Pheroid® technology as a tool to change the administration route of selected pharmaceuticals from intravenous to oral

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Thesis submitted for the degree *Doctor of philosophy* in Pharmaceutics at the North-West University

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Graduation: October 2018

Student number: 21090955

DEDICATION

Dedicated to my dad, Johannes Petrus Kleynhans (1946/12/22 – 2017/07/30), my biggest supporter who was denied by others to witness this final triumph of our journey just before we crossed the finish line.

Husband dies protecting wife

A BRAVE Hibberdene man was murdered and his wife wounded during a robbery at their Marianne Drive home last Sunday night.

Johannes Petrus Kleynhans (71) was killed while attempting to protect his wife during the ordeal. Police have reported that three men threw a piece of wood through a window and then entered the house while Mr Kleynhans and his wife were watching television at about 7.45pm.

"He fought the intruders in a valiant effort to keep them away from his wife. He was stabbed and was declared dead at the scene. His wife was also seriously wounded and was taken to hospital for treatment. She had also been stabbed. She has since been released from hospital," said police spokesman, Lieutenant-Colonel Zandra Wiid.

The men fled, taking a television and cellphones. A murder and robbery case is being investigated. No arrests have yet been made.

INSPIRATION

Want U is my lamp, o Here! En die Here laat my duisternis opklaar. Want met U loop ek ’n bende storm, met my God spring ek oor ’n muur.

Psalm 18:29-30

….it used to be so simple, once upon a time.

Because the universe was full of ignorance all around and the scientist panned through it like a prospector crouched over a mountain stream, looking for the gold of knowledge among the gravel of unreason, the sand of uncertainty and the little whiskery eight-legged swimming things of superstition. Occasionally he would straighten up and say things like “hurrah, I’ve discovered Boyle’s Third Law.” And everyone knew where they stood.

But the trouble was that ignorance become more interesting, especially big fascinating ignorance about huge and important things like matter and creation, and people stopped patiently building their little houses of rational sticks in the chaos of the universe and started getting interested in the chaos itself – partly because it was a lot more easier to be an expert on chaos, but mostly because it made really good patterns you could put on a t-shirt.

Terry Prachett, Witches Abroad

PERSONAL ACKNOWLEDGEMENTS

- First and foremost, I have to thank my Father in heaven for this opportunity and the courage to pursue it till the end.
- My mother and sister, words will never describe what you mean to me.
- I would like to thank my supervisor Prof. Anne Grobler and my co-supervisors Prof. Jan Rijn Zeevaart and Prof. Mike Sathekge for the opportunity to be part of such an amazing team. Thank you all for understanding the struggle and motivating me at the end when I wanted to give up on studies and life.
- Prof. Thomas Ebenhan for assisting me with gaining an understanding of the field of Nuclear Medicine by always being available for answering questions.
- Internetainers Rhett & Link for their Youtube channel Good Mythical Morning- thank you for bringing fun into my life during the though stages.
- Linkin Park, Three Days Grace, One Republic, Imagine Dragons, Twenty One Pilots, Sia and Audioslave for providing anthems to inspire me during the writing process.
- CX fitness and Virgin Active for providing me with the facilities to unload my stress.
- All my office friends, in no particular order. Andri, Henri and Ruan (office of 2016). Drieke, Stephanie, Linné, Bianca, Jennifer, Riaan, Palesa and Lerato (office of 2017). Janie, Vusi, Amanda, Johncy and Cathryn (students of Necsa and SBAH). Kobus, Jean and Henrico (Clicks pharmacy).
- My other friends for listening to my complaining: Sanet, Nkuli, Madelein and Pieter.
- The staff of LCL-Cynologics, thank you for your amazing assistance and kindness, the time spend with you in the beautiful Mauritius, I will treasure always.
- Anisa Wadee for providing me with the opportunity to locum part-time to keep the pot cooking. All the rest of the students and staff at Clicks, you have made it a pleasure to work during weekends.

ACKNOWLEDGEMENTS AND DECLARATION

I Janke Kleynhans, hereby declare that this thesis is a record of my own work (except where citations or acknowledgments indicate otherwise) and that the study in part or as a whole has not been submitted to any other university.

I would like to acknowledge the following individuals or organizations for their contributions to my study:

- Financial assistance was received from the National Research Foundation (NRF) of South Africa as well as the Nuclear Technologies in Medicine and Biosciences Initiative (NTemBI). Opinions expressed and conclusions arrived at, are those of the authors and are not necessarily to be attributed to the NRF and NTeMBI. Financial aid was also provided by the North-West University and the DST/NWU PCDDP.
- The North-West University for the facilities provided during all 9 years of studying at this institution.
- Dr. Matthew Glyn for providing assistance with all the Confocal Laser Scanning Microscopy analysis and Mr. Lesley Masetle for providing assistance with all the Malvern Mastersizing and zeta-potential measurement. The patient scanning for the clinical trial was performed by Ms. Dalene van Wyk at Steve Biko Academic Hospital.
- All the statistical analysis was performed by Prof. Faans Steyn from the Statistical Consultation Services of the North-West University.
- Part of the data (oral acute and subchronic evaluation and AMES test) for the article on the toxicity of the Pheroid® delivery system was provided by Dr. Dale Elgar.
- The handling of animals: Mr. Cor Bester, Mr. Kobus Venter, Mr. Hylton Buntting, Ms. Antionette Fick, Mr. Henri Dunn, Dr. Jenna Moonsamy, Mr. Deenoo Sathyam, Dr. Alexander Espitalier Noel, Mr. Neville Fitzroy and Dr. Stallone Tendai Terrera.
- Ms. Liezl Marie Scholtz for agreeing to be the study monitor for the clinical trials in humans.
- The International Atomic Energy Agency (IAEA) for provision of a grant to participate in the International Conference on Integrated Medical Imaging in Cardiovascular Diseases (IMIC 2016) and the for the honour of receiving the European Association of Nuclear Medicine (EANM) award for presenting distinguished work. This has been an opportunity of a lifetime. https://conferences.iaea.org/indico/event/100/contribution/103.

ABSTRACT

Key terms: Pheroid®, radiotracers, insulin, preclinical evaluation, clinical trial, toxicity testing

The Pheroid® drug delivery system can change the route of administration from the parenteral route to the oral route. This system is therefore investigated as a safe alternative formulation that can also contribute to better patient compliance. Pheroid® technology is currently on the verge of being applied in the clinical environment and an in-depth evaluation of this system's toxicity is provided as an original research article (submitted to Toxicology Reports). This is a prerequisite for any registration as a new pharmaceutical entity.

The oral formulation of ^{99m}Technetium methyl diphosphonate demonstrated potential in previous evaluations in Sprague Dawley rats as an alternative to intravenous injections of this radiopharmaceutical. This radiopharmaceutical was selected for evaluation based on clinical need (providing bone scans to patients contra-indicated for injections) as well as the high availability of ^{99m}Tc as radiotracer. A clinical trial was designed as a hybrid Phase I/II clinical trial with 16 volunteers. The trial was performed according to Good Clinical Practise regulations, and three patients were enrolled before the study was terminated due to lack of efficacy. Valuable information regarding the Pheroid® delivery system was gained and this application of Pheroid® will be refined and pursued further in the future. A review article is provided (submitted to the Journal of Controlled Release) regarding the application of drug delivery systems in nuclear medicine. This article provides insight into the shortcomings of nuclear medicine that can be addressed by the utilization of drug carrier systems such as Pheroid®.

A preclinical evaluation of the pharmacokinetics (for a 5 hour period) as well as a basic toxicology analysis, of oral insulin (entrapped in Pheroid®) was performed on Cynomolgus monkeys. Insulin was formulated in pro-Pheroid® capsules as well as a Pheroid® emulsion and administered through the oral route. The efficacy was evaluated based on a drop in blood glucose levels. A blood clinical biochemistry analysis was also performed to gain information regarding the physiological impact of this system (with the entrapped insulin). The pro-Pheroid® formulations lacked efficacy, but the Pheroid® emulsion was effective and demonstrated a longer-acting effect when compared to the short acting subcutaneous control insulin. The positive results gained in this study indicate that further investigations should be launched.

This thesis successfully demonstrated the safety of the Pheroid® delivery system for future applications, provided a summary regarding the nuclear application thereof and showed potential in second non-rodent model for the insulin Pheroid® formulation.

OPSOMMING

Sleutelterme: Pheroid®, radioaktiewe merkers, insulien, toksisiteits bepaling, prekliniese evaluering, kliniese proef,

Die Pheroid® geneesmiddelafleweringssisteem kan die roete van toediening van die parenterale roete tot orale roete verander. Hierdie sisteem word ondersoek as 'n veilige alternatiewe formulering wat ook kan bydrae tot beter pasiënte aanvaarding van terapie. Pheroid® tegnologie is op die punt van implementering in die kliniese omgewing en 'n in diepte evaluering van die sisteem se toksisiteit word verskaf as oorspronklike navorsingings artikel (ingestuur vir publikasie aan Toxicology Reports). Die toets van toksisiteit is 'n voorvereiste vir enige registrasie as 'n nuwe farmaseutiese entiteit.

Die orale formulering van ^{99m}Technetium metieldifosfonaat in Pheroid® het voorheen in pre-kliniese toetse in Sprague Dawley rotte om 'n effektiewe alternatief te wees vir intraveneuse inspuitings van hierdie radioaktiewemerker. Hierdie merker was gekies vir evaluasie op grond van kliniese aspekte (verskaf been skanderings aan pasiënte gekontradikteer vir intraveneuse toedienings bv. pediatriese pasiënte) asook die hoë beskikbaarheid van hierdie isotope (^{99m}Tc) in Suid-Afrika. Die kliniese proef was ontwerp as 'n hibried fase I/II kliniese proef met 16 pasiënte wat gewerf moes word. Good Clinical Practise standaarde was gevolg en drie pasiënte het deelgeneem aan die proef tot die voortydige staking as gevolg van 'n tekort aan effektiwiteit. Waardevolle inligting was gedurende hierdie evaluasie bekom en hierdie toepassing van Pheroid® sal verfyn en verder nagevolg word in die toekoms. 'n Opsommende artikel (ingedien aan die Journal of Controlled Release) oor die toepassing van geneesmiddel afleweringssisteme in kerngeneeskunde word ook verskaf. Hierdie artikel beskryf die tekortkominge van kerngeneeskunde wat deur die toepassing van geneesmiddel draers soos Pheroid® opgelos kan word.

'n Prekliniese evaluering van orale insulien (vasgevang in Pheroid®) was uitgevoer op Cynomolgus primate vir 'n tydperk van 5 ure, asook 'n basiese toksikologie analise. Hierdie hoofstuk word ook voorgedra as 'n oorspronklike navorsingsartikel. Insulien was geformuleer in pro-Pheroid® kapsules asook 'n Pheroid® emulsie en was toegedien deur die orale roete. Die effektiwiteit was gevalueer deur na die verlaging in bloedglukosevlakke te kyk. 'n Evaluasie van die bloed se kliniese biochemie eienskappe was gedoen om verdere inligting te verskaf aangaande die fisiologiese effek van hierdie formulering (met die inuslien geïnkorporeer in Pheroid®. Die pro-Pheroid® formulering het nie effektiwiteit getoon nie, maar die Pheroid® emulsie was effektief en het 'n langwerkende effek getoon in vergelyking met die kortwerkende

subkutaneuse kontrole insulien. Die postiewe resultate wat in hierdie studie verkry is dui aan dat verdere ondersoeke onderneem moet word.

Hierdie tesis het die veiligheid van Pheroid® bewys vir toekomstige toepassings daarvan, het ekstra inligting verskaf aangaande die kerngeneeskunde toepassing van Pheroid® asook potensiaal demonstreer in 'n tweede nie-knaagdier model van die insulien Pheroid® formulering.

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^{10}B	boron -10
^{111}In	indium-111
^{125}I	iondine-125
^{14}C-CQ	^{14}C-Choloroquine
^{153}Sm	samarium-153
^{15}O	oxygen-15
^{166}Ho	holmium-166
^{177}Lu	lutetium-177
^{188}Re	rhenium-188
^{188}W	tungsten-188
^{18}F	fluorine-18
^{18}F-FDG	fluorine-18-fludeoxyglucose
^{198}Au	gold-198
^{225}Ac	actinium-225
3R	Replace, Reduce and Refine
^{64}Cu	copper-64
^{89}Zr	zirconium-89
^{90}Y	yttrium-90
^{99}Mo	molybdenum-99
^{99m}Tc	technetium-99m
^{99m}Tc- HMPAO	^{99m}Tc-hexametazine

^{99m}Tc-HMPOA	technetium-99m-hexamethylpropyleneamineoxime
^{99m}Tc-MDP	^{99m}Technetium methylene diphosphonate
^{99m}Tc-MDP	technetium-99m-methyl diphosphonate
^{99m}Tc-MIBI	^{99m}Tc-Sestamibi
^{99m}Tc-MIBI	technetium-99m-hexakis-2-methoxyisobutylisonitrile
AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care International
ADME	Absorption, Distribution, Metabolism and Excretion
ALARA	As Low As Reasonably Achievable
API	Active Pharmaceutical Ingredient
ATC	Acute Toxic Class Method (OECD guideline 423)
BBSRC	Biotechnology and Biological Sciences Research Council
BMEDA	*N*,*N*-bis [2-mercaptoethyl]-*N′*,*N′*-diethylethylenediamine
CLI	Cerenkov luminescence imaging
CLSM	Confocal Laser Scanning Microscopy
CT	Computed tomography
DFO B	desferrioxamine B
DM	Diabetes Mellitus
DNA	deoxyribonucleic acid
DOTA	1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid
DST/NWU PCDDP	Department of Science and Technology/North-West University Preclinical Drug Development Platform
EGCG	epigallocatechin gallate

Elisa	Enzyme-linked immunosorbent assay
FDA	Food and Drug Administration
FDP	Fixed Dose Procedure (OECD guideline 420)
GCP	Good Clinical Practice
GRAS	Generally regarded as safe
HIV	Human Immunodeficiency Virus
IAEA	International Atomic Energy Agency
ICH	International Committee of Harmonization
ITLC	Instant Thin Layer Chromatography
IV	Intravenous
kPa	Kilopascal
LD_{50}	median lethal dosage
mCi	Milli Curie
MnMEIO	Md-doped magnetism engineered iron oxide
MRC	Medical Research Council
MRI	Magnetic Resonance Imaging
mV	Millivolt
MWNT	multi-walled nano particles
NECSA	The South African Nuclear Energy Corporation SOC Limited
NIRF	near infrared fluorescence
NOTA	1,4,7-triazacyclononane-1,4,7-triacetic acid
NRF	National Research Foundation

N-type Ca2+ channels	voltage-dependent calcium channels
OECD	Organisation for Economic Cooperation and Development
PEG	polyethylene glycol
PET	Positron Emission Tomography
PI	Principle Investigtor
PK-PD	Pharmacokinetics/ Pharmacodynamics
rhGH	recombinant human growth hormone
RIT	Radioimmunotherapy
Rpm	Rate per minute
SAHPRA	South African Health Product Regulatory Agency
SANAS	South African National Accreditation System
SBAH	Steve Biko Academic Hospital, Pretoria
SC	Subcutanous
SD	Standard Deviation
SEM	Scanning electron microscopy
SIRT	selective internal radiation therapy
SLN	sentinel lymph node
SPECT	Single Photon Emission Computed Tomography
SWNT	single-walled nano particles
TB	Tuberculosis
TEM	Transmission electron microscopy

UCL	Upconversion luminescence
UDP	Up and Down Procedure (OECD guideline 425)

CHAPTER 1: THESIS INTRODUCTION

The modern clinical environment is characterized by a movement away from traditional practice where disease management and therapy are measured objectively by clinical outcomes such as symptom manifestation, prognosis and disease progression. It is important to repair the increasingly negative public perception of treatment by altering options to incorporate convenience of therapy as well as quality of life (Seetharau *et al.*, 2007). Due to the availability of online health information (the e-patient revolution), patients are taking a more active role in the decision-making process and any negative perception of diagnosis and treatment can therefore motivate the patient to stop conventional treatment and pursue alternative options that are less established. (Liu, 1997; McKinstry, 2000; Shumay *et al.*, 2001; Bultz & Carlson, 2006; Seetharau *et al.*, 2007; Wald *et al.*, 2007; Al-Eidi *et al.,* 2016; Rocha *et al.,* 2017).

It is well known that patients prefer oral administration over parenteral treatments and the continuous administration of injections can demotivate patients (Delahantry *et al.,* 2007; Makine *et al.,* 2009). This study contributes to the conversion of the less satisfactory experience of receiving an injection to that of oral administration of various pharmaceutical active ingredients. This is applied in the field of nuclear medicine as well as diabetes treatment. The Pheroid® drug delivery system has been proven to be a safe alternative dosage form to the parenteral route. The use of the Pheroid® as carrier system has been shown to influence the properties of the entrapped drug in a large amount of pre-clinical research (summarized in Chapter 2). Since the Pheroid® drug delivery system has been investigated for active pharmaceutical ingredients (API's) other than those investigated in this study, data regarding its pharmacokinetics can be extrapolated to other applications. It is particularly useful when there is a need to enhance the absorption of an active pharmaceutical ingredient *via* a specific administration route or increase the delivery of an active ingredient to a target tissue within the human body (Grobler, 2009; Grobler *et al.,* 2014; Grobler & Zeevaart, 2015). This study aims to evaluate Pheroid® technology (both Pheroid® and pro-Pheroid® forms of this system) as a vehicle to alter the route of delivery of selected pharmaceutical agents. The Pheroid® system contains non-toxic ingredients and the method of manufacturing can be adapted to generate a system tailor-made to accommodate the API and address the shortcomings, either pharmacokinetic or treatment wise (e.g. poor oral absorption). This system is an oil-in-water microemulsion type system (Grobler, 2009).

1.1 Research problem

The research problem that was addressed during this study is if the Pheroid® delivery system can change the bioavailability of substances normally only available by parenteral routes, to the oral

route of administration. Two types of API's were selected namely radiotracers (^{99m}Tc-MDP) and therapeutically active proteins (insulin).

Radiotracers are radioactive substances used to diagnose disease by imaging the movement of these entities in the human body. These radiotracers are currently only available by the intravenous route, which makes this procedure unavailable to patients with poor vein quality as well as paediatric patients. Additionally, the risks associated with intravenous administration can be removed by use of an oral formulation. The first-in-human clinical trial therefore serves to answer the question if the oral Pheroid® formulation will provide oral administration of ^{99m}Tc-MDP and if the quality of scintigraphy scans will be as good as that provided by the gold standard.

Insulin (a key component in the treatment of diabetes mellitus) is degraded by the gastro-intestinal system and must be injected by means of multiple subcutaneous injections per day. This contributes to a high incidence of side-effects (e.g. inflammation at injection site) and distress to patients. This study will answer the question if an oral Pheroid® formulation will provide a substantial lowering of blood glucose levels.

A sub-research question was that before any potential medicinal product can be tested in man, a full investigation regarding safety is required. The evaluation of intravenously administered Pheroid® provides information regarding the possible side-effects of this system at absolute bioavailability.

1.2 Aim

To establish Pheroid® technology as a safe and effective drug delivery system and to change the administration route of radiopharmaceuticals and therapeutic proteins from the parenteral route to the oral route.

1.3 Justification of study

Some treatments for disease e.g. insulin for the treatment of Diabetes Mellitus is currently only available as treatment administered by the parenteral route. If the oral route is available, it has been determined that switching from parenteral administration to oral administration at the opportune moment can lead to a reduction in treatment cost as well as an increased effectiveness (Cyriac *et al.,* 2014; Eckmann *et al.,* 2014). Drug carrier systems are of great value in the provision of alternative routes of administration of API's (e.g. intra-nasal, oral, transdermal, buccal, ocular). The Pheroid® delivery system has applications in the delivery of API's through alternative routes; for example, the delivery of hormones such as calcitonin and insulin through the intra-nasal route,

the transdermal delivery of anti-cancer drugs and the oral delivery of radiopharmaceuticals (Oberholzer, 2009; Du Plessis, 2010; Chinembiri *et al.,* 2015; Grobler & Zeevaart, 2015).

Currently, nuclear medicine is mostly applied to the diagnosis of various diseases (myocardial disease, lung function, bone lesions, renal perfusion, thyroid pathology, and oncology) but can also be used as a treatment for cancer (Prvulvich & Bomanji, 1998; Lecouvet *et al,* 2014; Czernin, 2017). Valuable information regarding the bodily processes is provided by these techniques without any impact on the physiology of the patient. Nuclear imaging is also characterized by a low incidence of side effects while providing valuable clinical information. All nuclear imaging isotopes (with the exception of iodine) are currently delivered by the intravenous route and almost all side-effects are associated with the parenteral administration thereof, and not the characteristics of the isotope itself. For instance, a study by Kaushal and co-workers found that 54% of adverse drug reactions of all API's in a paediatric hospital patient were due to the use of the intravenous route and not the medication administered itself (2001). The delivery of radioisotopes through an alternative route (e.g. the oral route) should therefore contribute to a lower incidence of side-effects, higher patient comfort and an increase in the availability of this technology to all patients (e.g. paediatrics and geriatrics).

Type 1 diabetes mellitus (DM) always relies on multiple daily subcutaneous injections of insulin (nowadays commercially prepared by recombinant DNA technology), while insulin is also part of the treatment plan for most DM type 2 patients. Insulin is hampered, like peptides in general, by lack of efficacy when administered through other routes than the parenteral route. Various alternative routes have been investigated for insulin delivery namely the oral route, nasal route, buccal administration, pulmonary inhalation, transdermal diffusion, rectal administration and ocular administration. The development of a successful oral insulin formulation (through Pheroid® delivery) will have a great impact on the quality of life of patients with DM as well as possible delivery of other therapeutic proteins and peptides (Joshi *et al.,* 2007).

1.4 Objectives

Therefore, the following objectives were planned for this study:

- To determine whether the Pheroid®-entrapped ^{99m}Tc-MDP oral formulation is equal in efficacy as that of the gold standard, namely the ^{99m}Tc-MDP intravenous formulation. Efficacy was determined by the patient diagnosis based on the SPECT image obtained in a hybrid phase I/II clinical trial on 16 human volunteers with bone lesions.
- To evaluate the efficacy of an oral Pheroid®-based insulin formulation in Cynomolgus monkeys and prove the usability of this invention as a blood glucose lowering agent.

- As a prerequisite to human studies, to provide a toxicology evaluation of Pheroid® technology.

1.5 Hypothesis

Orally administered Pheroid®-based formulations will provide safe alternative dosage forms for pharmaceuticals currently only bioavailable by administration *via* the parenteral route.

1.6 Scope of study

This study covered an investigation into the application of Pheroid® as a delivery system to enable the administration of traditionally intravenous preparations, through the oral route. The toxicity of this system was also investigated. This thesis includes descriptions of pre-clinical and clinical testing with the focus on the effect of formulations on the outcomes of the investigation.

The clinical trial in humans (oral ^{99m}Tc-MDP in Pheroid®) took place in the Steve Biko Academic hospital, South Africa and rodent toxicity testing of Pheroid® at the vivarium of the DST/NWU Preclinical Drug Development Platform (PCDDP) in South Africa. The clinical trial took place over one month, with only three patients enrolled. The study was paused due to lack of efficacy requiring additional formulatory studies before further clinical testing. The preclinical evaluation in primates (oral insulin in Pheriod®) was performed at Les Campeches Ltd (LCL-Cynologics), Port Louis, Mauritius on a unique population of Cynomolgus Long Tail Macaques. These animals are Specific Pathogen Free species (free from SRV, SIV, STLV, B-Virus, filoviruses, rabies, malaria, dengue and chickungunya) and this results in samples that are non-medicated, non-immunized and non-infectious. The samples were analysed by the MRC Harwell Institute, United Kingdom.

1.7 Format of this thesis

This study encompasses four distinct topics all with the same goal, namely to apply Pheroid® as a tool to change the administration route of selected pharmaceuticals. This thesis therefore presented with unique challenges regarding the format of the chapters. Consequently, it is presented in four sections (chapter 2-5) each with its own literature study followed by the research presented as either an article (review or original) or in report format.

This thesis contains the following chapters:

- Chapter 1: Thesis introduction
- Chapter 2: A toxicity profile of Pheroid® technology
    - Pheroid® technology background
    - *A comprehensive toxicity profile of Pheroid® technology*

- Chapter 3: An introduction to nuclear imaging and the application of drug delivery systems
    - Literature regarding nuclear medicine
    - *Drug delivery systems: targeting new frontiers in nuclear medicine*
- Chapter 4: Clinical investigation of an oral ^{99m}Tc-MDP in Pheroid®
    - Literature on clinical trials and Good Clinical Practice
    - Report on the hybrid phase I/II clinical trial
- Chapter 5: Oral delivery of insulin with Pheroid® technology: an evaluation in primates
    - Literature on diabetes mellitus and alternative insulin delivery
    - Research article on oral Pheroid® insulin formulations
- Chapter 6: Conclusion drawn from new data generated

Annexures added include various ethical and scientific approval certificates including permissions to reprint figures from other sources. Also included are the instructions for authors for the 2 scientific journals the articles were submitted to.

It is envisioned that the reader will find this thesis to tell the story of a scientific investigation, not only including a selected outcome of positive data, but rather an inclusion of all data harvested during the duration of the candidate's PhD course. Care was taken to ensure sufficient positive data (the evidence of the safety of the Pheroid® system as demonstrated during the toxicity study as well as the evaluation of the oral insulin preparation in primates) is presented to contribute in a meaningful way to the bulk of scientific knowledge, but an active decision was made to report negative data also. It is the candidate's opinion that lessons learned from negative data is of equal importance as that of positive data.

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CHAPTER 2: A TOXICITY PROFILE OF PHEROID® TECHNOLOGY

The Pheroid® drug delivery system is a system that may be used to deliver pharmaceutical active ingredients (API) orally, that is currently only bioavailable by the intravenous route. The intravenous route is associated with many disadvantages, including infection and immunological responses with drugs that can elicit such a response (Dychter *et al.,* 2012). For any delivery system to be useful, it needs to be non-toxic and a completed dossier proving a formulation to be non-toxic is a prerequisite for clinical use of any pharmaceutical formulation, therefore a section on the toxicity screening of the Pheroid® system is included in article format. In addition, it is necessary for further clinical testing to provide evidence of biocompatibility to the various regulating authorities.

2.1 The history of Pheroid®

Pheroid® possesses distinctive features and does not always conform to the general characteristics attributed to lipid drug carriers. The first main difference is that the Pheroid® delivery system consists of the ethyl esters of natural and essential fatty acids that are physiologically acceptable (reducing the risk of allergic reactions). All the ingredients are listed by the Food and Drug Association (FDA) as Generally Regarded as Safe (GRAS). Due to the essential fatty components of Pheroid® the transport of drugs entrapped therein is increased over most physiological barriers (cells, tissue and organisms). The Pheroid® delivery system furthermore demonstrates high entrapment efficiencies (85%-100%), which is uncommon for lipid drug delivery systems (Uys, 2006; Grobler, 2009). Pheroid® is also manufactured during an environmentally friendly manufacturing process using no toxic components and resulting in no chemical waste.

The Pheroid® technology initially was developed as a topical base formulation under the trade name Emzaloid™ by MeyerZall (Pty) Ltd (founded by Johannes Petrus Meyer). Upon noticing that the peel of the banana fruit is used in Zulu traditional medicine for the treatment of skin conditions, Meyer analysed the components that might contribute to this activity. Meyer tested the formulation on himself as a cure for psoriasis and the results were remarkable. The distinct fatty acid composition of this remedy was formulated in a topical cream called Emzaloid™. Pheroid® technology was developed out of Emzaloid™ technology. The main differences between Emzaloid™ and Pheroid® technologies are the saturation level with nitrous oxide, the incorporation of α-tochopherol in Pheroid® technology and the manufacturing process (Meyer, 1993; Grobler, 2009; MeyerZall, 2012).

2.2 Classification of Pheroid® technologies

The Pheroid® system (Pheroid®, pro-Pheroid® or Pheroid® microsponges) consist of three phases namely an oil-phase (fatty acid based), aqueous-phase and gas-phase (nitrous oxide). Factors that influence the size and morphology of the individual Pheroid® vesicles are modifications to the method of manufacturing, components of the oil-phase as well as oil-to water ratio. The oil-phase has a standard base composition (Vitamin F ethyl ester, Kolliphor EL and α-tocopherol) but other ingredients can also be incorporated for example Incromega™ and polyethylene glycol. The water phase can include various buffer systems depending on the requirements of the application. The standard methods employed to analyse the properties of the vesicles formed are Confocal Laser Scanning Microscopy (CLSM) image analysis, zeta-potential measurement and particle size distribution (Grobler, 2009).

2.3 Factors influencing bioavailability of the formulation

The uptake of Pheroid® vesicles across cellular membranes are influenced by the structure of the vesicles as well as physiological factors. Structural factors include the size and morphology of the vesicles as well as the concentration and ratio of the fatty acid components. The pH and ionic strength of the physiological environment as well as the aqueous phase surrounding the vesicles also contribute to the absorption behaviour. After the cellular uptake of the vesicles, they are metabolized in the mitochondria or peroxisomes, depending on the particular composition of the vesicle in question and the contents are delivered at the site of action with increased bioavailability. The nitrous oxide and fatty acid components in the system influence barrier permeability and fluidity. Additionally, binding onto fatty acid membrane binding proteins on the cell's surface also increases the influx through membranes. The measurement of the zeta-potential of the formulation provides important insight in the interactions that will take place between the vesicle and membrane (Grobler, 2009).

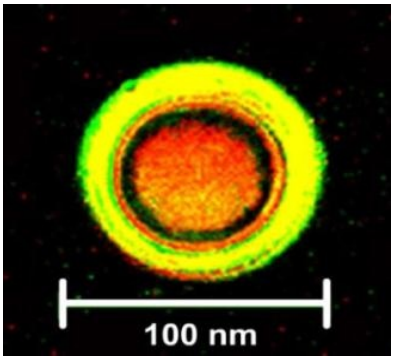
2.3.1 Approaches to the incorporation of the active ingredient in Pheroid®

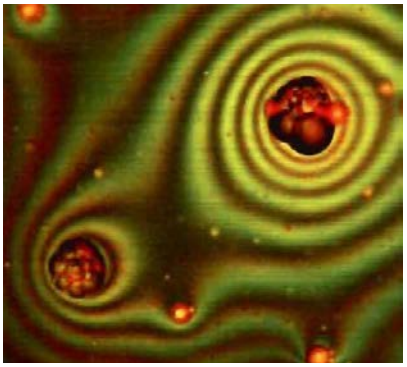
During the manufacture of lipid delivery systems standard methods to entrap or encapsulate the active ingredient in the drug carrier system exist. The higher the amount of API that is successfully entrapped in the drug delivery system, the more effective the drug delivery system will be in changing the pharmacokinetics of the API. There are two methods of passive entrapment, the first being the mixing of the active ingredient into either the lipid phase or the aqueous phase before the manufacturing process. Alternatively, the active ingredient can be entrapped after manufacturing by incubation with the already formed carrier system, provided it has an affinity for the lipids on the carriers' surface. Active entrapment involves the use of ligands for the entrapment

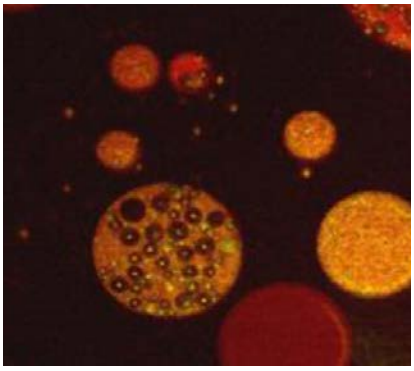
of the active ingredient (Phillips, 1999). Most Pheroid® technology applications utilize the passive method of entrapment of the API, either before, during or after the manufacturing process, although active entrapment is also under investigation. The selection of the method of manufacture as well as the entrapment time is based on a series of *in vitro* experiments combined with CLSM to determine the correct morphology.

2.3.2 Morphology of the Pheroid® vesicles

The morphology of the Pheroid® vesicles is analysed by CLSM. In some instances, SEM (Scanning Electron Microscope) and TEM (Tunnelling Electron Microscope) can also provide valuable morphological information (Slabbert *et al.,* 2011). The standard Pheroid® vesicles (Figure 2.1a) possess a lipid bilayer but cannot be grouped under the traditional liposomes) due to the lack of a phospholipid or cholesterol components. The formation of the vesicle is an autonomic process brought about by self-assembly due to unique interactions between the fatty acids and stabilization of the system by the nitrous oxide component. Pro-Pheroid® (Figure 2.1b) can be viewed as the pre-curser of Pheroid® and consists of the oil-phase only, which is gassed with nitrous oxide and administered in capsules or liquid form (buccal administration, paediatric oral drops, intra-nasal administration and ophthalmic formulations). Its application is in the packaging of active pharmaceutical ingredients that is labile in the presence of moisture. When ingested in capsule form, a Pheroid® forming zone is formed upon contact with gastric fluids when pro-Pheroid® is released out of the capsule. During this process active compounds are entrapped spontaneously in the formed vesicles and carried away from the area of formation. It was observed that the addition of long chain polyunsaturated fatty acids (e.g. eicosapentaenoic acid and decosahexaenoic acid) resulted in the formation of Pheroid® microsponges with small interior pockets giving it a sponge-like quality (Figure 2.1c). Hydrophobic active ingredients localize in these small interior spaces. The Pheroid® microsponge system (depot) displayed sustained release characteristics in topical formulations that are dependent on the concentration gradient of the pharmaceutical agent that exist between the skin surface and the Pheroid® formulation (Uys, 2006; Grobler, 2009).







a) Pheroid® vesicles **b)** Pro-Pheroid® reacting to water **c)** Pheroid® microsponges

Figure 2-1: An image of the different Pheroid® vesicles obtained by confocal scanning microscopy. (Reprinted from Grobler, 2009 with permission from the author).

2.3.3 Zeta-potential of the Pheroid® vesicles

Lipid based carrier systems all possess a charge on their surface area (measured as zeta-potential) that influences the stability of the systems as well as its interactions with the environment. In the case of liposomes, a negative charge provides a more stable system but at the same time increases the nonspecific cellular uptake of the carrier system. A zeta-potential that is high (±25 mV) indicates a stable emulsion where the repulsive forces overcome the attractive forces. If the zeta-potential is low, the attractive forces will overcome the repulsive forces and the system will become unstable. Neutral liposomes tend to aggregate more and are therefore unstable but possess a lower tendency to be cleared by the reticular endothelial system and a higher concentration of these drug carriers reach the target area. Liposomes with a positive charge interact with serum proteins and are therefore not useful and demonstrate low efficacy for the delivery of active pharmaceutical ingredients (Lian & Ho, 2000; Roland *et al.*, 2003). The manufacturing method of Pheroid® vesicles has a marked influence on the zeta-potential of the formulations (between -23.8 mV up to -35.7mV), with this range deemed as satisfactory in providing a stable formulation (Uys, 2006).

2.3.4 Particle size of the Pheroid® vesicles

The particle sizes that are targeted for Pheroid® vesicles range between 200 nm – 5 µm (Grobler, 2009). This range avoids the myriad of unknown and potentially dangerous physiological effects of particles with sizes below 100 nm (ultrafine particles) (Hoet *et al.,* 2004; Kreyling *et al.,* 2006). The size distribution of particles can also give an indication of emulsion stability, with aggregation of particles due to thermodynamic instability leading to an increase in particle size over time

(Billany, 2002). The particle size of Pheroid® microsponges is approximately twice that of normal Pheroid® vesicles (1.5 – 5 µm) and this may have an effect on the behaviour of the carrier system. In an optimization study it was found that the mean size of Pheroid® vesicles is decreased when the mixing rate during manufacturing (revolutions per minute of the homogenization) was increased. When the emulsification time was extended, the size of vesicles formed were bigger, but the loss of nitrous oxide associated with longer mixing periods should be considered (Uys, 2006). As with other lipid drug carrier systems the effect of the reticular endothelial system should be considered, with 50-100 nm vesicles avoiding uptake and removal by this system. Long circulating liposomes are larger than 500 nm (Lian and Ho, 2001), and the Pheroid® drug delivery system falls within these particle size ranges to extend the time the active ingredient spends in circulation. However, it is possible to manufacture the Pheroid® delivery system as a nano-emulsion if the application calls for it.

2.4 The pharmaceutical application of Pheroid® for oral delivery

2.4.1 Commercialized applications of Pheroid®

For a detailed discussion on the development of pharmaceutical topical applications of Emzaloid™ refer to previous publications (Saunders *et al.,* 1999; Gerber *et al.,* 2008; Grobler *et al.,* 2008; Fox *et al.,* 2011; Kilian *et al.,* 2015). Products based on this system that are currently marketed are Athru-derm™ (diclofenac as anti-inflammatory agent), Linotar™ (a psoriasis treatment containing coal tar) and Covarex (miconazole as anti-fungal). The Pheroid® delivery system has found application in the delivery of agents used in the agricultural sector including the optimization of crop production (Grobler, 2007; Grobler, 2009; Peters, 2016) as well as animal health (Krause *et al.,* 2015).

2.4.2 Future products in the drug development pipeline

a) Nutraceuticals

The oral bioavailability of EGCG (epigallocatechin gallate), an antioxidant obtained from green tea, was compared with EGCG-Pheroid® in a double blinded randomized cross-over study in 20 healthy human volunteers. The blood concentration of EGCG was measured over 8 hours post-administration and the maximum blood concentration of the Pheroid® formulation (224 ng/ml) was significantly higher (in a ratio of 10:6) than that of EGCG alone (139 ng/ml) (Moruisi, 2008).

b) Buccal administration of anti-inflammatory agents

The buccal and oral application anti-inflammatory drugs formulated in Emzaloid™ are patented and under investigation. Preliminary investigations substantiated the application thereof, with a

qualitative increase in pain relief experienced (Meyer, 1996). Since nitrous oxide is well known for its analgesic and anaesthetic properties, it might contribute to a synergistic mechanism of action against pain (Annequin *et al.,* 2000).

c) Delivery of hormonal therapy sensitive to gastric degradation

Pheroid® technology has an important application in the clinical environment to reduce the discomfort of patients that must undergo regular intramuscular or subcutaneous injections during treatment. During various *in vivo* investigations, Pheroid® increased the intestinal absorption of calcitonin, a peptide used in the treatment of osteoporosis and Paget's disease, up to useful concentrations (Du Plessis *et al.,* 2010). The intra-nasal administration of recombinant human growth hormone (rhGH) also provided *in vivo* results that validate further investigations in clinical trials in humans (Steyn *et al.,* 2010). The formulation of an oral insulin with Pheroid® and pro-Pheroid® technology is still under investigation in a primate animal model (refer to Chapter 5) and high efficacy was demonstrated in rodents (Oberholzer, 2009).

d) Optimization of anti-infective agents

The Pheroid® carrier system has been shown to increase the amount of anti-infective to which micro-organisms is exposed to, leading to a shorter treatment time and better adherence by the patient. One of the mechanisms for antibiotic resistance is caused by the development of efflux proteins on the bacterial cell membrane which transports the antibiotic out of the organism before it can accumulate in therapeutic amounts. Pheroid® can combat this by causing an increased influx of the drug through the cell wall and bypassing the efflux system. The effectiveness of oral Pheroid® formulations of intravenous anti-infective agents is also under investigation. The combination of more than one anti-infective in a single Pheroid® formulation is also possible to further reduce the development of resistance, providing a fixed dose combination formulation with pharmaceuticals targeting more than one cellular process. Pheroid® -artemisone had higher anti-malarial activity in C57BL6 mice and vervet monkeys than that of the unformulated drug. A better adverse effect profile with retained effectiveness of lumefantrine as well as mefloquine was confirmed *in vivo* when formulated in Pheroid® (Steyn *et al.,* 2011; Du Plessis *et al.,* 2013; Grobler *et al.,* 2014; Du Plessis *et al.,* 2015).

