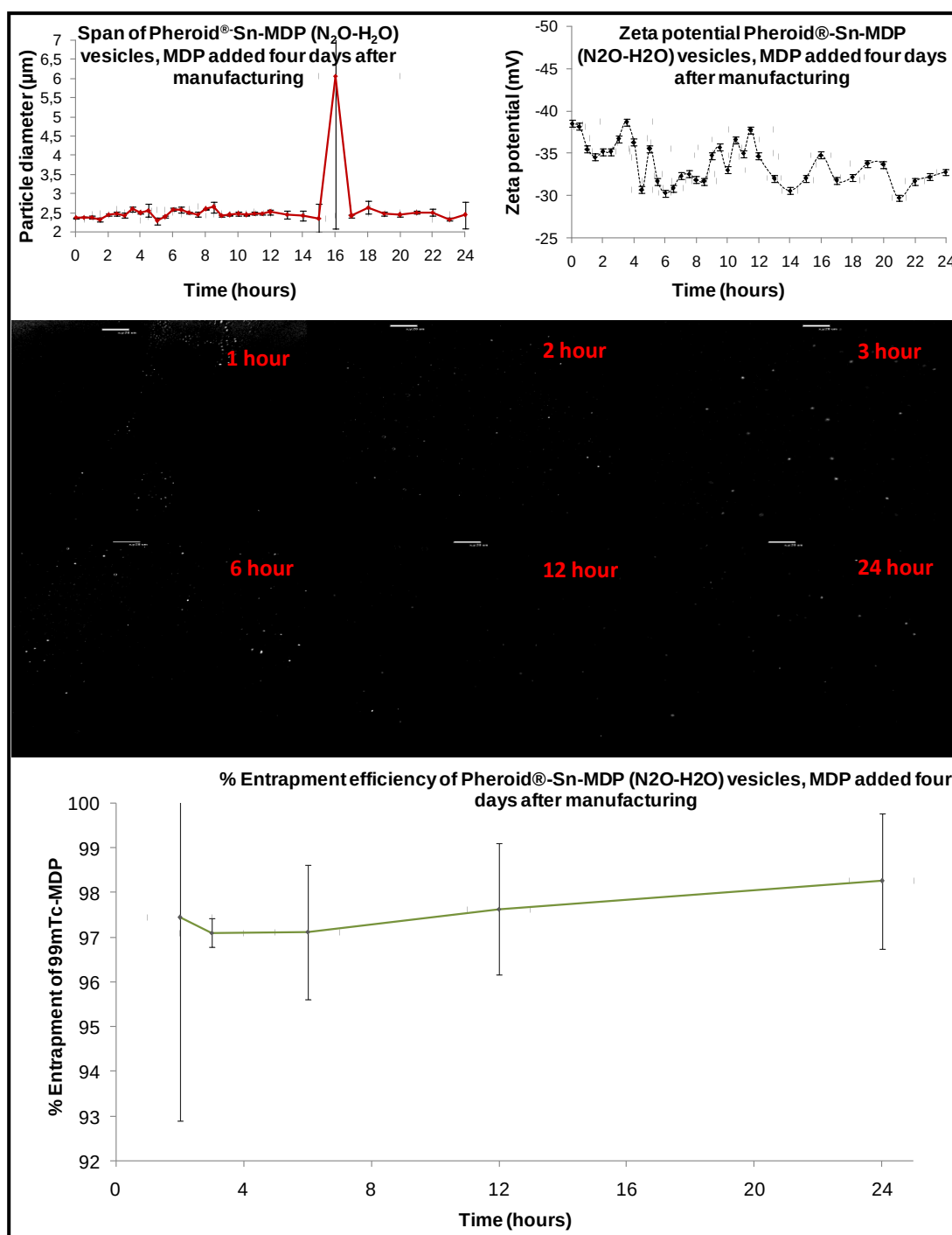


Figure 6.2. Particle size distribution span measured top left), zeta potential measured, reflectance spectroscopy measured (middle images) and % entrapment efficiency measured over 24 hrs. for pre-prepared Pheroid vesicles; Sn-MDP / ^{99m}Tc-MDP added 4 days after formulation.

Summarised data from aspects of this study not included in Chapters 4 and 5 may be found in Appendices 1 and 2.

6.2. Future prospects

The following are possible focus topics for future studies involving Pheroid-^{99m}Tc-MDP and the developed entrapment efficiency measurement method:

- Further animal trials, determining which of the formulations with good entrapment efficiencies provide the most appropriate biodistribution
- This could finally culminate in human clinical trials to test the viability of an oral Pheroid-^{99m}Tc-MDP dosage form.
- Expand the developed entrapment efficiency method so that it can be applied to other systems.

6.3 References

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APPENDIX | 1

Reproducibility of particle size distribution and Zeta Potential for formulations related to Chapter 4.

1. Particle size distribution ANOVA single factor

One way analysis of variance (ANOVA) was used to determine reproducibility of recorded measurements.

$H_0: \mu_1 = \mu_2$ vs. $H_a: \mu_1 \neq \mu_2$. A significance level $\alpha = 0.05$ was applied.

Reproducibility could only be claimed (H_0 was accepted) if the following held true: $p > \alpha$ and if $F \leq F_{crit}$.