The first-line anti-tuberculostatics (isoniazid, ethambutol, rifampicin and pyrazinamide) was formulated in pro-Pheroid® and based on positive *in vitro* and *in vivo* results, a Phase 1 cross-over clinical trial was undertaken in 16 healthy volunteers. A significant increase in the drug plasma levels of the rifampicin and isoniazid despite administration of only a 60% dose was observed. The plasma levels of ethambutol and pyrazinamide was not negatively influenced by

their formulation in Pheroid® and the half-life of ethambutol was greatly increased. Absorption of these drugs was faster and T_{max} values were reduced. An increase in the therapeutic window after administration was also observed. The Pheroid® formulation led to less side-effects compared to the standard drug, which predicts better patient compliance with therapy (Grobler, 2009). A phase 2 clinical study is necessary to determine the clinical efficacy of these formulations against tuberculosis and whether the duration of treatment may be shortened with this formulation.

e) Increased antigenicity of vaccines when formulated in Pheroid®

It is proposed that the formulation of antigens in the Pheroid® carrier system may result in a higher absorption rate through non-traditional vaccine delivery routes. Furthermore, the delivery of the antigen at the target site can be increased due to higher tissue penetration. Prolonged and controlled release may increase the magnitude of the immunological response against the antigen. It is also important to note that Pheroid® may unlock the oral or nasal delivery route for vaccines that must be injected due to stability issues. *In vivo* testing of the immunological response to Pheroid® entrapped vaccines against rabies, diphtheria and hepatitis B was carried out in support of a patent application and all demonstrated an optimized activity of the immune response (Grobler & Kotzé, 2006).

f) Application of Pheroid® in nuclear medicine

The development of a formulation whereby radiotracer diagnostics can be administered via the oral route can lead to increased ease of administration in the clinical setting and less emotional distress experienced by patients. The process of injection of pharmaceuticals is especially traumatic for critically ill patients who typically undergo these diagnostic procedures. An oral formulation can also make this procedure more accessible to paediatric patients and patients with poor vein quality. The effective oral absorption of two technetium radiotracers namely ^{99m}Tc-MDP (Technetium 99m-methyl diphosphonate) used to diagnose bone lesions and ^{99m}Tc-MIBI (Technetium 99m-methoxy-isobutyl-isonitrile) used to evaluate heart blood flow, was demonstrated *in vivo*. A hybrid phase I/II cross-over study in 16 human volunteers is currently underway (refer to Chapter 4) on the a oral ^{99m}Tc-MDP Pheroid® formulation (Grobler & Zeevart, 2015).

2.5 The drug development pipeline

The drug development pipeline is the process (refer to figure 2.2) utilized to take a drug candidate or formulation to the market. It is described as a flowing process, due to the fact that the order in which the phases is performed is not set in stone but is rather a continuous movement back and

forth with momentum gained towards the final registration phase, if the drug proves to be successful.

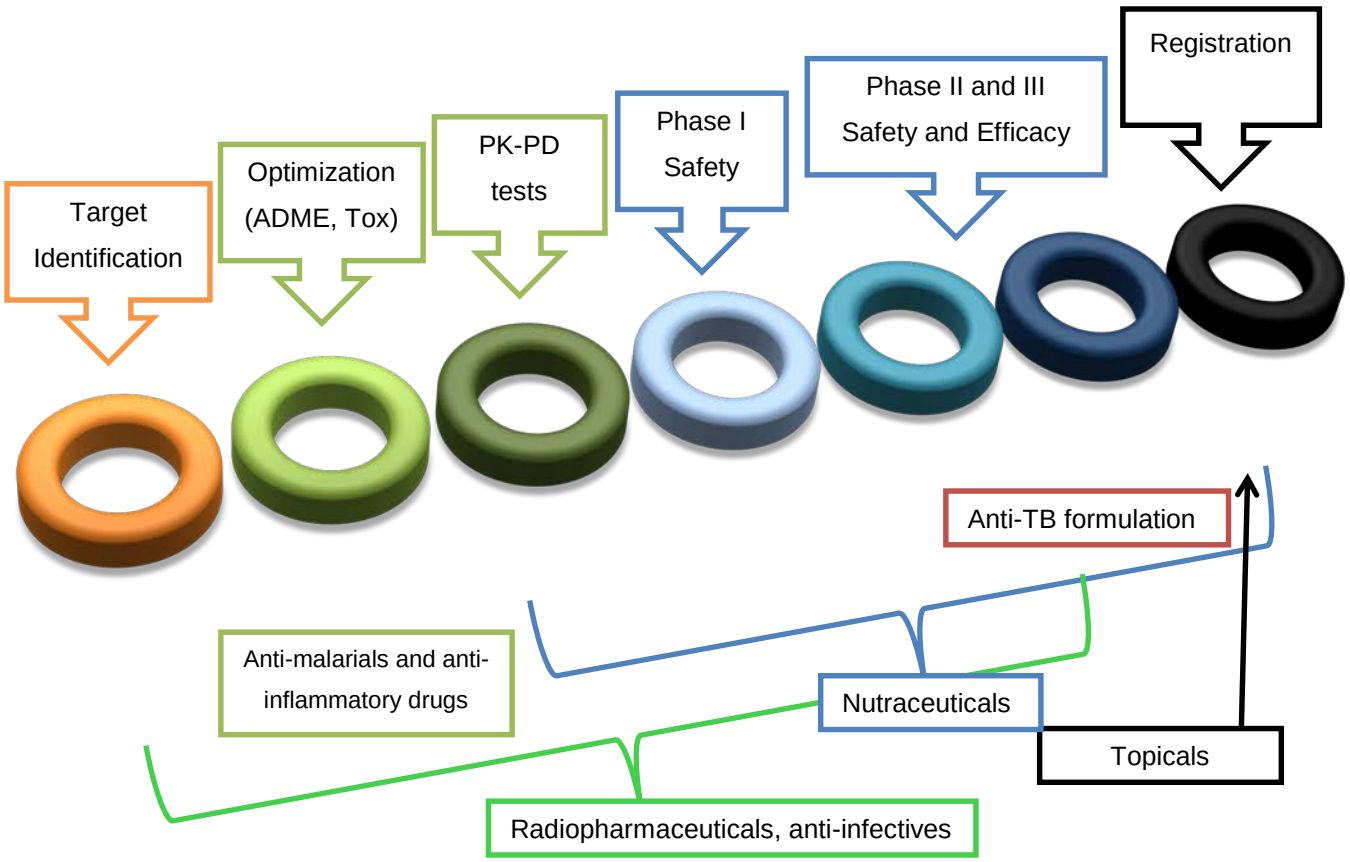


Figure 2-2: The stages of the drug development pipeline applied to Pheroid®. This figure is adapted from Ware & Khetani (2017) and Roses (2008). The orange and green coloured phases are the preclinical stages, and the blue phases indicate clinical application in humans. PK-PD: pharmacokinetic/pharmacodynamics; ADME: absorption, distribution, metabolism and elimination).

Although Pheroid® (with specific applications) has already been demonstrated as efficacious and safe (in Phase I and II human trials), the flow of the scientific knowledge of this system through the drug development pipeline necessitates an investigation into the toxicity of this system without an active ingredient present.

2.6 OECD guidelines for the evaluation of toxicity

Traditionally the toxicity of any pharmaceutical product was tested by the determination of the LD_{50} (median lethal dose) but this method has become obsolete due to the death of animals as the endpoint of this measurement. Currently three methods are widely used and are also prescribed by the Organisation for Economic Cooperation and Development (OECD) to evaluate the safety of new entities: the Fixed Dose Procedure (FDP) (OECD guideline 420), the Acute

Toxic Class Method (ATC) (OECD guideline 423) and the Up and Down Procedure (UDP) (OECD guideline 425). The FDP method avoids using the death of animals as endpoint by relying on clearly defined signs of adverse reactions after administration of fixed dose levels. With the evaluation of adverse reactions, the focus is on changes in the physical condition of the animal (fur, eyes, and mucous membranes), changes in the respiration, circulation, nervous system and behaviour patterns. The FDP results in lower animal numbers used per chemical or drug formulation evaluated as well as a decrease in animal suffering and mortality during testing. The ATC method still retains death of animals as the main endpoint and the UDP method also still aims to estimate an LD_{50} by adjusting the dose of each animal according to the outcome of the previous animal's administration. Table 2-1 provides a comparison between the three methods. Other methods of toxicity testing that do not involve animal models are currently not developed to the extent that it allows for an indication of the effect of pharmacokinetics on the toxicity of the substance. These methods are also not acknowledged by regulatory boards such as the FDA and the SAPHRA (South African Health Products Regulatory Authority) (Walum, 1998; OECD, 2001; Botham, 2004; Parasuraman, 2011).

Table 2-1: A comparison between FDP, ATC and UDP methods of toxicity testing (Adapted from Botham, 2004):

	FDP	ATC	UDP
Dose	Administered as a single bolus	Administered as a single bolus	Administered as a single bolus
Sighting study	A sighting study is included	Not included	Not included
Dose	5, 50, 300, 2000 and 5000 mg/kg	5, 50, 300, 2000 and 5000 mg/kg	Starting dose best estimate of LD_{50} (or 175 mg/kg) with dose adjustments
Animals	5 animals per dose	3 animals per dose	One animal is dosed at a time until study is terminated according to criteria.
Aim	Identification of lowest prescribed dose causing toxicity	Identification of lowest prescribed dose causing mortality	Provides an estimation of the LD_{50}
Output	Estimate of the LD_{50}, signs of acute toxicity and target organs of toxicity	Estimate of the LD_{50}, signs of acute toxicity and target organs of toxicity	Clear identification of the LD_{50}, signs of acute toxicity and target organs of toxicity.

The following section describes the methodology followed and results gained during the various investigations of the toxicity of Pheroid®. All the results are combined in a manuscript submitted for publication in Toxicology Reports (Elsevier) and formatted according to the instructions for authors (addendum 1 of this thesis).

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Toxicology Reports

A comprehensive toxicity profile of Pheroid® technology

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Abstract

The Pheroid® drug delivery system is now on the threshold of progressing into human clinical trials for various patented pharmaceutical applications and a systematic investigation of its toxicological properties *in vitro* and *in vivo* is thus a priority. *In vitro* testing included the Ames Assay. No genotoxicity was demonstrated during the analysis in *Salmonella typhimurium* strains TA98, TA100 and TA102. Acute and subchronic toxicity were investigated in two species of rodent. Observations focused on physical condition, blood biochemical analysis and haematological levels. Gross necropsy was performed on the test animals and organ weights as well as histopathology of selected organs. The acute toxicity study showed tolerance of the maximum prescribed dose of 2000 mg/kg in two rodent species after intravenous administration (absolute bioavailability). The oral formulation was tolerated without incidents. During both the acute and subchronic evaluations no physiological, chemical or haematological alterations were caused by the Pheroid® technology. Clear data is presented that the Pheroid® system will not contribute to toxicity during acute and prolonged administration (either through the oral or intravenous route) of the system. The toxicity of future treatments will consequently be dependent on the characteristics of the active ingredient entrapped in this system.

Keywords: Acute toxicity, subchronic toxicity, drug carrier system, genotoxicity, OECD guidelines, intravenous, oral

Abbreviations:

AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care International
API	Active Pharmaceutical Ingredient
LD_{50}	Median lethal dose
DST/NWU PCDDP	Department of Science and Technology/North-West University Preclinical Drug Development Platform
CLSM	Confocal Laser Scanning Microscopy

OECD	Organization for Economic Co-operation and Development
SANAS	South African National Accreditation System

1) Introduction

The advantages of microemulsions as carrier systems include thermodynamic stability, straightforward manufacturing processes and the ability to entrap either lipophilic, hydrophilic or amphyphilic drug molecules. The most user friendly microemulsions are the oil-in-water emulsions (such as the Pheroid® delivery system) due to the fact that the vesicle structure of the carrier system is not broken down when it is diluted by a biological aqueous phase. This allows for the administration of the oil-in-water microemulsion by both the oral or intravenous delivery routes (Paolino *et al.,* 2006; Solè *et al.,* 2012).

All Pheroid® formulations are manufactured from non-toxic ingredients (excluding the API) in a process designed to be environmentally safe with minimal waste production. Various formulations have been developed to allow for the oral delivery of traditionally intravenous pharmaceutical entities sensitive for gastric degradation. The Pheroid® system also targets the API to the organ of interest (during parenteral administration or oral administration) in a more effective manner reducing the exposure of healthy tissue. It is a stable micro- or nano-emulsion and consists of three phases namely an oil-phase (fatty acid based), aqueous-phase and gas-phase (nitrous oxide gas). Pro-Pheroid® (developed to accommodate pharmaceutical entities labile to moisture) contains the same fatty acid phase (packaged in hard gel liquid capsules), also gassed with nitrous oxide but this system excludes the water that is present in the Pheroid® system. The formation of Pheroid® vesicles occurs spontaneously when pro-Pheroid® is released out of the capsule and exposed to gastric contents (Grobler *et al.*, 2008; Steyn *et al.,* 2011; Grobler & Zeevart, 2015). It is critical to determine the safety of this technology before further clinical evaluation in patients occurs.

The aim of this study was to evaluate the toxicity of Pheroid® technology taking into account the foreseen applications thereof. Genotoxicity was evaluated *in vitro* by the AMES test. The Organization for Economic Co-operation and Development (OECD) Guideline 420 was applied for both the acute oral (pro-Pheroid®) and intravenous (Pheroid®) toxicity assays. This method is more humane than the LD_{50} procedure and incorporates the ethical principles of reduce, refine and replace during toxicity evaluations (OECD, 1992b). The acute evaluation for the intravenous study was performed on Sprague-Dawley rats and repeated in BALB/c mice (as a second animal model) to show that the formulation particle size does not contribute to any toxicity in the smaller veins of the mouse model. The OECD Guideline 408 was employed to evaluate repeated dose toxicity for oral applications of pro-Pheroid® (OECD, 1992a).

2) Materials and methods

2.1) Materials

The fatty acids used in the preparation of the Pheroid® vesicles and the pro-Pheroid® formulations were obtained as Vitamin F ethyl ester CLR (CLR Chemisches Laboratorium, IMCD), PEG 400 (Simga-Aldrich), Incromega E3322 and E7010 (Croda chemicals, South Africa). Other ingredients included dl-Alpha tocopherol (Chempure, South Africa, Kolliphor EL (BASF, Germany), preservatives (methylparaben and propylparaben, Sigma-Aldrich) and the antioxidants (butylated hydroxyanisole and butylated hydroxytoluene, Sigma Aldrich). The formulations were gassed with nitrous oxide gas obtained from Afrox (South Africa).

The supplier for most of the chemicals used in the mutagenicity assay (mutagens 2-acetylaminofluoroene, aflatoxicin B_1, biotin, histidine, nicotinamide adenine dinucleotide phosphate, glucose-6-phosphate and glucose-6-phosphate dehydrogenase) was Sigma Chemical Co. Another mutagen (cumolhydroperoxide) was obtained from Merck, dimethyl sulfoxide was obtained from BDH Laboratory suppliers, Agar-Bacto™ was sourced from Difco Laboratories and Oxoid nutrient broth #2 was purchased from Oxoid.

2.2) Methods

2.2.1) Study design

A systematic approach was followed for the evaluation of the toxicity of the Pheroid® system in a comprehensive manner, with the study design provided in Figure 1. The three formulations (formulation A, B and C) used for the different evaluations were modified to take into account the route of administration and the unique characteristics required by each.

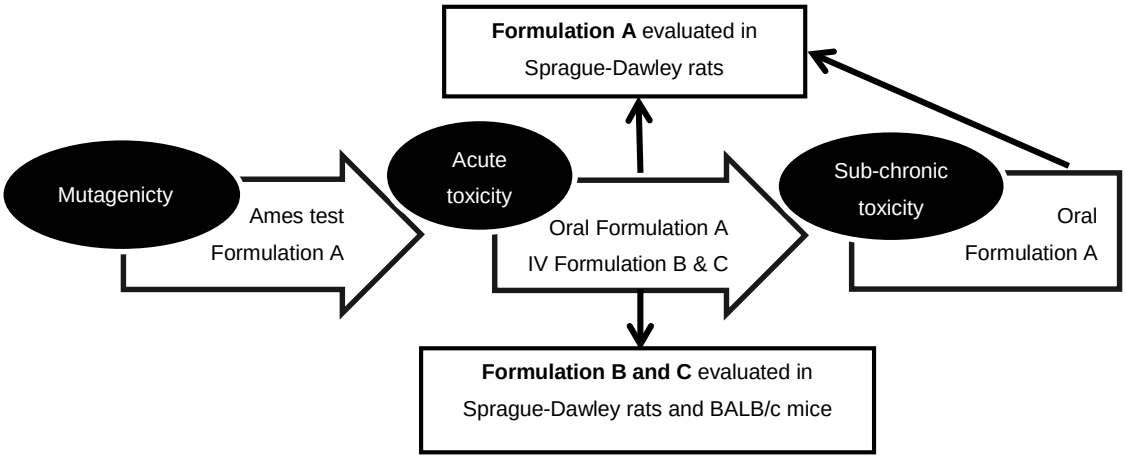


Figure 1: The work flow process followed to evaluate the toxicity of Pheroid®.

2.2.2) Formulation preparation and characterization

The characterization of the formulations is important to ensure repeatability between batches of administered formulations according to the standard formulation parameters for future applications, with regards to the safety thereof. The particle sizes of the vesicles of the different formulations and the overall distribution was determined by laser diffraction (Malvern Mastersizer Hydro 2000, Malvern instruments, Worcestershire) and is indicated as the polydispersity index. Each sample was measured six times and the mean and standard deviation was determined. The zeta-potential of the samples was measured on the Malvern Zetasizer Nano ZSP (Malvern instruments). Morphological conformation was obtained by confocal laser scanning electron microscopy (CLSM, Nikon D-eclipse C1 confocal scanning microscope) as per the method outlined by Slabbert and coworkers (2011) to allow visualizing of the Pheroid® drug carrier system. The pro-Pheroid® formulation was prepared for physical characterization by mixing it with a 0.1 N hydrochloric acid diluent, to simulate the acidic environment of the stomach (Grobler *et al.*, 2014). The presence of bacterial endotoxins in the raw materials was determined by the point-of-use Endosafe®-PTS™ system (Charles River Laboratories).

The composition of the test formulations (formulation A, B and C) is provided in table 1. The different ingredients for each formulation was heated by microwave and added in a stepwise process talking into account the thermo-stability of each. All the formulations were subjected to gassing with nitrous oxide for a total of 4 days. For the Pheroid® formulations (formulation B and C) the water gassed with nitrous oxide (± 170 kPa) was mixed with the oil phase by homogenization (13500 rpm with a Heidolph Diax 600 homogenizer, Labotec South Africa) for 4 minutes. As a final step for the intravenously administered Pheroid® the formulations were filtered

through a series of filters – the smallest 0.22 µm to result in emulsions safe for intravenous administration.

2.2.3) Toxicity assays methods

a) *In vitro* toxicity assays

The mutagenic effects of pro-Pheroid® was tested on *Salmonella typhimurium* strains TA98, TA100 and TA102 according to the method proposed by Maron and Ames (1983). Undiluted formulation A (100 µL per plate) as well as various dilutions with sterile water (20 µL, 10 µL per plate) were evaluated. The mutagens used as the positive control were cumolhydroperoxide (100 ng/plate for TA102), 2-acetylaminofluoroene (5 µg/plate for TA98) and aflatoxicin B_1 (10 ng/plate for TA100). Culture tubes were filled with 2 ml of the top agar, 0.1 ml of a fresh overnight culture of one of the strains of bacteria and 0.1 ml of the test formulation. Testing was performed in the absence or presence of metabolic activation (either 0.5 ml water or 0.5 ml S9). S9 is a liver homogenate obtained from Aroclor 1254-induced male Fisher rats, as described by Maron and Ames (1983), to provide cytochrome P450 enzyme activity. The addition of P450 enzyme activity allows for the prediction of the effect of metabolism on the toxicity of a formulation. The final mixture in each culture tube was transferred onto sterile growth media plates and incubated for 3 days at 37°C.

b) *In vivo* toxicity assays

The animals (Sprague Dawley rats and BALB/c mice) were obtained from and housed in the Vivarium of the DST/NWU PCDDP. This facility is AAALAC accredited (Association for Assessment and Accreditation of Laboratory Care International) as well as being a SANAS certified (South African National Accreditation System) Good Laboratory Practice compliant facility to conduct toxicity studies. The animals were housed in group cages (based on treatment and dose received) with maintenance of an artificial 12-hour day and night cycle and *ad libitum* access to a conventional rodent diet and water. Temperature was kept at 21 ± 2°C and a relative humidity of 55 ± 10% was maintained prior and throughout the study. The ethical aspects of this study were approved by the AnimCare committee of the North-West University (NWU-00493-16-A5) and the Ethical committee of the North-West University (06D01). All animals weighed within a ± 20 % of the mean weight of all study animals enrolled of that particular species.

For all animal studies the following haematological parameters were analysed: haemoglobin, white cell count and the red blood cell count. For clinical biochemistry the following were measured: proteins as indicator of liver function (total serum protein, bilirubin, albumin, globulin); hepatic enzymes (alanine transaminase, alkaline phosphatase, aspartate transaminase);

electrolyte levels (sodium, potassium, calcium); indicators of kidney function (urea, creatinine); amylase, glucose and a full lipid panel (triglycerides, low-density lipoprotein, high-density lipoproteins and cholesterol) (Stogdale, 1981). After euthanasia (by decapitation) the organs were removed during the gross necropsy in a manner that ensured the integrity of the organs, but also included the removal of any irrelevant tissue that could influence the weight. The organs for histopathology were stored in 10% formalin and embedded in paraffin wax, sectioned at 5 µm and stained with haematoxylin and eosin (H & E). After fixation, digital images of the samples were taken with a model PCM 2000 confocal laser scanning microscope connected to a Nikon TE300 inverted microscope equipped with a Nikon 60x/1.40 Apo Planar oil objective and/or a Nikon 40X/0.75 dry objective. A Nikon DXM 1200 digital camera in combination with ACT-1 software was used to capture images. The H & E stained sample was excited with the helium-neon ion and argon ion lasers and a neutral density filter was utilized to obtain distinction between the different regions of the sample. Photobleaching was minimalized with a pinhole size of ¼ airy units.

Acute oral toxicity study (Sprague Dawley Rats)

For the oral acute and subchronic studies the control group received 300 µL of normal drinking water administered through oral gavage.

For the oral evaluation of toxicity formulation A were used. The Sprague Dawley rats were 7 weeks old during enrolment in the study and three treatment groups (n=20 per group) with equal members of both sexes of animals was composed by random selection. The different treatments were administered through oral gavage and the animals were observed multiple times (with special emphasis during the first 4 hours) for clinical signs of adverse events during the first 24 hours post administration. The dose of 50 mg/kg was selected since this system is the pre-assembled form of Pheroid® and this dose is the upper limit foreseen to be administered for other applications. The functional assessments performed during these observations included changes in physical condition (skin, fur, eyes, and mucous membranes), cardiovascular systems (respiratory and circulatory), excretion (presence of abnormal urinary symptoms and diarrhoea) as well as behavioural changes. The Humane Endpoints Guidance Document was adhered to during the performance of this study (OECD, 2000). For the next fourteen days these daily observations were performed, as well as daily weighing and food consumption monitoring. Blood samples were collected 24 hours after the administration of the acute pro-Pheroid® dose in EDTA and lithium-heparin tubes. Animals were sacrificed on day 14 and a gross necropsy was performed on each animal and organ weights (brain, thyroid, heart, lungs, stomach, liver, spleen, kidneys) were determined (OECD, 1992b).

Acute Intravenous study (Sprague Dawley rats and BALB/c mice)

The control groups of the intravenous studies received saline for injection at a dose of 2000 mg per kg of bodyweight. Formulations B and C (refer to Table 1 for formulation characterization parameters) were evaluated during this acute intravenous toxicity assay. As per OECD guidelines, only female Sprague Dawley rats with ages ranging from 8 to 10 weeks were selected, since female rats are generally more susceptible to adverse reactions (unless previous data indicates otherwise for a specific compounds) (Lipnick *et al.,* 1995). Animals were randomly selected and acclimatized in study housing for 5 days prior to administration of test formulations. A control group (n=5) was injected with saline to provide a baseline for general conditions as well as the influence of the injection procedure. A dose of 2000 mg/kg (n=5) for each test formulation was evaluated; this is the highest dose prescribed by the OECD guidelines. Animals were monitored 3 times in the first 15 minutes and hourly thereafter for the first 8 hours. Daily monitoring was performed as described for the oral acute study for a span of 2 weeks where after animals were euthanized and a gross necropsy (including measuring of organ weights) was performed. This study was repeated on BALB/c mice (also 2000 mg/kg and n=5) with blood samples only taken from the rats due to the small blood pool of mice. Organs obtained from all animals (both rats and mice) were preserved in a 10% formalin solution to allow for histopathology if gross necropsy findings or haematological and biochemical analysis were to be deemed abnormal (OECD, 1992b).

Subchronic oral toxicity evaluation (Sprague-Dawley rats)

The duration of the subchronic evaluation was 90 days according to the procedure prescribed by the OECD guidelines (OECD, 1992a). This study was performed on Sprague Dawley rats and only formulation A (refer to table 1) was administered. The control group and the pro-Pheroid® treatment group contained 30 animals (male n=15, female n=15). Animals were randomly assigned to treatment groups and received 50 mg/kg pro-Pheroid® as daily dose, made up to 300 µl with water as treatment, or just the control water (300 µl) administration through oral gavage. Body-weight determination of the animals as well as functional assessments of overall health was performed prior to enrolment and at least once a week during the span of the study. Food consumption was measured weekly per group cages; additional feed administered between formal weighing times was taken into account. After 90 days, animals were euthanized, organs were removed, and blood was collected. Urine for analysis was collected for the sub-chronic study between days 81 to 85, were obtained overnight and analysed with Multistix™ test strips and the ClinitekStatus® analyser, both for the analysis of the presence of glucose, bilirubin, ketones, specific gravity, erythrocytes, proteins, pH, nitrites and leukocytes in rat urine (Bayer HealthCare). The animals in the sub-chronic study were placed individually in metabolic cages (being fasted

for 12 hours prior) and urine samples were collected overnight. During the stay of animals in metabolic cages, *ad libitum* access was provided to water, but no food was administered to prevent contamination of urine samples. The samples were kept cold and analysed the following day. Differences between the groups of the same gender were analysed by means of a frequency count of parameters. All the blood samples from rats were obtained by tail vein incision and collected in EDTA tubes (for haematological analysis), lithium-heparin tubes (clinical chemistry) and SST tubes (blood glucose levels), immediately processed and sent for analysis.

2.2.4) Statistical analysis

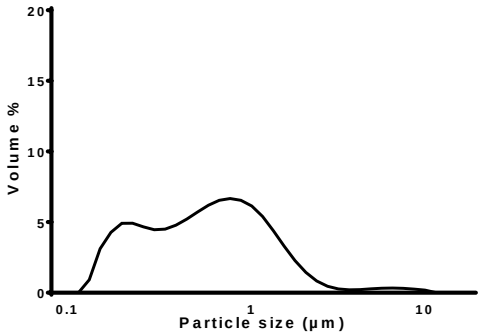
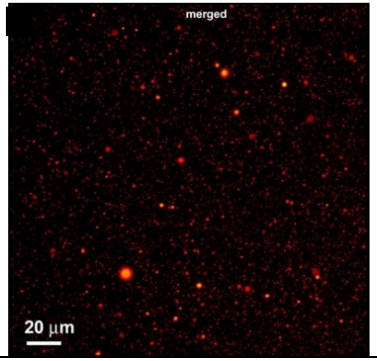
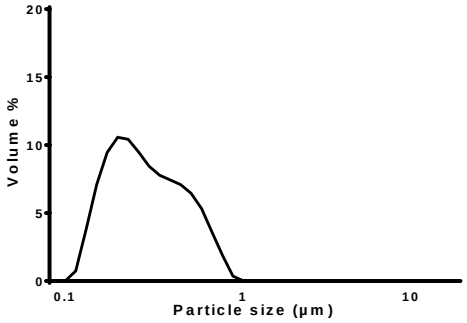
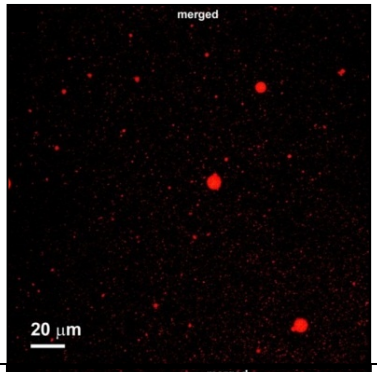
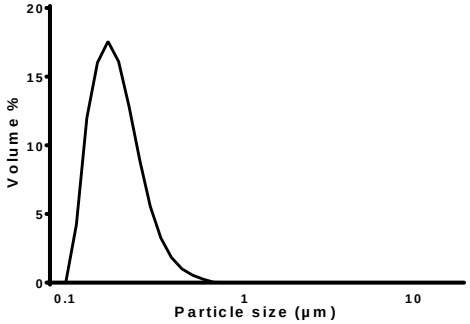
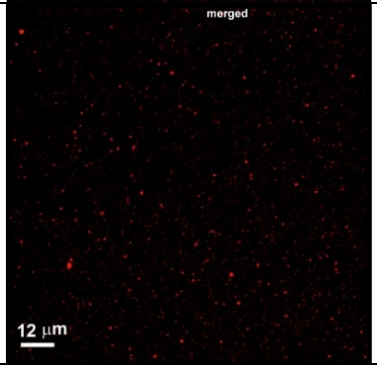
Statistical analysis was performed using Statistica (StatSoft Inc.) and Prism (GraphPad). Analysis of all data was done by one-way ANOVA, with evaluation for normality in distribution. Statistical significant differences were evaluated using the Tukey HSD test or Unequal N HSD test. Abnormality in data was analysed with the Dunnett's test or Kruskal-Wallis Anova and a p-value < 0.05 was taken as statistically significant. The Bonferroni test permitted the analysis of weekly bodyweight and food consumption changes within groups.

3) Results

3.1) Formulation preparation and characteristics

The formulations (A, B and C) were prepared successfully, demonstrating the correct internal structure that is characteristic of all Pheroid® technology. Table 1 provides the product specifications for each formulation evaluated during this study. The larger population of each formulation was measured to be below 2 µm which is typical for Pheroid® technology (200 nm – 5 µm). Formulations B and C did not exceed the upper limit of 5 µm deemed as the maximum safe size of individual emulsion drops, for intravenous administration.

Table 1: The specification of the formulations evaluated for toxicity

Formulation components	Particle Size Distribution	Additional information	CLSM (Morphology)
Formulation A (pro-Pheroid®) • Vitamin F Ethyl Ester (65%) • Kolliphor El (22%) • Polyethylene glycol 400 (10%) • Incromega E3322 (1%) • Incromega E7101SR (1%) • Dl-Alpha Tocopherol (1%)		• Particle size span: 2.22 µm • Median particle size: 0.93 µm • Distribution of 80% of vesicles: 0.22 µm-1.7 µm • Zeta-potential: -23.3 ± 0.8 mV • Endotoxin levels: less than 5 EU/ml • pH measured: 6.05	
Formulation B (Pheroid®) • Vitamin F ethyl Ester (14%) • Kolliphor EL (5%) • Dl-Alpha tocopherol (1%) • Nitrous oxide water (40%) • Saline (40%)		• Particle size span: 1.53 µm • Median particle size: 0.37 µm • Distribution of 80% of vesicles: 0.18 µm- 0.65 µm • Zeta-potential: -21.1 ± 1.1 mV • Endotoxin levels: less than 5 EU/ml • pH measured: 6.45	
Formulation C (Pheroid®) • Vitamin F ethyl Ester (67%) • Kolliphor EL (28%) • Dl-Alpha tocopherol (1%) • Nitrous oxide water (4%)		• Particle size span: 0.88 µm • Median particle size: 0.23 µm • Distribution of 80% of vesicles: 0.15 µm-0.33 µm • Zeta-Potential -24.2 ± 0.6 mV • Endotoxin levels: less than 5 EU/ml • pH measured: 6.35 .	

3.2) Toxicity assays

a) *In vitro* assays

A pre-incubation assay was performed in the presence of S9 due to the higher sensitivity of this assay. The positive controls added to the test strains (*Salmonella typhimurium* strains TA98, TA100 and TA102) did provide the expected increased histadine positive revertants under control conditions (indicated as statistically different, Table 2). The positive controls therefore provided good benchmark for positive mutagenicity. The metabolised derivatives of the pro-Pheroid® did not induce base-pair or frame shift mutagenesis during this analysis.

Table 2: Mutagenicity of the standard plate incorporation assay of pro-Pheroid® to *Salmonella typhimurium* test strains

	TA 98		**TA100**		**TA102**	
	-S9	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**
DMSO	36.2 ± 7.0	40.2 ± 8.4	136.4 ± 5.3	140 ± 14.4	581.0 ± 24.2	555.5 ± 60.1
Positive control	38.0 ± 5.8	**355.6 ± 82.5#**	162.4 ± 30.2	**355.6 ± 17.5#**	1789 ± 356#	**2039 ± 219#**
Pro-Pheroid® 10 µL	26.3 ± 3.2	29.3 ± 7.1	88.3 ± 4.6	81.3 ± 8.1	290.7 ± 2.2	318.7 ± 9.0
Pro-Pheroid® 20 µL	19.3 ± 5.8	25.0 ± 6.1	95.0 ± 17.4	93.7 ± 21.6	324.0 ± 34.9	313.3 ± 4.5
Pro-Pheroid® 100 µL	18.7 ± 5.7	29.3 ± 5.5	115.3 ± 6.0	111.0 ± 4.1	327.7 ± 9.3	273.3 ± 8.2

#p<0.001, *p<0.05, significant different from DMSO control groups for each study

b) In vivo toxicity assays

Food consumption and monitoring of condition

No animals (Sprague Dawley rats or BALB/c mice) died or exhibited any adverse events, during any the acute (oral and intravenous) or the subchronic evaluations. No abnormal changes in body weight, food consumption, respiration, coat condition, movement and behaviour were present in any of the animals during scheduled examinations in all the studies. The initial and final body weights of animals in different test groups are presented in Table 3 with no significant differences present between test and control groups. The body weights of animals in the acute intravenous study (both BALB/c mice and Sprague Dawley rats) as well as that of the sub-chronic oral study are presented in Figure 2.

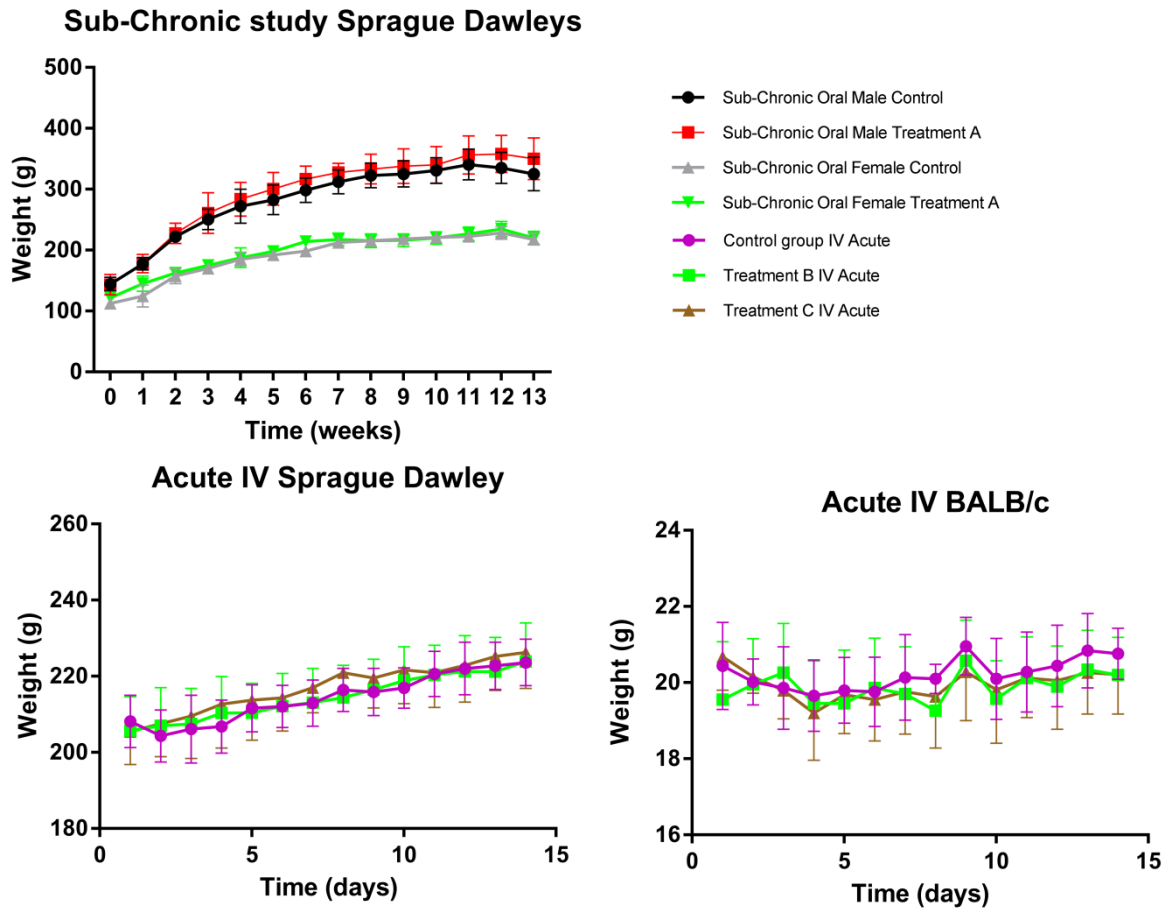


Figure 2: The body weight distribution of animals included in the acute and subchronic studies.

c) *Haematology, clinical biochemistry and urine analysis*

The organ weights were determined after euthanasia of all the subjects included in the different study groups (Table 3). Note that due to the difficulty of identifying the thyroid of BALB/c mice, this organ was not isolated during the investigation. The only statically significant differences present were in weight of the full stomach during the intravenous acute administration evaluations in both mice and rats, and the spleens of the mice receiving treatment B. The effects of the three treatments on all the parameters is presented in Table 3 with the haematological and blood chemistry data as part of the supplementary section of this article. No statistical significant changes in blood parameters were present in any of the treatments. The white blood cell count was however increased in all the animals (both control and test groups) included in the acute study of oral pro-Pheroid®. The white blood cell counts all the animals in intravenous acute evaluation and the oral sub-chronic evaluations was normal. A statistical significant decrease in

alanine transferase was indicated for the animals of formula C during the acute intravenous evaluation (Figure 3). Whilst an increase in ALT is associated with liver damage and the destruction of hepatocytes, a decrease is associated with healthy liver function.

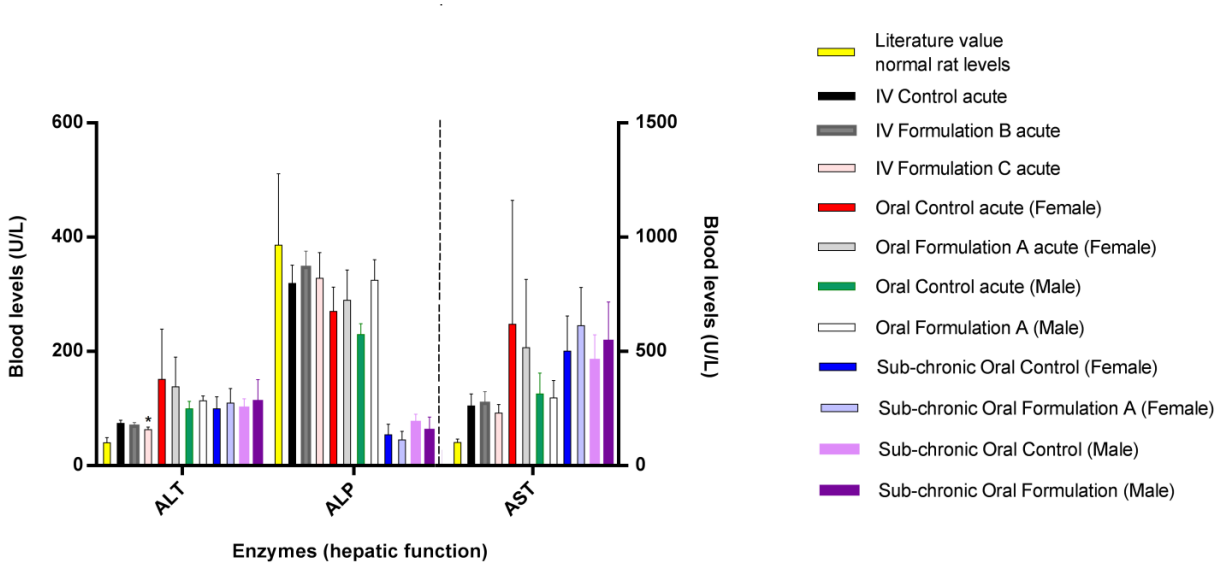


Figure 3: Hepatic enzyme levels of Sprague-Dawley rats during acute intravenous and oral toxicity evaluation. A statistical significant difference is reported for the ALT level of the IV formulation B group (Literature values obtained from Matsuzawa *et al*., 1997).

Urea was also lower in the animals receiving formulation B (Figure 4) during the acute intravenous dosing. This is not considered significant with regards to toxicity since an increase is usually associated with malfunction. In the subchronic study creatinine (Figure 4) was statistically significantly increased for the female test animals, indicating a lowered clearance of creatinine by the kidneys. To evaluate whether Pheroid® related renal toxicity was present, histopathology and urine analysis was included in this study. This increase in creatinine was not present in any other test group and all the histopathology reports came back as negative for treatment related toxicity with no malfunction of organs present. Figure 5 provides a selection of microscopy images taken of major organs (including kidney tissue) removed from animals in the pro-Pheroid® treatment group part of the subchronic study.

No significant differences were present in the urine analysis results. All urine samples tested negative for glucose and no significant levels of bilirubin were present in samples. The levels of ketones, specific gravity of samples, erythrocytes, pH levels, proteins, nitrites and leukocytes in test and control animals similarly showed normal distribution with no disparities bearing any

statistical significance. The frequency table of the results is included in the supplementary data of this article.

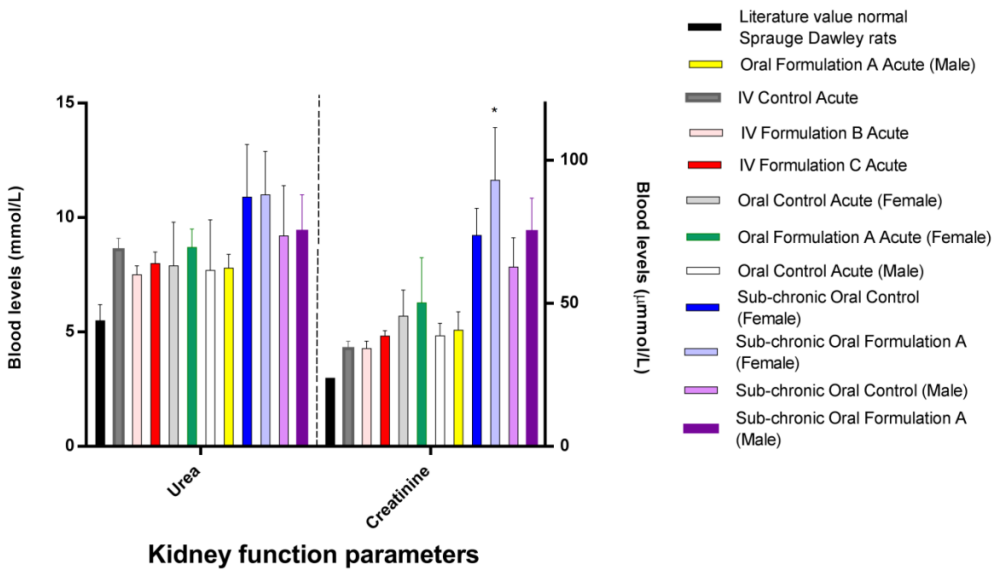


Figure 4: Urea (mmol/L) and creatinine (µmol/L) levels of Sprague-Dawley rats during acute and subchronic evaluations. A statistical significant difference is reported for the urea levels of the IV formulation B group and creatinine levels of the oral subchronic female test group (literature values obtained from Matsuzawa *et al.*, 1997).

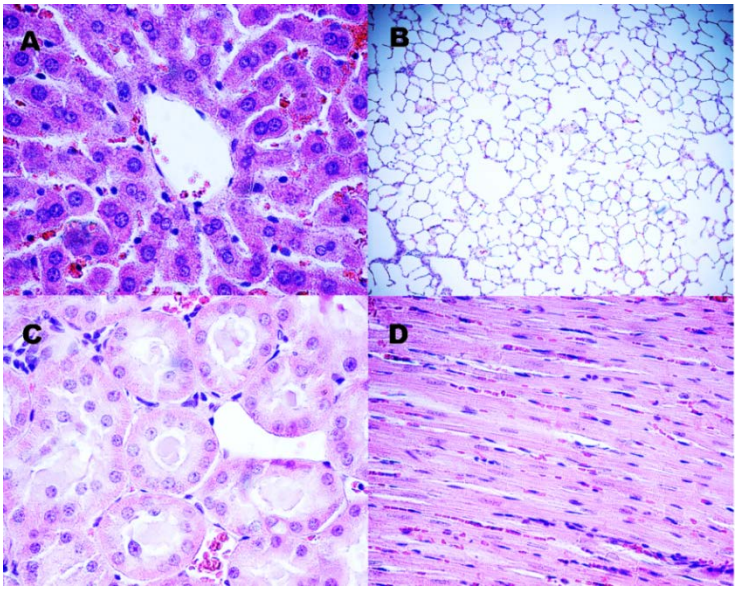


Figure 5: Light microscopy images of organs from the pro-Pheroid® subchronic treatment group with a) liver tissue, b) lung tissue, c) the kidney and d) heart muscle (all stained with haematoxylin and eosin).

Table 3: A summary of findings of toxicity evaluations

In vitro evaluations	Acute oral toxicity	Acute IV toxicity	Sub-chronic toxicity
- No statistical significant genotoxicity present.	- ↓ Cholesterol levels female rats receiving formulation A	- ↓ Spleen size BALB/c mice receiving formulation B	- ↑ Blood creatinine levels in female rats receiving formulation A
	- Normal organ morphology	- ↓ Full stomach size BALB/c mice formulation B and C	- Normal organ morphology
		- ↓ Urea concentration in rats receiving formulation B	
		- ↓ ALT levels of rats receiving formulation C	
		- Normal organ morphology	.

4. Discussion

The Pheroid® and pro-Pheroid® systems both contain the same oil components which differ from liposomes by the absence of phospholipids and cholesterol. The pro-Pheroid® system is a precursor form of the Pheroid® technology, with the only difference being the lack of the water phase, with the nitrous oxide being incorporated in the oil phase. All the formulations evaluated during this study demonstrated emulsion particles mostly smaller than 1 µm in diameter with none exceeding 5 µm. For intravenous emulsion formulations the particle size of individual droplets are of particular importance due to the propensity of large droplets to cause emboli blocking small blood vessels with lethal results. The total volume of the emulsion administered as well as the concentration of large particles present in these formulations is important contributors to the incidence of adverse events (Hörman & Zimmer, 2016). To focus only on a reduction of particle size will therefore not guarantee the safety of the formulation. Traditionally 5 µm is considered as the upper limit (no more of 0.05% of the formulation may exceed this) allowed for particle size of intravenous emulsions and is indicated as such in older versions of the British Pharmacopoeia. It was contradictorily determined that commercially available total parenteral nutritional formulations do not always conform to these parameters since it was demonstrated that emulsion droplets of larger diameters (exceeding 7.5 µm) may deform and pass through pulmonary blood vessels depending on their consistency. It is proposed by Koster and coworkers (1996) that the individual characteristics or components of the formulation may contribute to the degree of toxicity associated with larger particle size. No agreement on the influence of particle size on adverse events or the exact ranges that intravenous emulsions should abide by, are available in literature (Burnham *et al.*, 1982; Koster *et al.*, 1996; Metha *et al.*, 1992; Hörman & Zimmer, 2016). Our aim was to restrict the particle size of the intravenous formulations in this study to the smallest

possible, economically viable particle size, while concomitantly filtering formulations to provide sterility. Although the zeta-potential measured for the formulations is lower than the ± 25 mV stated in literature as beneficial, the formulations did demonstrate sufficient stability based on particle size distributions evaluated over a 7-day period (Roland *et al.*, 2003). We do suggest that the formulations should be filtered just before administration to ensure that the particle sizes are narrowly restricted (below 1 µm) and sterility is maintained. Another option to ensure sterility would be the use of gamma irradiation and Pheroid® has been proven to be stable during this process. However, the API can be sensitive to degradation by gamma irradiation and this can also be a factor (Botha, 2007; Van der Merwe, 2008). The pH of the formulations as well as presence of bacterial endotoxins were monitored to ensure the safety of the formulations for intravenous administration. CLSM demonstrated that satisfactory Pheroid® vesicles were formed presenting with internal structure and the correct morphology.

The pro-Pheroid® did not demonstrate mutagenic effects in the AMES test. This comes as no surprise since formulations containing similar fatty acid ingredients in literature also were void of mutagenicity (Arterburn *et al.*, 2000; O'Hagan & Menzel, 2003; Matulka *et al.*, 2006). Another component of the delivery system, alpha-tocopherol, also demonstrated no mutagenicity in a study by Karekar and co-workers (2000). The testing for mutagenicity in the presence of cytochrome P450 enzymes (S9) demonstrated that both Pheroid® as well as any breakdown products are safe, bearing no effect on the structural integrity of cellular DNA.

The highest dose prescribed by OECD guideline 420 (2000 mg/kg) was reached during the acute evaluation of the intravenously administered Pheroid®, with no adverse events identified. During intravenous administration to BALB/c mice and Sprague Dawley rats, care was taken to monitor animals intently for acute shock symptoms due to intravenous administration and animals were unhindered by this administration. Animals were in good health during all the scheduled examinations of both the acute and subchronic studies, and final body weights of all the groups were characteristic for animal species. During these studies the aim was mainly to compare the parameters of the control groups, with that of the test groups and not comparing them with literature values. It was previously demonstrated that housing, breeding and biological rhythm of animals housed in animal facilities have a large influence on haematology and blood chemistry levels (Braun *et al.*, 1993). Our studies therefore relied on the differences between the control groups and the test groups to increase accuracy, although none of the parameters evaluated was abnormal to the extent that indicates disease or distress.

The following differences were noted when compared with the control values (refer to table 3).

i) The increased white blood cell count in control and test groups of the acute oral study was determined not to be related to treatment with Pheroid®. This phenomenon was present in all four groups evaluated during the study and not the tests groups only. Furthermore, it was not present in any of the acute intravenous study test groups (with the formulation being 100% bioavailable) or the subchronic study (with continuous administration). No other significant changes in blood chemistry were present.

ii) The decrease in alanine transferase demonstrated as a statistically significant decrease during the blood chemistry analysis of acutely intravenously treated animals (formulation C) and is not associated with any disease process. A positive correlation between lowered cholesterol and ALT also exist, with high levels of cholesterol and fatty liver disease associated with high levels of ALT. It is notable that the Pheroid® delivery system incorporates essential fatty acids, which is well known for the treatment of distorted lipid profiles.

iii) The decrease in cholesterol levels was therefore not an unexpected result, which was present in the male group of the oral acute study.

iv) The statistical significant increase in creatinine levels in the subchronic study for the female treatment group is noteworthy. To investigate this, the histopathology examinations as well as the urine analysis was performed, which are more sensitive indicators of kidney function. Both urine analysis and morphology demonstrated normal function. There was an absence of any pathology-related differences in organ weights determined after termination of animals. No treatment related pathology was identified present in any of the histopathology examinations.

5. Conclusion

Based on this evaluation, we conclude that the Pheroid® delivery system is safe for administration intravenously and orally for acute as well as repeated dosing. The maximum dose tested in Sprague Dawley rats and BALB/c mice was 2000 mg/kg Pheroid® and 50 mg/kg pro-Pheroid® and this dose should be taken into account. It should also be stressed that the normal precautions for intravenous safety of emulsions (particle size, bacterial endotoxin measurement and sterility) should always be adhered to for ensuring the safety of the formulation. The Pheroid® system also does not present with any mutagenicity. Future studies might be needed to determine the subchronic safety of the intravenous administered Pheroid® in the case of such an application for the system nessesitating multiple intravenous administrations. The effect of the system on the developing animal foetus should be evaluated prior to administration during pregnancy and lactation.

It is required practise to evaluate the toxicity of the drug delivery system with the selected pharmaceutical entities entrapped to ensure that alterations in biodistribution and possible slow release mechanisms brought about by Pheroid® does not alter the toxicity profile of the pharmaceutical ingredient in a negative way.

It is however clear that no potential health risks are associated with the Pheroid® drug delivery system when administered without any active ingredient incorporated.

6. Acknowledgements and declaration of interest

Financial assistance was received from the National Research Foundation (NRF) of South Africa (grant number SFH 14070573914), the Innovation Fund as well as the Nuclear Technologies in Medicine and Biosciences Initiative (NTemBI). Funding for the study was also provided by NTemBi and the DST/NWU PCDDP. Opinions expressed and conclusions arrived at, are those of the authors and are not necessarily to be attributed to the NRF and NTeMBI. Various patents regarding applications of the Pheroid® technology are registered by the authors. The authors declare that they have no additional conflicts of interest. We also thank Dr. Matthew Glynn and Mr. Lesley Masetle for assisting with the analysis of the various formulations. We would also like to thank Mr. Cor Bester, Mrs. Antionette Fick and Mr. Kobus Venter for assistance in the handling of the animals. Lastly we would like to thank Prof. Faans Steyn for assisting with the statistical analysis of data.

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Supplement 1: The body weights and organ weights (mean ± SD) of animals during acute and subchronic toxicological evaluations

	Rats 2000 mg/kg IV Acute Pheroid®			BALB/c mice 2000 mg/kg IV Pheroid®			Subchronic Sprague Dawley rats 50 mg/kg oral pro-Pheroid®			
	Control (n=5) ♀	**Formulation B (n=5) ♀**	**Formulation C (n=5) ♀**	**Control (n=5) ♀**	**Formulation B (n=5) ♀**	**Formulation C (n=5) ♀**	**Control** (n=15) ♀	**Formulation A (n=15) ♀**	**Control (n=15) ♂**	**Formulation A (n=15) ♂**
Body weight										
Initial	202.6 ± 6.6	205.8 ± 8.9	205.5 ± 8.6	19.9 ± 1.3	19.5 ± 1.4	20.1 ± 0.6	137.8 ± 4.6	136.0 ± 8.4	113.6 ± 7.2	114.3 ± 5.3
Terminal	227.8 ± 3.4	226.4 ± 7.6	228.5 ± 8.6	20.8 ± 0.7	20.2 ± 0.9	20.1 ± 0.9	219.6 ± 12.8	222.1 ± 12.5	131.6 ± 10.0	147.6 ± 23.1
Organ weights as % of final body weight										
Brain	0.7 ± 0.1	0.7 ± 0.0	0.7 ± 0.1	1.8 ± 0.2	1.6 ± 0.2	2.0 ± 0.2	0.6 ± 0.1	0.5 ± 0.1	0.7 ± 0.1	1.2 ± 0.9
Heart	0.4 ± 0.1	0.4 ± 0.0	0.4 ± 0.0	1.0 ± 0.2	0.7 ± 0.2	0.8 ± 0.1	0.4 ± 0.1	0.4 ± 0.0	0.4 ± 0.0	0.6 ± 0.4
Kidney (L)	0.3 ± 0.1	0.3 ± 0.0	0.4 ± 0.0	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.2	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.6 ± 0.3
Kidney (R)	0.3 ± 0.1	0.4 ± 0.0	0.4 ± 0.0	1.0 ± 0.3	0.8 ± 0.2	0.9 ± 0.2	0.5 ± 0.0	0.5 ± 0.1	0.5 ± 0.2	0.7 ± 0.4
Liver	3.3 ± 0.4	3.5 ± 0.1	3.5 ± 0.2	5.8 ± 0.7	5.3 ± 0.4	5.3 ± 0.8	2.3 ± 0.2	2.3 ± 0.2	2.1 ± 0.4	4.1 ± 1.9
Lungs	0.6 ± 0.1	0.6 ± 0.1	0.8 ± 0.1	1.1 ± 0.2	0.8 ± 0.3	1.0 ± 0.2	0.5 ± 0.1	0.5 ± 0.2	0.6 ± 0.1	1.1 ± 0.8
Spleen	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.1	0.8 ± 0.2	**0.5 ± 0.1***	0.6 ± 0.2	0.4 ± 0.1	0.3 ± 0.1	0.4 ± 0.0	0.5 ± 0.3
Stomach (full)	1.9 ± 0.5	**2.0 ± 0.2***	1.7 ± 0.3	1.9 ± 0.3	**1.4 ± 0.3***	**1.1 ± 0.2***	0.6 ± 0.2	0.5 ± 0.1	0.6 ± 0.0	1.2 ± 0.7
Thyroid	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	-	-	-	0.2 ± 0.1	0.1 ± 0.0	0.2 ± 0.1	0.3 ± 0.2

*p<0.05 significant different from control groups for each study

Supplement 2: The haematological profiles (mean ± SD) of Sprague-Dawleys during acute and subchronic toxicity

	2000 mg/kg IV Acute Pheroid®			2000 mg/kg Oral Acute Pheroid®				50 mg/kg oral sub-chronic pro-Pheroid®			
	Control (n=5) ♀	**Formulation B (n=5) ♀**	**Formulation (n=5) ♀**	**Control (n=10) ♀**	**Formulation A (n=10) ♀**	**Control (n=10) ♂**	**Formulation A (n=10) ♂**	**Control (n=15) ♀**	**Formulation A (n=15) ♀**	**Control (n=15) ♂**	**Formulation A (n=15) ♂**
Haemoglobin (g/L)	151.1 ± 4.0	146.5 ± 12.3	150.9 ± 5.1	161.5 ± 9.6	166.7 ± 8.1	161.0 ± 5.1	155.4 ± 5.4	168.4 ± 13.1	167.8 ± 7.0	177.9 ± 5.3	175.2 ± 8.7
Red blood cells ($x10^{12}$/L)	7.3 ± 0.4	7.4 ± 0.7	7.6 ± 0.3	8.2 ± 0.2	8.1 ± 0.3	8.0 ± 0.3	7.5 ± 0.3	9.1 ± 7.8	8.9 ± 0.3	9.8 ± 0.3	9.6 ± 0.5
Haematocrit (L/L)	0.5 ± 0.0	0.5 ± 0.1	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.4	0.5 ± 0.0	0.6 ± 0.0	0.5 ± 0.0
MCV (fl)	65.4 ± 1.1	66.3 ± 1.7	64.8 ± 0.8	51.5 ± 1.8	52.8 ± 0.6	52.4 ± 1.4	53.3 ± 1.4	59.3 ± 0.0	58.9 ± 1.5	56.2 ± 1.6	56.0 ± 1.0
MCHC (g/dl)	31.6 ± 1.3	30.0 ± 0.8	30.4 ± 0.5	32.0 ± 1.9	32.5 ± 0.5	31.6 ± 0.6	31.8 ± 0.7	31.2 ± 2.5	31.9 ± 0.6	32.5 ± 0.3	32.5 ± 0.3
Red cell distribution (%)	14.6 ± 0.7	15.4 ± 0.7	14.3 ± 1.0	14.5 ± 0.9	14.2 ± 1.1	15.0 ± 0.9	14.8 ± 1.1	15.7 ± 1.0	15.5 ± 1.1	17.6 ± 0.6	17.3 ± 0.8
Platelet count	727 ± 282	653 ± 124	969 ± 107	475 ± 131	484 ± 190	508 ± 168	303 ± 185	465 ± 143	448 ± 226	373 ± 88.8	517.1 ± 175.8
White cells ($x10^9$/L)	3.9 ± 1.8	2.8 ± 2.4	2.1 ± 0.7	10.6 ± 3.5	13.9 ± 2.1	12.2 ± 2.1	11.6 ± 3.6	5.9 ± 1.6	6.3 ± 2.0	6.2 ± 1.2	7.5 ± 2.1

*p<0.05 significant different from control groups for each study

Supplement 3: The clinical chemistry (mean ± SD) of rats included in the acute and subchronic studies

	Acute study Sprague Dawley rats 2000 mg/kg IV Pheroid (*n*=5)			Acute study Sprague Dawley rats 50 mg/kg oral pro-Pheroid® (*n*=10)				Subchronic study Sprague Dawley rats 50 mg/kg oral pro-Pheroid® (*n*=15)			
	Control (n=5) ♀	**Formulation B (n=5)♀**	**Formulation C (n=5) ♀**	**Control (n=5) ♀**	**Formulation A (n=5) ♀**	**Control (n=5) ♂**	**Formulation A (n=5) ♂**	**Control (n=15) ♀**	**Formulation A (n=15) ♀**	**Control (n=15) ♂**	**Formulation A (n=15) ♂**
Serum proteins											
TSP (g/L)	66.8 ± 2.6	65.8 ± 4.4	64.0 ± 1.6	56.2 ± 4.9	57.5 ± 3.6	56.4 ± 2.0	53.1 ± 3.2	76.4 ± 4.8	77.5 ± 4.1	78.0 ± 4.2	80.2 ± 6.4
ALB (g/L)	35.8 ± 1.5	34.8 ± 2.4	34.0 ± 0.7	46.3 ± 4.1	43.4 ±1.8	41.9 ± 2.1	41.3 ± 2.1	44.9 ± 2.4	48.0 ± 3.9	43.3 ± 2.7	45.1 ± 3.6
Globulin (g/L)	31.0 ± 1.2	31.0 ± 2.1	30.0 ± 1.0	12.7 ± 2.7	14.1 ± 2.9	14.5 ± 2.2	11.8 ± 2.4	31.5 ± 3.5	29.5 ± 5.0	34.6 ± 3.0	35.1 ± 5.8
A/G	1.2 ± 0.1	1.1 ± 0.0	1.1 ± 0.1	3.6 ± 0.8	3.2 ± 0.7	3.0 ± 0.5	3.7 ± 0.9	1.4 ± 0.2	1.7 ± 0.5	1.3 ± 0.1	1.3 ± 0.3
Total bilirubin (µmol/L)	<2	<2	<2	10.7 ± 6.0	10.6 ± 5.0	7.6 ± 1.2	8.0 ± 1.3	15.2 ± 4.4	15.8 ± 6.2	13.5 ± 4.0	16.5 ± 5.5
Hepatic function											
ALT (U/L)	74.2 ± 5.3	69.4 ± 6.3	**63.4 ± 4.2 ***	151.2 ± 88.0	138.3 ± 51.7	99.7 ± 12.8	113.5 ± 8.7	99.5 ± 21.2	109.6 ± 25.6	100.7 ± 16.4	112.0 ± 38.5
ALP (U/L)	319.0 ± 32.1	347.4 ± 28.3	328.0 ± 45.1	270.2 ± 42.7	289.6 ± 53.3	229.6 ± 19.0	324.5 ± 36.2	54.1 ± 18.7	45.1 ± 15.3	75.9 ± 14.2	61.6 ± 23.5
AST (U/L)	261.6 ± 53.5	273.4 ± 50.9	231.2 ± 36.6	618.8 ± 543	517.2 ± 299	314.9 ± 91.2	296.7 ± 76.4	501.9 ± 153.7	613.6 ± 166.8	461.1 ± 111.9	544.7 ± 172.7
Electrolytes											
Na^+ (mmol/L)	142.0 ± 1.2	140.4 ± 2.6	142.4 ± 1.1	131.2 ± 2.2	132.4 ±1.8	133.1 ± 1.8	132.9 ± 1.3	137.1 ± 1.5	135.9 ± 2.8	136.5 ± 1.6	137.3 ± 2.6
K^+ (mmol/L)	6.4 ± 0.8	5.7 ± 0.6	6.2 ± 0.4	9.3 ± 1.1	9.6 ± 0.9	8.8 ± 0.4	8.7 ± 0.6	7.1 ± 1.3	8.0 ± 1.2	8.0 ± 0.6	8.2 ± 1.2
Ca^{2+} (mmol/L)	2.6 ± 0.1	2.5 ± 0.1	2.5 ± 0.1	0.9 ± 0.3	0.7 ± 0.2	0.9 ± 0.2	1.0 ± 0.2	0.8 ± 0.3	0.9 ± 0.2	0.8 ± 0.2	0.8 ± 0.2
Kidney function											
Urea (mmol/L)	8.6 ± 0.5	**7.5 ± 0.4 ***	8.0 ± 0.5	7.9 ± 1.9	8.7 ± 1.8	7.7 ± 2.2	7.8 ± 0.6	10.9 ± 2.3	11.0 ± 1.9	9.2 ± 2.2	9.4 ± 1.6
Creatinine (µmol/L)	34.2 ± 3.6	34.2 ± 2.6	38.6 ± 1.9	45.6 ± 9.1	50.2 ± 15.8	38.7 ± 4.4	40.7 ± 6.4	73.8 ± 9.5	**93.1 ± 18.4 ***	62.7 ± 10.3	75.1 ± 11.7
Pancreatic function											
Amylase (U/L)	2772 ± 332	2831 ± 235	2777 ± 630	1905 ± 48	2024 ± 342	2219 ± 304	1988 ± 227	2381 ± 1843	1989 ± 1160	3871 ± 2467	3356 ± 1184
Nutritional status											
Glucose (mmol/L)	6.5 ± 0.8	6.4 ± 0.5	6.5 ± 0.3	7.2 ± 0.8	7.2 ± 0.3	7.0 ± 0.5	7.5 ± 0.7	3.6 ± 0.7	3.7 ± 0.6	3.2 ± 0.7	3.5 ± 0.5
Serum lipids											
Triglycerides (mmol/L)	0.8 ± 0.2	1.0 ± 0.3	0.8 ± 0.1	2.1 ± 0.4	2.3 ± 0.3	2.3 ± 0.4	2.3 ± 0.3	1.2 ± 0.3	1.1 ± 0.2	0.9 ± 0.2	1.0 ± 0.2
LDL (mmol/L)	<0.1	<0.1	<0.1	0.3 ± 0.1	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.0	0.3 ± 0.1
HDL (mmol/L)	1.7 ± 0.2	1.7 ± 0.3	1.7 ± 0.2	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	1.0 ± 0.2	1.0 ± 0.1	0.7 ± 0.1	0.7 ± 0.1
Cholesterol (mmol/L)	2.8 ± 0.3	2.7 ± 0.4	2.2 ± 1.3	1.7 ± 0.2	1.6 ± 0.1	1.8 ± 0.1	**1.6 ± 0.2***	1.8 ± 0.3	1.8 ± 0.3	1.6 ± 0.2	1.9 ± 0.6

*p<0.05 significant different from control groups for each study

Supplement 4: Urine analysis results (represented as a frequency table).

	Female animals		Male animals	
	Control (*n*=15)	Pro-Pheroid® (*n*=15)	Control (*n*=15)	Pro-Pheroid® (*n*=15)
Glucose				
Negative	15	15	15	15
Positive	0	0	0	0
Bilirubin				
Negative	15	15	15	13
Trace	0	0	0	2
Ketones				
Negative	12	15	1	4
Trace	3	0	5	4
15 (mg/dL)	0	0	9	7
Specific gravity				
≤1.005	0	2	0	0
1.010	6	5	4	4
1.015	5	5	7	6
1.020	2	2	4	4
1.025	2	1	0	2
Erythrocytes				
Negative	11	12	6	5
Trace – lysed	3	0	5	6
Trace – intact	0	1	1	1
Ca 25 (Ery/µL)	0	1	2	3
Ca 80 (Ery/µL)	1	1	1	0
Ca 200 (Ery/µL)	0	0	0	0
pH				
6.0	0	1	0	0
6.5	7	5	9	3
7.0	7	9	0	11
7.5	1	0	2	1
8.0	0	0	0	0
8.5	0	0	1	0
Proteins				
Negative	2	4	0	0
15 (mg/dL)	6	1	0	2
30 (mg/dL)	5	8	11	7
100 (mg/dL)	2	2	4	6
Nitrites	0			
Negative	14	15	14	15
Positive	1	0	1	0
Leukocytes				
Negative	4	3	0	0
Ca 15 (Leu/µL)	1	0	0	0
Ca 70 (Leu/µL)	5	7	7	6
Ca 125 (Leu/µL)	2	5	7	8
Ca 500 (Leu/µL)	3	0	1	1

2.7 Conclusion of this chapter

Previous preclinical work indicates that Pheroid® can dramatically enhance the acceptability of a product by for example allowing the oral administration of an intravenous product. Furthermore, the Pheroid® has now been demonstrated to be a very safe carrier system (unlike other systems for example nanoparticles that is suspect to physiological incompatibilities) during *in vitro* and acute and subchronic *in vivo* evaluations.

Future products (as discussed in Section 2.4.2) will subsequently be progressed to further stages of the drug development pipeline, such as the application of Pheroid® to nuclear medicine and peptide drug delivery.

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CHAPTER 3: AN INTRODUCTION TO NUCLEAR IMAGING AND THE APPLICATION OF DRUG DELIVERY SYSTEMS

This chapter introduces the field of nuclear imaging as well as a review of the current state of utilization of drug delivery systems in nuclear medicine. The review article was submitted to the Journal of Controlled Release, it was not accepted but the candidate was invited to resubmit the article to this journal provided certain changes were incorporated. These changes are incorporated in the current article included here and will be sent again to this journal.

3.1 Overview of the history of Nuclear Medicine

Nuclear imaging (with radioisotopes), magnetic resonance imaging, optical imaging and ultrasound imaging are grouped under the scope of molecular imaging. Molecular imaging is defined as a study (visualization, characterization and measurement) of processes in a living entity at cellular and molecular level (Mankoff, 2007). Nuclear imaging is grouped together with nuclear therapy under the specialist area of nuclear medicine, which is defined as the implementation of radioisotopes to diagnose, treat or generate information about diseases (Wagner, 2009).

The father of nuclear medicine, Georg Charles de Hevesy recorded the first use of a natural radioisotope ^{212}Pb to study the uptake of lead in the various plant organs by analysing ashed samples with an electroscope. The concept of “radioelements as indicators” was coined by De Hevesy and he performed studies on rabbits to follow the movement of bismuth containing antisyphilitic medication (^{214}Bi) and ^{212}Pb in biological systems. De Hevesy famously used his new discovery to expose his landlady who he suspected served him the same meat day after day in various dishes. He doused his left-overs with radioactivity and demonstrated a few days later, by means of an electroscope, that his so-called freshly prepared food was radioactive, contaminated by himself at a previous meal (Meyer, 1979). In 1925 the first use of nuclear imaging in humans was recorded when Hermann Blumgart injected radium-C (^{214}Bi) into himself to start a trial on both healthy and diseased human volunteers to assess the dynamics of blood flow. Blumgart and his co-worker Yenz also fashioned the first instrumentation to detect radiotracers in humans. Accordingly, Blumgart is seen as the father of diagnostic nuclear medicine (Patton, 2003; Blumgart & Yens, 1926; Wagner, 2009).

Blumgart and Yenz stipulated the following requirements for an optimal biological indicator (e.g. contrast agents, radiotracers) (Blumgart & Yens, 1926):

- The indicator must not be toxic.

- The indicator must be foreign to the body or biological system.
- The indicator must allow for the study of a homeostatic system without disruption of physiological function. The biological event that is followed with the radiotracer should not be influenced by the procedure or the indicator utilized.
- The indicator must disappear rapidly from the system thereby allowing for repeat administrations and measurements.
- The indicator must be detectable in small quantities.

The requirements for clinical use of such an indicator were defined as (Blumgart & Yens, 1926):

- The measurement must be objective, and no cooperation must be needed from the test subject.
- The method must be non-invasive (apart from the administration via injection).
- The method of detection must be rapid to give a real-time indication of the movement of the tracer.

In the early 1930's Frédéric and Irène Joliot-Curie demonstrated the creation of artificial radioactivity which led to the possibility of the production of radioisotopes of most elements. This extended the range of isotopes that the field of nuclear medicine could choose from (Joliot & Curie, 1934; Becker & Sawin, 1996). The production of these artificial radioisotopes were made possible soon after, with the invention of the cyclotron (circular particle accelerator) by Ernest Lawrence (Wagner, 2009). In 1936, based on the discovery of iodine radioisotopes, the thyroid group of Massachusetts General Hospital investigated the use of radioactive iodine for the treatment and diagnosis of thyroid disease. Due to delays caused by shortage of radioiodine and the start of World War II halting most research, radioiodine would only become widely used in clinical practice after 1946. To this day, radioiodine is still one of the first-line treatments for hyperthyroidism (Becker & Sawin, 1996).

The last component that was needed for the development of nuclear medicine as a field was the gamma camera or scintillation camera invented by Hal Anger in 1957. Anger described this instrument as an electronic device that takes images of the location of gamma-ray and positron-emitting isotopes within a living organism (Anger, 1964). This device detects the minute entities which form the core of nuclear medicine, namely the radionuclide.

3.2 The radionuclide

A radionuclide is defined as an atom that is unstable due to a surplus of nuclear energy which is released by emitting an appropriate ionizing radiation when it degenerates (Theobold, 2010). The ideal radionuclide for medical purposes has the following characteristics (Theobold, 2010):

- The decay half-life of the radioactive isotope should be suitable allowing for the proper time for the evaluation of the biological effect studied.
- The isotope should provide the suitable radiation (gamma photons for diagnostic purposes and alpha and beta particles for therapy).
- It must be easily available, and the production method should be accessible.
- The chemistry of the isotope should allow for bonding to the selected pharmaceutical ligand.

In order to be used as an agent for following biological processes (both normal and abnormal) *in vivo*, the radionuclide is bound to a selected pharmaceutical ligand to form a radiotracer (Theobold, 2010).

3.3 The radiotracer

The ideal radiotracer contains a radioisotope with the necessary qualities for radioimaging combined with a molecule that is identical (physically and chemically) to the natural molecule to be investigated in the system. The radioisotope that is used as label must be firmly associated with the ligand so as not to become dissociated during the investigation (Theobold, 2010). The radiotracer should also conform to the ideal characteristics of a molecular imaging agent as stated by Blumgarth. Table 3-1 provides a non-exhaustive list of radionuclides used in clinical practice, their half-life data, along with some examples of coupled ligands and their indications.

During imaging, the images are captured by one of the two major imaging systems, namely SPECT (Single Photon Emission Computed Tomography) or PET (Positron Emission Tomography).

Table 3-2: Radionuclides used in clinical practice (Fernande, 2010)

Radionuclide	Half-life	Ligand and exemplary indications
Carbon-11	20 min	^{11}C-flumazenil (evaluate cerebral function) ^{11}C-Choline (prostate cancer evaluation)
Chromium-51	27.7 days	^{51}Cr-EDTA (measurement of glomerular filtration rate)
Fluorine-18	110 min	Most widely used PET isotope. ^{18}F-fludeoxyglucose (glucose utilization of major organs and identification of tumours)
Gallium-67	78.3 hr	^{67}Ga citrate (detection of infection, tumours and inflammation)
Gallium-68	68 min	^{68}Ga-DOTA (detection and staging neuroendocrine tumours)
Iodine-123	13.2 hr	^{123}I (diagnosis of thyroid disease)
Iodine-125	59.4 days	^{125}I-albumin (blood and plasma volume determination)
Iodine-131	8.02 days	^{131}I (diagnosis of treatment of thyroid disease) ^{131}I-norcholestrol (evaluation of adrenal cortical tissue)
Indium-111	67.3 hr	^{111}In-pentetreotide (investigation of neuroendocrine tumours) ^{111}In-oxine and ^{111}In-tropolone (labelling blood cell components)
Krypton-81m	13 sec	^{81m}Kr (used for imaging of airways)
Oxygen-15	122 sec	^{15}O (measurement of oxygen consumption)
Rhenium-186	3.7 days	^{186}Re-HEDP (bone pain palliation)
Rhenium-188	17 hr	^{188}Re-DMSA (bone pain palliation)
Samarium-153	1.9 days	^{153}Sm-EDTMP (relieve of bone pain from metastasis)
Strontium-89	50.6 days	^{89}Sr (relieve bone pain from metastasis)
Technetium-99m	6.01 hr	Most widely used SPECT agent. Some examples provided: ^{99m}Tc-HM-POA (diagnose abnormalities of cerebral blood flow) ^{99m}Tc-MDP (bone imaging) ^{99m}Tc-MIBI (cardiovascular imaging and hyperthyroidism)
Thallium-201	3.04 days	^{201}TI (myocardial perfusion)
Yttrium-90	64 hr	Ibritumomab tiuextan (treatment of non-Hodgkin lymphoma) DOTATOC (treatment of somatostatin receptor-positive neuroendocrine tumours).
Lutetium-177	6.65 days	^{177}Lu-EDTMP (relieve of bone pain)

3.4 The imaging techniques: Planar bone scintigraphy, SPECT and PET

There are important differences with regard to chemical properties between SPECT and PET isotopes. It is however the differences in nuclear decay mechanisms of the selected medical isotopes that influence the choice imaging system (Blower, 2010).

3.4.1 Planar gamma imaging and SPECT

When radioisotope accumulates in a certain area, the gamma rays produced from the isotope will be these specific locations. The gamma rays are captured by the SPECT camera rotating around the patient taking multiple images at different angles through a collimator. The collimator is a lead shield that is manufactured to absorb gamma rays. This apparatus has uniformly distributed openings that face the crystal of the camera in a parallel fashion. All rays that are not projected

parallel to the camera crystal, are removed from the image (Figure 3-1) resulting in a more accurate capturing of the area from which the rays originate (Keller, 1968; Tsialas & Hine, 1970).