1.1 Pheroid[®] vesicles (N₂O-H₂O).

SUMMARY

Groups	Count	Sum	Average	Variance
Row 1	74	556,231	7,619603	91,95921
Row 2	74	595,086	8,151863	108,3048
Row 3	74	669,506	9,171315	148,2636

ANOVA

Source of Variation	SS	Df	MS	F	P-value	F _{crit}
Between Groups	90,77294	2	45,38647	0,39067	0,67708	3,037667
Within Groups	25093,99	219	116,1759			
Total	25184,77	221				

1.2 Pheroid[®] vesicles with Sn-MDP added to aqueous phase (N₂O-H₂O) before preparation.

SUMMARY

Groups	Count	Sum	Average	Variance
Row 1	74	387,716	5,239405	31,51786
Row 2	74	453,683	6,130851	33,55844
Row 3	74	575,592	7,77827	73,13967

ANOVA

Source of Variation	SS	Df	MS	F	P-value	F _{crit}
Between Groups	245,5443	2	122,7722	2,66479	0,071871	3,037088
Within Groups	10089,77	219	46,07199			
Total	10335,31	221				

1.3 Pheroid[®] vesicles with Sn-MDP added to aqueous phase (N₂O-H₂O) during preparation.

SUMMARY

Groups	Count	Sum	Average	Variance
Row 1	74	578,814	7,821811	140,2242
Row 2	74	529,577	7,156446	81,91894
Row 3	74	588,356	7,950757	120,2509

ANOVA

Source of Variation	SS	Df	MS	F	P-value	F _{crit}
Between Groups	26,89326	2	13,44663	0,117817	0,888915	3,037088
Within Groups	24994,76	219	114,1313			
Total	25021,66	221				

1.4 Pheroid[®] vesicles with Sn-MDP added to aqueous phase (N₂O-H₂O) directly after preparation.

SUMMARY

<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
Row 1	74	2066,383	28,30662	843,5038
Row 2	74	1718,419	23,53999	505,8432
Row 3	74	2177,775	29,83253	758,5896

ANOVA

<i>Source of Variation</i>	<i>SS</i>	<i>Df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F_{crit}</i>
Between Groups	1573,037	2	786,5184	1,119367	0,328373	3,037667
Within Groups	151771,4	219	702,6455			
Total	153344,5	221				

1.5 Pheroid[®] vesicles (N₂O-saline).

SUMMARY

<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
Row 1	73	325,595	4,460205	119,2156
Row 2	73	339,66	4,652877	60,46467
Row 3	73	408,479	5,595603	95,32873

ANOVA

<i>Source of Variation</i>	<i>SS</i>	<i>Df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F_{crit}</i>
Between Groups	53,89788	2	26,94894	0,293979	0,74559	3,037667
Within Groups	19800,65	219	91,66966			
Total	19854,54	221				

1.6 Pheroid[®] vesicles with Sn-MDP added to aqueous phase (N₂O-saline) before preparation.

SUMMARY

<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
Row 1	74	457,024	6,260603	231,1867
Row 2	74	410,49	5,623151	184,732
Row 3	74	504,6	6,912329	248,289

ANOVA

<i>Source of Variation</i>	<i>SS</i>	<i>Df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F_{crit}</i>
Between Groups	60,66475	2	30,33238	0,137001	0,872045	3,037667
Within Groups	47822,95	219	221,4025			
Total	47883,61	221				

1.7 Pheroid[®] vesicles with Sn-MDP added to aqueous phase (N₂O-saline) during preparation.

SUMMARY

<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
Row 1	74	800,473	10,96538	260,0752
Row 2	74	958,191	13,1259	304,6194
Row 3	74	937,185	12,83815	303,6103

ANOVA

<i>Source of Variation</i>	<i>SS</i>	<i>Df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F_{crit}</i>
Between Groups	200,9424	2	100,4712	0,347129	0,707108	3,037667
Within Groups	62517,95	219	289,435			
Total	62718,9	221				

1.8 Pheroid® vesicles with Sn-MDP added to aqueous phase (N₂O-saline) directly after preparation.**SUMMARY**

<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
Row 1	74	1171,45	15,83041	399,5673
Row 2	74	1189,748	16,07768	496,0827
Row 3	74	1397,604	18,88654	213,5296

ANOVA

<i>Source of Variation</i>	<i>SS</i>	<i>Df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F_{crit}</i>
Between Groups	426,5071	2	142,169	0,382769	0,765522	2,646014
Within Groups	80970,11	218	371,4225			
Total	81396,62	221				

1.9 Pheroid® vesicles (N₂O-H₂O) , 4 days after manufacturing.**SUMMARY**

<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
Row 1	74	181,235	2,449122	0,019842
Row 2	74	196,787	2,659284	1,371012
Row 3	74	193,5	2,614865	0,672896

ANOVA

<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F_{crit}</i>
Between Groups	1,815763	2	0,907881	1,319755	0,269317	3,037088
Within Groups	150,6537	219	0,687917			
Total	152,4695	221				

1.10 Pheroid® vesicles (N₂O-H₂O) , MDP added 4 days after manufacturing.**SUMMARY**

<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
Row 1	74	321,904	4,409644	133,8838
Row 2	74	333,141	4,563575	153,03
Row 3	74	341,682	4,680575	140,1323

ANOVA

<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F_{crit}</i>
Between Groups	2,695836	2	1,347918	0,009469	0,990576	3,037667
Within Groups	30747,32	219	142,3487			
Total	30750,02	221				

1.11 Pheroid[®] sponges (N₂O-H₂O) , MDP added 4 days after manufacturing.**SUMMARY**

<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
Row 1	74	192,681	2,752586	12,54423
Row 2	74	208,135	2,973357	23,01814
Row 3	74	205,159	2,810397	19,63898

ANOVA

<i>Source of Variation</i>	<i>SS</i>	<i>Df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F_{crit}</i>
Between Groups	1,838523	2	0,919261	0,049911	0,951326	3,038877
Within Groups	3867,81	219	18,41814			
Total	3869,648	221				