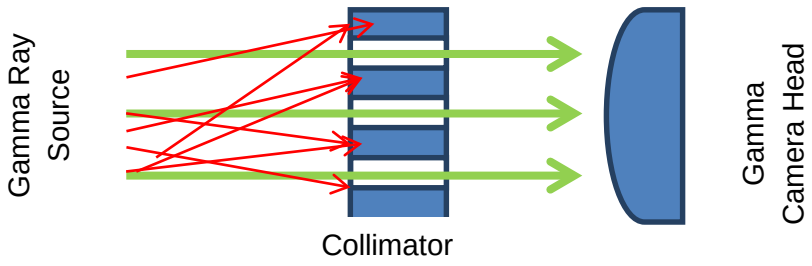


Figure 3-1: A schematic representation of the collimator adapted from Keller (1968) and Tsialas and Hine (1970). The red arrows represent gamma rays that are scattered and not included in the image due to the inability to pinpoint the origin accurately. The green arrows are gamma rays that are parallel to the collimator holes and the gamma camera head crystal.

The gamma rays travel through the collimator and hit a crystal in the gamma camera head which produces scintillation of light (converted from photons). The phototubes connected to the crystal measure the amount of light received and convert this into a voltage pulse (proportional to the amount of light received). These pulses are computed by pulse height analysers or multichannel analysers and mapped into a digital matrix by a computer. The resulting image can be enhanced by functions of the computer software, for example image filtering, contrast modification and background suppression. The software also includes quantitative tools where the regions of interest (e.g. the tumour) can measured (Hutton, 2010).

Whereas planar gamma imaging provides a two-dimensional flat image (Figure 3-2), SPECT results in a three dimensional image composed of a series of planar images (Figure 3-3). This system has one or more gamma cameras that are mounted to rotate around the subject. The captured images can be selected on the computer to provide a single cross-sectional slice of the patient (Hutton, 2010). SPECT/CT is a relatively new system where the CT (Computed Tomography) provides a method of anatomical localization. CT produces a computer-reconstructed image of the patient composed from parallel x-ray images taken in quick succession. The end result is a full transectional sliced image of the patient that enables the physician to scroll through the whole body to see abnormalities (Theobold, 2010). When the CT image is associated with a nuclear image (either SPECT or PET) the areas of abnormal metabolism is highlighted to distinguish between malignant and benign lesions (Figure 3-3).

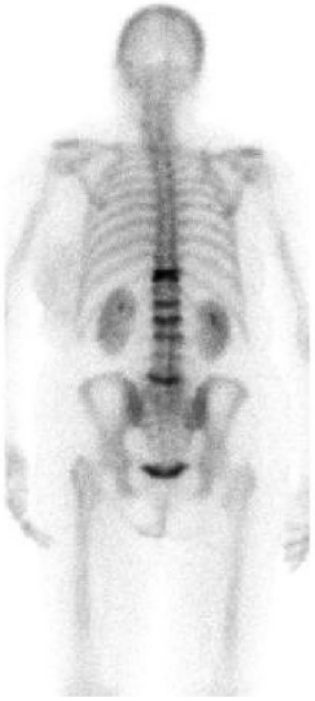


Figure 3-2: A planar bone scintigraphy image obtained after injection of ^{99m}Tc-MDP indicating spinal fractures (thoracic and lumber). (Reprinted with permission from the Publisher. Original source, Cook, G.R., Gnanasegaran, G., Chua, S. 2010. Seminars in Nuclear Medicine, 40: 52-61. ©2009 Elsevier.

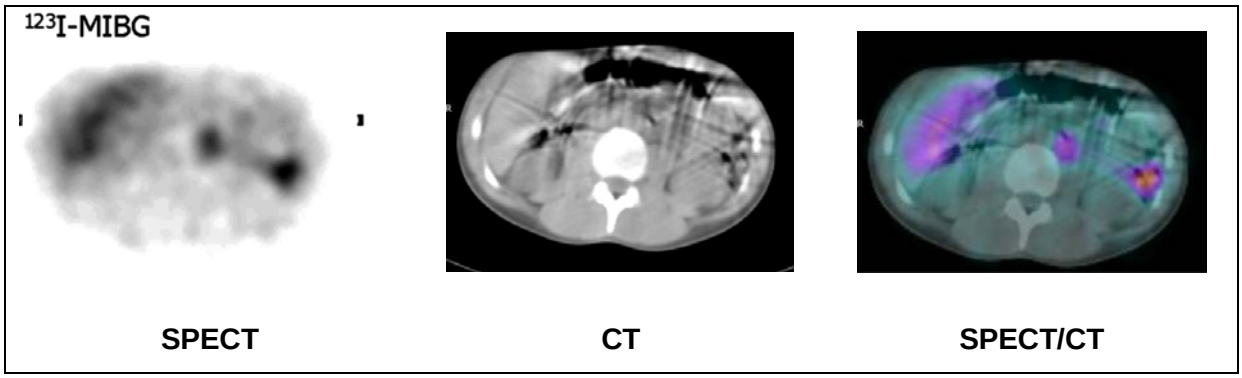


Figure 3-3: A combined SPECT/CT image of a pheochromocytoma indicating metabolic information as well as anatomical localization (Reprinted with permission from the Publisher. Original source: Delbek, D., Schöder, H., Martin, W.H., Wahl, R.L. 2009. Seminars in Nuclear Medicine, 39: 308-340. ©2009 Elsevier).

3.4.2 PET imaging

PET isotopes are unstable with a tendency to discharge positrons to change to a more energetically stable state (i.e. radioactive decay). These released positrons (antiparticles) collide with electrons (particles) in its vicinity. During this process of decay, a pair of rays moving in exact opposite directions (photons with an energy of 511 keV) are emitted by the radioisotope. The photons are captured by the PET camera (Figure 3-4), which consists of detectors placed in an ordered arrangement of panels around the subject. Only paired photons captured within a short time period of each other are detected. When a decay event is detected, the line joining the two detectors involved provides the origin of the annihilation event. The information gathered by the detectors are arranged with a computer program to provide a set of parallel projections. These projections can be sectioned similar to SPECT. A three dimensional image (Figure 3-5) is composed from all these individual reactions and indicates the location of radiotracer molecule accumulation and consequently of the lesion in question (Warwick & Sathekge, 2011; Zhu *et al.*, 2011; Alauddin, 2012).

The radioisotopes used for PET imaging are short lived. The most used isotopes are ^{11}C, ^{13}N, ^{15}O and ^{18}F that easily form covalent bonds with small organic molecules (Blower, 2010). Almost 90% of PET imaging uses the radioisotope fluorine-18; a typical example is ^{18}F-fluorodeoxyglucose as a tumour diagnostic for the evaluation of abnormal metabolism (cardiac and neural). The uptake of FDG (structurally similar to glucose) is markedly higher in tumour cells due to an increased consumption of glucose by malignant cells. The FDA approved FDG for diagnosis, staging and monitoring of tumours (Zhu *et al.*, 2011).

To provide comparison of the energy a patient is exposed to during ^{99m}Technetium scans the organ dose of ^{18}F-FDG is provided in Table 3-2. Dosimetry is a measure of the absorbed dose of radiation per organ and it can be used as an indicator of the safety of a radiotracer as well as the organs which are most prone to be affected by radiation (Theobold, 2010).

Figure 3-4: **The PET camera at Steve Biko Academic Hospital, South Africa (Image is the personal property of the author).**

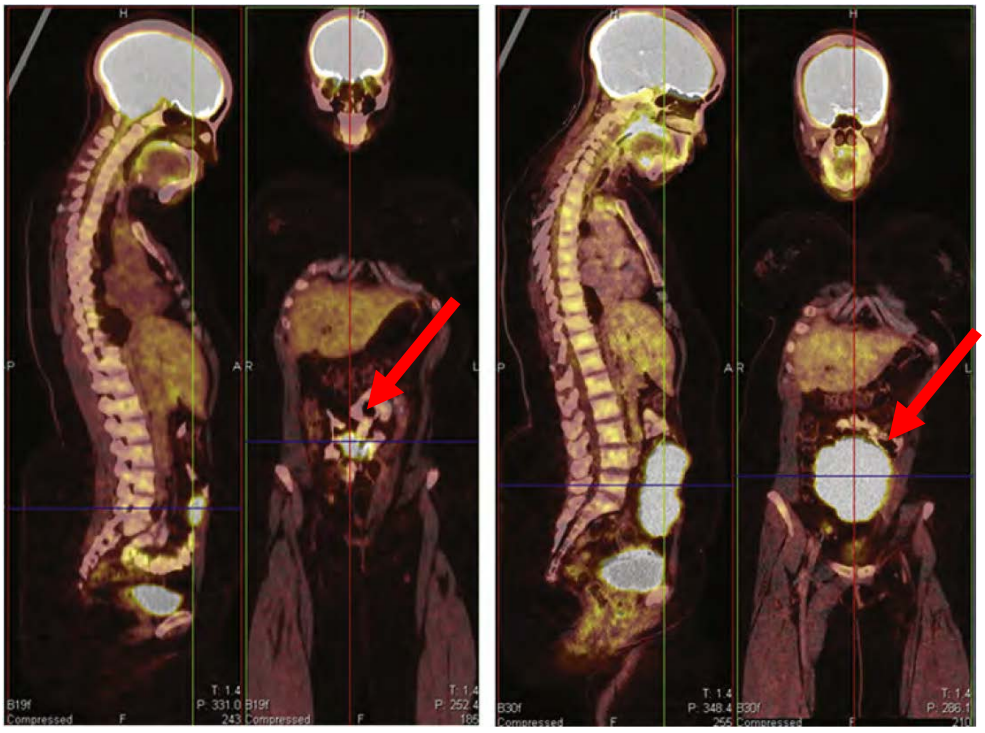


Figure 3-5: **An ^{18}F-fluorodeoxyglucose PET scan of an HIV positive patient with cervical cancer. On the left is the initial scan and on the right is the follow-up scan obtained after 6 months, indicative of fast progressing cancer growth (Reprinted with permission from the Publisher. Original source: Sathekge, M., Maes, A., Van de Wiele, A. 2013. Seminars in Nuclear Medicine, 43: 349-366. ©2013 Elsevier).**

Table 3-2: Dosimetry of 18F-FDG (Hayes *et al.*, 2002).

Organ	Absorbed dose per unit activity administered (rad/mCi)
Brain	0.17
Heart wall	0.25
Kidneys	0.078
Liver	0.088
Lungs	0.056
Pancreas	0.052
Red marrow	0.040
Spleen	0.27
Urinary bladder wall	0.27
Ovaries	0.041
Testes	0.041

Both SPECT and PET clearly contribute to nuclear imaging in unique ways and it is necessary to weigh the advantages and disadvantages of each imaging technique.

3.4.3 A comparison between SPECT and PET

If the advantages of both imaging techniques are evaluated (Table 3-3), it is clear that both techniques are required in a nuclear medicine facility; the techniques are complementary to each other rather than competitive. The amount of exposure of the patient to radiation should also be considered (Table 3-4).

Table 3-3: A comparison between SPECT and PET

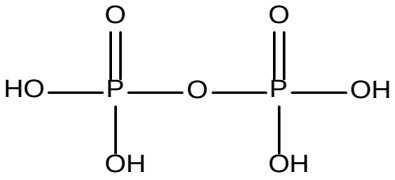
Advantages of SPECT	Advantages of PET
Wider observational window compared to PET (longer half-life of single photon emitters).	Improved imaging quality and fewer artefacts present on images.
Technology is improving with advances made to better image quality to become comparable with PET.	Scans the entire patient for metastases (identify all types of tumours).
Dual tracer imaging is a possibility with radiotracers of different energies.	Shorter scan acquisition time compared to SPECT.
Gamma emitters (due to molecular size) are used, which is most suitable for labelling of peptides, proteins and larger ligands.	Positron emitters are used, which is more suitable for the labelling of small organic metabolites and drugs.
Low costs (entry and on-going) when compared to PET. If only hardware is considered, a SPECT camera costs about $600 000 while a PET camera costs $ 2 million.	More suitable for scanning obese patients than SPECT.
Wide availability of SPECT cameras compared to PET technology.	More accurate and sensitive in the identification of small abnormalities.

Table 3-4: A comparison of whole body dose radiation received from PET and SPECT agents (Maltby *et al.,* 2010).

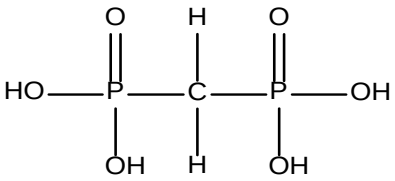
Radiotracer	Whole body dose (Rads/mCi)
^{18}F-FDG (PET tracer)	0.070
^{99m}Tc-MDP (SPECT tracer)	0.016
^{99m}Tc-MIBI (SPECT tracer)	0.043

^{99m}Technetium is a SPECT tracer that is historically the most important but also currently still the most utilized radioisotope in Nuclear Medicine (Duatti, 2010). It is also the radioisotope that is the focus of this study.

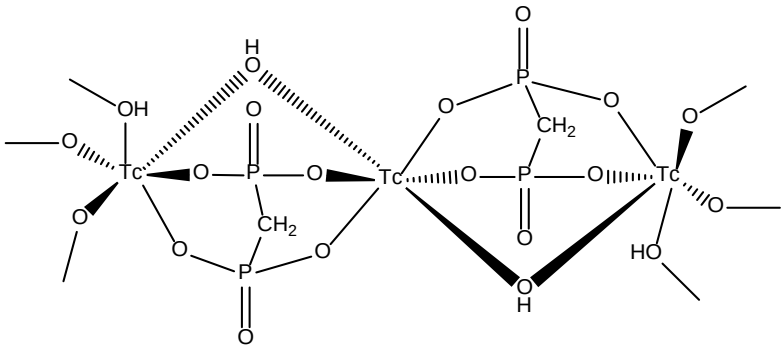
The bone seeking ability of bisphosphonates is due to the formation of a complex between the available phosphonate oxygen atoms present on the radionuclide-bisphosphonate complex and the hydroxyapatite. The resulting hydroxyapatite-MDP complex is a ligand for the bare Ca^{2+} ions present in high concentrations at sites of bone tissue formation. The complex accumulates in the areas with pathological amounts of bone turnover. If the MDP is bound to ^{99m}Tc this accumulation will be detected by scintillation during planar gamma imaging or SPECT (Subramian *et al.*, 1975; Abram & Alberto, 2006; Lewington, 2015).



A) Pyrophosphate



B) Methylene Diphosphonate



C) ^{99m}Tc-Methylene Diphosphonate complex

Figure 3-6: Bisphosphonates A) pyrophosphate, B) methylene diphosphonate and C) the formation of complexes between ^{99m}Tc and MDP (adapted from Abram & Alberto, 2006 and Blake *et al.,* 2001).

A variety of pathologies may give rise to increased osteogenisis, some more common than other. When ^{99m}Tc is coupled to a bone seeking agent the radioisotope is accumulated at areas of abnormal osteogenesis and bone metabolism. Various bone seeking agents have been coupled to ^{99m}Tc, but ^{99m}Tc-MDP still remains the gold standard radiotracer of bone imaging. Due to strong binding of bisphosphonates like pyrophosphate to calcium, methyl diphosphonate was developed as an analogue with the P-O-P bond replaced by a P-C-P bond (Figure 3.7). This resulted in a compound less prone to metabolism. These compounds in their unlabelled state are indicated for the treatment of osteoporosis and Paget's disease due to their inhibition of resorption (Blake *et al.*, 2001).

The applications for ^{99m}Tc-MDP bone imaging are:

a) Bone tumours

The most common indication for a bone scintigraphy imaging procedure is the diagnosis of bone tumours, most often metastases of breast, prostate or lung cancer (Love *et al.*, 2003). Bone scintigraphy provides a method to evaluate metastatic bone lesion progression and the localization of these lesions. It also enables the prediction of the risk of contracting crippling skeletal fractures, in order to facilitate timely preventative measures (Subramian *et al.*, 1975; Even-Sapir *et al.*, 2006; Iagaru *et al.*, 2012). When the presence of metastatic bone lesions is confirmed, planar bone scintigraphy may be employed to determine the rate of disease progression and efficacy of the therapy utilized (Even-Sapir *et al.*, 2006; Iagaru *et al.*, 2012). Up to 75% of cancer patients complaining of skeletal pain present with metastasis; similarly, 25-45% of patients without pain demonstrates metastasis (Figure 3-7). This method can provide timely diagnosis of metastasis in the bones for appropriate management. When the degree of metastasis demonstrates a higher distribution, nearly all the radiotracer accumulates in the bone tissue and no radioactivity is measureable in the soft tissue or other organs and lesser renal activity is present. This is a very characteristic presentation called a superscan (Brenner *et al.*, 2012).

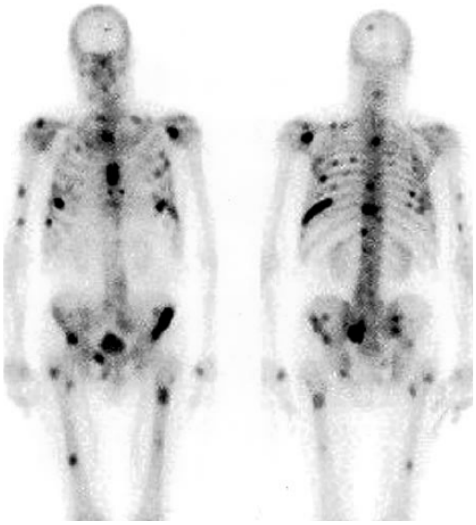


Figure 3-7: A bone scintigraphy image indicating metastasis in a patient with primary lung cancer (Reprinted with permission from the Publisher. Original source: Love, C., Din, A.S., Thomas, M.B., Kalapparambath, T.P.I., Palestro, C.J. 2003. RadioGraphics, 23: 341-358. ©Radiographic Society of North America).

b) Other applications

Fractures show abnormal bone scintigraphy images after approximately 24 hours and may identify lesions not presented on radiographs. The shortest time-frame for lesions to return to a normal undetectable state is 6 months and bone scintigraphy can be utilized to determine the time at which trauma occurred (Love *et al.*, 2003). In race horses bone scintigraphy has unique application in the early detection of stress fractures. Due to the fact that physiological changes are detected at a metabolic level by bone scintigraphy (higher bone turnover in areas of distress), the stress fractures in race horses can be diagnosed at an earlier stage than by normal x-ray imaging, resulting in early treatment (Mackey *et al.,* 1987). Osteomyelitis in humans, presenting with focal hyperperfusion, focal hyperaemia and focally increased bone uptake, can be identified with three phases of scanning. Bone scintigraphy imaging can also be used to evaluate whether joint replacements have become infected if pain is experienced at the joint (Love *et al.*, 2003; Love *et al.*, 2009; Van der Bruggen *et al.*, 2010). Paget's disease is indicated by a characteristic bone scan image demonstrating increased uptake of the radiotracer through large areas or even the whole of the affected bone while the normal anatomy of the bone is preserved. The borders between the normal and abnormal bones are clearly defined and Paget's disease is easily differentiated from other types of bone pathologies. This disease is caused by abnormal bone metabolism of unknown cause and is treated with bisphosphonate therapy (Ryan & Fogelman, 1997; Love *et al.*, 2003; Cook *et al.*, 2010).

Other less common indications for bone scintigraphy is determining the origin of hypocalcaemia (hyperparathyroidism vs. malignancy), renal osteodystrophy, osteoporotic fractures, osteomalacia, evaluation of bone trauma (e.g. sports injuries), evaluation of paediatric skeletal development, osteoarthritis and sympathetic dystrophy of the hand (Demangeat *et al.*, 1988; Ryan & Fogelman, 1997; Kiuru *et al.*, 2002; Love *et al.*, 2003; Kim *et al.*, 2008; Cook *et al.*, 2010; Nadel, 2010; Van der Wall *et al.*, 2010).

3.5 Safety of ^{99m}Tc-MDP

No more than 1 per 200 000 adverse events are reported with injection of ^{99m}Tc-MDP and reported cases are usually mild and anaphylactoid in category (e.g. nausea, rash, hypotension and arthralgia). Interactions that may reduce the efficacy of this diagnostic method are concomitant use of bisphosphonates, tetracycline, iron and chelates. Antacids increase the biodistrubtion of ^{99m}Tc-MDP to the liver. The radiation dosimetry of ^{99m}Tc-MDP is provided in Table 3-5 for comparison with other radiotracers (Maltby *et al.*, 2010).

Table 3-5: Dosimetry of ^{99m}Tc-MDP (Subramian *et al.,* 1975).

Organ	Absorbed dose per unit activity administered (rad/mCi)
Kidneys	0.047
Liver	0.014
Skeleton	0.038
Average soft tissue	0.009
Red marrow	0.025
Urinary bladder wall	0.44
Ovaries	0.017
Testes	0.012

About 50% of the injected dosage (600 – 800 MBq for SPECT) ^{99m}Tc-MDP accumulates in the skeleton with maximum bone accumulation taking place one hour after injection. The unbound radiotracer complexes are excreted through the kidneys (Maltby *et al.*, 2010).

3.6 Summary of the background of nuclear medicine

The fast pace of development in the field of nuclear medicine is very noticeable when the history of its development is considered. The incidence of adverse events developed due to administration of radiopharmaceuticals are rare and none of the side-effects reported to date required hospitalization, which makes it a very attractive field for new diagnostic methods and even treatments. The frequency of adverse events reported for radiopharmaceuticals is a 1000 times lower than those reported for x-ray contrast media and other medicine administered in the hospital setting (Silberstein & Ryan, 1996; Hesselwood & Keeling, 1997; Silinidir & Özer, 2008;

Santos-Oliveria, 2009). Current research focus on the field of theranostics, where the diagnostic radiotracer has a dual ability to treat the disease in question (Denoyer & Pouliot, 2013). The nuclear medicine field has shortcomings that will be discussed in the next section of this chapter – along with the application of drug delivery systems to this field.

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Drug delivery systems: targeting new frontiers in nuclear medicine

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Abstract

Introduction: Drug delivery systems are entities designed to alter the biological behaviour of the pharmaceutical active ingredients that they carry to afford more beneficial biodistribution and safety profiles. Many problems currently faced by the field of nuclear medicine (e.g. developing new theranostics, utilizing multimodal imaging platforms and providing targeted delivery) can be solved by applying drug delivery systems that has been proven successful in other fields of medicine, to radiopharmaceuticals. This review describes the advancements being made towards this goal.

Areas covered: The applications of drug delivery systems (liposomes, nanoparticles, microspheres) in the field of nuclear medicine are discussed. Only systems with foreseen or confirmed clinical applications in nuclear medicine are discussed, not instances where nuclear imaging is merely a tool to evaluate the biodistribution of novel delivery technologies.

Conclusion: Great advancements have been made with the development of novel systems incorporating nuclear entities in drug delivery systems, with the possibility of reshaping the nuclear medicine landscape. Nonetheless, translation from preclinical evaluations to clinical use is lacking and serious investment is needed to achieve this goal.

Keywords: Nuclear medicine; drug carrier systems; radiopharmaceuticals; theranostics; multi-modality imaging

Acknowledgements

J. Kleynhans received financial assistance from the National Research Foundation (NRF) of South Africa (grant number SFH 14070573914) as well as the Nuclear Technologies in Medicine and Biosciences Initiative (NTemBI) and the DST/NWU PCDDP. Opinions expressed, and

conclusions arrived at, are those of the authors and are not necessarily to be attributed to the NRF and NTeMBI or the DST/NWU PCDDP.

Conflicts of interest

The authors declare that they have no conflict of interest.

Abbreviations

^{198}Au	gold-198
^{10}B	boron -10
^{111}In	indium-111
^{125}I	iondine-125
^{15}O	oxygen-15
^{166}Ho	holmium-166
^{177}Lu	lutetium-177
^{188}Re	rhenium-188
^{188}W	tungsten-188
^{18}F	fluorine-18
^{18}F-FDG	fluorine-18-fludeoxyglucose
^{225}Ac	actinium-225
^{64}Cu	copper-64
^{89}Zr	zirconium-89
^{90}Y	yttrium-90
^{99}Mo	molybdenum-99
^{153}Sm	samarium-153
^{99m}Tc	technetium-99m
^{99m}Tc-HMPOA	technetium-99m-hexamethylpropyleneamineoxime
^{99m}Tc-MDP	technetium-99m-methyl diphosphonate
^{99m}Tc-MIBI	technetium-99m-hexakis-2-methoxyisobutylisonitrile
ALARA	As Low As Reasonably Achievable
BMEDA	*N*,*N*-bis [2-mercaptoethyl]-*N*′,*N*′-diethylethylenediamine
CLI	Cerenkov luminescence imaging
CT	Computed tomography
DFO B	desferrioxamine B
DNA	deoxyribonucleic acid
DOTA	1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid
FDA	Food and Drug Administration

MnMEIO	Md-doped magnetism engineered iron oxide
MRI	Magnetic Resonance Imaging
MWNT	multi-walled nano particles
NIRF	near infrared fluorescence
NOTA	1,4,7-triazacyclononane-1,4,7-triacetic acid
N-type Ca2+ channels	voltage-dependent calcium channels
PEG	polyethylene glycol
PET	Positron Emission Tomography
RIT	Radioimmunotherapy
SIRT	selective internal radiation therapy
SLN	sentinel lymph node
SPECT	Single Photon Emission Computed Tomography
SWNT	single-walled nano particles
UCL	Upconversion luminescence

1) Introduction

Drug delivery systems can provide solutions to the continuous growing social and economic problems faced by modern health care [1-3]. Currently there is great concern about the decrease in the number of new pharmacological agents registered with the FDA (Food and Drug Administration, USA), which may very well be a symptom of a reduction in the number of drugs entering all the different phases of the drug development pipeline. The reason most frequently provided for this trend in literature is a diminishing drug development budget allocated by pharmaceutical companies due to a low return on investment of new pharmaceutical entities. Substantial numbers of tested entities fall out at various stages of the development process due to toxicity, low activity at the target site, unsuitable pharmacokinetic profiles and lack of movement across biological membranes [4-7]. Drug delivery systems promote the concentration of the drug reaching the target site and the improvement of less than favourable pharmacokinetic profiles. This allows the revisiting of previously failed compounds with the opportunity to adjust the relationship between safety and efficacy. Due to the fact that a higher concentration of the active ingredient reaches its target, the dosage administered can be lowered, which reduces the risk of adverse effects. If the dosage is less, the cost of the therapy also falls, and the economic burden of disease is decreased. With the higher delivery across membranes, drugs normally administered intravenously also become bioavailable through the gastrointestinal system [1, 8, 9, 10].

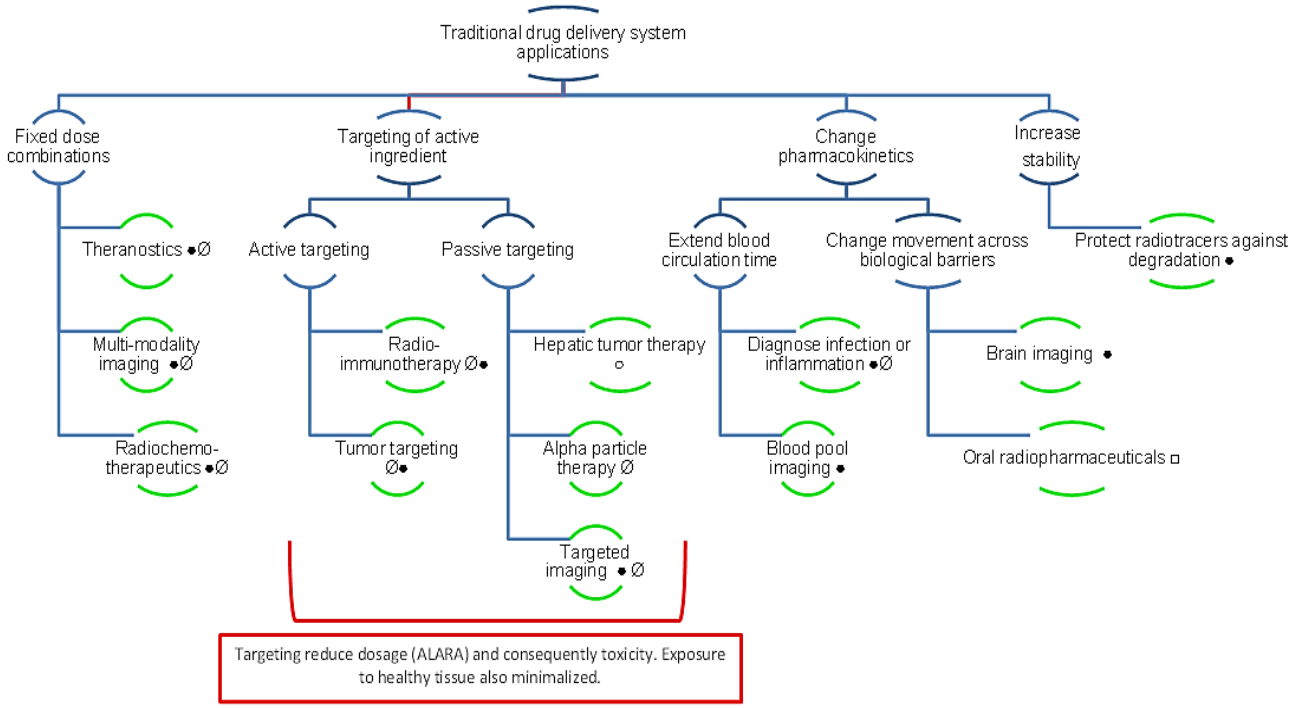
Problems faced by the health sector regarding the economic burden of disease are by no means limited to therapeutic agents alone and the same plague is hampering radiopharmaceutical

production [11]. Due to the escalating shortage of medical isotopes, in particular molybdenum-99 (^{99}Mo) and the subsequent generation of technetium-99m (^{99m}Tc), the more effective delivery of radiopharmaceuticals will ensure a decrease in the demand for these agents. Whilst enormous amounts of capital are invested in the development of more sensitive detecting equipment to allow for a decrease of isotope dosage administered per patient scan (reducing risk of exposure as well as cost), drug delivery systems may provide a similar dosage reduction without the need of any investment in hardware by medical facilities. Diagnostic imaging has an even greater need of selective targeting to the regions of interest compared to normal pharmacological agents, since any radiopharmaceutical molecule that fails to accumulate at the target site, will reduce image quality and result in the presence of artefacts. Since the ALARA (As Low As Reasonably Achievable) principle will always be an important goal in the medical application of nuclear isotopes, the reduction of the dosage administered per diagnostic procedure made possible by drug delivery systems will contribute further to the safety of medical personal and patients. With the development of new theranostic radiopharmaceutical agents with application in both diagnosis and treatment, the targeting becomes a priority due to the high amounts of irradiation used or employed during the treatment phase (associated with repeated dosing) and possible side-effects in areas of unspecific delivery. Except for a few isotopes, for instance iodine-131(^{131}I), most radiopharmaceuticals are only available as parenteral formulations. Research to develop new preparations that allows for the administration of these entities through the oral route are underway and aims to reduce the distress experienced by patients during administration. Drug carrier systems allow for the concurrent movement of two agents (either two diagnostics or combinations of therapeutic and diagnostic agents), which is optimal for multimodality imaging strategies and some theranostic applications [11-16]. Drug delivery systems clearly have great significance in combatting the shortcomings of current nuclear medicine.

Almost every group of drug delivery system developed has been applied in the radiopharmaceutical field [17, 18], most probably because these systems are routinely labelled with an isotope to follow their movement in living organisms without disturbing the natural functioning of the system in question [19-21]. Often, it then becomes apparent that the properties of the isotope employed to evaluate the drug carrier system deviates from its normal behaviour when entrapped in this entity, with potential for adding to the arsenal of diagnostic entities available to the medical world [20, 22]. Only drug delivery systems paired with radiopharmaceuticals that demonstrate foreseen diagnostic value is included in this review; the use of radiopharmaceuticals purely for the sake of evaluation of the biological functioning of the drug delivery system is excluded. It is noteworthy that in a sense radiopharmaceutical tracers can already be viewed as a carrier system that locates the selected isotope at a specific target site due to ligand interactions. This review will focus exclusively on known drug carrier systems

traditionally applied in pharmaceutical delivery (for example liposomes and nanoparticles), with application in the field of nuclear medicine. Refer to Figure 1 for a basic outline of possible applications of drug delivery systems in nuclear medicine as well as an indication of the types of carrier systems that has been investigated in certain areas.

Figure 1: The applications of traditional drug delivery systems (blue) with the promising solutions for nuclear medicine (green). The different drug carrier systems that have been investigated include liposomes (●), microspheres (○), nano-particles (Ø) and Pheroid® (□).



The drug carrier systems discussed in this review are liposomes (Section 1.1), microspheres (Section 1.2) and nanoparticles (Section 1.3). Refer to Table 1 for a description of the parameters that classifies the different systems.

Table 1: Characteristics of drug carrier systems [23-26]

	Liposomes	Microspheres	Nanoparticles
Components	Phospholipid vesicles with one or more lipid bilayers encompassing an aqueous phase.	Manufactured from various material, most commonly glass, polymer and ceramic.	Unlimited materials may be used if the material exhibits novel properties due to the reduction in size.
Size	Small (≤ 100 nm) Intermediate (100-250 nm) Large (≥ 250 nm) Giant (> 1 µm)	10 nm – 2000 µm	1-100 nm
Pros	Very adaptable system	Easy manufacturing Biocompatible Slow-release capability	Very adaptable and easily functionalized with targeting entities
Cons	Mostly unstable Repeatability of production and quality control difficult	Drug release from particle not always controlled	Questions remain regarding safety of these systems

1.1) Liposomes

Liposomes are drug carrier systems that incorporate bilayers of lipids encapsulating a hydrophilic interior where the active pharmaceutical ingredient accumulates. Other variations of this technology occur (for example oil-emulsions type systems) with different interplays between hydrophobic and hydrophilic layers. Liposomes are extremely versatile and can be tailor made to suit the entity to be delivered as well as the characteristics of the cellular target to be reached. The consensus is that the hydrophilic pharmaceutical active ingredient will become entrapped in the system whilst the lipid component will allow for the transportation through hydrophobic biological barriers to release the load at the area of interest. Liposomes as drug delivery systems have produced substantial results with various products reaching the marketing phase. There is however a large discrepancy between the amount of preclinical work and products reaching the market [27-31].

1.2) Microspheres

Included in this review is microsphere technology which, although not in the nanoscale range, is still an effective delivery system that changes the behaviour of the materials they carry. These entities are small spherical structures with dimensions between 10 nm to 1000 µm made of a wide range of materials that integrates the active ingredient in a stable solid form of uniform size [32, 33].

1.3) Nanoparticles

Further down the size range, nanotechnology is a rapidly expanding frontier of modern research that managed to reach the common household. These structures can be manufactured from a myriad of materials (including metals in different states, polymers, lipids and larger biological molecules) if their size are restricted to the nanometer range and they exhibit novel properties that differ from that of the parent substances. [34-36]. Although nanoparticles have great capacity to incorporate different types of imaging and therapeutic entities in one system, development of these agents are hampered by additional intensive investigations required to determine pharmacokinetics, biocompatibility, accumulation and safety (nanotoxicology) due to the undefined characteristics of these units [18, 37].

2) Fixed dose combinations

2.1) Theranostics in nuclear medicine

Theranostics, a term proposed by Funkhouser in 2002, describes the combination of the outcomes provided by both a therapeutic agent and a diagnostic agent when combined in one single pharmaceutical entity. Theranostics opens new frontiers in the arena of personalized medicine, which strives to adapt to the uniqueness of each patient during the treatment of disease, especially regarding choice of the therapeutic agent, mode of action and dosage selection. In the context of radiopharmaceuticals, a theranostic agent will combine a PET (Positron Emission Tomography) or SPECT (Single Photon Emission Computed Tomography) isotope to act as a biomarker (for imaging) of the target but simultaneously incorporate a therapeutic entity that will be delivered based on the distribution of the marker. Although radiopharmaceutical theranostics are mainly focused on the treatment of cancer, other applications are currently under investigation, such as agents developed to diagnose and treat infectious diseases [38-40].

a) Rhenium isotopes

Since liposomes for non-nuclear applications have been extensively characterized it is no surprise that the Institute of Nuclear Energy Research (Taiwan) has already advanced a lipid theranostic agent into phase 1 clinical testing (ClinicalTrails.gov identifier NCT02271516), although the trial is currently not yet in the recruiting stage. The liposome system contains rhenium-188 (^{188}Re) as a dual imaging (155 keV gamma emission) and therapeutic isotope (2.12 MeV beta emission) linked to BMEDA (*N*,*N*-bis [2-mercaptoethyl]-*N′*,*N′*-diethylethylenediamine) acting as chelator. The chelator-isotope complex is entrapped in the fully assembled nanopegylated liposome vesicles by a remote loading method before administration [41]. Preclinical studies demonstrated the ability of the liposomes to passively target ^{188}Re (due to the enhanced permeability and

retention effect of liposomes) to various neoplasms in the gastrointestinal tract, including tumour associated ascites. The survival rate was increased in a C26 colon carcinoma ascites mice model, with inhibition of both the tumour and subsequent ascites [42, 43]. Compared to the gold standard treatment of 5-fluorouracil (a chemotherapeutic agent) for colon carcinoma as well as colonic peritoneal carcinomatasis, ^{188}Re-BMEDA liposomes exhibited a higher therapeutic efficacy in rodent xenografts [43, 44]. Acute toxicity studies published on this theranostic ascertained no severe toxicity in the selected rodent models. High dosages were associated with short term weight-loss, a temporary reduction in hematologic parameters and a cytogenetic risk [45, 46]. Synergistic enhancement of the efficacy of this system is reported with concomitant administration of doxorubicin liposomes as well as external beam radiotherapy [47, 48]. The short physical half-life of this isotope as well as the availability of a ^{188}W/^{188}Re (tungsten-188/rhenium-188) generator bodes well for its clinical application as theranostic. Remote loading is associated with high entrapment efficiency that can be adapted according to the need. It is however important to ensure that the system (especially in the case of liposomes) is stable, so that all instances of the entrapped isotope is distributed in the carrier system, and that leakage of the isotope is minimized.

Other preclinical applications of rhenium isotopes entrapped in liposomes showing promise *in vivo* include pancreatic cancer, glioma, head and neck cancer, lung metastases, breast cancer and intraperitoneal treatment of ovarian cancer [49-56]. ^{188}Re has been incorporated in various magnetic nanoparticles to allow for targeting of these particles by exposing the patient to an external magnetic field [57, 58]. The incorporation of ^{188}Re into microspheres is also under investigation [59, 60]. Again, a lack of clinical translation is evident since only one of the various *in vivo* applications is currently transitioning into the clinical testing phase.