1.12 Pheroid[®] vesicles (N₂O-H₂O) Vit F: Koll. EL ratio 1 : 2.8, MDP added 4 days after manufacturing.**SUMMARY**

<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
Row 1	74	651,941	8,810014	292,9249
Row 2	74	406,951	5,499338	10,35839
Row 3	74	426,897	5,768878	9,968818

ANOVA

<i>Source of Variation</i>	<i>SS</i>	<i>Df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F_{crit}</i>
Between Groups	500,2826	2	250,1413	2,395591	0,093503	3,037088
Within Groups	22867,4	219	104,4174			
Total	23367,69	221				

1.13 Pheroid[®] vesicles (N₂O-H₂O) Vit F: Koll. EL ratio 1 : 1, MDP added 4 days after manufacturing.**SUMMARY**

<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
Row 1	74	698,513	9,439365	63,4487
Row 2	74	706,05	9,541216	83,94921
Row 3	74	675,478	9,128081	55,63559

ANOVA

<i>Source of Variation</i>	<i>SS</i>	<i>Df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F_{crit}</i>
Between Groups	6,856148	2	3,428074	0,050653	0,95062	3,037088
Within Groups	14821,45	219	67,67784			
Total	14828,3	221				

1.14 Pheroid[®] vesicles (N₂O-H₂O) Vit F: Koll. EL ratio 2.5 : 1.3, MDP added 4 days after manufacturing.**SUMMARY**

<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
Row 1	74	427,952	5,783135	110,1732
Row 2	74	458,186	6,191703	119,1174
Row 3	74	466,858	6,308892	119,6058

ANOVA

<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F_{crit}</i>
Between Groups	11,27466	2	5,637331	0,048473	0,952693	3,037088
Within Groups	25469,44	219	116,2988			
Total	25480,72	221				

2. Zeta potential measurement ANOVA single factor

One way analysis of variance (ANOVA) was used to determined reproducibility of recorded measurements.

$H_0: \mu_1 = \mu_2$ vs. $H_a: \mu_1 \neq \mu_2$. A significance level $\alpha = 0.05$ was applied.

Reproducibility could only be claimed (H_0 was accepted) if the following held true: $p > \alpha$ and if $F \leq F_{crit}$.

2.1 Pheroid[®] vesicles (N₂O-H₂O).**SUMMARY**

<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
Row 1	74	-982,66	-13,2792	39,64476
Row 2	74	-931,99	-12,5945	32,51894
Row 3	74	-924,52	-12,4935	35,13785

ANOVA

<i>Source of Variation</i>	<i>SS</i>	<i>Df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F_{crit}</i>
Between Groups	27,04284	2	13,52142	0,37804	0,68565	3,037088
Within Groups	7833,013	219	35,76718			
Total	7860,056	221				

2.2 Pheroid[®] vesicles with Sn-MDP added to aqueous phase (N₂O-H₂O) before preparation.**SUMMARY**

<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
Row 1	74	-1856,14	-25,083	46,3827
Row 2	74	-1912,16	-25,84	40,87693
Row 3	74	-1945,96	-26,2968	47,91773

ANOVA

<i>Source of Variation</i>	<i>SS</i>	<i>Df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F_{crit}</i>
Between Groups	55,61918	2	27,80959	0,61718	0,540399	3,037088
Within Groups	9867,947	219	45,05912			
Total	9923,567	221				

2.3 Pheroid[®] vesicles with Sn-MDP added to aqueous phase (N₂O-H₂O) during preparation**SUMMARY**

<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
Row 1	74	-990,66	-13,3873	70,60564
Row 2	74	-1069,2	-14,4487	70,54943
Row 3	74	-1061,99	-14,3513	60,5149

ANOVA

Source of Variation	SS	Df	MS	F	P-value	F _{crit}
Between Groups	50,94317	2	25,47159	0,37891	0,685055	3,037088
Within Groups	14721,91	219	67,22332			
Total	14772,85	221				

2.4 Pheroid[®] vesicles with Sn-MDP added to aqueous phase (N₂O-H₂O) directly after preparation.

SUMMARY

Groups	Count	Sum	Average	Variance
Row 1	74	-512,79	-6,92959	7,752749
Row 2	74	-537,403	-7,2622	8,81077
Row 3	74	-523,523	-7,07464	7,105692

ANOVA

Source of Variation	SS	Df	MS	F	P-value	F _{crit}
Between Groups	4,115547	2	2,057774	0,260816	0,770661	3,037088
Within Groups	1727,852	219	7,889737			
Total	1731,968	221				

2.5 Pheroid[®] vesicles (N₂O-saline).

SUMMARY

Groups	Count	Sum	Average	Variance
Row 1	74	-1114,97	-15,0671	32,49462
Row 2	74	-1162,43	-15,7085	34,84953
Row 3	74	-1225,47	-16,5604	38,9406

ANOVA

Source of Variation	SS	Df	MS	F	P-value	F _{crit}
Between Groups	83,05139	2	41,5257	1,172107	0,311649	3,037088
Within Groups	7758,787	219	35,42825			
Total	7841,838	221				

2.6 Pheroid[®] vesicles with Sn-MDP added to aqueous phase (N₂O-saline) before preparation.

SUMMARY

Groups	Count	Sum	Average	Variance
Row 1	74	-1856,14	-25,083	46,3827
Row 2	74	-1912,16	-25,84	40,87693
Row 3	74	-1945,96	-26,2968	47,91773

ANOVA

Source of Variation	SS	Df	MS	F	P-value	F _{crit}
Between Groups	55,61918	2	27,80959	0,61718	0,540399	3,037088
Within Groups	9867,947	219	45,05912			
Total	9923,567	221				

2.7 Pheroid[®] vesicles with Sn-MDP added to aqueous phase (N₂O-saline) during preparation

SUMMARY

Groups	Count	Sum	Average	Variance
Row 1	74	-1077,16	-14,5562	60,492
Row 2	74	-1058,52	-14,3043	58,22497
Row 3	74	-1118,51	-15,115	60,74905

ANOVA

Source of Variation	SS	Df	MS	F	P-value	F _{crit}
Between Groups	25,4778	2	12,7389	0,212947	0,808366	3,037088
Within Groups	13101,02	219	59,82201			
Total	13126,5	221				

2.8 Pheroid[®] vesicles with Sn-MDP added to aqueous phase (N₂O-saline) directly after preparation.

SUMMARY

Groups	Count	Sum	Average	Variance
Row 1	74	-1022,79	-13,8215	29,72505
Row 2	74	-1072,85	-14,498	32,10801
Row 3	74	-1113,95	-15,0534	30,62333

ANOVA

Source of Variation	SS	Df	MS	F	P-value	F _{crit}
Between Groups	56,32979	2	28,16489	0,913887	0,402486	3,037088
Within Groups	6749,316	219	30,8188			
Total	6805,646	221				

2.9 Pheroid[®] vesicles (N₂O-H₂O) , 4 days after manufacturing.

SUMMARY

Groups	Count	Sum	Average	Variance
Row 1	74	-2172,06	-29,3521	25,92266
Row 2	74	-2107,83	-28,4842	22,22273
Row 3	74	-2105,25	-28,4493	22,16201

ANOVA

Source of Variation	SS	Df	MS	F	P-value	F _{crit}
Between Groups	38,71481	2	19,3574	0,825976	0,439167	3,037088
Within Groups	5132,44	219	23,4358			
Total	5171,155	221				

2.10 Pheroid[®] vesicles (N₂O-H₂O) , MDP added 4 days after manufacturing.

SUMMARY

Groups	Count	Sum	Average	Variance
Row 1	74	-2549,14	-34,4478	7,810735
Row 2	74	-2495,38	-33,7214	8,401864
Row 3	74	-2498,65	-33,7655	6,75285

ANOVA

<i>Source of Variation</i>	<i>SS</i>	<i>Df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F_{crit}</i>
Between Groups	24,54987	2	12,27493	1,603487	0,203547	3,037088
Within Groups	1676,478	219	7,65515			
Total	1701,028	221				

2.11 Pheroid[®] sponges (N₂O-H₂O) , 4 days after manufacturing**SUMMARY**

<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
Row 1	74	-2094,17	-28,2996	11,08393
Row 2	74	-2068,87	-27,9577	13,68872
Row 3	74	-2058,36	-27,8157	11,73798

ANOVA

<i>Source of Variation</i>	<i>SS</i>	<i>Df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F_{crit}</i>
Between Groups	9,156815	2	4,578408	0,376198	0,686909	3,037088
Within Groups	2665,275	219	12,17021			
Total	2674,432	221				

2.12 Pheroid[®] sponges (N₂O-H₂O) , MDP added 4 days after manufacturing**SUMMARY**

<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
Row 1	74	-1912,74	-25,8479	45,13503
Row 2	74	-1850,33	-25,0045	45,6886
Row 3	74	-1889,08	-25,5281	42,67115

ANOVA

<i>Source of Variation</i>	<i>SS</i>	<i>Df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F_{crit}</i>
Between Groups	26,83321	2	13,41661	0,301509	0,740008	3,037088
Within Groups	9745,119	219	44,49826			
Total	9771,952	221				

2.13 Pheroid[®] vesicles (N₂O-H₂O) Vit F: Koll. EL ratio 1 : 2.8, MDP added 4 days after manufacturing.**SUMMARY**

<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
Row 1	74	-1296,61	-17,5218	12,61785
Row 2	74	-1411,51	-19,0745	18,53383
Row 3	37	-667,027	-18,0277	6,170533

ANOVA

<i>Source of Variation</i>	<i>SS</i>	<i>Df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F_{crit}</i>
Between Groups	91,36637	2	45,68319	3,330783	0,037958	3,045588
Within Groups	2496,212	182	13,71545			
Total	2587,578	184				

2.14 Pheroid[®] vesicles (N₂O-H₂O) Vit F: Koll. EL ratio 1 : 1, MDP added 4 days after manufacturing.**SUMMARY**

<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
Row 1	74	-1031,8	-13,9432	2,992991
Row 2	74	-1047,54	-14,1559	3,864951
Row 3	74	-1026,3	-13,8689	3,649899

ANOVA

<i>Source of Variation</i>	<i>SS</i>	<i>Df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F_{crit}</i>
Between Groups	3,284588	2	1,642294	0,468877	0,626331	3,037088
Within Groups	767,0724	219	3,502614			
Total	770,357	221				

2.15 Pheroid[®] vesicles (N₂O-H₂O) Vit F: Koll. EL ratio 2.5 : 1.3, MDP added 4 days after manufacturing.**SUMMARY**

<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
Row 1	74	-1320,7	-17,8473	12,62855
Row 2	74	-1381,8	-18,673	10,62611
Row 3	74	-1405,3	-18,9905	16,37539

ANOVA

<i>Source of Variation</i>	<i>SS</i>	<i>Df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F_{crit}</i>
Between Groups	51,54333	2	25,77167	1,950918	0,144606	3,037088
Within Groups	2892,994	219	13,21002			
Total	2944,537	221				

APPENDIX | 2

Statistical comparison of formulations and data not presented in Chapter 5.