2.2) Multi-modality imaging

Nowadays diagnosis is mostly based on the information received from more than one modality to increase the accuracy of clinical outcomes. Information provided by different imaging modalities such as computed tomography (CT) providing anatomical information and PET providing functional imaging can be utilized in a complimentary fashion. Fusion of images by using computer software has the limitation of having to counteract variations in positioning of the patient due to the involvement of different scanning equipment and often imaging at different times. The hardware approach was developed in 1996, when the first tandem SPECT/CT was developed (PET/CT followed shortly thereafter) that enabled a single scanning procedure. The concurrent measurement of both anatomic and physiological data solves the issues of accurate localization of nuclear signals [59-64]. The combination of PET/CT and SPECT/CT has since become standard practice and individual scanners are rarely employed [65]. Due to the successes of the tandem SPECT/CT, various other combinations of modalities are under development and even

in clinical employment, sometimes necessitating the combination of more than one tracer, *e.g.* a radiopharmaceutical as well as a contrast agent [66-70]. To ensure that all the administered tracers behave in the same pharmacokinetic pattern and that the results are interconnected, these individual entities can be packaged in one drug delivery system. During the hybridization of two imaging techniques it is important that the system will, above all, provide added clinical benefit that cannot be obtained with other interventions [71].

a) Combining nuclear imaging with magnetic resonance imaging

Although PET or SPECT imaging gives unsurpassed non-invasive insight on biological processes through the tracking of radiotracers, the one big shortcoming thereof is the lack of anatomical information provided. This is largely side stepped by concomitant CT in the clinical setting but combining PET or SPECT with MRI will in addition provide the benefit of high quality information of soft tissue structures. The replacement of CT with MRI should also remove the risks associated with the ionizing radiation to the patient. Current research is aimed at updating hardware to enable the tandem use of these two technologies and this ignited the search for dual modality probes that can cause co-localized signals for monitoring by both techniques. Drug carrier systems are uniquely qualified to incorporate tracers for both these modalities in one single operating system [72 -78]. The debate on whether or not simultaneous PET/MRI is a need, or a novelty was explored in depth by Yankeelov and co-workers and it is clear that currently only a few applications validates the expensive addition of an MRI scanner rather than making use of co-registration alone. However, drug carrier systems can provide multi-modality probes, which should allow elucidation of fundamental clinical questions, and consequently an unequivocal place for combined PET/SPECT/MRI in the arsenal of scanning equipment. An example of such will be providing a detailed anatomical characterization of a lesion afforded by the contrast agent incorporated in the system, as well as PET imaging allowing for concurrent disease staging [79]. Various dual modality agents have researched different stages of development with exciting possibilities opening up.

a.1) PET/MRI

Since the development of the combination PET/MRI hardware has already progressed to useable systems, a great amount of research is focused on the development of dual modality probes for this type of imaging system.

Lipid based PET/MRI agents

Liposomes have also found application in this region of interest. As with other applications, the probes can either be incorporated in the lipid component used for formation of the liposome or be

entrapped in the water component inside the liposome. Paramagnetic radiopharmaceutical ^{89}Zr labelled octreotide containing gadolinium showed good targeting for the somatostatin receptor subtype 2, with both probes incorporated in the lipid phase during the manufacturing of the liposome. This combination can therefore provide high-resolution anatomical information as well as functional information offered by the PET component [80]. Various ^{64}Cu labelled liposomes are also under investigation for possible clinical usefulness. These can be manufactured by covering the superparmagnetic iron core with PEG phospholipids which allows for chelating with ^{64}Cu as PET imaging agent [81, 82]. Iron oxide nanoparticles were coupled with NOTA (1,4,7-triazacyclononane-1,4,7-triacetic acid) to gallium-68 (^{68}Ga) resulting in dual function probes substituted to the functional amine groups of PEG phospholipids. PET and MRI were established successfully in mouse xenograft models and this system therefore has dual-imaging potential [83]. Due to the adaptability of liposomes for targeting, the ability to entrap multiple probes and a good track record in other applications, it is quite possible that many more such agents will be developed in the near future. Again, it is necessary that both the contained imaging agents are distributed exactly the same, as leakage of the one agent out of the system in regions of non-interest will distort the information provided and fail the goal for which these agents are developed.

Dextran based PET/MRI agents

Dextran coated iron oxide particles can be adapted to permit for the modification of the surfaces of these nanoparticles and allow coupling to PET agents. This method has been successfully applied in iron containing nanoparticles linked with ^{64}Cu for the targeting of vascular inflammation [84, 85] and ultra-small paramagnetic iron oxide particles with the ability to bind to various isotopes (^{89}Zr, ^{64}Cu and ^{111}In) [86].

Protein based PET/MRI agents

Protein based nanoparticles can also be employed to develop dual-modality imaging agents. MnMEIO (Md-doped magnetism engineered iron oxide) probes were functionalized with serum albumin to provide nanoparticles that can be used as probes for magnetic resonance imaging. To these nanoparticles, Iodo-beads (^{124}I) were coupled to provide dual PET imaging capabilities [87]. In similar research, polyaspartic acid coated iron oxide nanoparticles conjugated with arginine-glycine-aspartic peptides were synthesized, providing a nanoparticle platform for coupling with ^{64}Cu for PET imaging as well magnetic contrast. This probe showed potential in the detection of tumours that demonstrate integrin expression [88].

Although PET combination probes with MRI are currently dominating the multifunctional imaging landscape, SPECT/MRI is not far behind.

a.2) SPECT/MRI

Successful imaging of cancers expressing mesothelin was performed *in vivo* by ^{111}In-mAbMb conjugated to dextran coated super magnetic iron oxide nanoparticles. These particles also demonstrate the optimal size distribution for their application as dual-SPECT/MRI nanoprobes [89]. To allow direct radiolabeling of the supermagnetic iron oxide nanoparticles with a radiolabeled tracer, De Rosales and co-workers removed the dextran core of commercially available Endorem SPION's and bound ^{99m}Tc-alendronate directly to the core of the iron oxide particle. It is envisioned that the bisphosphonate of the radiotracer (alendronate) can be further adapted for SPECT or PET combined with MRI [90]. An even smaller bisphosphonate iron oxide nanoparticle for functionalizing with SPECT agents has been developed [91]. All the aforementioned nanoparticles have not yet been modified to target specific disease mechanisms and needs further development to allow for clinical use. To target angiogenesis of tumour tissue, Lijowski and colleagues produced nanoparticles containing ^{99m}Tc (for SPECT imaging), gadolinium (for MR imaging) and an $\alpha_v\beta_3$-integrin antagonist. This was a further modification of an imaging probe investigated for PET imaging only. *In vivo* testing showed a high targeting of tumours and this probe therefore has the potential to be used for tumour localization with SPECT as well as visualization of tumour vasculature by MRI [92]. Lee and co-workers synthesized supermagnetic iron oxide nanoparticles labelled with ^{99m}Tc and coupled with lactobionic acid to target hepatocytes. In a bio-distribution study these nanoparticles accumulated in liver tissue [93]. Although some clinicians might deem PET more advanced and informative than SPECT, there is no denial as to the unique contribution information obtained by SPECT contributes to the diagnostic process. If this technology can be improved upon, with the employment of drug carrier systems amongst other strategies, it can provide equal imaging compared to PET for certain therapeutic applications, accompanied with much less expense than that of PET imaging.

b) Combining optical imaging with nuclear imaging

To allow for imaging of patients, imaging probes used in optical, including multimodal systems all emit in the near-infrared region. One such dual PET-optical probe has been determined safe with favourable pharmacokinetic characteristics in a microdose first-in-human investigation. This tracer is composed of inorganic silica nanoparticles (C-dots) combined with ^{124}I for PET imaging, Cy5 dye for fluorescence and cRGDY as targeting peptide component for the imaging of integrin expressing tumours. This technology can provide PET imaging and also allow for image guided surgery [93,94]. These C-dot probes can be adapted to target a variety of biological processes and has been proven effective and safe in animal xenografts [95, 96]. Similar silica nanoparticles containing other fluorescent dyes and PET agents are also under investigation. A probe for tumour imaging with a quantum dot core possessing DOTA as chelator (to allow for binding of

^{64}Cu and vascular endothelial growth factor for targeting), was determined to be a viable probe during *in vivo* studies [97]. The same principle was followed in the development of another probe coupling quantum dots to ^{64}Cu with $\alpha_v\beta_3$-integrin to allow for tumour targeting [98]. Liposomes also have the ability to entrap radiotracers and fluorophores without having an effect on *in vivo* distribution characteristics allowing for adaptability of this system for various targets as needed [99]. Micelles are also being researched as possible carriers for dual optical/nuclear imaging probes, with all published studies demonstrating coinciding of both probes at tumour tissue [100, 102].

Upconversion luminescence (UCL) is the ability of a particle to internalize two or more received photons and subsequently emit fluorescent light of high energy that is sufficient for the optical imaging of biological systems. Lanthanides are very popular UCL agents that provide strong enough signals for clinical application. A study investigated the incorporation of Samarium-153 (^{153}Sm) into nanoparticles containing lanthanide but these nanoparticles need optimization since they were mostly accumulated by the liver and spleen *in vivo* [103].

Cerenkov radiation is the phenomena of the emission of photons by certain radioisotopes whilst decaying. These events can be captured by Cerenkov luminescence imaging (CLI). Cherenkov luminescence is present in certain positron emitters (e.g. ^{18}F [fluorine-18], ^{64}Cu, ^{89}Zr,^{124}I), beta emitters (^{131}I) as well as alpha emitters (^{225}Ac) [104, 105]. To allow for the emission of Cerenkov radiation, the isotope should have a minimum beta energy yield of 263 keV [106]. To allow for the production of an image, these events need to be propagated additionally since Cerenkov luminescence is absorbed by the tissue surrounding these probes. A possible strategy for CLI is to convert this radiation to fluorescence and this was done successfully by utilization of europium oxide nanoparticles. This is an example where near infrared emitting nanophosphors are activated, not by outside applied energy, but from photons originating from nuclear isotopes incorporated in the same nanoparticle. Other lanthanide containing nanoparticles can exhibit similar ability, as well as noble metal nanoclusters [107-110]. A study evaluating the CLI provided by ^{198}Au (gold-198) incorporated in gold nanocages, provided evidence of *in vivo* efficacy to image breast cancer xenografts with this noble metal system optimizing its own luminescence [111]. Quantum dots can also be activated by internal radiation to produce fluorescence for the imaging of biological systems and can therefore be utilized as a carrier system for multi-modal imaging [112].

Combining the modalities of optical imaging (or optical visualization with dyes) with nuclear imaging has very tangible advantages when applied to the mapping of the sentinel lymph node (SLN) during the evaluation of metastases. Currently in clinical practice a ^{99m}Tc radiotracer is injected into tumour lesions and the SLN (Sentinal Lymph Node, first node draining from the

tumour site) is identified with SPECT imaging. Thereafter a dye may be injected into the same tumour site during the surgical procedure to provide optical guidance for the removal of the correct lymph node. The removed lymph nodes are then evaluated for the presence of malignant cells and this predicts the clinical staging of the patient as well as probable life expectancy [113]. The entrapment of a dye in this system has generated scientific interest since simultaneous localization will be of great clinical value [114]. Iron oxide nanoparticles functionalized with ^{99m}Tc is proposed, contributing magnetic resonance imaging to the system as well as visualization by black colouring of lymph tissue due to accumulation of iron oxide nanoparticles [115]. Liposomes functionalized with ^{99m}Tc could also be employed to entrap dyes or optical agents [116]. Nanoprobes could be synthesized to contain multiple optical imaging agents and radionuclides and for application in SLN imaging [117]. In the case of SLN imaging, the size of the carrier system is more important than in other applications since smaller particles will be cleared away from the injections site more efficiently, but larger particles will lead to less leakage at the SLN and therefore a balance needs to be found. No exact specifications are found in literature, and trial and error is currently the only way to identify the correct dimensions for each developed carrier system.

c) Combining more modalities

The logical next reasoning will be if two imaging modalities can be combined, why not combine even more? Care should be taken that the approach is not that it can be done, but if it should be done, since there should still be additional benefit obtained by the combination.

For SNL imaging Kim and co-workers developed a PET/MRI/optical nanoprobe with a silica core incorporating a near-infrared dye and ^{68}Ga with positive *in vivo* imaging supporting the potential of further clinical application [118].

A liposome carrying hexadecyl-4-^{124}iodine-iodobenzoate as well as a gadolinium-1,2-dipalmitoyl-*sn*-glycero-3-phosphoetahnolamine-complex is also under development. The choice of radioisotope is based on the long half-life of ^{124}I (4.2 days) which allows ample time for production and follow-up scan procedures post-administration. This isotope also emits strong Cerenkov luminescence that allows for optical imaging. MRI is provided by the inclusion of a gadolinium complex and both these entities are distributed identically *in vivo* to allow for equivalent tumor imaging. The components of this liposome were adapted to enable fast removal of carriers from the reticuloendothelial system and prolonged retention at the tumour site resulting in clear images with minimal background interference [119].

To combine PET, MR and fluorescence molecular tomography, ^{18}F-CLIO was developed as a new trimodal probe. This nanoparticle has a dextran and superparamagnetic iron core and is

labelled with ^{18}F by so called “click” chemistry. This system is biodegradable *in vivo*, and no long-term toxicities are envisioned for these tracers, but extensive biological evaluations are necessary [120]. Xie and co-workers developed a trimodal PET/MR/Optical nanoprobe with iron oxide nanoparticles functionalized by dopamine to afford the incorporation of other probes (^{64}Cu-DOTA and Cy5.5). Substantial accumulation at tumour sites was recorded with low accumulation by macrophages which show promise for the clinical use thereof [121]. Yang and co-workers combined gold and iron-oxide to form a nanoparticle functionalized with ^{64}Cu and a targeting affibody providing a trimodal tumour targeting imaging agent [122]. The use of porphyrins to manufacture ^{64}Cu containing nanoparticles (pophyrosomes) results in a dual-modality probe, due the intrinsic fluorescence nature of porphyrin, with *in vivo* efficacy for applications in prostate cancer and imaging of micro-metastases [123, 124].

UCL can be combined with PET and MR to add high sensitivity and spatial resolution to the system but negate the limited penetration normally provided by fluorescence. Various lanthanides (gadolinium-3^+, ytterbium-3^+ and erbium-3^+) was combined with ^{18}F as PET agent in nanphosphor particles ($NaYF_4$) in research done by Zhou and co-workers. The incorporation of gadolinium afforded magnetic properties for MRI. The *in vivo* evaluation provided optimal results for both ultra-sensitive investigations on a molecular level as well as whole-body evaluation [125]. Other manufactured UCL/PET/MRI nanophosphor probes incorporating lanthanides were developed to contain ^{124}I and also provides tumour specificity [126].

e) Multimodal theranostics

Li and colleagues designed a liposome to carry a magnetic resonance agent (gadolinium), near infrared fluorescence (NIRF) dye (IRDye-DPSE) and either ^{99m}Tc as SPECT tracer or ^{64}Cu as PET tracer. To make this system a theranostic, doxorubicin could be post-loaded into the system to target tumour cells. The different imaging combinations and applications will be discussed in section 3 on multimodality imaging, but the liposome carrier system offers unique capabilities to entrap as many different probes and therapeutic entities into one core as is desired [127]. Another example is lutetium-177 (^{177}Lu) labelled cerasomes that entrap indocyanine green to enable optical imaging (NIRF), nuclear imaging as well as photothermal ablation of tumours [128].

Wu and co-workers synthesized ^{177}Lu phosphate nanoparticles with apoferritin as core with the aim of providing an agent suitable for RIT that allows for imaging as well. The apoferritin core can be functionalized with different monoclonal antibodies to target tumour cells [129]. Zielhuis and colleagues also synthesized liposomes to be loaded with holmium-166 (^{166}Ho) to provide beta- and gamma emission as a therapeutic component, ^{99m}Tc for SPECT and paramagnetic gadolinium for MRI (Magnetic Resonance Imaging). *In vivo* studies are yet to be undertaken [130].

Maldonado and co-workers have developed a micelle that can harness the ability of quantum dots to generate light for the activation of a cisplatin pro-drug (also incorporated in the micelle) resulting in targeted chemotherapy. Furthermore, the quantum dots also add heat induction for photodynamic cancer therapy and the generation of singlet oxygen as two additional cancer treating mechanisms. Finally, ^{99m}Tc is incorporated as SPECT component to provide nuclear imaging additionally to the optical imaging provided by the quantum dots [131]. If this probe can progress to clinical usage it will provide not only a three-pronged therapeutic attack, but also a dual imaging modality.

2.3) Radiochemotherapeutics

Since liposomal nanocarriers can entrap more than one entity it provides the opportunity for the co-administration of a chemotherapeutic drug with an imaging radioisotope in a single system providing concurrent distribution and therefore a theranostic system. The combination of scintigraphic agent indium-111 (^{111}In) and vinca alkaloid vinorelbine, which inhibits cell growth, was proposed as a theranostic agent for colon carcinoma. Promise was recognized in three studies on xenograft rodents, with passive targeting of tumour cells allowed by the polyethylene glycol (PEG) nanoliposomes entrapping the combination. The system was adapted to allow for the evasion of the reticuloendothelial system and animals showed an increased reduction in tumour growth and less toxicity with this system, compared to unformulated vinorelbine [132-134]. Since rhenium isotopes (^{186}Re and ^{188}Re) have intrinsic theranostic capabilities due the release of energy that is capable of both being cytotoxic and being useful for imaging, the combination with a chemotherapeutic agent like doxorubicin can have substantial benefits. Incidentally, doxorubicin is already commercially available in liposome entrapped form providing some previous knowledge regarding the biological behaviour of such an entity (Doxil®). This system was evaluated for the treatment of head and neck cancer in rat xenografts and displayed a reduction in tumour size with a good toxicity profile [135]. Another agent for colorectal cancer, sorafenib, was combined with ^{188}Re in a liposome carrier and demonstrated viability in a mouse cancer model [136]. Since the combination of Samarium-153 (^{153}Sm) with chemotherapy is quite common in clinical practice, it is quite surprising that few theranostic agents explore this particular combination. One such instance is microspheres (bioactive glass) combining ^{153}Sm with doxorubicin for the treatment and palliation of bone cancer. These microspheres are however still in the development phase and have not been evaluated preclinically [137].

The concept of theranostic radiochemotherapeutics is not limited to cancer therapy and demonstrates possibilities in anti-infective therapy. Kaul and co-workers developed liposomal radiochemotherapeutics for mycobacterial infections utilizing ofloxacin and rifampicin combined with ^{99m}Tc for SPECT imaging. In this instance the application might not truly be purely

theranostic, but also that of a companion diagnostic depending on the outcome of clinical trials. Preliminary studies on this system show *in vitro* efficacy against mycobacteria as well as *in vivo* targeting efficacy confirmed by SPECT imaging in a mouse model of infection [138]. A companion diagnostic is a diagnostic agent used pre-treatment to determine the applicability of the therapeutic drug as treatment. The aim of these companions to therapy might be to determine efficacy or safety thereof or both. These agents are developed based on the biomarkers that predict the efficacy and toxicity of the planned treatment [139]. Chen and co-workers developed a chemotherapeutic nanoparticle that has the capacity for adding a PET companion diagnostic for quick evaluation of the accumulation at tumour sites, before treatment is initiated. The possible therapeutic advantages were demonstrated successfully *in vivo* [140]. When a theranostic agent is developed which entraps two different entities in one drug carrier system, it is important that the dosing characteristics of these agents (e.g. dosage frequency required) are marriageable to avoid overexposure to a particular entity. In the case of the packaging of radioisotopes in a system with other pharmaceuticals, this becomes even more important due to ALARA principles and safety of the patient. However, if managed correctly, these agents can become powerful combatants in the treatment of resistant diseases like colon carcinoma and tuberculosis.

3) Targeting of active ingredients

3.1) Active targeting

a) Radioimmunotherapy

Radioimmunotherapy (RIT) involves the isotope providing therapeutic radiation being coupled to a monoclonal antibody with affinity for tumour tissue, directly targeting the malignancy. The use of liposomes for optimization of RIT is currently under development. The concept is mainly focused on the passive targeting ability of liposomes to accumulate more RIT molecules at the tumour site. Jestin and co-workers manufactured various liposome nanocapsules containing haptens with affinities for bispecific anti-bodies in tumour tissue. This system was evaluated with ^{99m}Tc complexes and showed stability and the possibility exists to further functionalize this system with therapeutically suitable radioisotopes [141, 142].

Radioimmunoconjugates can also be combined with magnetic nanoparticles which can then be localized to tumour sites with external magnetic fields during clinical use. An example of this is an ^{131}I-anti-vascular endothelial growth factor monoclonal antibody that is combined with magnetized dextran nanoparticles. This technology provides tumour reduction *in vivo* with no bone marrow suppression present [143]. Our search did not uncover any drug carrier systems containing a RIT agent that has been clinically evaluated in humans up to date. These systems rely on the active targeting provided by an outside source (monoclonal antibody or magnetic field) to localize the

nuclear isotope to the area of interest rather than passive targeting inherent to the system itself. Overall, active targeting provides more control over the distribution of the isotope than demonstrated with passive targeting.

b) Tumor targeting

Carbon nanotubes are one of the most scientifically valued nanostructures due to a variety of applications based on the physical strength of this material. This system has an above average electrical and thermal conductivity, an ordered structure, is highly elastic and flexible while at the same time exhibiting considerable strength and a high surface area. It is made up of interconnected benzene rings forming a sheet-like structure that is rolled up into a tube. This system is categorized into the carbon allotropic category of fullerenes. There are two types of structures namely single walled (SWNT) that has one rolled up sheet of carbon and is the smaller sized tube, and multi-walled (MWNT) which contains multiple rolled up sheets. Carbon nanotubes have a unique contribution to drug delivery because this system does not illicit an immune reaction when introduced into biological systems. This system is also very adaptable to incorporate various entities but is hampered by its hydrophobic nature which commands the need for surface modification to allow for biological applications [144-146]. Ruggiero and co-workers reports a SWNT made out of carbon that utilize radiometal-ion chelates to allow better solubility and coupling to radioisotopes whilst simultaneously incorporating an antibody (E4G10) to target tumour vasculature. It was demonstrated that chelators 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) and desferrioxamine B (DFO B) increased solubility of the SWNT substantially so that biological application is feasible. Due to the high increase in blood vessel formation of tumour tissue, these tissues exhibit a high amount of structural error which includes expression of the monomeric vascular endothelial-cadherin epitope on epithelial cells to which the antibody E4G10 binds exclusively. The carbon tubes are therefore targeted to tumour vasculature by this antibody where the radioisotope is then subsequently delivered. For PET imaging zirconium-89 (^{89}Zr) is coupled to the SWNT and for therapy following imaging the ^{89}Zr can be substituted with alpha particle-emitting ^{225}Ac to deliver highly concentrated ionizing radiation. This system showed high efficacy and an acceptable safety profile in *in vivo* animal studies and is currently under investigation for clinical use [147]. Other tumour xenograft studies on carbon nanotubes coupled to radioisotopes involved copper-64 (^{64}Cu) and ^{111}Indium (^{111}In). In general, it was determined that carbon nanotubes clear rapidly from the blood pool to the target area and have ideal qualities as a drug carrier system for radiotheranostic agents [148, 149].

Gold nanoparticles have also been implemented to stabilize and allow the targeting of various isotopes including ^{64}Cu and iodine-125 (^{125}I). *In vivo* efficacy of alloyed gold nanoparticles containing ^{64}Cu was demonstrated for tumour cells in a mouse breast cancer model. Targeting of

radioimmuno-gold-nanoparticles to lung cancer tissue was achieved *in vivo* by the labelling of gold nanoparticles with the cetuximab antibody and ^{131}I. Additionally, gold nanoparticles can be excited through the surface plasmon resonance phenomenon to produce photo-thermal heating locally thereby destroying the cancer cells. The theranostic capabilities of a PET agent combined with gold nanoparticles will therefore allow for imaging of the accumulation of these particles at the tumour site and activation thereof at the most opportune moment for therapy. Animal xenograft studies have confirmed the potential of such an application [150-153]. Gold nanorods labelled with ^{125}I have been developed for the imaging of infection and inflammation but is yet to be applied as a theranostic agent [154]. Another study investigates the therapeutic application only (and not diagnostic) use of radioactive gold-198 (^{198}Au) nanoparticles for thermoablation [155]. This has been investigated for prostate tumours as well as liver neoplasms with success demonstrated *in vivo*. Al-Yasiri and colleagues developed a stable ^{198}Au nanoparticle containing mangiferin from phytochemical origin. This unique approach to a more environmentally conscious application of nanotechnology is worth mentioning since this allows the reduction of the gold precursor to nanoparticles without the use of additional toxic reducing agents. During the *in vivo* evaluation no toxicity was found in the animals and a 5-fold reduction in tumour volume over three weeks was demonstrated [156, 157]. Although gold is a very useful agent for thermoablation of cancer tumours, other types of nanoparticles have been developed for this application and will be discussed in the next section. It is important to note that the use of radioactive nanoparticles will come with unique challenges to the regulatory and safety measurements to be prescribed and taken during manufacturing. Not only will safety precautions exist regarding the toxicity due to the nuclear energy used, but also due to the unique properties (different biodistribution and additional toxicity) of nano-particles, further contributing to safety concerns. It might very well be that the process of creation of the necessary regulatory platforms with the capacity to monitor this technology might slow down the conversion thereof into clinical use.

Photo-thermal agents can convert externally applied electromagnetic radiation to release heat that destroys target cells in the vicinity of the molecule. The combination of such a nanoparticle with a radiopharmaceutical provides the ability to monitor the distribution inside the patient and monitor biodistribution before the external electromagnetic energy is applied. DeNardo and co-workers synthesized an iron oxide nanoparticle linked with a monoclonal antibody L6 analogue and ^{111}In for scintigraphy imaging as a breast cancer theranostic. Xenograft studies in mice showed accumulation at the target site as well as subsequent tumour growth delay, but acute animal deaths were present in some test groups [158]. For combination of PET with photothermal ablation therapy, Zhou and co-workers synthesized ^{64}Cu containing copper monosulfide nanoparticles. These nanoparticles are distributed by passive accumulation to the tumour site and absorb near-infrared light. *In vivo* efficacy was verified in glioblastoma xenografts [159]. It is

however notoriously difficult to convert results from xenograft animal models to human efficacy due to different blood diffusion rates and biodistribution to organs. It is therefore important that thorough clinical investigations are performed, and side-effects are reported meticulously before the technology is evaluated as unfeasible or indeed viable. Furthermore, since *in vivo* studies are the mainstay of preclinical evaluation, adverse-events or deaths in animals need to be serious indicators of failure, even though these might not be present in humans. Until better predictors of toxicity exist (especially for nano-technology), animal data are high indicators of the future of the invention under investigation.

3.2) Passive targeting

a) Hepatic tumor therapy

For application of selective internal radiation therapy (SIRT) in hepatocellular carcinoma, two types of microspheres are currently used in the clinical setting namely the TheraSphere® and SIR-Sphere®. The TheraSphere® was developed as an insoluble, damage and irradiation resistant carrier of yttrium-90 (^{90}Y) developed due to previously investigated resin-bead microspheres resulting in fatal adverse events. These were caused by the leaching of ^{90}Y out of the system and subsequent distribution to the bone marrow [160]. The TheraSpheres® are pre-manufactured with the ^{89}Yttrium captured in non-toxic glass microspheres (20-30 µm), which is bombarded post-manufacture with neutrons to produce ^{90}Y microspheres. This isotope is a 2 MeV beta exclusive emitter with a half-life of 2.7 days, making it ideal for local irradiation of tumours. Since external beam irradiation is insufficient in liver tumours, these spheres are injected intra-arterial and trapped in the vascular system of the tumour to deliver a high local dosage. The first investigations in animals (canines, rodents and rabbits) confirmed efficacy and tolerable side-effects but a system to deliver the TheraSpheres® whilst still in suspension had to be developed [160, 161]. TheraSpheres® were registered by the FDA under the Humanitarian Device Exemption Guidelines and extensive clinical trials before adoption in clinical practice was therefore not required. An exhaustive number of publications followed (with only some referenced in this article) regarding the safety and efficacy of this invention with the consensus that administration with adherence to proper protocol leads to a reasonable effective and non-toxic treatment option. Non-life threatening gastrointestinal complications, lymphoenia without clinical significance, pneumonitis, cholecystitis, acute pancreatitis, radiation dermatitis and gastro-intestinal ulceration was reported in some cases. These side-effects are also reported with resin ^{90}Y microspheres (SIR-Sphere®) and are related to the irradiation dosage received [162-168]. SIR-Spheres®, the second FDA registered ^{90}Y-sphere to reach the stage of clinical use, has a wider particle size distribution (20-60 µm) when compared to TheraSphere® technology and contains less activity (40-70 Bq per SIR-Sphere® compared to the 2500 Bq per TheraSphere®). These spheres are

non-degradable plastic resin spheres into which ^{90}Y is incorporated. The possible competitive edge for SIR-Spheres® are a lower activity dosage as well as lower specific gravity compared to the TheraSphere® but this does not clearly translate to more beneficence in clinical practice. The clinical results of SIR-Spheres® demonstrated efficacy and safety equal to TheraSpheres® and the system design allows for the effective containment of ^{90}Y with no leaching [169-172]. Considering the limited options available in the treatment of hepatic malignancy, which is an extremely aggressive cancer, radioembolization with either type of ^{90}Y microbeads are a viable choice of agent in the clinical setting. The urgency for the provision of a treatment of this cancer contributed to the fast tracking of the use of this technology in the clinical environment where other applications of these novel drug delivery strategies still have failed to be converted from preclinical investigations to the market. In applications where other treatment options (although not optimal) is available, a more rigorous testing is required along with the presence of only very mild side-effects allowed and great clinical effect, which lead to the failure of many new systems investigated.

b) Alpha particle therapy

Alpha-particle emitters are potent cytotoxic agents (due to high linear energy transfer) with a DNA damage capacity far greater than that of beta emitters. Furthermore, the damage to healthy cells can be limited if the alpha emitter is successfully targeted to tumour tissue due to the small radiation distance exhibited by these agents. Although clinical trials with alpha-particle emitting agents have given beneficial results, clinical use is limited by the shortage of the radionuclide supply [173, 174]. Research is currently being done on the development of a liposome containing actinium-225 (^{225}Ac) but no animal evaluations or human trails seem to have been published at this stage. The adaptability of liposomes to hone in on the tissue to be targeted may very well be the key to make therapy with alpha-particle emitters viable [175- 177]. Liposomes have a natural tendency to accumulate in tumours based on their specific size and it is therefore possible to fine-tune this system to deliver alpha emissions without collateral damage. ^{225}Ac was successfully incorporated into lanthanum phosphate nanoparticles (linked to monoclonal antibodies for targeting) and *in vivo* targeting efficacy was established. The system was further optimized to reduce the leakage of daughter isotopes and reduce possible toxicity [178, 179]. Since a preparation containing radium-223 (^{223}Ra) was approved by the FDA (Xofigo®), the optimization of this alpha-emitter is under investigation. This isotope demonstrates distinct advantages over ^{225}Ac such as higher availability (a $^{227}Ac/^{223}Ra$ generator is more accessible) as well as a lower amount of leakage of daughter isotopes due to the more intrinsic stable ^{223}Ra. Current research regarding incorporation of this isotope in drug delivery systems is focused on developing a stable complex and no *in vitro* evaluations are available at this stage. Systems under evaluation include iron oxide nanoparticles, nanozeolite bioconjugates, hydroxyapatite particles and lanthanum

phosphate nanoparticles [180-184]. Many techniques and substances (if biocompatible) for use in nanomedicine can be borrowed from other areas of science and there is a myriad of possible fully developed materials to apply to the development of novel pharmaceuticals. According to the literature, various techniques and materials can be used to coat nanoparticles and stabilise these systems to stop leakage of the entrapped alpha-emitter from the drug delivery system. The decay of ^{225}Ac leads to concern about its usefulness due to the recoil energy (Szilard Chalmers effect): the amount of energy produced during decay may cause a severance in the metal-ligand bonding and destroy the bioconjugate used to target this isotope. The usability of a multi-shell nanoparticle, to contain the isotope and daughter nuclides, while targeting the nanoparticle with other moieties attached to it has been evaluated [189]. Astatine-211 is another very attractive alpha-particle emitter than may be used for this type of therapy, although no drug delivery systems have applied this particle. This might be a useful future line of research [190].

c) Targeted imaging

Vescan® was an agent developed in the 1980s for the imaging of tumours by entrapping ^{111}In in liposomes. This agent was proven effective in various clinical trials including a Phase II and Phase III trial but was never commercialized [185-188]. Stealth liposomes containing ^{111}In was also investigated clinically in human trials with positive results, indicating accumulation at tumor sites and reduced accumulation by the reticuloendothelial system [191, 192].

To change the pharmacokinetic properties of ^{99m}Tc-MIBI (^{99m}Tc-hexakis-2-methoxyisobutyl-isonitrile) to allow for tumour imaging, Belhaj-Thayeb and colleagues entrapped it in PEG-liposomes. During an *in vivo* biodistribution studie in xenografted mice, a higher accumulation of the tracer was present in tumors as well as longer retention of the tracer in blood circulation and it is a feasible option for the imaging of cancer lesions [193]. The linking of liposomes with pure pertechnetate was also investigated in animal models and avoidance of the reticuloendothelial system was accomplished with the adaptation of the surface charge of particles [194]. Incorporating ^{99m}Tc in a nanosystem comprising of folated-γ-glutamic acid and chitosan had positive effects on intratumoral applications in an *in vivo* study [195]. Liposomes linked to vasoactive intestinal peptide (expressed in breast cancer) also showed a higher accumulation of their entrapped ^{99m}Tc-HMPOA (99mTc-hexamethylpropyleneamineoxime) in cancer xenografts [196]. To predict the efficacy of therapy by liposomes containing chemotherapeutic drugs (for example Doxil®), Kleiter and co-workers evaluated the effects of co-administered ^{99m}Tc-liposomes. It was verified that both types of liposomes accumulated similarly without having any effect on one another and this is therefore a valid method for prediction of therapy outcomes [197]. The pre-administration of a labelled liposome might therefore afford the same qualities as

theranostic agents in the case of Doxil® therapy success prediction and the provision of possible side-effect expectations.

For optimization of PET imaging various systems are being researched in literature. Long-circulating nanoparticles containing ^{64}Cu in a cross linked system was functionalized with folate and this system demonstrated passive accumulation, albeit hindered by extensive sequestration by the reticuloendothelial system [198]. Liposomes have been developed to target ^{64}Cu to neuroendocrine tumours, breast cancer lesions, head and neck cancer with all these systems demonstrating promising enhancement of accumulation in tumour tissue *in vivo* [199-201]. Liposomes containing ^{67}Ga was developed with surface charge variations by Ogihara and colleagues and it was shown that these systems can differentiate between tumours and inflammation during *in vivo* evaluations in xenografted mice. Various modifications were made to enhance tumour delivery but these liposomes never translated to clinical use [202-204].

The use of liposomes in tumour imaging has been impeded by the slow clearance of these drug carriers from the blood circulation resulting in a high background noise during imaging. A method was developed to remove liposomes from circulation once tumour accumulation is sufficient. This system is based on the strong affinity of biotin to bind to avidin. The radiotracer containing liposomes were functionalized with biotin and upon administration of avidin 2 hours later, all residual liposomes were collected and removed from the circulation by the reticuloendothelial system, providing a neat solution to reduce background interference [205, 206].

4) Enhancing pharmacokinetics

A more traditional use of drug carrier systems in the pharmaceutical industry is to harness the ability of these systems to allow for the selective accumulation of entities at the site of action or by a registered event. This phenomenon can be useful when information on a certain biological process needs to be imaged with nuclear imaging. The drug delivery system may therefore enhance accumulation at the site of action (e.g. the tumour), be involved in a certain biochemical process (e.g. lipid metabolism) and reduce adverse events due to less exposure of sites outside the region of interest.

4.1) Extend blood circulation time

a) Diagnose infection or inflammation

The differentiation between infection and sterile inflammation is always a conundrum in the clinical setting. Nuclear imaging plays an integral role in the localization of these lesions as well as distinguishing between the two disease processes. When the cause of the lesion is found and the

position or absence of distant metastatic lesions is known, the physician can prescribe the most appropriate therapy for the particular manifestation [207].

A first-in human-study determined that ^{99m}Tc labelled liposomes with a negatively charged surface demonstrates a tendency to localize at inflammation sites caused by rheumatoid arthritis. No side-effects were experienced by the test subjects after a slow intravenous injection. This system uses the normally unwanted effect of reticuloendothelial system to accumulate liposomes due to a higher presence of these cells in the inflamed synovial tissue. The same research group performed a phase I clinical trial for these liposomes on infections and joint abscesses, with similar positive results. It is therefore important to note that this system does not distinguish between infection and sterile inflammation [208-210]. Dams and co-workers also verified the efficacy of PEG liposomes labelled with ^{99m}Tc to visualize infection and inflammation, establishing this system as an effective imaging agent with attractive safety and supply characteristics which proved effective in human trails. However, again no distinction between infection and inflammation exists [211]. A first-in-human trial evaluating ^{99m}Tc-PEG-liposomes for imaging inflammation due to Chron's disease showed efficacy, but side-effects caused the termination of the investigation. Side-effects noted was dyspnoea and facial erythema upon administration of the tracer intravenously. Modification of the system is needed before further application can take place [212].

Various animal studies are published on the visualization of infection in animal models by ^{99m}Tc or ^{111}Indium containing liposomes without determining the selectivity for infection imaging or inflammation. All studies demonstrate efficacy in localization of infections sites [213-216]. Various research groups evaluated the efficacy in sterile inflammation but not infection [217, 218]. One group did determine that liposomes do target infection and inflammation with equal efficacy. These liposomes therefore do provide an alternative for leukocyte scintigraphy in infection but lack selective discrimination between disease processes due to the generic nature of targeting processes present in both inflammation and infection [217, 218]. Macrophage targeting dextran nanoparticles containing ^{18}F and ^{89}Zr also provided *in vivo* efficacy to detect local inflammation [221, 222].

In an effort to develop nuclear imaging agents that distinguish between bone infection as opposed to aseptic bone inflammation, Ferreira and co-workers linked ceftizoxime to long-circulating pH-sensitive liposomes, since this antibiotic has previously been proven to target infection and not inflammation. The broad-spectrum antibiotic properties of this molecule were harnessed in this liposome system to target bacterial cell walls and accumulate by preference at areas of infection. Selectivity for infection was ascertained *in vivo* in rats and was subsequently further enhanced by performing two imaging procedures, 8 hours apart [223]. It is important that targeting strategies

such as these, which focus on disease specific mechanisms, are further developed to provide agents that effectively distinguish between infection and inflammation.

b) Blood pool imaging

Liposomes linked to ^{99m}Tc were developed as blood-pool imaging agents that provide a system that does not rely on the injection of radiolabeled red blood cells. These liposomes did show good stability and prolonged staying time in the circulation and modification with PEG ensured that liver accumulation is reduced [224, 225]. Prolonged circulation half-life was found for stealth liposomes and such liposomes can therefore be very appropriate as blood pool imaging agents.

4.2) Change movement across biological barriers

a) Brain imaging

Although ^{18}F-deoxyfluoroglucose (^{18}F-FDG) is currently used as the standard imaging agent for visualization of brain tumours, controversy surrounds its usefulness, since brain tissue (like tumours) exhibits high glucose consumption. A search for new agents that might prove more accurate and useful is therefore on-going. Liposomes that contain 1-[^{18}F]fluoro-3,6-dioxatetracosane exhibit a higher contrast *in vivo* during the imaging of brain glioma compared to that of the gold standard ^{18}F-FDG. The liposomes did not distribute to normal brain tissue due to an inability to cross the blood-brain barrier, but even the smallest tumour lesion was picked up with this liposomal tracer [226].