1. Entrapment efficiencies: ANOVA single factor to compare formulations

One way analysis of variance (ANOVA) was used to compare data sets of entrapment efficiencies for different formulations..

$H_0: \mu_1 = \mu_2$ vs. $H_a: \mu_1 \neq \mu_2$. A significance level $\alpha = 0.05$ was applied.

Statistically significant difference could only be claimed (H_0 was rejected) if the following held true: $p < \alpha$ and if $F \geq F_{crit}$.

SUMMARY						
Groups	Count	Sum	Average	Variance		
MDP added to saline before homogenising	6	422.2420778	70.37367963	7.492975847		
MDP added to saline before homogenising, shaken 2 hrs after homogenising	6	412.9614988	68.82691647	70.5590246		
MDP added during homogenising, shaken 2 hrs after homogenising	6	510.1562107	85.02603511	60.50081988		
Pre-made Pheroid with Vit f: Kolliphor 1:1	6	376.2182727	62.70304546	95.73069762		
Pre-made Pheroid sponges	6	396.7351427	66.12252378	198.4369622		
Pre-made Pheroid Vesicles	6	574.0498955	95.67498259	20.34764584		
Freshly made Pheroid, MDP added to water before homogenising	6	589.1544298	98.19240497	0.124050516		
ANOVA						
Source of Variation	SS	df	MS	F	P-value	F _{crit}
Between Groups	7720.560449	6	1286.760075	19.87527806	5.94X10 ⁻¹⁰	2.371781196
Within Groups	2265.960882	35	64.7417395			
Total	9986.521331	41				

	Treatment pairs	Tukey HSD Q statistic	Tukey HSD p-Value	Tukey HSD Inference
1	MDP added to before homogenising, vs MDP added to saline before homogenising, shaken 2 hrs after homogenising	0.4709	0.8999947	Insignificant
2	MDP added to saline before homogenising vs MDP added during homogenising, shaken 2 hrs after homogenising	4.4606	0.0467921	* p<0.05
3	MDP added to saline before homogenising vs Pre-made Pheroid with Vit f: Kolliphor 1:1	2.3351	0.6328813	Insignificant
4	MDP added to saline before homogenising, vs Pre-made Pheroid sponges	1.2942	0.8999947	Insignificant
5	MDP added to saline before homogenising, vs Pre-made Pheroid Vesicles	7.7024	0.0010053	** p<0.01
6	MDP added to saline before homogenising, vs Freshly made Pheroid. MDP added to water before homogenising	8.4688	0.0010053	** p<0.01
7	MDP added to saline before homogenising, shaken 2 hrs before homogenising vs MDP added during homogenising, shaken 2 hrs after homogenising	4.9315	0.0205993	* p<0.05
8	MDP added to saline before homogenising, shaken 2 hrs before homogenising vs Pre-made Pheroid with Vit f: Kolliphor 1:1	1.8643	0.8188101	Insignificant
9	MDP added to saline before homogenising, shaken 2 hrs before homogenising vs Pre-made Pheroid sponges	0.8233	0.8999947	Insignificant
10	MDP added to saline before homogenising, shaken 2 hrs before homogenising vs Pre-made Pheroid Vesicles	8.1733	0.0010053	** p<0.01
11	MDP added to saline before homogenising, shaken 2 hrs before homogenising vs Freshly made Pheroid. MDP added to water before homogenising	8.9397	0.0010053	** p<0.01
12	MDP added during homogenising, shaken 2 hrs after homogenising vs Pre-made Pheroid with Vit f: Kolliphor 1:1	6.7957	0.0010053	** p<0.01
13	MDP added during homogenising, shaken 2 hrs after homogenising vs Pre-made Pheroid sponges	5.7547	0.0043528	** p<0.01
14	MDP added during homogenising, shaken 2 hrs after homogenising vs Pre-made Pheroid Vesicles	3.2418	0.2761461	Insignificant
15	MDP added during homogenising, shaken 2 hrs after homogenising vs Freshly made Pheroid. MDP added to water before homogenising	4.0082	0.0966105	Insignificant
16	Pre-made Pheroid with Vit f: Kolliphor 1:1vs Pre-made Pheroid sponges	1.0410	0.8999947	Insignificant
17	Pre-made Pheroid with Vit f: Kolliphor 1:1vs Pre-made Pheroid Vesicles	10.0376	0.0010053	** p<0.01
18	Pre-made Pheroid with Vit f: Kolliphor 1:1 vs Freshly made Pheroid. MDP added to water before homogenising	10.8039	0.0010053	** p<0.01
19	Pre-made Pheroid sponges vs Pre-made Pheroid Vesicles	8.9966	0.0010053	** p<0.01
20	Pre-made Pheroid sponges vs Freshly made Pheroid. MDP added to water before homogenising	9.7629	0.0010053	** p<0.01
21	Premade Pheroid Vesicles vs Freshly made Pheroid. MDP added to water before homogenising	0.7664	0.8999947	Insignificant

APPENDIX | 3

Author's guidelines for preparing article for submission (Chapter 4) to the International Journal of Pharmaceutics and (Chapter 5) Journal of Pharmaceutical and Biomedical Analysis as presented in (Elsevier, 2017).

DESCRIPTION

The *International Journal of Pharmaceutics* is the journal for **pharmaceutical scientists** concerned with the physical, chemical and biological properties of devices and **delivery systems** for **drugs**, **vaccines** and **biologicals**, including their design, manufacture and evaluation. This includes evaluation of the properties of drugs, **excipients** such as surfactants and polymers and novel materials. The journal has special sections on **pharmaceutical nanotechnology** and personalized medicines, and publishes research papers, reviews, commentaries and letters to the [editor](#) as well as special issues.

Editorial Policy

The over-riding criteria for publication are originality, high scientific quality and interest to a multidisciplinary audience. Papers not sufficiently substantiated by experimental detail will not be published. Any technical queries will be referred back to the author, although the Editors reserve the right to make alterations in the text without altering the technical content. Manuscripts submitted under multiple authorship are reviewed on the assumption that all listed authors concur with the submission and that a copy of the final manuscript has been approved by all authors and tacitly or explicitly by the responsible authorities in the laboratories where the work was carried out. If accepted, the manuscript shall not be published elsewhere in the same form, in either the same or another language, without the consent of the Editors and Publisher.

Authors must state in a covering letter when [submitting](#) papers for publication the novelty embodied in their work or in the approach taken in their research. Routine bioequivalence studies are unlikely to find favour. No paper will be published which does not disclose fully the nature of the formulation used or details of materials which are key to the performance of a product, drug or excipient. Work which is predictable in outcome, for example the inclusion of another drug in a cyclodextrin to yield enhanced dissolution, will not be published unless it provides new insight into fundamental principles.

AUDIENCE

Pharmaceutical Scientists, Clinical Pharmacologists, Chemical Engineers, Biotechnologists.

GUIDE FOR AUTHORS

INTRODUCTION

Types of paper

(1) Full Length Manuscripts

(2) Rapid Communications

- (a) These articles should not exceed 1500 words or equivalent space.
- (b) Figures should not be included otherwise delay in publication will be incurred.
- (c) Do not subdivide the text into sections. An Abstract should be included as well as a full reference list.

(3) Notes

Should be prepared as described for full length manuscripts, except for the following:

- (a) The maximum length should be 1500 words, including figures and tables.

- (b) Do not subdivide the text into sections. An Abstract and reference list should be included.

(4) Reviews and Mini-Reviews

Suggestions for review articles will be considered by the Review-Editor. "Mini-reviews" of a topic are especially welcome.

PREPARATION

Use of word processing software

It is important that the file be saved in the native format of the word processor used. The text should be in single-column format. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. In particular, do not use the word processor's options to justify text or to hyphenate words. However, do use bold face, italics, subscripts, superscripts etc. When preparing tables, if you are using a table grid, use only one grid for each individual table and not a grid for each row. If no grid is used, use tabs, not spaces, to align columns. The electronic text should be prepared in a way very similar to that of conventional manuscripts (see also the [Guide to Publishing with Elsevier](#)). Note that source files of figures, tables and text graphics will be required whether or not you embed your figures in the text. See also the section on Electronic artwork.

To avoid unnecessary errors you are strongly advised to use the 'spell-check' and 'grammar-check' functions of your word processor.

Article structure

Subdivision - numbered sections

Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2, ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to 'the text'. Any subsection may be given a brief heading. Each heading should appear on its own separate line.

Introduction

State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Material and methods

Provide sufficient detail to allow the work to be reproduced. Methods already published should be indicated by a reference: only relevant modifications should be described.

Results

Results should be clear and concise.

Discussion

This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

Conclusions

The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

Appendices

If there is more than one appendix, they should be identified as A, B, etc. Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq. (A.2), etc.; in a subsequent appendix, Eq. (B.1) and so on. Similarly for tables and figures: Table A.1; Fig. A.1, etc.

Essential title page information

- **Title.** Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.
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Abstract

A concise and factual abstract is required. The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, References should be avoided, but if essential, then cite the author(s) and year(s). Also, non-standard or uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself.

The abstract must not exceed 200 words.

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(PubChem CID: 15865)

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Strunk Jr., W., White, E.B., 2000. *The Elements of Style*, fourth ed. Longman, New York. Reference to a chapter in an edited book:

Mettam, G.R., Adams, L.B., 2009. How to prepare an electronic version of your article, in: Jones, B.S., Smith, R.Z. (Eds.), *Introduction to the Electronic Age*. E-Publishing Inc., New York, pp. 281–304. Reference to a website:

Cancer Research UK, 1975. Cancer statistics reports for the UK.
<http://www.cancerresearchuk.org/aboutcancer/statistics/cancerstatsreport/> (accessed 13.03.03).

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APPENDIX | 4

Abstract of and poster presented at the annual SAAPI conference, 2015.

Entrapment determination of ^{99m}Tc -MDP containing Pheroid[®] formulations

A van As^a, AF Grobler^a, JR Zeevaart^{a,b} and N I Barnard^a

^aDST/NWU Preclinical Drug Development Platform, North-West University, Potchefstroom Campus, 2520, South Africa.

^bRadiochemistry, South African Nuclear Energy Corporation SOC Limited (Necsa), Pretoria, 0001, South Africa

Purpose: ^{99m}Tc Technetium Methylene-Diphosphonate (^{99m}Tc -MDP) is a bone targeting radio tracer used as a diagnostic tool (Subramanian *et al.*, 1975), while Pheroid[®] is a drug delivery system capable of entrapping both hydrophilic as well as lipophilic active pharmaceutical ingredients and delivering them in biological systems (Grobler *et al.*, 2008; Steyn *et al.*, 2011; Grobler *et al.*, 2014). The combination of ^{99m}Tc -MDP and Pheroid[®] presents the possibility of oral, rather than standard IV, administration of radiotracers. Hydroxyapatite was used as a model to determine entrapment of ^{99m}Tc -MDP in Pheroid[®] formulations. Entrapment in Pheroid[®] prevents ^{99m}Tc -MDP adsorption onto the inorganic hydroxyapatite model. This study assessed Pheroid[®] entrapment efficiency of MDP for a range of formulations over time.