A novel application of liposomal ^{18}F would be the imaging of synaptic density in the brain by targeting voltage-dependent calcium channels (N-type Ca^{2+}). Neurodegeneration and evaluation of cognitive function can be monitored through this tracer during therapy. A derivative of ω-conotoxin is linked to ^{18}F to form K24-{18F]-GVIA, a tracer that has affinity for $Ca2^{+}$ channels. This tracer has been successfully entrapped in liposomes. The liposomes were modified to enhance brain penetration by incorporation of cerebroside 3-sulfate – a constituent of myelin sheath tissue. The viability of this research needs to be proven *in vivo* and clinically, but this is a good example of the versatility of modifiable liposomes in targeting different regions of interest [227].

It is possible to label whole blood with ^{15}oxygen-oxygen (^{15}O) to measure the oxygen consumption in humans, but this has the propensity to damage red blood cell function and necessitate removal of the protein fraction by the washing of red blood cells. Tiwari and colleagues are proposing the use of liposomes containing haemoglobin labelled with ^{15}O-oxygen to be injected as a type of surrogate red-blood cell for the measurement of brain oxygen consumption. This method provides

a tracer that effectively measures the cerebral metabolic rate of oxygen consumption in rats and may have further applications in the clinical setting [228]. It is however important to note that the half-life of ^{15}O is extremely short (2.04 minutes) and restrictions therefore exist regarding clinical usage [229]. Furthermore, if this isotope is to be used effectively for imaging, the labelling process needs to be extremely effective due to time constraints and “afterloading” of the liposome post-manufacture is the only viable option.

b) Oral radiopharmaceutics

In order to allow for the administration of radiotracers by the oral administration route rather than the traditional intravenous route, Grobler and Zeevaart patented a formulation that entraps a radiotracer of choice in Pheroid® technology. The system is a nano- or micro-emulsion and Pheroid® vesicles possess a lipid bilayer but cannot be grouped under the traditional liposomes due to lack of a phospholipid or cholesterol component. While the unformulated ^{99m}Tc-MDP (^{99m}Tc-methyl diphosphonate) radiotracer offered no absorption out of the gastro-intestinal tract during the *in vivo* rodent evaluations, the ^{99m}Tc-MDP formulated in Pheroid® provided blood circulation levels comparable to that obtained after the gold standard ^{99m}Tc-MDP intravenous injections. This technology may enable the oral administration of radiotracers that can reduce stress in children, patients receiving multiple nuclear imaging procedures, patients that fears needles and patients with collapsed arteries. Clinical evaluation still needs to be performed [9]. The Pheroid® employs passive entrapment with a very high rate of success compared to normal liposomes. Active entrapment is however another possibility that can be explored to enhance clinical efficacy.

5) Increase stability

5.1) Protect radiotracers against degradations

Certain peptides are unstable when circulating in body fluids. For instance, the use of bombesin, which binds to gastrin-releasing peptide receptors that are overexpressed in certain tumours, is hampered by the susceptibility of this peptide to destruction by biological fluids. To utilize ^{99m}Tc-linked bombesin as a radiotracer, De Barros and colleagues formulated long circulating liposomes containing this pair and demonstrated the applicability of these systems to imaging breast cancer tumours *in vivo*. Drug carrier systems can therefore provide additional protection of sensitive proteins and enable their use as tracers for nuclear imaging [230].

6) Discussion

6.1) Current challenges

Exciting preclinical work in this field rarely seems to reach clinical translation and this is a concerning trend (refer to table 2). Therefore, the most important current challenge is the translation of the vast amount of *in vivo* successes in animal models to clinical use. Similar alarm is expressed in other fields where drug carrier systems are applied as is evident in literature reviews on non-nuclear applications. Some of the reasons provided in literature are the difficulty of up-scaling complex manufacturing processes associated with these systems, additional costs of quality assurance, resistance offered by government regulatory boards, lengthy registration processes as well as intellectual property challenges [231-234]. It is our opinion that a lack of understanding of the complex interactions of these novel technologies with the biological environment and incorporated nuclear agents also leads to under optimization, with the result that these systems fall short of their potential.

Table 2: A summary of the different stages of the drug development pipeline that nuclear medicine applications of drug delivery systems discussed in this review.

	Therapeutic NM	Theranostics	Multimodality imaging	Enhancement of pharmacokinetics
Develop-ment phase	① ^{225}Ac liposomes (alpha emitter) [175-17] ②^{223}Ra nanoparticles (alpha emitter) [180-184] ③ Radioimmunotherapy [59-61]			
Preclinical phase	① ^{225}Ac liposomes (alpha emitter) [178-179]	① ^{188}Re nanoparticles (beta & gamma emitter) [81-82] ②^{188}Re microspheres (beta & gamma emitter) [83-84] ③ Radiochemotherapeutics [132-139] ④ ^{99m}Tc anti-TB rifampicin & ofloxacin [140] ⑤ Thermal ablation gold nanoparticles with ^{64}Cu and ^{125}I [150-157] ⑥ Multimodal imaging theranostics [110-114]	①Liposomes for PET/MRI [80-] ②Nanoparticles for PET/MRI [84-88] ③ Nanoparticles for SPECT/MRI [89-93] ④ Nanoparticles for PET/optical [93-98] ⑤ Liposomes for PET/optical [99] ⑥ Micelles for PET/optical [100-102] ⑦ Nanoparticles for SPECT/optical [103] ⑧ SLN imaging nanoparticles [115,117] ⑨ SLN imaging liposomes [116] ⑩ More than 2 modality imaging [118-126]	①^{18}F brain imaging [226-227] ②^{99m}Tc infection and inflammation imaging [213-220] ③Nanoparticles PET imaging infection and inflammation [221-222] ④Distinguishing inflammation from infection [223] ⑤^{99m}Tc liposome blood pool imaging [224-225] ⑥Increased stability of labelled tracer with liposomes [230] ⑨PET tracer targeting with nanoparticles [218] ⑩Oral administration of tracers [9]
Clinical trial phase		①^{188}Re-BMEDA liposomes [41-60] ② ^{89}Zr/^{225}Ac nanoparticles [147]		① Targeting tracers with stealth liposomes [208-212]
Clinical use	① ^{90}Y TheraSpheres [160-161] or ^{90}Y SIR-Spheres (beta emitter) [162-172]			

Abbreviations: ^{225}Ac: actinium-225; ^{223}Ra: radium-223; ^{186}Re: rhenium-186; ^{188}Re: rhenium-188 ^{99m}Tc: technetium-99m ; TB: tuberculosis; ^{18}F: fluorine-18; PET: Positron Emission Tomography; SPECT: Single Photon Emission Computed Tomography, MRI: Magnetic Resonance Imaging; ^{89}Zr: Zirconium-89; SNL: Sentinel Lymph Node; ^{90}Y: yttrium-90.

6.2) Limitations to successful application of drug delivery systems to nuclear medicine

Although the vast amount of preclinical work did not directly translate to clinical usability, the literature produced contributes to the broad understanding of these systems by the scientific community. To define the ideal characteristics of a drug carrier system in the field of nuclear medicine, it is worth to consider both the limitations dictated by the radiotracer as well as those contributed by the characteristics of the drug carrier system itself. We therefore propose some limitations for an ideal drug carrier system if it is to be utilized to deliver radiotracers.

Particle size

- The drug carrier system size needs to be restricted to below 400 nm otherwise the system will be removed from the blood circulation by the reticular endothelial systems before it can accumulate at the target site [235]. Other systems can be utilized to circumvent the reticular endothelial system (e.g. negatively charged liposomes) for larger entities.
- If, however the enhanced permeability and retention effect (EPR) associated with tumour vasculature, inflammation and other lesions is to be used for passive accumulation, macromolecules with high molecular weights (sized between 10-500 nm and restricted below 40 kDa) should be used and the drug delivery system should contribute to increasing the size of the radiotracer [236].

Stability

- The system should be stable and not leach isotope at non-target areas. Rapid release (also called “burst release”) is unacceptable and the carrier system should only release its load at the target site [237].
- The drug carrier system and the radiopharmaceutical should be coordinated in such a way that the radiotracer is delivered at the target site in a manner that allows accumulation of the required amount to allow imaging/therapeutic activity within the constraints of the decay half-life of the isotope in question.
- The drug delivery system should be stable in the blood circulation and not aggregate since particles larger than 5 μm can cause emboli and cardiovascular events.
- When the drug carrier system reaches the target site, the radiotracer should be released efficiently and effectively to ensure high image quality or therapeutic activity. If the drug carrier system fails to release the tracer upon arrival at the region of interest, the whole aim of the system fails [238].

Surface charge

- A negative surface charge of particles reduces removal by the reticular endothelial systems and this should therefore be considered during the development of the drug carrier system [239, 240]
- If the surface charge is overtly negative they can have the tendency to accumulate in the liver. It is therefore important to strike a balance between the surface charge and the excretion functions of the body [240].

Physiological acceptability

- In active targeting, the ligands (e.g. antibodies) used to target the drug carrier system and entrapped radiotracer should not illicit adverse events in the biological system.
- The components or ingredients of the drug carrier system should be Generally Regarded as Safe (GRAS) compounds.
- The drug carrier system should not increase the toxicity of the radiotracer due to changes in biodistribution. An example of increased side-effects in conventional pharmaceuticals is the additional side-effect of hand-foot syndrome when doxorubicin is delivered with liposomes. This was attributed to the extended circulation of the drug in the bloodstream resulting in a higher delivery of the drug to the skin surface [241].
- Drug delivery vehicles that failed to deliver their radiotracer load at the target site should be removed and excreted effectively since undelivered tracer can reduce image quality or expose the patient to higher radiation levels.
- Combination of two entities (e.g. radiotracer and a chemotherapeutic agent) should be compatible.
- A complete understanding of the drug carrier system's biodistribution, toxicity and pharmacokinetics is essential [242]. Furthermore, the available modifications to the system as well as the biological effects should be quantified so that the system can be tailor made for the radiotracer that needs to be delivered. Before the basic characterization of the drug delivery system is not complete, it should not be utilized for clinical application.
- Biological constraints imposed by the target site should be kept in mind when formulating radiotracers into drug carrier systems. For example, if the blood brain barrier needs to be crossed, the drug delivery system should comply with the specifications that will allow it to carry the radiotracer across this biological barrier [243].
- Radiotracer pharmacokinetics and decay rates need to be compatible with the drug delivery system and the biological effect required [238].

6.3) Future perspectives of drug delivery systems in nuclear medicine

Since the time Ehrlich famously kicked off the search for the “magic bullet” to treat disease effectively and efficiently, the progress of drug delivery systems can be described as slow but sure. From the literature it is clear in that there is a general disillusionment regarding the outcomes obtained from liposome research when the efforts and economic investments in this area of research are evaluated. The delivery of pharmaceutical active ingredients by nanoparticles also lacks clinical translation with the failure of most of these agents to accumulate at the target site in therapeutic quantities and not distribute to other tissue [244-246]. It is however important to realize that every failed development does still contribute to an understanding of these systems and which characteristics do not contribute to biological effectiveness.

Cell mediated drug delivery is a promising area of drug delivery systems that might be investigated in the future for the delivery of radiopharmaceuticals. These systems use specific cells loaded with the pharmaceutical (or in this case radiotracer) and since the cells retain their function, their distribution will mimic their natural movement to disease areas. These carrier systems have advantages over artificially produced systems including well known biodistribution behaviours, reduced side effects as well as reduced clearance by the reticular endothelial systems [247].

The funding for nanotechnology products for all applications is ample with the National Nanotechnology Initiative of the United States contributing $1.5 billion per year and the private sector even more than that [248]. Although this budget is targeted at research and development in general (with $400 million dollars dedicated to health research in 2011), the investigation of the properties of these technologies can be applied not only to basic research, but also be translated to the health sector [249]. Currently this field progress through trial and error and with the vast amounts of literature regarding the behaviour of these systems as well as economic investment therein the future does look promising. It is therefore not a question of “if”, but rather of “when” the rewards for the investment and research will start to provide benefits in quick succession.

7) Conclusion

Drug delivery systems have great potential when applied to nuclear medicine and will most probably be used to solve some of the important hurdles that are faced by this field. It is however important that the drug delivery system that is applied to a specific clinical use should be characterized in depth. The parameters that can be customized to suit the unique needs of the radiopharmaceutical in question and what type of biological behaviour can be expected from the drug carrier system when administered must be known. This will propel the preclinical research into more clinical successes in the future.

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3.7 Summary of this chapter

The Pheroid® delivery system provided a substantial change in the pharmacokinetics of the ^{99m}Technetium radiotracers when evaluated in rodent *in vivo* studies. The literature review of the current state of nuclear medicine demonstrated clear gaps in this field that can be addressed by the application of drug carrier systems. It was therefore decided to progress into first-in-human clinical trials and evaluates the Pheroid® system as a vehicle for oral delivery of radiopharmaceuticals.

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CHAPTER 4: CLINICAL INVESTIGATION OF AN ORAL ^{99M}TC-MDP IN PHEROID®

This chapter describes the application of Pheroid® technology to the area of nuclear medicine within the context of the quality system(s) used and the legislations governing clinical trials. The clinical trial protocol followed during this investigation in accordance with the principles of Good Clinical Practice (GCP), the manufacturing of the formulation administered in compliance with Good Manufacturing Practice (GMP), and the results are described and discussed. Less obvious, the principles of Good Laboratory Practice (GLP) are relevant during the preclinical characterization of therapies and in the interpretation of results obtained. Whilst no therapy should progress to the clinical trial stage unless supported by preclinical evidence performed according to GLP, specific attention is accorded to GCP in the section below.

The Good Practices are set standards that provides guidelines proceeding, during and after clinical trials that involve human participants. GCP standards ensure that the human participants' rights and health are protected and that the data generated is transparent and trustworthy (Dongen, 2001). The International Conference on Harmonization (ICH) provides the following definition of GCP (ICH, 1996):

> "*Good Clinical Practice is an international ethical and scientific quality standard for designing, conducting, recording and reporting trials that involve the participation of human subjects. Compliance with this standard provides public assurance that the rights, safety and well-being of trial subjects are protected, consistent with the principles that have their origin in the Declaration of Helsinki, and that clinical trial data are credible."*

GCP is an example of learning from mistakes and evolved from incidents that demonstrated the need for clearer guidelines whereby the procedures during clinical trials are regulated. These guidelines are constantly revised to adapt to the progress made in the science that it governs.

4.1 Are clinical trials ethical or scientific?

When the history of the testing of medicine is regarded, it is often asked whether clinical trials are necessary and ethical and whether such trials do not place the study participants in harm's way. In his exposé on clinical trials, Passamani (1991) argues that any test on human subjects can be performed in an unethical manner and that the regulations governing these trials should ensure that they are carried out in an ethical and scientifically sound manner. The improvement of these regulations for the treatment and prevention of disease is seen as the duty of scientists.

Passamani proves that observational methods relying on the monitoring of patients in an uncontrolled manner are unethical and ineffective. It is in the poor planning, inadequate documenting and compromising of the rights of study participants that the ethicality of clinical trials becomes tainted (Passamani, 1991). Clinical trials that adhere to Good Clinical Practice (GCP) principle provide the soundest results, both ethically and scientifically. Although the implementation of GCP is contributing to the ethicality of clinical trials, legislation is still of evolving through the identification of possible unethical practices and the adaptation of guidelines. The focus is currently on the registration and reporting of clinical trial results – with the emphasis on the reporting of negative data. Failure to report data can affect the body of scientific knowledge as well as potentially distort future regulatory and health decisions (Moorthy *et al.,* 2015).

As to whether clinical trials can be seen as part of science, it must be evaluated if clinical trials incorporates the basic concepts of science. Most definitions of science include systematic study and observation and experiment. It is therefore quite clear that clinical trials form part of the empirical field of scientific medicine. The following basic concepts of scientific method are built in to the clinical trial process (Piantadosi, 2005):

- Performing perception or measurement with instruments.
- Repeatability and quantification.
- Recording to facilitate referencing and defence of results.
- Control of extraneous factors (e.g. control groups and the reducing of bias).
- Providing a complete report for external evaluation, verification or disproof.

It is important that trial participants are not influenced to participate in the clinical trial in an unethical manner and it is prescribed as such by the International GCP guidelines. Participants will however inevitably be influenced to participate by factors outside the control of the study team. It is important to plan the study to allow prompt and adequate enrolment, but still to the benefit of the participants (Passamani, 1991).

4.2 The evolution of Good Clinical Practise

The first acknowledgement of the rights of the hospitalised patient was issued by the government of Prussia in 1891 by stating that no patient may receive treatment with tuberculin for tuberculosis against their will (Berghammer, 2014). The movement to start regulating pharmaceuticals (along with the food industry) and the manufacturing thereof was a result of the publication of a book (*The Jungle)* written by Upton Sinclair, published in 1905. This book described the unethical practices in the meat packing industry where ground meat was contaminated with rotten or diseased meat and sometimes even the remains of ill-fated workers who fell in the machinery.

Public uproar moved the American Congress to pass the Pure Food and Drug Act in 1906, which ordered the truthful labelling of all products including pharmaceuticals. This Act led to the formation of the Bureau of Chemistry, U.S. Department of Agriculture, later to be renamed as the Food and Drug Administration (FDA) (Immel, 2001).

In 1931 the Weimar Republic introduced the Guidelines for New Therapy and Human Experimentation that defined the difference between therapeutic and non-therapeutic research. These guidelines were the first to mention the concept of informed consent. The main reason for the development of these guidelines were the shock expressed by the public after learning of some experiments that was undertaken on humans at that time. An example of such research was committed by Neisser and his co-workers who experimented on a cure for syphilis. During this study the serum from infected patients was injected in patients hospitalized for other reasons and some of these vaccinated patients contracted the disease. The Guidelines for New Therapy and Human Experimentation further prohibited experimentation on dying patients due to their vulnerability; this was the first statement in clinical trial guidelines that some groups of patients are more vulnerable in terms of informed consent (Berghammer, 2014).

In 1935 a manufacturer of sulphanilamide used diethylene glycol as an additive without determining its toxicity and this led to the death of 107 people. This incident led to the Federal Food, Drug and Cosmetic Act (1938) forcing companies to test products for safety before selling them to the public. Another such incident was the production of sulpha-drugs contaminated by phenobarbital that injured 300 people resulting in many fatalities. The regulation of GMP was established to ensure safe manufacturing (Immel, 2001).

One of the most notable cases of failure to regulate pharmaceuticals was the Thalidomide Incident in the 1960s. Thalidomide was indicated for the treatment of morning sickness, but it demonstrated teratogenicity and caused deformities in approximately 10 000 children in Europe. Legislation was instated that required medicine to be tested on animals (preclinical studies) before administration to humans. The informed consent of participants and the responsibility of investigators to report adverse events to the FDA were also introduced. In 1979 the regulation of GLP was finalised to govern the laboratory tests that were used to confirm whether tested drugs were safe for use in humans (Immel, 2001; Berghammer, 2014).

After the Second World War once again the exposure of the horrifying experiments conducted on prisoners in the Nazi concentration camps under Hitler's rule led to the promulgation of new regulations. All these experiments were performed on vulnerable people without their consent. The medical professionals standing trial argued that no legislation was in place to stop experimentation on humans and this was rectified by the Nuremberg Code in 1947 (Hardicre,

2014; Berghammer, 2014). The following principles were proposed by the Nuremberg Code (Hardicre, 2014):

- The obtainment of consent that is voluntary and informed.
- Research on humans should be necessary for the good of society and not pointless and available by other means.
- Research on humans should be based on prior knowledge of the disease (e.g. animal research).
- The research method should avoid pointless physical or mental harm.
- No act should be done if there is a prior reason to predict death or disability.
- The degree of risk taken should not exceed the importance of the problem solved.
- Proper provision should be made to protect the participant against risks of injury, disability or death.
- Experiments should be conducted by scientifically qualified persons.
- The participant may withdraw at any stage.
- The scientist in charge must be prepared to terminate the study due to probable injury, disability or death associated with continuation of the study.

Another study that necessitated amendments to the GCP principles was the Tuskegee Syphilis study on participants from poor socio-economic backgrounds. The study participants were not provided with information on the disease they were carrying and had to provide blood samples and received placebo treatment. When penicillin became available in 1947 as an effective treatment for syphilis, the study participants did not receive the new treatment and were not informed of the availability thereof since the researchers still wanted to determine the progression of the untreated disease. This failure to provide treatment led to the death of 128 of the study participants. In answer to these incidents the World Medical Association published the Declaration of Helsinki, which was later adopted by the International Conference of Harmonization (ICH) (Hardicre, 2014). The general principles of this declaration are (WMA, 2013):

- The physician must protect the health of the patient.
- Research on medical substances must include human subjects and these interventions must be evaluated continually for safety, effectiveness, efficiency, accessibility and quality.
- Science may never take precedence over the rights of the participants.
- Ethical, legal and regulatory norms for research, nationally and internationally, must be abided to.

- Research must be scientific and ethical and under the supervision of qualified investigators.
- Study animals and the environment must be protected.
- Underrepresented groups in medical research should be provided access to participate.
- Medical care and medical research may be combined.
- The compensation and treatment of participants harmed in research must be ensured.

The Declaration of Helsinki also describes the risk benefit analysis principle*, i.e.* that the importance of the research should outweigh the risks. Procedures should also be in place to reduce risks and no physician should perform research if doubts exist about adequate risk management (WMA, 2013).

Equally unethical was the Willowbrook Study performed on mentally disabled children. The aim of the Willowbrook Study was to evaluate the difference between untreated hepatitis and the effects of gamma globulin treatment. There were no other institutions available to care for these children and the school only enrolled children if their parents signed consent to allow their participation in the study. Uninfected children were inoculated with hepatitis, and although this study provided a vaccine for this disease, it violated the rights of these mentally ill children. During the Jewish Chronic Disease Hospital Study in 1965, 22 bedridden patients were injected with cancer cells, without their consent, to determine if their low immunity would allow the formation of tumours (Piantadosi, 2005, Hardicre, 2014). These two incidents led to the inclusion of additional principles regarding vulnerable individuals in the Declaration of Helsinki (WMA, 2013).

- All vulnerable participants should receive special protection.
- Medical research may only be done on vulnerable groups if characteristics deem that the research cannot not be carried out on a non-vulnerable group.
- There must be a high likelihood that this research group receives benefits from the research results.

The Declaration of Helsinki states that the subjects must all be volunteers and that they must be adequately informed of the whole of the study and then provide consent. The informed consent must state why the study is being done, what procedures will be done during the trial, the duration of the trial, the aim of the trail, benefits expected, risks involved and other treatments available. The Declaration of Helsinki also provides clear guidelines for obtaining consent if the research involves minors or mentally incompetent subjects. The patient-physician relationship must not be affected by the participant providing consent or refusal to participate in the study and a clearly defined line should exist between areas of care related to the study and that which is not related (Robertson & Gan, 2001, WMA, 2013).

If an effective intervention is established during the clinical trial, the sponsors of the trial must provide participants with access to these interventions. The Declaration of Helsinki prescribes that all research should be registered on a database that is accessible to the public and that both positive and negative results should be published (WMA, 2013).

In 1974 the National Research Act was instated in America. This act prescribes that all research where human subjects are involved should be approved by Institutional Review Boards (IRBs). The Belmont Report in 1979 provided additional basic ethical principles namely respect for people, beneficence and justice. Additional guidelines were contributed by the Council for International Organizations of Medical Science to provide for the application of the Nuremberg Code and the Declaration of Helsinki. All of these principles were incorporated in the Good Clinical Practice Guidelines of the International Conference of Harmonization (Piantadosi, 2005).

4.3 Current guidelines: The International Conference of Harmonization and Good Clinical Practice

The following 14 principles were adopted by the ICH-GCP (ICH, 1996; Vijaynanthan & Nawawi, 2008):

1. The ethical principles that originated in the Declaration of Helsinki and are consistent with regulations and GCP should be adhered to.
2. All the predicted risks should be weighed against the benefits (subject and society) before the trial starts, and benefits should outweigh the risks.
3. The rights, safety and health of participants should prevail over benefits to society and science.
4. Nonclinical and clinical information on the investigational product should be adequate.
5. A detailed protocol that is scientifically sound, describing the trial, should be available before the start of the clinical trial.
6. The protocol should be reviewed by the Institutional Review Board or Independent Ethics Committee (IEC) and approved before the start of the trial.
7. A qualified physician should always decide over the medical care given to study participants.
8. All persons involved in conducting a clinical trial should be appropriately qualified.
9. Voluntary informed consent should be obtained from every study participant before allowing them to enrol in the trial.
10. All data generated in the trial should be recorded and stored to ensure accurate reporting, interpretation and verification.
11. Confidentiality of the patient should be protected including their medical records.

12. Investigational products should be manufactured according to Good Manufacturing Practices.
13. Quality monitoring should be done on every aspect of the clinical trial,

As with any guidelines, criticism towards GCP can be found in literature. A deficiency that is raised is that these guidelines were developed through informal consensus and not through evidence-based research processes. This could lead to guidelines that may not be as effective in ensuring ethical research in practice as was hoped. The cost of administration to maintain the vast amount of documentation necessary to comply, especially during multi-site trials involving large patient groups, increases the cost of research. During the development of these guidelines the intention was mainly to provide procedures aimed at the industry and these guidelines do not necessarily provide for supplementary research originating from academic centres. Another concern raised is that the focus on written records should not reduce the focus on the actual protection of the human participant. It is proposed that ICH-GCP still only provides a general framework for ethical research and that the individual still fills in the gaps based on their own ethical framework. The solution put forward is that the guidelines should be updated often to adapt to the fast-paced research environment. Care should be taken that these guidelines remain up to date, scientifically sound, flexible and simple to implement (Shekelle *et al.*, 1999, Grimes *et al.*, 2005; Vijaynanthan & Nawawi, 2008).

4.4 GCP in the context of South Africa

Due to its unique combination of an adequate infrastructure (research and health) coupled with a high disease burden, South Africa is a popular setting for clinical trials. Furthermore, the diversity found in the South African population regarding socio-economic status, educational status, social development status and cultural differences make for a rich environment to study environmental influences on disease. The South African population should be protected against exploitation either intentionally or non-intentionally (researchers not familiar with the environment) with additional GCP guidelines. Additional to the ICH-GCP, the South African GCP (SA-GCP) states that minors, women, people with disabilities, substance abusers, prisoners, people in dependent relationships (aged, students, employees, patients) and patients highly dependent on medical care are vulnerable and should receive added protection. Research fields that require extra care are the study of indigenous medical systems, innovative therapy or interventions and emergency care. Precaution should also be taken when groups with a collective quality are studied to respect their traditional beliefs, leadership structure and social structures. The limited economic development and lack of availability of health care should not be exploited in vulnerable communities. Research on HIV receives special mention in the SA-GCP concerning the handling of post-trial therapy, withdrawal of subjects and the confidentiality regarding their HIV status. The

Principle Investigator must be of South African nationality to ensure an understanding of the unique context the research is conducted in. The informed consent should also be provided in the language of the study participant and the level of education of the participant should be considered to ensure full understanding of the information provided. The National Health Research Ethics Council (NHREC) was established in 2006 (DOH-SA, 2006, DOH-SA, 2015).

A study by Burgess and co-workers (2009) evaluated the recruitment of patients in a South-African context. This study provides a regional perspective, recorded during a local cardiovascular trial, on the reasons that motivate South Africans to participate in such studies. Due to the high volume of patients serviced by the health sector in South Africa, resources are often not distributed equally amongst populations and study participants indicated that access to high-quality medical care and medication was a great motivation in participating in clinical trial research. Patients also indicated that participation in a research trial gave them a sense of belonging to a group and 81% of participants indicated that they experienced the meeting of people with similar health problems as an emotional benefit. Surprisingly 98% of participants indicated that they participated in the clinical trial to learn more about their disease. It is therefore important that clinical trial personnel accommodate clinical trial participants in this regard. Of the participants in this study, 80% indicated that patient remuneration does not influence their decision to participate in clinical trials. Contrary to developed countries, 78% of participants indicated that they do not perceive additional threats to their health due to participation in clinical trials (Burgess *et al.*, 2009).

4.5 GCP study documents

All the procedures during a clinical trial should be documented to adhere to ICH-GCP and SA-GCP. This enables the appropriate parties to evaluate whether the trial has been conducted in a scientifically and ethically sound manner. There are certain traditional documents that form part of every clinical trial. The South African GCP guidelines group the essential documents for the conduct of a clinical trial in three categories based on the stage when they are generated, namely: 1) before commencement of the clinical trial, 2) during the conduct of the clinical trial, and 3) after completion or termination of the trail (DOH-SA, 2006).

Pre-trial documents include the following: Investigator's brochure, signed protocol, a sample case report form, advertisement for participant recruitment (if applicable), financial aspects of the trial, insurance statements, signed agreement between involved parties (e.g. investigator and sponsor), approval of the protocol, informed consent forms, case report forms and other documents needed, the composition of the ethics committee, regulatory approval of protocol, CV's of investigators, description of laboratory test and procedures, samples of investigational

product labels, shipping records for investigational product, decoding procedures for blinded trials, master randomisation list (if needed), pre-trial monitoring report and the trial initiation monitoring report (DOH-SA, 2006).

During the conduct of the trial the following documents are required: Investigator's brochure updates, any revisions (to protocol, CRF, informed consent), CV's of new investigators, certificates of analysis for new batches of investigational products, monitoring visit reports, signed Informed consent forms, source documents, complete CRFs, notifications of Adverse Events and Serious Adverse Events, interim reports to authorities, participant screening log, participant identification code list, participant enrolment log and investigational products accountability at the site (DOH-SA, 2006).

After completion of the trial the following documents are required to finalise the trial: investigational product accountability, documentation of investigational product destruction, final trial close-out monitoring report, treatment allocation and decoding (if applicable), final report by investigator and a clinical study report.

Examples of the study documents used during this study are available on request.

4.6 Responsibilities of the study team members

Certain roles are traditionally present in the study team and members fulfilling these roles carry certain responsibilities.

- The Principal Investigator (PI)

According to GCP-SA the principal investigator must be a South African-based scientist and this investigator is responsible for the design, conduct, analysis and reporting of the trial. The Principal Investigator may delegate tasks to other team members but remains accountable to regulatory authorities to ensure adherence to GCP. Consequently it is important that the PI has a thorough knowledge of the investigational product, especially its safety and toxicity (ICH, 1996; Switula, 2000; DOH-SA, 2006).

It is further the responsibility of the PI to ensure that approvals from the appropriate ethics committees and scientific committees are obtained and if applicable, registration at the Medicine Control Council (MCC) / South African Health Products Regulatory Agency (SAHPRA) and the Clinical Trial Register of the Department of Health. The PI should read the information on the investigational product provided by the sponsor and have knowledge of the protocol and regulatory requirements. The PI must adhere to the Declaration of Helsinki, ICH guidelines for GCP, and other legislation as applicable. The investigative product must only be used for trial

purposes according to the study protocol and the PI must take responsibility for the control of this product. The documentation for every aspect of the clinical trial should be thorough and the availability of facilities, equipment and other resources is the responsibly of the PI. The PI must ensure that informed consent is ethically obtained. The PI must involve monitors to ensure a proper review of quality control and data verification and facilitate audits from the sponsor, ethics committee, regulatory and other applicable authorities. The PI should make trial results (positive and negative) available to the public and share the benefits of the research with the trial participants. The PI is in charge of the safety reporting procedures (ICH, 1996; Switula, 2000; Dongen, 2001).

The PI makes the trial related medical decisions and must ensure the appropriate handling of Adverse Events. Should a participant need additional medical care, the PI has to ensure that the participant /patient is advised thereof. The PI should confirm the reason for early withdrawal from the study by participants, to ensure these are not due to an unreported adverse event (ICH, 1996; Switula, 2000).

- Study Coordinator (SC) or Research Coordinator

Most of the PI responsibilities may be delegated to the Study Coordinator (SC). This might include the informed consent procedures and ensuring product accountability, but the PI remains ultimately legally accountable for these responsibilities. The SC is typically central in trial activities as it is his/her responsibility to act as liaison between the different study team members. It is consequently important for the SC to find a balance between the role of medical caregiver and researcher (Orentlicher, 2002). It was demonstrated during a survey that the inclusion of a SC on the research team improves participant recruitment rate, participant retention and study efficacy (Cooper & Lomax, 1989; Good & Schuler, 1997; McKinney & Vermeulen, 2000). The SC must enrol (screening, informed consent) and manage study participants (follow-up care, report events) according to ethical, regulatory and protocol-specific requirements.

The SC is also in charge of maintaining records of all the clinical trial procedures in the Master File and Site File as well as ensuring compliance with regulatory policies. The SC facilitates quality assurance activities like monitoring visits and audits. The SC must further ensure that the PI is informed of all study related activities and be knowledgeable of the study protocol. The SC bears responsibility for compliance with Good Clinical Practice during research activities (Orentlicher, 2002).

- Study Monitor

The Study Monitor is a person/ company appointed to monitor all the study activities and documentation thereof. The Study Monitor reports to the Study Sponsor. The activities of the Study Monitor includes clinical trial site inspections, inspection of records, correlation of all events reported, monitoring investigational medicine accountability, measuring compliance with the study protocol, evaluating the informed consent process and other appropriate activities (ICH, 1996).

4.7 The phases of the clinical trials

Traditionally clinical trials progress along fixed phases from phase I to phase IV. Phase one studies are performed after adequate animal testing and is performed primarily evaluate pharmacokinetics with adverse event reporting. This study involves healthy volunteers (20-80). Phase II clinical trials are used to evaluate a dose response relationship to effect, identify an optimal dose and investigate safety issues. Phase III studies involves a large group of patients and tests for efficacy, side-effects and superiority to other available agents. The can be seen as “confirmatory trials” or “registration trials”. Based on the outcome of phase III studies, the drug/medicine can be approved by the regulating authority (MCC/ SAHPRA or FDA). After registration of the drug/medicine, phase IV studies are utilized to evaluate the long term efficacy of the drug/medicine and its effects on quality of life. These trials are used to gain marketing information or broader clinical expertise (Robertson & Gan, 2001; Evans, 2010).

Since advances in technology necessitated certain flexibility in the planning of clinical trials to suit the need of the agent tested clinically, the traditional phase naming of clinical trials is no longer set in stone. The clinical trial design is based on purpose, convenience and structure rather than a traditional pathway. Common practice is to circumvent this structure by combining phases (e.g. hybrid phase I/II trial or even phase I/II/III) or alternatively (but less common) to use descriptive titles (dose-finding trial, comparative trial, safety trial) (Piantadosi, 2005). For example dose-toxicity and dose-efficacy can be determined during a phase IA trial. Further evaluation of chronic administered dosages can be evaluated during a phase Ib trial as well as the effect of concomitant food administration with the API in question. Phase II clinical trials will be a pilot study (phase IIa) to evaluate efficacy on a small scale followed by ‘n more intensive study (phase IIb) on unhealthy patients. Phase III studies can also be divided in phase IIIa (before submission for FDA approval) and a further phase IIIb (performed after the dossier is submitted to the FDA to answer further questions) (Mahan, 2014).

4.8 The study design

In the parallel group design, study participants are divided into groups that each receives a single therapy and then the outcomes of the different groups are compared. Conversely in the crossover design each participant receives more than one treatment at different times of the study (with a normalization period in between) and the differences between treatments are then compared for each patient. There are some advantages and disadvantages associated with this cross-over design (Piantadosi, 2005; Li *et al*., 2015).

Advantages of the crossover study design (Piantadosi, 2005; Li *et al.,* 2015):

- Evaluation of the different treatments is precisely – each patient is their own control and inter-subject variability is not present.
- Sample size can be reduced due to the lack of inter-subject variability and a smaller number of study participants are needed.
- In some cases, recruitment for crossover trials are easier because all study participants will receive treatment and have a possible benefit.

Disadvantages (Piantadosi, 2005; Li *et al.,* 2015):

- In some instances more time of study participants will be needed to receive both formulations, and this may lead to difficulties during recruitment. This could also lead to a progression in disease between treatments.
- Carry-over effects from one treatment to another may be a problem. It is of critical importance that the “wash-out” period between treatments is of sufficient duration.
- The premature removal of a study participant can have a more drastic effect on the data loss (data from both treatments are lost) when compared to parallel groups.
- If the treatment with one agent (for example a vaccine reducing chances of infection) affects the possible data with the other agent, then the crossover study design is inappropriate.

If a crossover design is preferred, the investigators should be confident that the disease will not have a large change in the level of intensity throughout the clinical trial. The study should also be designed to ensure that the treatment is confined to a certain time-frame and does not carry over to the next treatment. The wash-out period is required to be at least three times the blood-plasma elimination half-life or three times the half-life of the decay of the immediate pharmacological effect in a single-dose crossover study. When evaluating the efficacy of radiotracers by comparison with the current gold standard radiotracer, the crossover study design is very suitable and indeed standard practise. It is ideal due to the short half-life of these agents and also the fact

that radiotracers by definition evaluate biological process without changing disease characteristics (Chow & Liu, 1999, Piantadosi, 2005).

4.9 The clinical application of ^{99m}Tc-MDP in South Africa

According to the South African National Cancer Register (2012) the estimated lifetime risk of developing bone malignancy is 1:3013 in South African males and 1:4050 in South African females (National Register, 2012). Oncology patients are a vulnerable patient group with a high incidence of emotional distress (Seetharamu *et al.*, 2007). The current gold standard radiotracer for planar bone scintigraphy is ^{99m}Technetium methylene diphosphonate (^{99m}Tc-MDP or ^{99m}Tc-medronic acid), which is injected intravenously.

The intravenous route of delivery is effective in cases where immediate action is required from the pharmaceutical agent (e.g. during emergency) or when the pharmaceutical agent is poorly absorbed from the gastro-intestinal tract. A preparation that is administered *via* this route must comply with strict regulations regarding sterility. There is a well-documented negative perception of this route by the patient due to pain, side-effects and inconvenience (York, 2007). The oral route is the most popular route of administration due to high patient tolerability, convenience and safety. Unfortunately, some pharmaceutically active ingredients cannot be administered by this route due to lack of absorption from the gastro-intestinal tract or due to a deficit in stability when exposed to the harsh gastro-intestinal fluids. Other disadvantages of this route include a delay in onset of action due to the timeframe needed for absorption as well as possible irregular absorption of the pharmaceutical agent (Borner *et al.*, 2001; York, 2007). Over 40 million nuclear medicine procedures (with a market value of 9.6 billion dollar) are performed every year with a demand that increases 5% annually. Of the 40 million procedures, 80% of these are utilizing ^{99m}Technetium as isotopes. South Africa provides 15% of the world's supply of ^{99m}Technetium, produced by NTP (affiliated to Necsa) using the SAFARI-1 reactor at Pelindaba. NTP sells 7500 vials of MDP kits for bone scintigraphy annually (World Nuclear Association, 2017).

Preliminary preclinical studies were performed at the DST/NWU PCDDP to determine the biodistribution of the Pheroid® delivery system with ^{99m}Tc-MDP entrapped in the Pheroid® entity/vesicle. The *in vivo* biodistribution of the Pheroid® in rodents was investigated by SPECT. In an unanticipated development, the Pheroid® formulation of this radiopharmaceutical demonstrated an enhanced uptake after *per os* administration when compared to the standard ^{99m}Tc-MDP formulation alone (Grobler & Zeevaart, 2015).