Methods Four different Pheroid[®] formulations incorporating Sn-MDP (an analogue for ^{99m}Tc -MD) as well as ^{99m}Tc -MDP were prepared and characterized. Characterisation methods included particle size distribution measurement, Zeta potential measurement, Confocal laser microscopy and pH measurements. Formulations were lastly also characterised in terms of entrapment efficiency of ^{99m}Tc -MDP in Pheroid[®].

For the entrapment efficiency study these formulations, along with a control, were incubated at 37°C on hydroxyapatite with a specific surface area of 14.45 m²/g.

Formulation 1 Pheroid[®] vesicles with ^{99m}Tc -MDP added to aqueous phase (N₂O-H₂O) before preparation

Formulation 2 Pheroid[®] vesicles with ^{99m}Tc -MDP added to aqueous phase (N₂O-saline) before preparation

Formulation 3 Pre-prepared Pheroid[®] vesicles with Vitamin F: Kolliphor EL 1:1 ratio; ^{99m}Tc -MDP added 4 days after formulation

Formulation 4 Pre-prepared Pheroid[®] vesicles; ^{99m}Tc -MDP added 4 days after formulation

The specific activity on the hydroxyapatite was measured after 1,2,3,6,12 and 24 hours. The amount of activity on the hydroxyapatite was expressed as percentage of total activity. The amount of activity remaining in supernatant solutions (i.e. entrapped, un-adsorbed ^{99m}Tc -MDP) was expressed as percentage entrapment in the Pheroid[®].

Results: The results indicated that different preparation methods for Pheroid with ^{99m}Tc -MDP had a significant effect on both the amount and time of ^{99m}Tc -MDP entrapment.

Formulation	Max entrapment (%)	Time to max entrapment (Hrs)
1	97.67	3 hour
2	73.75	12 hours
3	78.99	24 hours
4	98.26	12 hours

Conclusions: Pheroid[®] vesicles with ^{99m}Tc -MDP added to aqueous phase (N₂O-H₂O) before preparation as well as pre-prepared Pheroid[®] vesicles; ^{99m}Tc -MDP added 4 days after formulation,

showed the best entrapment overall. They displayed stable high entrapment %, and therefore shows the most promise for clinical applications.

Grobler, A., Kotzé, A. & Du Plessis, J. 2008. The design of a skin-friendly carrier for cosmetic compounds using Pheroid[®]™ technology. *Science and Applications of Skin Delivery System Technologies Allured*. Ed. Johann Wiechers:283-311.

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Entrapment determination of ^{99m}Tc -MDP containing Pheroid[®] formulations

Adele van As¹, Anne Frederica Grobler¹, Jan Rijn Zeevaart^{1,2} and Nicola Isabel Barnard¹

¹ DST/NWU Preclinical Drug Development Platform, North-West University, Potchefstroom Campus, 2520, South Africa.

² Radiochemistry, South African Nuclear Energy Corporation SOC Limited (Necsa), Pretoria, 0001, South Africa.

Introduction

Pheroid[®] drug delivery system

Pheroid[®], a drug delivery system, entraps both hydrophilic and lipophilic active pharmaceutical ingredients (API), delivering them in biological systems. Types of Pheroid[®]: Pheroid[®] vesicles, Pheroid[®] micro-sponges and Pro-Pheroid[®].

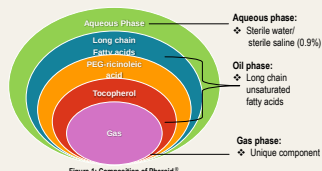


Figure 1: Composition of Pheroid[®]

- ^{99m}Tc Technetium Methylene-Diphosphonate (^{99m}Tc -MDP)
- A bone targeting radio tracer used as a diagnostic tool.
- ^{99m}Tc -MDP has a high affinity for bone and bone-like compounds such as Hydroxyapatite (HAP)

Why Pheroid[®], ^{99m}Tc -MDP and HAP?

- Combining ^{99m}Tc -MDP and Pheroid[®] could make oral administration of radiotracers possible
- HAP was used to determine entrapment of ^{99m}Tc -MDP in Pheroid[®]
- Entrapment in prevents ^{99m}Tc -MDP adsorption onto HAP
- This study assessed Pheroid[®] entrapment efficiency of ^{99m}Tc -MDP for a range of formulations over time.

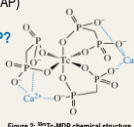


Figure 2: ^{99m}Tc -MDP chemical structure

Methods

Pheroid[®], ^{99m}Tc -MDP formulations preparation

- Pheroid[®] vesicles and Pheroid[®] micro-sponges were made using a patented method
- The following parameters were varied:
 - Aqueous phase (sterile water / 0.9 % saline)
 - Addition of ^{99m}Tc -MDP (before / during / after mixing of Pheroid[®] components)
 - Shaking of formulation before incubation
- For characterization non-labeled Pheroid[®]-MDP formulations were prepared
- ^{99m}Tc in saline as well as MDP kits were obtained from Necsa^{*}

Table 1: Summary of different Pheroid[®] - ^{99m}Tc -MDP formulations

Aqueous phase	Pheroid type	When was MDP added?
$\text{N}_2\text{O}-\text{H}_2\text{O}$	Vesicles	Before mixing phases
$\text{N}_2\text{O}-\text{Saline}$	Vesicles	Before mixing phases
$\text{N}_2\text{O}-\text{H}_2\text{O}$	Vesicles, Vitamin F: Kolliphor EL 1:1 ratio	4 days after manufacturing
$\text{N}_2\text{O}-\text{H}_2\text{O}$	Vesicles	4 days after manufacturing

Characterization

- Non-labeled Pheroid[®]-MDP formulations were characterized:
 - Particle size distribution over 24 hours after addition of MDP
 - Zeta potential over 24 hours after addition of MDP
 - pH
- HAP was characterized:
 - BET surface analysis (using N_2)

Entrapment study using HAP:^{*}

All experiments done in triplicate

- 3 mL Pheroid[®] - ^{99m}Tc -MDP formulations incubated (37 °C) on 50 mg HAP
- Samples centrifuged (5000 rpm, 5 min) at time points 0, 1, 2, 3, 6, 12 and 24 hours.
- HAP residual dried at 90 °C.
- All specific activities corrected for decay

Initial specific activity $\propto$ [MDP]

% Entrapment and [MDP] entrapped calculated as follows:

$$\% \text{ Entrapped} = \frac{\text{Total activity in sample} - \text{Activity on HAP}}{\text{Total activity in sample}} \times 100$$

$$[\text{MDP}] \text{ Entrapped} = \frac{\text{Total activity in sample} - \text{Activity on HAP}}{\text{Total activity in sample}} \times [\text{MDP}]$$

^{*} All radioactive experiments performed at Necsa

Results

1 Pheroid[®] vesicles with ^{99m}Tc -MDP added to aqueous phase ($\text{N}_2\text{O}-\text{H}_2\text{O}$) before preparation

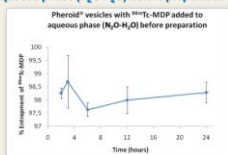


Figure 3: Entrapment % of Pheroid[®] vesicles with ^{99m}Tc -MDP added to aqueous phase ($\text{N}_2\text{O}-\text{H}_2\text{O}$) before preparation

- Maximum entrapment @ 3 hours (97.67%)
- Stable throughout first 24 hours

2 Pheroid[®] vesicles with ^{99m}Tc -MDP added to aqueous phase (N_2O -saline) before preparation

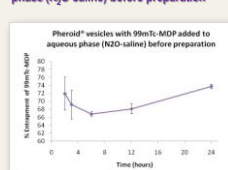


Figure 4: Entrapment % of Pheroid[®] vesicles with ^{99m}Tc -MDP added to aqueous phase (N_2O -saline) before preparation

- Maximum entrapment @ 12 hours (73.75%)
- Not very stable during first 24 hours

3 Pre-prepared Pheroid[®] vesicles with Vitamin F: Kolliphor EL 1:1 ratio; ^{99m}Tc -MDP added 4 days after formulation

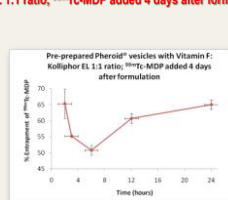


Figure 5: Entrapment % of pre-prepared Pheroid[®] vesicles with Vitamin F: Kolliphor EL 1:1 ratio; ^{99m}Tc -MDP added 4 days after formulation

- Maximum entrapment @ 24 hours (78.99 %)
- Not very stable during first 24 hours

4 Pre-prepared Pheroid[®] vesicles; ^{99m}Tc -MDP added 4 days after formulation

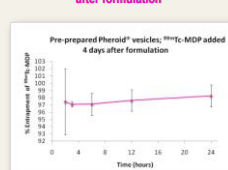


Figure 6: Entrapment % of pre-prepared Pheroid[®] vesicles; ^{99m}Tc -MDP added 4 days after formulation

- Maximum entrapment @ 12 hours (98.26 %)
- Stable over 24 hours (Difference of 1 % between maximum and minimum entrapment)
- Easy to prepare radio therapeutic formulation, only requires addition of ^{99m}Tc -MDP to formulation before administration

Discussion

Influence of oil : surfactant ratio on entrapment

- Comparing 1 to 3
- An oil ratio of 1:1 (opposed to 2.8 :1) causes lower entrapment and prevents stabilization of entrapment
- This can be attributed to compromised vesicle integrity; too high surfactant ratios prevent formation of stable vesicles.

Pre-made Pheroid[®] vs. Freshly prepared Pheroid[®]

- Comparing 1 to 4
- Both showed excellent entrapment, this formulation parameter does not influence the entrapment efficiency.
- Long term stability of formulations is a concern, and literature has shown that formulations need at least 4 days to stabilize.
- 1 might therefore present storage stability issues, which would make 4 a more desirable formulation

Influence of saline / sterile water on entrapment

- Comparing 1 to 2
- Presence of saline reduced % entrapment by as much as 15%
- Entrapment % stability was also negatively influenced, with the saline formulation not stabilizing at all during first 24 hours.
- Reduced entrapment could be due to higher ion concentration reducing the probability of ^{99m}Tc -MDP being entrapped

Conclusions

Various different Pheroid[®] formulations were investigated in terms of optimized entrapment in the shortest time possible

The following parameters formulations showed the most promise for future applications as orally administered radio tracers:

- Pheroid[®] Vesicles pre-made (sterile water as aqueous phase), ^{99m}Tc -MDP added 4 days after manufacturing
- Pheroid[®] Vesicles freshly prepared (sterile water as aqueous phase), ^{99m}Tc -MDP added to aqueous phase before mixing

When used in medical facilities the pre-made formulations will be best suited because:

- Formulation stable beforehand, can be stored for days before introducing ^{99m}Tc -MDP.
- Minimal preparations required by medical staff
- Sufficient entrapment achieved after 1st hour, maintained for at least 12 hours

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- Necsa for use of their facilities

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