A phase IIa clinical trial will establish whether a Pheroid® ^{99m}Tc-MDP formulation will unlock the oral route for administration of the radiotracer during the bone scintigraphy procedure in humans.

4.10 The phase IIa clinical trial study design of oral ^{99m}Tc-MDP Pheroid®

The phase IIa clinical trial was designed as a cross-over study with 16 patients to be enrolled. The patients were to be scanned on day one with the standard ^{99m}Tc-MDP radiopharmaceutical followed by a wash-out period of a minimum of two days. The wash-out period is required to be at least three times the blood-plasma elimination half-life or three times the half-life of the decay of the immediate pharmacological effect in a single-dose crossover study (Chow & Liu, 1999:38). On the final day the Pheroid® entrapped radiopharmaceutical (as an oral emulsion with a volume of approximately 4 ml) was administered. Randomization regarding the order of the scan procedures (gold standard vs. test formulation) was not possible due to the fact that the gold standard scan image was needed immediately to plan the therapy of the patient; it was deemed unethical to delay the start of oncology treatment in order to randomize the formulation administered in the case of a severely ill patient. Furthermore, since the study participants were government funded patients, it was necessary to provide them with a diagnosis as soon as possible (preferably on the first day of admission) since failure to show up for the second appointment due to lack of funding (e.g. an inability to get leave from work or pay for travel) was a high probability. If the patient received the test formulation on the first day and did not attend the second appointment, a failure of both the study aims (receiving a control to compare to) and treatment aims (providing the patient with a diagnosis) would not be obtained. However, since there is a high volume of bone scintigraphies performed at the facility (4-7 patients per scan day), it was not foreseen that enrolment of 16 patients would be a problem.

The clinical trials for ^{99m}Tc-MDP and ^{99m}Tc-MIBI formulated in Pheroid® were approved by The Research Ethics Committee, Faculty Health Sciences, University of Pretoria (159/2015) as well as the Human Research Ethics Committee, North-West University (NWU-0183-15-A1). Scientific approval for the study was obtained from the GXP Research Committee as well as the Pharmacen Research Committee, both situated at the North-West University. Study monitoring was performed according to GCP standards by an independent researcher from the DTS/NWU PCDDP not involved in the study. The Nuclear Medicine Department of Steve Biko Academic Hospital where the clinical trial took place is registered for handling medical isotopes at the Department of Health (M/0001/12/1148). Remuneration was provided for patients for each of the study days with emergency services as well as follow up services (including the services of a social worker at SBAH), which was to be provided free of charge if requested, although no incidents occurred.

The choice of 16 patients per clinical trial was based on a review of literature (Subramanian *et al.,* 1975; Fogelman *et al.,* 1979; De Murphy *et al.,* 1997; Gilson *et al*., 1999; Press *et al*., 2000; Maricinow *et al.,* 2013; Bretin *et al.,* 2015). It is more accurate to use a sample size based on

similar clinical trials in literature than using a calculation (numerous statistical assumptions are made to enable the quantification of clinical outcomes) (Senn, 2007: 277).

Pregnancy and breastfeeding had to be excluded in any female participant, and participants had to be older than 18 years of age. This radiotracer should only be used in groups of patients when benefits outweigh risks, it would have been unethical to expose paediatric patients or unborn foetuses to additional radioactivity. In order to be included in this study, the patient had to be deemed in need of a bone scintigraphy procedure by his primary physician or oncologist to diagnose bone pain of unknown origin or to determine the presence of bone metastases. The study participant further had to provide informed consent in the language of their choice (informed consent was provided in the spoken languages of the region namely English, Afrikaans and Sesotho). History of any gastro-intestinal dysfunction due to disease (either acute or chronic) or concomitant pharmaceutical agents was cause for exclusion. A normal haematological profile, hepatic function, renal function and vital signs had to be demonstrated before participation in this trial. The patient had to be capable of lying in a supine position for 45 minutes without moving. Appointments were made with two days in between the first and second appointment (as washout period) and had to be honoured by the participant. After being included in this study, reasons for removal from the study was noncompliance with the study protocol, failure to adhere to appointments, adverse reactions of a serious nature, serious adverse events, removal requested by participant and any indication that constraints of the study or interventions during the study could affect the patient's health negatively. None of the enrolled participants were removed from the study.

4.11 Manufacturing of formulations

The lyophilized MDP kit was reconstructed with the ^{99m}Tc-eluent on-site, the normal procedure followed at the hospital. To verify the quality of the reconstituted ^{99m}Tc-MDP, silica-gel instant thin-layer chromatography (ITLC) was performed with saline and acetone as migrating solutions to distinguish between ^{99m}Tc-MDP, unbound pertechnetate and reduced forms of ^{99m}Technetium (also part of normal procedure followed).

To prepare the Pheroid® test formulation, 500 mCi of the ^{99m}Tc-MDP (eluated in 5 ml saline) was added to pre-gassed (nitrogen oxide) water to result in 10 g of water phase. The water phase was heated to 70°C. The oil-phase contained Vitamin F ethyl ester (heated no more than 70ºC), Kolliphor EL (heated to 120ºC) and dl-α-tocopherol (heated to no more than 55ºC). The oil-phase (cooled down) was added to the water-phase and self-emulsification was enhanced by homogenization at 13 500 rpm (Heidolph Diax 600 homogenizer, Labotec South Africa) until the mixture has cooled. The concentrations of oil and water phase was 4% and 96% respectively.

Thereafter entrapment of the ^{99m}Tc-MDP was promoted by stirring the formulation with a magnetic stirrer for the designated entrapment time (18 hours) according to protocol used in previous *in vivo* rodent studies. Passive accumulation of the ^{99m}Tc-MDP in the water phase of the vesicles was therefore applied, with entrapment post-manufacture. All Pheroid® formulations were characterized after radioactivity had decayed completely, to determine the particle size of the emulsion droplets, the stability (zeta-potential) of the formulations as well as microscopic investigation of the vesicles through confocal laser scanning microscopy.

4.12 Characterization parameters of the clinical trial formulations

The particle size distribution was typical of Pheroid® formulations manufactured with this method, destined for oral administration. The mean particle size of emulsion droplets was 1.98 µm with 80% of the droplets distributed between 0.20 µm and 4.75 µm. The particle distribution graph is presented in Figure 4-1.

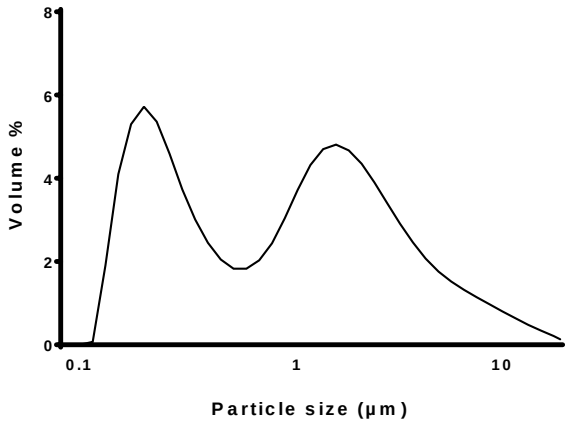


Figure 4-1: Particle size distribution of the oral Pheroid® ^{99m}Tc-MDP formulation administered to patients during the hybrid phase I/II clinical trial. The graph reflects the means of 6 measurements.

The zeta potential of the formulation was deemed satisfactory, with a value of -22.1 ± 0.8 mV – slightly lower than the optimal -25 mV. It is our opinion that the amount of saline included in this formulation may have a slightly detrimental effect on this measurement. The fact that the stability of the Pheroid® is influenced by the presence of saline is in this case not a major concern since the addition of the ^{99m}Tc-MDP (in saline) and the entrapment process takes place directly before administration and not weeks beforehand. The standard Pheroid® in general is a highly stable formulation with zeta-potential measurements far above the standard ±25 mV. The CLSM measurements confirmed that Pheroid® vesicles with the correct morphology and internal structure were formed during the manufacturing (see Figure 4.2). Overall a standard formulation

was presented, although there is currently the fluorescence-based method of determining the entrapment efficacy (amount of active ingredient internalized by the Pheroid® vesicles) of the formulation manufactured but it was not appropriate for this study.

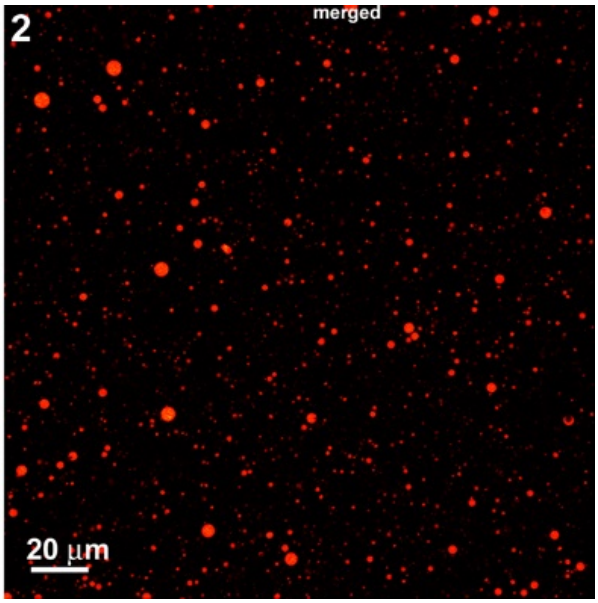


Figure 4-2: The confocal micrograph (merged from images taken at wavelengths of 568-642 and 500-530 nm) of the Pheroid® formulation administered to the first two patients that participated in the clinical trial of oral ^{99m}Tc-MDP.

4.13 Imaging protocol

A Siemens E.Cam (Signature Series) whole body SPECT scanner (Figure 4-3) that is based at Steve Biko Academic Hospital (Nuclear Medicine Department) was used for all scanning procedures during this study.

The patients were fasted overnight before the scanning procedure but could drink as much water as needed. A blood sample was taken for blood glucose monitoring before the scanning procedure to ensure their glucose levels were indicative of fasting. If a hypoglycaemic blood sugar level was measured below 3 mmol/L, the study participant would have been referred to the emergency unit for immediate care. If a hyperglycaemic level is measured, the participant's personal physician would have been informed (if the participant consented).

Figure 4-3: The SPECT scanner used for all scans during this trial (image was obtained by the author herself, with permission from SBAH).

The gold standard radiotracer was administered by the intravenous route with a dosage of no more than 25 mCi. During the waiting stages of the procedure, patients could consume adequate fluids and void their bladder regularly in the designated bathroom assigned for disposal of urine contaminated by radioactive waste. Scanning was performed at the usual time (approximately 3 hours of waiting time) after administration. After the scanning procedure, the patients were consulted and vital signs were checked to ensure that it was safe to release the patient from care.

The patient was similarly prepared for the second scanning procedure. The dosage of the oral formulation also did not exceed 25 mCi. Due to the fact that the volume of preparation does not give an indication of the amount of radioactivity present (e.g. a formulation of ^{99m}Tc-MDP that has the correct dosage of 25 mCi can be either 1 ml or 2 ml depending on the activity of the reactor at that stage), the dosage was not measured according to volume or chemical composition, but rather according to radiation dosage as measured by amount of radioactivity (the Capintec cr-15 dose calibrator). The residual radioactivity in the syringe is measured after administration and subtracted to provide the administrated dose. This dosage was administered to the patient through a syringe at the back of the oral cavity with sufficient water provided to wash the residual formulation down. Scanning times were 0 min, 1 hour, 2 hours, 3 hours and 4 hours. At this stage of the investigation it was decided that the capturing of images at later times was not useful since the half-life of the radiotracer is 6.02 hours. After the scanning procedure the patients were consulted, remuneration was provided, and vital signs were checked to ensure that it was safe to release the patients from care.

4.14 Results: Patient 1 and 2 enrolled in the study

4.14.1 Patient population

As a pilot, two patients were successfully recruited, and the informed consent process was performed as prescribed. Patient 1 was a reasonably physically active male aged 66 years old and the standard (intravenous) ^{99m}Tc-MDP scan demonstrated that the patient did not have any lesions (Figure 4-4). It was however decided to include this patient in the trial since this is a hybrid phase I/II trial and that it was the first-in-human administration of this new formulation. Patient 2 was a 58-year-old female patient; unable to walk. This patient presented with severe metastatic disease in the gold standard ^{99m}Tc-MDP scan (Figure 4-4).

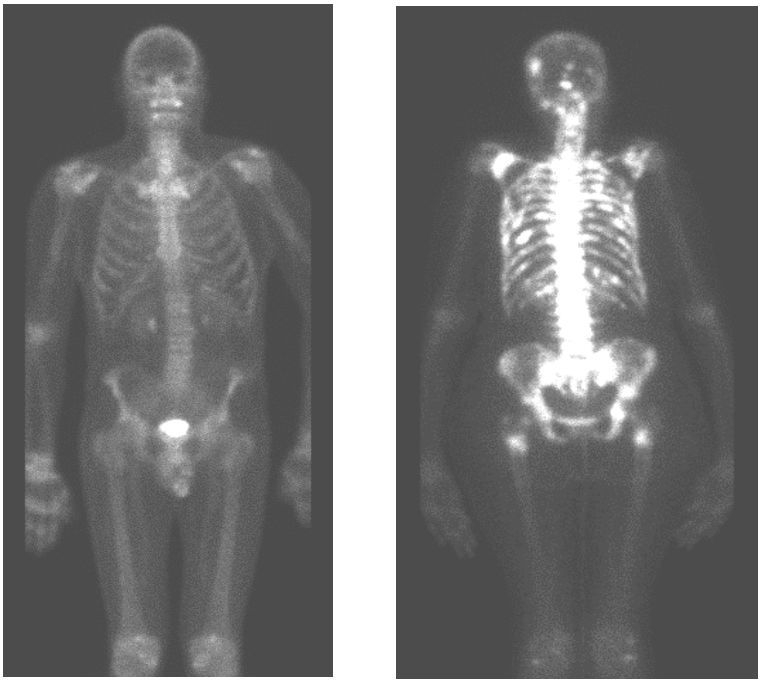


Figure 4-4: The gold standard scan of patient 1 (left) without disease and patient 2 (right) with extensive metastatic disease of the bone.

Both patients presented themselves for the second study day to receive the test formulation. The administration of the test formulation was done by using a syringe to administer the formulation at the back of the throat. The patients received water to wash down the formulation. None of the patients complained about the taste of the formulation.

4.14.2 Results and discussion

The scan data received for both patients are provided in Figure 4-5 with the concentration of radioactive compound ^{99m}Tc-MDP clearly limited to the gastro-intestinal tract. No lesions were highlighted which indicates that the formulation in its present state is ineffective. Note the prominence of the thyroid in both patients.

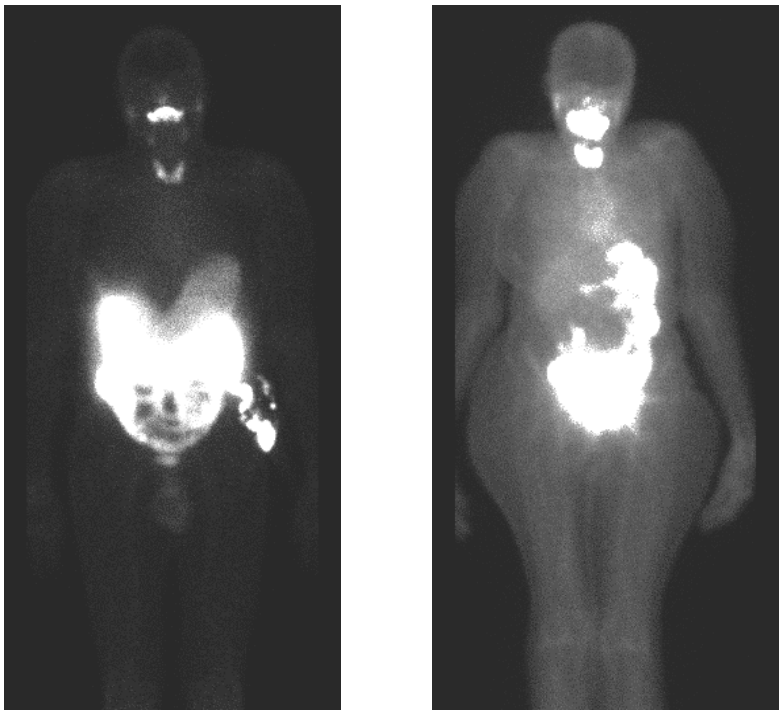


Figure 4-5: The patients' scans at 3 hours after receiving ^{99m}Tc-MDP in Pheroid® via the oral route. Note the high presence of the radioactivity in the gastro-intestinal system, as well as the thyroid gland.

A very prominent demonstration of the thyroid in the scans (see Figure 4.5) raised the question on the stability of the ^{99m}Tc-MDP incorporated in the formulation, as free ^{99m}Tc (known as pertechnetate and noted as $^{99m}TcO_4^-$) accumulates in the thyroid, stomach and saliva glands (Figure 4.6). Intravenously administered free $^{99m}TcO_4^-$ is clinically used to image thyroid functioning. Pertechnetate is used worldwide as a substitute to ^{123}Iodine for the imaging of the thyroid because of similar biodistribution based on the isotope's volume and chemical charge. The clear demonstration of thyroid uptake indicated the presence of free $^{99m}TcO_4^-$ that entered the blood stream. We therefore investigated the effect of the pH in the stomach on the ^{99m}Tc-MDP by exposing the radiotracer to different concentrations of hydrochloric acid (HCl) and performing ITLC after 5 minutes of exposure. It was furthermore demonstrated that pertechnetate does form after being left overnight (purity degrades form 99% to about 60%) and since the Pheroid®

formulation tested was stirred overnight this could further have contributed to the formation of pertechnetate. Free pertechnetate is absorbed from the gastrointestinal tract although in an erratic way (Hays, 1973; Ramos et al., 2002).

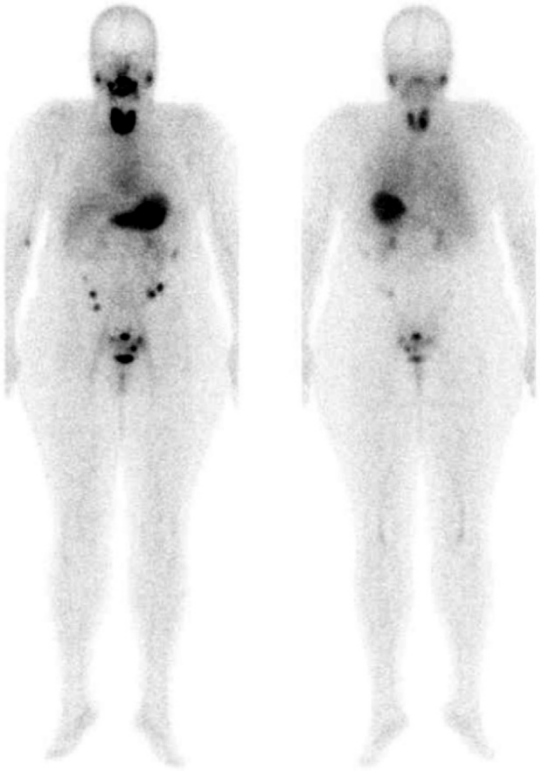


Figure 4-6: A whole body pertechnetate scan demonstrating accumulation in the thyroid and saliva glands (published with permission of the Publisher. Original source: Navaro, P., López, L., González, M., Liévano, P., Sangrós., Ȧlvarez, S et al., Peritoneal strumosis: an extension study with 99mTc-pertechnetate. R Rev Esp Med Nucl Imagin Mol, 2012, 31(2) 97-100. © 2011. Elsevier Espana. S.L. Semnim. All rights reserved).

4.15 Investigation into the stability of the formulation

After the lack of efficacy demonstrated in the first two patients, the clinical trial was halted to evaluated gathered evidence and replan the workflow. A formulatory study was undertaken to provide for formulations that might be more effective. The focus was to increase the stomach pH clinically and develop a method which will allow the immediate administration of the formulation to remove the degradation effect of ^{99m}Tc-MDP to pertechnetate overnight.

To evaluate the effect of the stomach contents on the formulation, we conducted a preliminary screening on the effect of the pH level only. Although this was a very primitive investigation, a clear tendency was observed.

4.15.1 Methods and materials

Hydrochloric acid (obtained from Sigma-Aldrich, 37% reagent grade) was added to purified water and the pH was adjusted to different acidic gradients (0.5, 1, 2, 3, 4 and 5) to evaluate the stability of the radiotracer at these levels. An amount of 200 µL of ^{99m}Tc-MDP (tested 99% pure by ITLC) was added to 10 ml of the hydrochloric acid-water and the mixture was shaken lightly and incubated at room temperature for 5 minutes. Thereafter ITLC was performed and the different parts of the plate were read by the Hidex® 600 SL automatic gamma counter (Hidex Oy, Finland). The counts were then adjusted for decay. All measurements were done in triplicate and are presented in Figure 4-7 with standard deviation reported.

4.15.2 Results

A clear tendency for the tracer to disintegrate at lower pH values was observed (see Figure 4.7) producing free $^{99m}TcO_4^-$. These results might indicate a tendency of the low pH of the stomach leading to disintegration of the ^{99m}Tc-MDP to free $^{99m}TcO_4^-$, which will then accumulate in the thyroid gland. It was therefore decided to provide the next patient with an antacid before administration of the test formulation. The initial formulation was stirred for 16 hours, and this might have contributed to further instability of the ^{99m}Tc-MDP tracer. It was decided to manufacture a formulation that does not necessitate overnight stirring. This would also be a much safer and practical formulation since the amount of radioactivity needed to compensate for overnight decay is impractical.

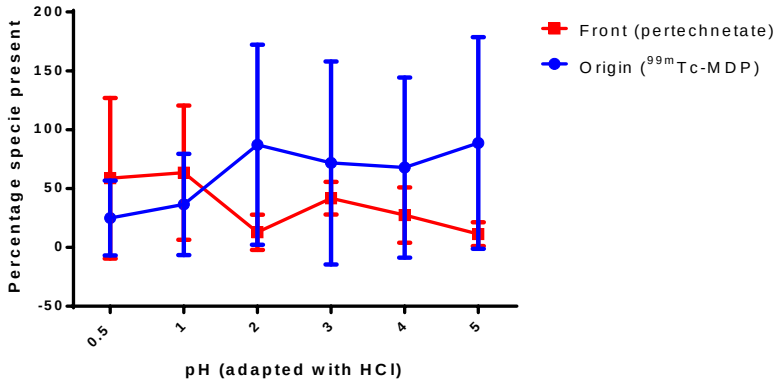
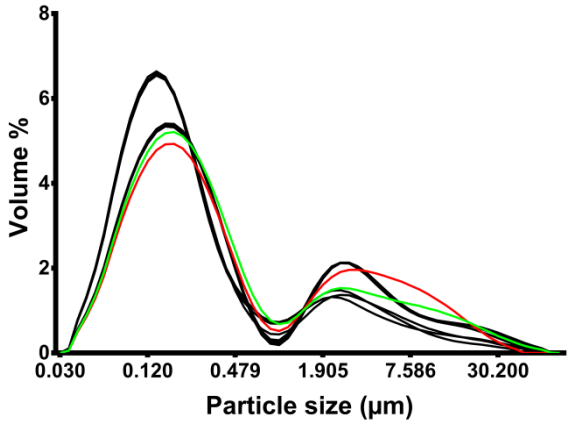
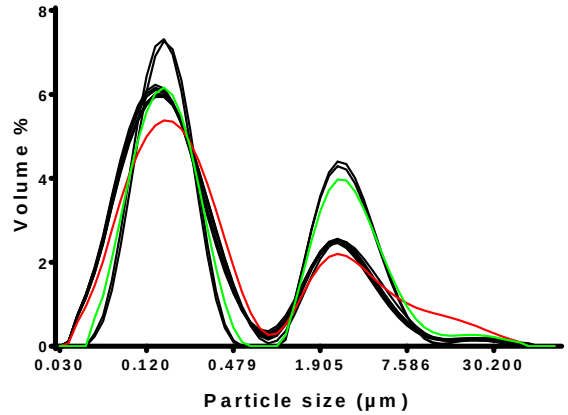


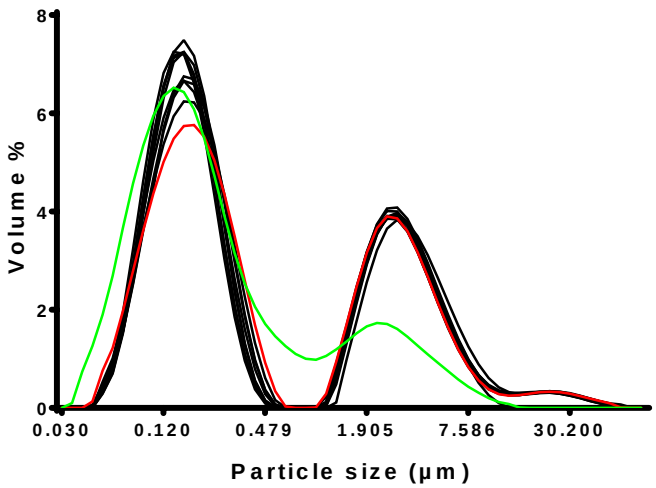
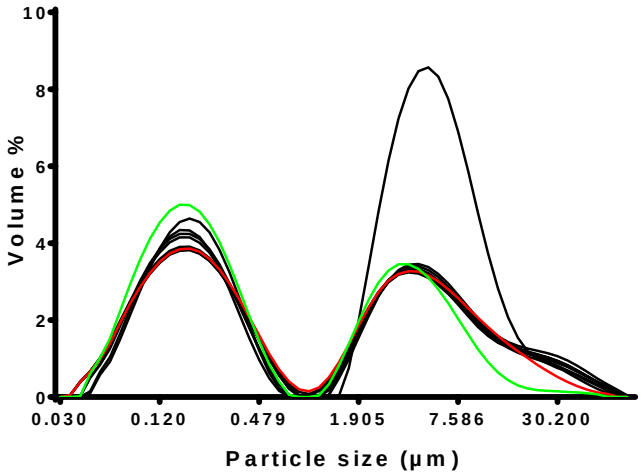
Figure 4-7: The amount of radiotracer still intact (indicated in blue) present after exposure to different pH levels for 5 min. The red curve represents free pertechnetate.

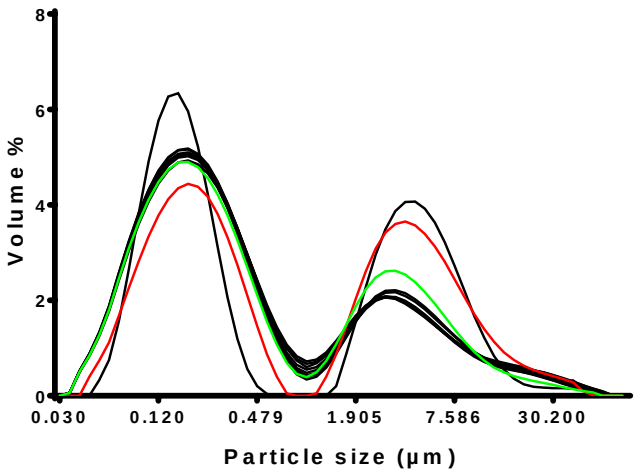
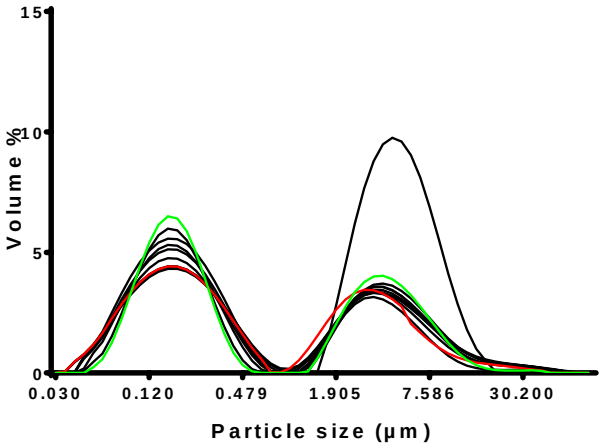
4.16 Formulation study

All formulations (see Table 4-1) were manufactured by addition of 0.4 ml saline containing the cold MDP kit to the nitrous oxide gassed water phase (96-87% depending on the formulation studied). Then the gassed pro-Pheroid® was added (either containing Incromega E7010 and Incromega TG3332, or not containing these ingredients as specified in table 4.1) and the formulations were vortexed for 1 minute after addition. Particle size distribution was measured with the Malvern Mastersizer Hydro 2000 (Malvern instruments, Worcestershire) at times (0 min, 15 min, 30 min, 1 hours, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 24 hours). The particle size distribution graphs were evaluated for changes that might indicate instability or entrapment of the radiotracer. Take note that the concept of this investigation was to search for hints about the behaviour of this formulation, and not to provide systematic empirical information. The aim was to increase the probability of passive entrapment by adding the active ingredient (after MDP has been labelled with ^{99m}Tc) to the water phase (it is hydrophilic in nature) before manufacturing.

Table 4-1: Six different formulations were investigated and the details about these formulations are presented (logarithmic scale x-axis):

The contents of the formulation	Particle size distribution chart over 24 hours (red is after 15 minutes, green is the control without 99mTc-MDP).	Comments
Formulation A **Gassed Pro-Pheroid 4%** (containing standard amounts of Vitamin F Ethyl Ester, Dl-alpha-tocopherol and Kolliphor EL). **MDP in saline 0.4 ml** **Gassed water 96%**		In this formulation the concentration of the particles at 15 minutes (red line) has a smaller population in the region of under 1 µm and more above 1 µm when compared to the population of the control formulation (green line) that does not contain ^{99m}Tc-MDP. The formulation is relatively stable between 1 - 6 hours, with the measurement at 24 hours having substantially smaller particles.
Formulation B **Gassed Pro-Pheroid 10%** (containing standard amounts of Vitamin F Ethyl Ester, Dl-alpha-tocopherol and Kolliphor EL). **MDP in saline 0.4 ml** **Gassed water 90%**		The formulation does differ substantially at 15 minutes (red line) when compared to the control formulation (green line). The formulation is stable at the time points measured (1 hours and 24 hours).

Formulation C **Gassed Pro-Pheroid 13%** (containing standard amounts of Vitamin F Ethyl Ester, Dl-alpha-tocopherol and Kolliphor EL). **MDP in saline 0.4 ml** **Gassed water 87%**		The formulation is again different at 15 minutes (red line) when compared with the rest of the times as well as the control formulation (green line). However, the control formulation also shows a large difference compared to all the rest of the measurements.
Formulation D **Gassed Pro-Pheroid 4%** (containing standard amounts of Vitamin F Ethyl Ester, Dl-alpha-tocopherol and Kolliphor EL). Also containing Incromega E7010 and incromega TG3332. **MDP in saline 0.4 ml** **Gassed water 96%**		This formulation demonstrated a shift in particle sizes from the control formulation (green) to the measurement of 15 minutes post ^{99m}Tc-MDP addition. Note that at 24 hours the formulation demonstrated just one peak (all particles above 1.9 µm), which most probably indicate aggregation or less probably the exclusive presence of microsponges.

Formulation E **Gassed Pro-Pheroid 10%** (containing standard amounts of Vitamin F Ethyl Ester, Dl-alpha-tocopherol and Kolliphor EL). Also containing Incromega E7010 and incromega TG3332. **MDP in saline 0.4 ml** **Gassed water 90%**		This formulation demonstrated a shift in particle sizes from the control formulation (green) to the measurement of 15 minutes (red line) post ^{99m}Tc-MDP addition.
Formulation F **Gassed Pro-Pheroid 13%** (containing standard amounts of Vitamin F Ethyl Ester, Dl-alpha-tocopherol and Kolliphor EL). Also containing Incromega E7010 and incromega TG3332. **MDP in saline 0.4 ml** **Gassed water 87%**		This formulation demonstrates a shift in particle sizes from the control formulation (green) to the measurement of 15 minutes post ^{99m}Tc-MDP addition. As for Formulation D, the formulation demonstrated just one peak (all particles above 1.9 µm) at 24 hours, which can indicate aggregation or the exclusive presence of just microsponges.

The cold kit MDP is a good approximation of the formulation that will result with the labelled kit. This was confirmed by analysis after formulations used in the clinical trial decayed; the cold kit is associated with tin (which is replaced by ^{99m}Tc upon labelling) which provides a molecule in the same chemical state as when bound to ^{99m}Tc.

4.17 Discussion and conclusion after first 2 patients and formulatory study

It became clear that the clinical setting adds certain constraints that must be considered when the choice of formulation is made to ensure future marketability.

The amount of radioactivity (^{99m}Tc-MDP) should be kept as low as possible to reduce the risks associated with compounding as well as the costs associated with the procedure. To have a formulation stand for 18 -24 hours before administration necessitate loading with a high dosage of radioactivity (more than 500 mCi) when compared to immediate administration (30 mCi). High amounts of radioactivity increase the risk during compounding, the risk of radiation exposure to the radiopharmacist or radiochemist at the facility as well as the risk of spilling.

In radiation work it is always preferred to strive for ALARA. When a high amount of radioactivity is reserved for one patient scan, this increases the cost of the procedure and also reduces the number of patients that can be accommodated. If the Pheroid® system is loaded with radiotracer that decays overnight, the full capacity of the carrier system is not fully utilized because a substantial number of the vesicles will carry decayed tracer by the time the formulation is administered. The overnight entrapment time may also contribute to degradation of the radiotracer to free pertechnetate, which can result in pronounced thyroid gland imaging.

It was therefore decided to administer formulation F (see Table 4-1) to another volunteer, immediately upon compounding (e.g. approximately 5-10 minutes of entrapment time). It was also decided that this patient will receive an antacid prior to the treatment to try for a neutral pH of the stomach to evaluate the influence thereof on efficacy.

4.18 The final patient enrolled in the study

To prepare the Pheroid® test formulation 30 mCi of the ^{99m}Tc-MDP (eluated in 5 ml saline) was added to pre-gassed (nitrogen oxide) water (made up to 4.4 ml). The gassed oil-phase (0.6 ml) contained Vitamin F ethyl ester, Kolliphor EL, Incromega TG 3332, Incromega E7010 and dl-α-tocopherol. The oil-phase was added to the water-phase and the formulation was shaken for 1 minute in a closed container. The ratio of the oil to water phase was 13:87.

4.18.1 Patient preparation

The patient received Nexium® 40 mg (esomeprazole) at 12 pm on the afternoon of the previous day as well as at 7 am on the morning of the scan day to decrease basal acid secretions as well as a feedback acid secretion that could result after the administration of magnesium hydroxide.

On the study day the patient received 15 ml of magnesium hydroxide (milk of magnesia) 45 minutes before the administration of the test formulation to neutralize the gastric contents as far as possible. The dosage of the oral formulation again did not exceed 25 mCi. Scanning times were 0 min, 1 hour, 2 hours, 3 hours and 4 hours. The patient was consulted after the scanning procedure, and vital signs were checked to ensure that it is safe to release the patient from care.

4.18.2 Results and discussion

Although the administration of antacids does have a pharmacokinetic interaction on the biodistribution to the liver, it was decided to first evaluate whether the absorption of the formulation is affected by the gastric pH and thereafter, if successful, study the possible change in pharmacokinetics. The image procured at 3 hours (Figure 4-8) is presented for comparison with previous scanning images. The lack of efficacy is still present – no uptake outside the intestinal tracts, although the involvement of the thyroid gland and saliva glands have been eliminated. The presence of pertechnetate due to degradation in the stomach in previous scans is therefore confirmed by the lack thereof in this scan. Further measures were taken to mask the high amount of the radioactivity in the stomach by utilizing a lead shield. However, no difference was seen in the scan since the activity imaged through the lead shield was still very high. Furthermore, such a practice would shield any presence of bone cancer in the ribcage, an area which is of clinical importance in bone cancer metastasis.

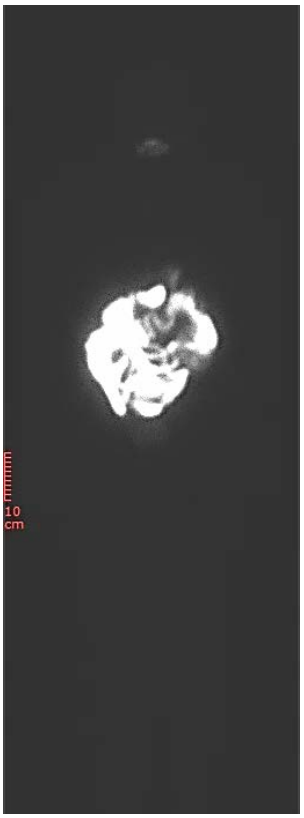


Figure 4-8: SPECT scan of the final patient performed during this study (note only the GI tract is visible – no other patient tissue was picked up).

4.19 Final conclusions and future studies

New insight regarding the Pheroid® delivery system was gained during this investigation and although the use of this technology in nuclear medicine is currently not yet clinically viable, further studies are planned. It is evident that the translation of efficacy from animal models to human clinical trials is not a straightforward process.

During this investigation it was determined that the degradation of the fraction of radiotracer not entrapped by the Pheroid® delivery system might in fact contribute to some of the artefacts identified (e.g. the accumulation in thyroid and saliva glands) during the first two patient scans. Furthermore, as mentioned in the introduction of this chapter, the oral route may lead to a delay in the onset of action or biodistribution of the product when compared to intravenous administration. Irregular absorption may also be present in some patients due to factors unknown at this stage. Due to the restrictions of the half-life (6 hours) of the selected isotope (^{99m}Tc), any manufacturing methods that involves a more labour and time intensive process are not viable. If time intensive strategies are to be investigated, it may be an option to switch the radiotracer to ^{153}Sm-EDTMP, which contains an isotope with a longer half-life (1.9 days) and has additional pain

relief capabilities in the case of bone metastasis. This will enable the patient to completely pass the administered oral dose through the intestines before imaging and allow for imaging without a very “hot” intestine with potential visualisation of previously “masked” body compartments (due to intense activity in the GI tract) such as bone. Another option will be to lower the entire dosage of ^{99m}Tc-MDP, therefore reducing the activity present in the gastrointestinal system. This may result in a higher entrapment percentage and efficiency vs. dosage administered might be increased with a lower amount of radiotracer added to the formulation.

Afterloading as preferred manufacturing process may also be investigated, whereby a ligand to which the isotope is attracted is entrapped in the encapsulated water phase of the Pheroid®. The affinity of the tracer to the ligand will provide the energy for the isotope to move to the inside of the vesicle. Theoretically it might be possible to include the tracer in the water phase during manufacturing of a concentrated Pheroid®. After the system is stable, the water phase on the outside can be diluted with more water to result in a concentration gradient with a diluted concentration on the outside of the vesicles and a concentrated tracer on the inside. Previous studies using fluorophores indicate that the label becomes concentrated in the interior of the vesicles (personal communication; A Grobler). The pertechnetate can be added to the formulation with the concentration gradient resulting in the inclusion of ^{99m}Tc into the vesicles and the formation of the radiotracer. Administration of the formulation may need to take place immediately after addition of the pertechnetate, to ensure that the formed radiotracer does not leach out of the vesicles.

Then again, the current MDP kit will not allow for afterloading, as it contains Sn(II) as reducing agent, which is readily oxidised to Sn(IV) in solution (pH >1) and air (hence the lyophilized packing in a glass vial for storage). Sn(II) is needed for the reduction of Tc from Tc (VII) (pertechnetate) to Tc(V) that enables the binding of Tc to molecules such as MDP. To circumvent this, separately reduced ^{99m}Tc (for instance via the ^{99m}Tc Red blood cell kit) can be added during this final step.

Yet another option would be to use a different tracer for afterloading as a manufacturing method. Alternatively, an additional ligand may be added to the interior of the vesicles, if this does not inhibit the efficacy of the radiotracer. It is a well-established fact that the size of liposomes (not like Pheroid®) is critical to the passive distribution of these systems as well as the elimination and absorption characteristics. It is quite possible that the size of the Pheroid® carriers can have a similar impact and the use of both larger (homogenous distribution) as well as smaller vesicles need to be evaluated.

Future work might include revisiting the rodent studies (especially SPECT scanning on a micro-SPECT specifically used for rodents). Other animal work might include an evaluation of this

system in primates with additional adaptations done that can contribute to a better formulation. Future studies also include the entrapment of other radiopharmaceuticals containing ^{99m}Tc-MDP (for example albumin labelled with ^{99m}Tc) to evaluate the effect of the size of the pharmaceutical entity on the efficacy of entrapment and oral absorption. Other radiotracers can also be evaluated to result in a wider size range. Different radiotracers, for example PET radiotracer ^{18}F-FDG, can also be entrapped in the Pheroid® delivery system for further evaluations. Such studies can lead to a larger database of physicochemical properties of entrapped tracers taking into account *in vivo* data.

The above studies will assist in further elucidation of the characteristics and possible applications of the Pheroid® system. It is however clear that a thorough *in vivo* investigation should be launched before further human testing continues on this particular application.

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CHAPTER 5: ORAL DELIVERY OF INSULIN WITH PHEROID® TECHNOLOGY: AN EVALUATION IN PRIMATES

This chapter describes the oral delivery of insulin that is normally sensitive to degradation by the gastric content, by means of entrapment in the Pheroid® system. This application is patented and has been investigated in rodents. Pre-clinical testing of medicines requires proof of efficacy in two animal species: the larger of which is either the dog or a primate model (FDA, 2015). This chapter introduces the physiological role of insulin (its effect on blood glucose and associated disease conditions). The data of a bioavailability and efficacy study in a primate model is presented in article format (though not for publication purposes).

Insulin has been described as a very fragile molecule with a small therapeutic index (with great toxicity in overdose) and great variance in blood glucose lowering with the same dosage. The method of administration of this protein is distressing to patients due to being only available parenterally. Furthermore, the production of insulin is complicated greatly by its physical instability. The study of methods to better the therapy of Diabetes Mellitus (DM) patients dependent on insulin is a very important area of scientific research (Mayer *et al.,* 2007).

5.1 Blood glucose homeostasis by insulin

Insulin, amongst other functions, increases the uptake of glucose as a nutrient to cells by the muscle, liver and adipose tissue. It is the hormone most responsible for anabolic functions through the increasing of synthesis of adipose tissue, muscle tissue and carbohydrates in the body. The non-functional proinsulin is produced and stored as a hexamer (Figure 5-1), a very stable molecule whereas the active insulin is in the form of a monomer (Figure 5-2) which is far more unstable but results in quick biological actions (Dunn, 2005; Dimitriadis *et al.,* 2011).

Different stimuli can release insulin from the β-cells located in the islets of Langerhans in the pancreas. By far the most important stimuli to the β-cells that increase the secretion of insulin is high levels of glucose. Low levels of glucose inhibit the secretion of insulin and stimulate the α-cells to secrete glucagon into the blood, thereby increasing glycogenolysis and gluconeogenesis. Insulin binds to the receptors on the cellular membranes of target cells (Figure 5-3) resulting in an increase in RNA and DNA synthesis, protein synthesis, glycogenesis, lipogenesis and cell growth. Inhibitory actions of insulin are a reduction in lipolysis, proteolysis, glycogenolgysis, gluconeogenesis and ketogenesis. The effects of insulin are therefore not simply to increase absorption of glucose, as is the public perception, but the lack of insulin also leads to an overproduction of glucose by the liver. Consequently, a deficit thereof do not only result in the

build-up of glucose in the blood, but has a far-reaching impact on the anabolic processes in the body (Sonksen and Sonkensen, 2000; Koeslag *et al.*, 2003; Neal, 2016).

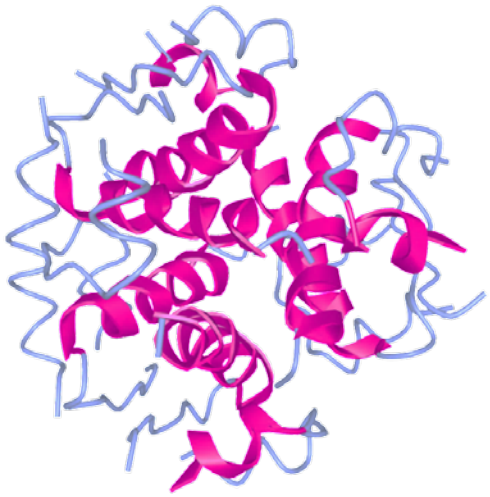

Figure 5-1: The structure of insulin as a hexamer (MMDB ID:7045; PDB: 1ZNI, adapted with iCn3D PDB file viewer provided by NCBI).

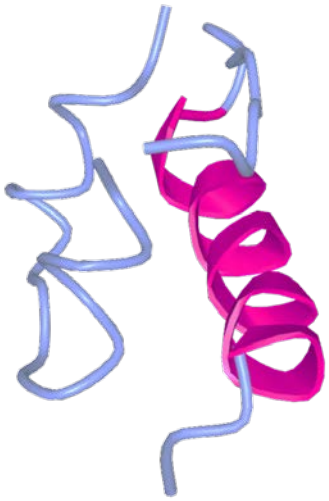

Figure 5-2: The structure of insulin as a monomer (MMDB ID: 61024 PDB ID:2M1, adapted with iCn3D PDB file viewer provided by NCBI).

5.2 Diabetes Mellitus (DM)

The current consensus is that DM is a group of metabolic diseases with the main characteristic of hyperglycaemia. This can be caused by faulty insulin secretion, a lack of action of secreted insulin or a combination of both processes. Inflammation is implicated in the pathology of insulin resistance in type 2 DM. When hyperglycaemia is present for a substantial period, key organs are

damaged (e.g. eyes, heart, nerves) leading to end stage organ failure (Koeslag *et al*., 2003; American Diabetes Association, 2014; Esser *et al.,* 2014).

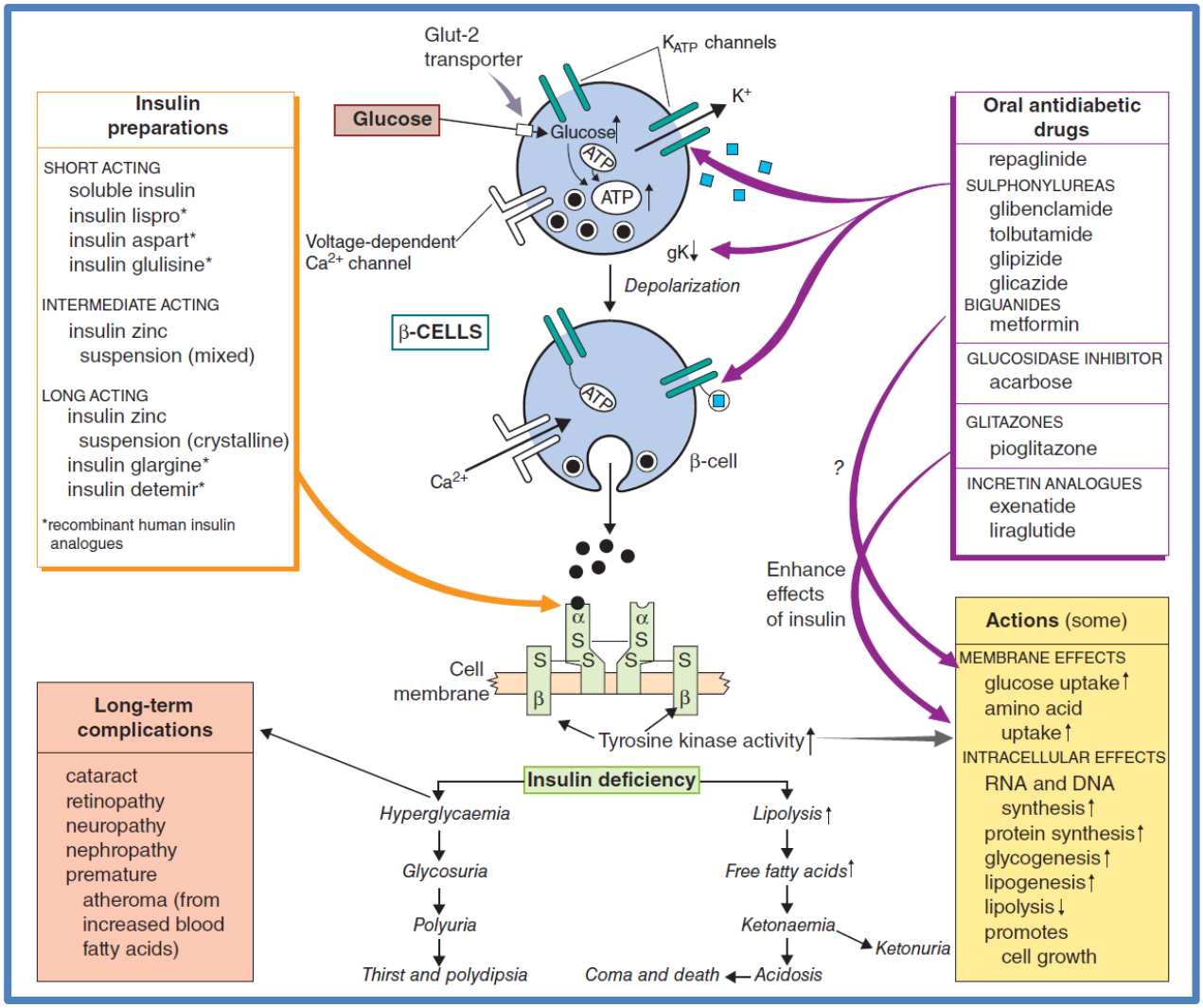

Figure 5-3: Blood glucose homeostasis and mechanisms of action of diabetes medications (Reprinted with permission from the Publisher. Original Source, Neil, 2017. Section 36 in Medical Pharmacology at a glance, John Wiley and Sons ©2015).

The current treatment of Diabetes mellitus will be discussed later in this chapter. Figure 5-3 represents the action of insulin and the effects that result when a deficit or malfunction thereof is present. Note that this figure indicates glucose as the main stimulus for insulin secretion. When insulin binds to the membrane receptors, the tyrosine kinase activity increases and this leads to all the anabolic effects (in this figure categorised as membrane effects and intracellular effects). The autoimmune destruction due to inflammation of pancreatic β-cells may lead to a decrease in the amount of insulin secreted or an abnormality in the function of the produced protein. At the

target organs, the tissue may exhibit a decreased sensitivity to insulin. It is a complex disease, with many stages in the insulin metabolic pathway being sensitive for abnormalities.

Two broad types of DM occur namely, type 1 (an absolute deficiency of insulin secretion) and type 2 (combination of insulin resistance and compensatory insulin secretion). Type 1 DM accounts for 5-10% of diagnosed patients, but no measures are available to prevent the development of this disease if susceptibility is suspected. The identification of a specific type of DM is often difficult since the factors causing the disease can be a mixture of before-mentioned factors. The fact that the same families have genetic family histories of both type 1 DM and type 2 DM indicates that it is possible that most patients have a combination of these two DM types and that the traditional classification might not be relevant any longer. Treatment for this disease is therefore highly individualized (Atkinson & Eisenbarth, 2001; Tuomi, 2005; Tripathi & Srivastava, 2006; American Diabetes Association, 2014).

Patients diagnosed with DM usually present with polyuria, orthostatic hypotension, weight loss, malaise, polydipsia or vision abnormalities. Other acute neurological symptoms include confusion and altered mental state, lethargy, loss of alertness, seizures and in severe cases coma. Acute presentations that can be lethal are hyperglycaemia with concurrent ketoacidosis or nonketotic hyperosmolar disease. Chronic complications include vascular (micro- and macrovascular) as well as non-vascular complications. Microvascular complications include neuropathy (loss of peripheral sensation), nephropathy (kidneys are very susceptible to hyperglycaemia) and retinopathy. An increased incidence of infections can also be present if chronic hyperglycaemia is left untreated. Other concurrent cardiovascular complications like hypercholesterolemia and hypertension are also present and myocardial infarction is a great cause of morbidity in DM. It is estimated that patients with Diabetes Mellitus type 2 usually have the disease for approximately 12 years before clinical presentation leads to diagnosis. By then some irreversible damage is already present (Tripathi & Srivastava, 2006; Aschner *et al.,* 2014; American Diabetes Association, 2014). Other types of DM include diabetes caused by pancreatitis, cystic fibrosis, hemochromatosis, Cushing syndrome, acromegaly, pheochromocytoma, drug induced DM (e.g. by glucocorticoids), genetically caused dysfunction of β-cell function, infections and auto-immune mediated types of DM (Kerner & Brückel, 2014).

Both the risk for developing DM and the diagnosis thereof are highly dependent on the blood glucose levels of the individual. The blood glucose parameters prescribed for diagnosis are as follows (Bolen *et al.,* 2007; Aschner *et al.,* 2014; American Diabetes Association, 2014; Kerner & Brückel, 2014).

- Pre-diabetes: Fasting blood glucose at levels of 5.6 to 6.9 mmol/L. Post meal levels (2 hours) of blood glucose at 7.8 to 11 mmol/L.
- Diabetes: Fasting blood glucose at levels higher than 7.0 mmol/L. Post meal levels (2 hours) higher than 11.1 mmol/L.
- A HbA_{1c} value of ≥ 6.5% per 48 mmol/mol also indicates positive diagnosis for DM.

Additional risk factors in overweight adults are lack of exercise, a family history of DM, ethnic background, women diagnosed with gestational DM, concurrent hypertension or high colestrol, females diagnosed with polycystic ovary syndrome, glucose intolerance and a history of cardiovascular disease. Methods of diagnosis include the oral glucose tolerance test or the glycated haemoglobin blood test (HbA_{1c}). The HBA_{1c} is generally considered as more reliable since the average plasma glucose concentration of the preceding three months is measured (Bolen *et al.,* 2007; Aschner *et al.,* 2014; American Diabetes Association, 2014).

5.3 Incidence of DM

The incidence of DM has more than doubled over the last 30 years, making the treatment and prevention of this disease a priority. A further disquieting development is a significant increase in the incidence of this disease amongst the younger population. Another study indicated that the incidence of type 2 DM is also increasing in children younger than 15 years. In a study published in 2014 there was a 21.1% increase in type 1 DM and a 30.5% increase in type 2 DM in the United States amongst children and adolescents (Dabelea *et al.,* 2014). It is estimated that the amount of people diagnosed with DM in Africa will increase from 27.5 million in 2012 to 49.7 million in 2030, with 522 million cases expected worldwide (Chen *et al.,* 2011a; Kengne *et al.,* 2013; Patterson *et al.,* 2014; Dabelea *et al.,* 2014).

5.4 Current treatment

The treatment of DM is a complicated process and variables have been identified which contribute to the effective control of blood glucose levels: the age at which the patient is diagnosed with DM, the family history of the disease, the duration of the disease, the ethnic background of the patient as well as the body mass index (Hsieh *et al.,* 2014). Type one DM is always treated with injectable insulin, either as multiple injections per day or through automatic administration via infusion. Type 2 DM has an additional oral therapy component with metformin as the first line of therapy (see Figure 5-3). Metformin reduces the production of glucose in the liver and increases the uptake of glucose in muscle tissue and it is also the safest oral agent since it does not have the risk for hypoglycaemia with overdose (Bolen *et al.,* 2007). Other additional agents that can be used concomitantly are sulfonylureas, thiazolidinediones, dipeptidyl peptidase-4 inhibitors, sodium-glucose cotransporter-2 inhibitors and glucagon-like peptide-1 receptor agonists. The most

reported side-effects for DM oral medications are a high risk for hypoglycaemia (reported in sulfonylureas and meglitinides), gastrointestinal adverse effects (metformin) and an increased risk for heart failure (with thiazolidinediones). If the patient with type 2 DM becomes unmanageable on oral therapy alone, insulin therapy should be added to the treatment plan (Bolen *et al.,* 2007; Bennett *et al.,* 2011; American Diabetes Association, 2015). Refer to Table 5-1 for a summary of oral agents, mechanism of action and side-effects.

Table 5-1: A summary of the oral agents used for treatment of DM 2*

Class and example	Mechanism of action	Side-effects
Biguanide (Metformin)	Counter insulin resistance by reducing gluconeogenesis and glucogenolysis. Increase uptake of glucose into skeletal muscle.	- Lactic acidosis - Diarrhoea & weight loss - Neuropathy (Vit 12 deficiency)
Dipeptidyl peptidase 4 inhibitor (Vidagliptin)	It inhibits dipeptidyl peptidase-4 secretion consequently increasing glugcagon-like-peptide-1 levels. Pancreatic response to glucose is therefore increased.	- Pancreatitis - Upper respiratory tract infections
Sodium-glucose cotransporter inhibitor (Canagliflozin)	Blocks the subtype-2 sodium-glucose transport proteins and reduce renal glucose reabsorption.	- Ketoacidosis - Genital mycosis - Bone density reduction - Increase LDL levels
GLP-1 agonists (Exenatide)	Acts like glucagon-like-peptide-1 and increase insulin secretion, suppress glucagon secretion, reduce absorption of glucose and appetite.	- Thyroid cell tumour - Pancreatitis - Gastrointestinal side-effects & weight loss
Sulphonyl Ureas (Glimepiride)	Increase insulin secretion. Directly stimulate insulin secretion at the islet β-cells.	- High risk of hypoglycaemia - Cardiovascular events - Weight gain
Thiazolidinediones (Rosiglitazone)	Increase insulin sensitivity. Increase the production of certain insulin-sensitive genes and reduce insulin resistance.	- Cardiac failure - Bladder cancer - Bone density decrease - Weight gain & oedema - Anaemia
A-glycosidase inhibitors (Acarbose)	Decrease digestion of carbohydrates. Inhibits α-glycosidase which is responsible for gastro-intestinal uptake of polysaccharides and sucrose. Reduce post-prandial increase in blood glucose levels.	- Gastrointestinal side-effects

*Adapted from Tripathi & Srivastava, 2006; Mathieu & Degrande, 2008; Aroda *et al.,* 2012, Kaushal *et al.,* 2014 and Chaudhury *et al.,* 2017).

5.5 Insulin

The first patient was treated successfully with an insulin extract from bovine origin in 1922. Insulin is a protein consisting of 2 polypeptide chains linked with disulphide bridges. As mentioned in the introduction, the hexamer (pro-hormone) needs to be cleaved enzymatically until it is in active monomer form (American Diabetes Association, 2015). Different insulin preparation results in

different pharmacokinetics (Table 5-2) and therapy for each patient can be individualized according to specific needs.

Table 5-2: Insuin preparations on the market and their characteristics (De Witt & Hirsch, 2003)

Class	Type	Trade names	Onset	Peak	Duration
Ultra rapid	Aspart Glulisin Lispro	Humalog NovoRapid	5-15 min	30-90 min	5 hrs
Short	Regular insulin		30-60 min	2-3 hrs	5-8 hrs
Intermediate	Isophane (NPH) Zinc	NPH or Humalin N Humalin L	2-4 hrs 4-12 hrs	4-10 hrs 4-12 hrs	10-16 hrs 12-18 hrs
Long acting	Glargine Detemir	Ultralente Lantus	6-10 hrs 2-4 hrs	10-16 hrs No peak	18-24 hrs 20-24 hrs
Combination	70% NPH/30% regular 50% NPH/50% regular 75% NPH/25% Aspart 70% NPH/30% Aspart	Humalin 70/30 Humalin 50/50 Humalog Mix 75/25 NovoLog Mix	30-60 min 30-60 min 5-15 min 5-15 min	Dual Dual Dual Dual	10-16 hrs 10-16 hrs 10-16 hrs 10-16 hrs

Although a wide range of preparations are available, great shortcomings are still present.

5.6 Shortcomings of insulin therapy

Currently insulin is only available for the parenteral route. The parenteral route is notorious for its poor patient compliance due to pain, allergic reactions and risk of hypoglycaemia (Chaturvedi *et al.,* 2012). It is suspected that DM type 1, which progresses by immune-mediated mechanism due to its autoimmune nature, can be aggravated by parenteral administration of insulin. Alternative methods of insulin administration will have an added benefit in this patient group (Ergun-Longmire, 2004). Peripheral hyperinsulinemic side effects should be relieved by an alternative form of administration (especially oral administration) that transports the administered insulin directly to the liver upon dosing (Wilcox, 2005).

5.7 Alternative insulin delivery systems

Insulin is hampered, like peptides in general, by lack of efficacy when administered through other routes than the parenteral route. Furthermore, insulin needs to be administered multiple times per day which leads to great discomfort experienced by DM patients. Various alternative routes have been investigated for insulin delivery, namely the oral route, nasal route, buccal administration, pulmonary inhalation, transdermal diffusion, rectal administration and ocular administration (see Figure 5-4).

A few alternative insulin formulations have reached the market (see Table 5-3). Exhubera®, an inhalable insulin was the first product to be sold but was retracted after poor marketing strategies failed to recruit users. A product that also reached the market was Oral-lyn which is a buccal administered micellar solution. Macroflux® is under development for application in insulin delivery by utilization of microprojection array patch technology (Hultström *et al.,* 2014). The latest product, Afrezza® an inhalable insulin, was introduced to the market in 2017 with no patient acceptability feedback available yet (Goldberg & Wong, 2015). The advantages and disadvantages associated with the different routes of administration is summarized in Table 5-4.

Table 5-3: A summary of alternative insulin delivery systems to reach the market.

Name	Route	Comments
Nectar/Exubera	Powder pulmonary inhalation	Big cumbersome device, poor marketing. Was retracted 1 year after launch (Mack, 2007).
AERx®	Liquid aerosol inhalation	Not yet marketed (Heineman, 2011; Heineman & Jacques, 2009).
Afrezza® (MannKind)	Dry powder inhalation	Currently marketed. Smaller device with effective lowering in blood glucose values. May carry a risk of lung cancer (Kim *et al*., 2003; Goldberg & Wong, 2015).
Oral-lyn	Buccal administration spray	Rapid absorption and 2-hour effect. Little literature available. This product is registered in Ecuador. Trials still under way for FDA approval (Heineman, 2011; Heineman & Jacques, 2009).
Aerodose/ Aerogen	Inhalation insulin	Last positive literature published in 2003 (Owens, 2003).
Macroflux®	Microprojection array patch technology - transdermal	Preliminary results demonstrate efficacy when compared to subcutaneous injection (Hulström *et al.,* 2014).
AIR Insulin	Dry powder inhalation	Phase 2 study in 2007 demonstrated efficacy. No further literature available (Muchmore *et al.,* 2007).
Capsulin	Oral enteric coated insulin for release in small intestines	Still in clinical trial phase. Demonstrates a hypoglycaemic action of 6 hours in duration (Heineman, 2011; Heineman & Jacques, 2009).
ORMD-0801	Oral combination of insulin with enzyme inhibitors.	Still in clinical trial phase. No adverse events.
IN-105	Insulin analogue with increased stability and solubility	Biocon had to stop development after Phase II trial due to lack of efficacy (Heineman, 2011; Heineman & Jacques, 2009).
Eligen	Oral carrier agent that conform insulin in a complex that protects it against GI tract	Phase 2 clinical trial in 2008 was successful, no further developments (Hoffman & Qadri, 2008).

Other therapies to increase the internal production of insulin are the transplantation of a healthy pancreas into the DM patient as well as stem cell therapy. Pancreas transplantation mostly

concurs with kidney transplantation in end-stage renal failure. Organ rejection is quite commonplace and immunosuppressive drugs must be administered to the patient (Rekittke *et al.,* 2016).

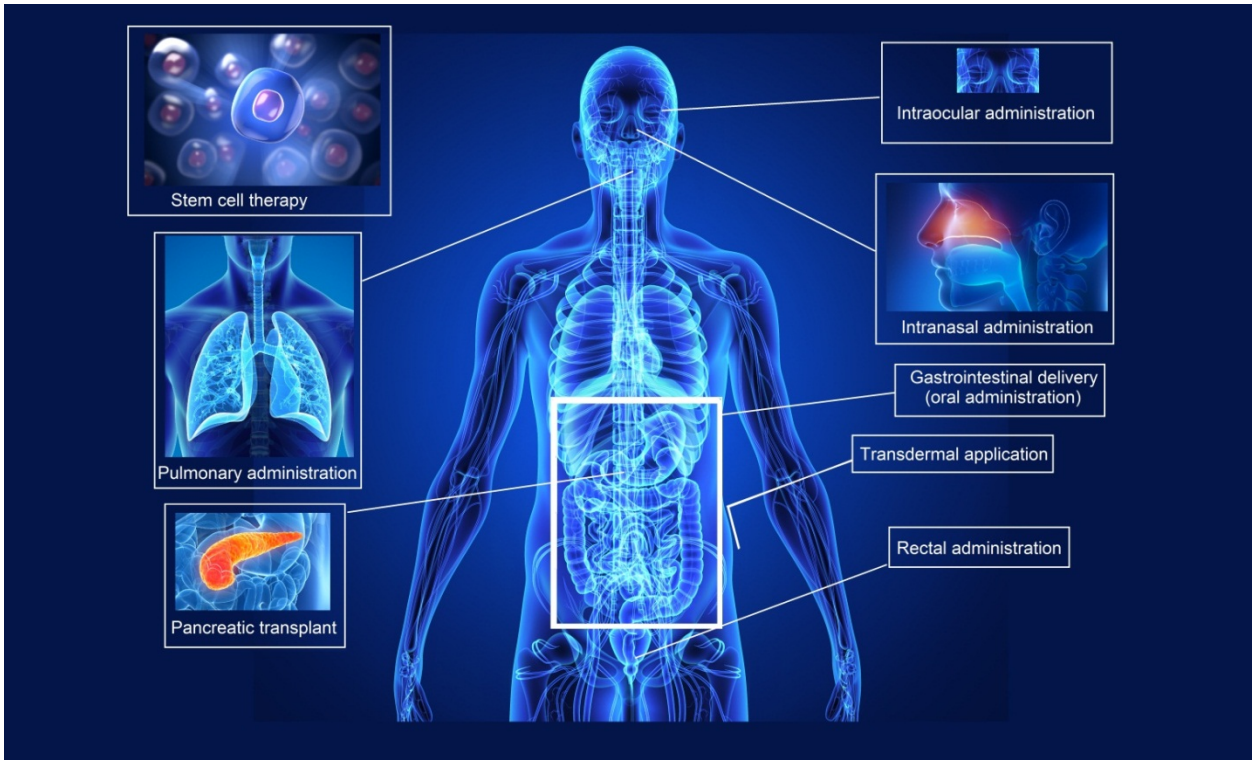

Figure 5-4: A summary of strategies to increase insulin concentration other than subcutaneous injections (adapted from Owens, 2002; Mayer *et al.,* 2007; images bought from Shutterstock – royalty free images, licence information included in addendum).

5.7.1 The oral route of administration

This route is an accepted route by both manufacturers and patients alike (Carino & Mathiowitz, 1999). Formulations administered through this route do not have to adhere to the strict safety measurements regarding sterility which is required from parenteral formulations. The development of an oral insulin is eagerly anticipated by DM patients and this route will improve patient compliance far more than any other strategies There is therefore sufficient demand for such a product, but the accompanying technology needs infrastructure, economic and human resource investments. It is associated with fewer risks of adverse events and has the highest patient compliance of all routes.

Table 5-4: Different alternative routes and their advantages and disadvantages (adapted from Kafagy *et al.,* 2007; Shah *et al.,* 2016).

Route	Advantages	Disadvantages
Oral	Increased portal insulin Good acceptance by patient	Lower bioavailability
Buccal	Bypass first-pass metabolism	Presence of saliva and swallowing
Intraocular	Bypass first-pass metabolism No immunological reactions Rapid absorption	Blinking and tearing Rapid loss of active ingredient through drainage
Intranasal	Large area for absorption rich in blood vessels	Nasal irritation Great variation in absorption
Transdermal	If no needles are involved – very acceptable to patients.	Local irritation Formation of redness and blisters
Rectal	Bypass first-pass metabolism	Local irritation and patient compliance
Pulmonary	Rapid onset of action No enzymatic barrier Better patient compliance	Inhalers not optimized Decrease lung function Side-effect of coughing Cancer risk undetermined

If insulin is delivered through the oral route the result might be a more physiological release profile of the active insulin in contrast to the immediate availability of parenteral formulations. It will result in a more portal availability of insulin rather than peripheral that leads to less side-effects. The risk of side effects associated with injections for example immediate hypoglycaemia, peripheral hyperinsulinemia, pain at the injection site and so forth, will be reduced. The challenges faced by oral insulin administration is the gastro-intestinal enzymes (proteolysis) to be overcome, the pH differences throughout the gastro-intestinal tract, as well as impediments to absorption caused by mucus and epithelial barriers (Carino & Mathiowitz, 1999; Kafagy *et al.,* 2007; Shah *et al.,* 2016; Muheem *et al.,* 2016).

Since insulin was discovered, 90 years of search for an oral insulin followed with the pilot first in man evaluations in 1922. Today there are numerous oral formulations in the preclinical and clinical phases, but an oral agent is yet to reach the market. The high variability in the gastro-intestinal physiology and transit time of patients in clinical trials result in many promising agents failing in phase III clinical trials. It is therefore important that the chosen drug delivery system leads to a predictable physiological response (Owens, 2002; Ziljstra *et al.,* 2014).

The challenges for oral protein absorption are as follows (Muheem *et al.,* 2016):

- The high molecular weight structures and both hydrophilic and hydrophobic areas in the protein results in poor absorption over biological membranes.
- These molecules loose function when small changes in structure are made which can be caused by changes in pH and enzymes and they are therefore unstable.
- Most proteins possess short biological half-life with rapid clearance by the liver.
- Great amounts of proteins are degraded in the stomach and duodenum due to pH changes.
- The great biological potency of these molecules requires precise dosing.
- Proteins can illicit an immune response more easily than other chemicals.
- Some proteins need to be activated by enzymatic cleaving and these enzymes are present only in certain areas of the body.
- Proteins are expensive and difficult to manufacture.

Different technologies have been developed to overcome these barriers, with various levels of success. Capsulin is an oral insulin formulation (enteric coated insulin) that has demonstrated efficacy in clinical trials, but no further developments are available in literature (Luzio *et al.,* 2010; Shah *et al.,* 2016). The different technologies are as follows:

a) Absorption enhancers

Absorption enhancers increase the delivery of pharmaceutical active ingredients across epithelial barriers. One of the strategies that has been used with some success during *in vivo* evaluations is based on a physical change to these absorption barriers through changing membrane fluidity, decreasing mucus viscosity, increasing the leakage of proteins through membranes or by opening up tight junctions present in the epithelial lining. The main problem with this strategy is that any physiological change brought about to allow for the absorption of insulin, also allows for the increased transport of other unwanted molecules normally blocked by the barriers in the gastro-intestinal tract (Radwan, 2002; Kafagy *et al.,* 2007).

b) Enzyme inhibitors

Enzyme inhibitors may be used to slow down the degradation of insulin in the gastro-intestinal system, thereby raising the insulin concentration available for absorption. Again, various studies have proven beneficence. Problems associated with this approach are possible side-effects, since other peptides or protein molecules that is normally degraded by normal physiological functioning, will also be absorbed in higher concentrations. A high incidence of intestinal discomfort due to reduced digestion of food proteins is associated with this method (Kafagy *et al.,*

2007). Refer to Table 5-2 regarding ORMD-0801, an oral insulin which incorporates enzyme inhibitors for the oral delivery of insulin (Eldor *et al.,* 2013).

c) Mucoadhesive polymeric systems

Upon administration certain polymeric drug carrier systems become adhesive in the gastro-intestinal system and can bond to gastric mucosa with concurrent hydration. This allows for prolonged intensive contact of the insulin, entrapped in this system, to the mucus and epithelial barrier of the stomach as well as less exposure to the gastric fluids and enzymes present. Different mucoadhesive polymeric systems exist and different studies investigated the use thereof in insulin delivery. There are currently no major concerns regarding the safety of these systems and it is seen as a viable option for insulin administration (Whitehead *et al.,* 2004; Krauland *et al.,* 2004; Kafagy *et al.,* 2007). All research on mucoadhesive polymer systems currently published is still in the preclinical phase (e.g. Andreani *et al.,* 2015; Kim *et al.,* 2015; Mukhopadhyay *et al.,* 2015)

d) Particulate carrier delivery systems

Particles smaller than 10 µm enter the blood circulation through gut-associated lymphoid tissues. Included in this group of systems are liposomes, microcapsules, nanocapsules and polyacrilic acid. The Pheroid® technology described in the main part of this dissertation falls into this category. The problems associated with these technologies include the effective entrapment of the polyphylic insulin in these systems, the control of the release of pharmaceutical ingredients out of these systems, the stability of the system as well as the prevention of aggregation. The toxicity of these systems need in depth evaluation for each application (Kafagy *et al.,* 2007; Builders *et al.,* 2008). An in-depth review on the oral delivery of insulin through nanoparticles was provided by Chen and co-workers (Chen *et al.,* 2011b). None of these systems has progressed to the clinical trial phase yet.

5.7.2 Buccal administration

This transmucosal route allows for the absorption of the protein through the internal jugular vein providing high bioavailability and a bypass of the hepatic first-pass metabolism. It has been shown that the buccal administration of insulin provides dependable constant concentrations in the blood circulation (Xu *et al.,* 2002; Kafagy *et al.,* 2007).

Absorption enhancers however need to be included in formulations that are destined for the buccal route and these agents have been shown to cause local irritation of the mucosa at the site of administration. Another limitation is the presence of saliva and the probability of premature swallowing of the system. In order to solve some of these problems, an insulin buccal spray has

been developed. This is marketed under the trade name Oral-Lyn™ (Xu *et al.,* 2002; Kafagy *et al.,* 2007). For a thorough review of Oral-Lyn™ and clinical trials thereof refer to an article written by Pozzilli and co-workers (Pozzilli *et al.,* 2010).

5.7.3 Nasal administration

An attractive route for the administration of insulin is the nasal route. Scientists at the North-West University have investigated this route for the administration of Pheroid® entrapped insulin with promising results (Oberholzer, 2009). The nasal tissue is a large area for absorption rich in blood vessels, with avoidance of the first-pass metabolism by the liver.

The limitations of this route for the absorption of proteins include clearance by the mucuciliary system, some enzyme activity present on the nasal surface and epithelial barriers. Absorption enhancers must again be incorporated in the system with the concurrent possibilities of side-effects. Damage to the nasal mucosa may present with particularly uncomfortable side-effects and poor patient compliance. This route of administration is also characterized by a large variety of physiology between patients. Other variations like presence of infection, higher excretion of mucus and allergies might result in day to day variations of bioavailability in the same patient (Kafagy *et al.,* 2007).

Human studies have provided conflicting results, with some advantages still well proven e.g. improvement of hepatic energy metabolism as well as increased cerebral perfusion and better cognition in geriatric DM 2 patients (Novak *et al.,* 2014; Gancheva *et al.,* 2015).

5.7.4 Pulmonary administration

The lungs consist of a large surface area that is rich in blood flow from which pharmaceutical ingredients can be absorbed and it also bypasses the first-pass metabolism. This route of administration for insulin has received a lot of interest as a possible target for delivery (Goldberg & Wong, 2015; Shah *et al.,* 2016).

Drugs can be delivered as aerosol formulations if particle sizes are less than 5 µm. The enzymatic differences of the lungs compared to the gastrointestinal system are aimed at different targets and also lack peptidases and as a result proteins can be administered due to an absence of enzymes degrading them. Formulations for this route include dry powders and particulate carrier systems like liposomes and nanoparticles. It is believed that insulin absorption by this route is through transcytosis and paracellular mechanisms. Indeed, two drug delivery systems (Exhubera® and Afrezza®, both dry powder formulations) that is administered through this route of administration have reached the market (Goldberg & Wong, 2015; Shah *et al.,* 2016).

Disadvantages of this administration route are the impact of lung physiology affecting absorption for instance asthma, smoking, and the greater weight gain reported for patients receiving inhaled insulin compared to patients on normal insulin injections. Higher incidences of hypoglycaemia were also reported for inhalant insulin therapy. The safety of chronic inhalation is still under investigation, although vast amounts of data may become available soon since such a formulation is on the market for clinical use (Patton *et al.,* 1999; Skyler *et al.,* 2001; De Fronzo *et al.,* 2005; Freemantle *et al.,* 2005; Rosenstock *et al.,* 2005; Kafagy *et al.,* 2007).

An in-depth review regarding inhalation insulin with specific focus on Exubera® and the results of various clinical trials, patient compliance and effects on physiology, is provided by Barnett. It is necessary to take heed of the mistakes made during the development and marketing of Exhubra® to circumvent similar mishaps in the future since this product was clinically viable (Barnett, 2004). Another in depth-evaluation is provided by Mack (2007) regarding the decision of Pfizer to stop the marketing of Exubera®. Pfizer is blamed for not having a good enough marketing strategy and the sales topped only $12 million after 9 months compared to the estimated $ 1 - 4 billion expected. The reasons given were device was also far more costly than normal insulin therapy and that physicians were hesitant to change the therapy of patients who were already stabilized on subcutaneous therapy. Furthermore, the inhaler device was also very big, and patients indicated that they were uncomfortable to use it in public. Mack concludes that caution should be taken to ensure that the device is acceptable to patients and that clinicians are provided with enough data and marketing information to ensure that they are willing to prescribe alternative dosage forms (Mack 2007; Shah *et al.,* 2016).

Afrezza®, marketed by MannKind and Sanofi-Aventis, employs a new smaller inhaler (Gen2) and Technosphere® formulation of insulin as a dry powder. This device is smaller and is proposed to be more acceptable to patients. The side-effects identified during clinical trials were hypoglycaemia, a decline in pulmonary function from baseline (over two years), coughing, diabetic ketoacidosis, lung cancer, hypokalaemia and oedema. Listed as an interaction with thiazolidinediones, Affreza® can cause heart failure when administered concomitantly (Goldberg & Wong, 2015; Kim & Polsker, 2015; Shah *et al.,* 2016). Lay-man's articles demonstrate a low and steadily decreasing amount of scripts and a reduction in stock value for MannKind. MannKind has recently appointed a new Chief Medical Officer to assist with the marketing of Afrezza® so that this product can reach its full market potential. (Osborne, 2018; MannKind, 2018).

This route is highly researched for alternative delivery of insulin, but is hampered by the quality of inhaler devices, costliness and questions regarding long-term safety.

5.7.5 Ocular administration

Another novel route of administration is the eye. There is an astonishing low incidence of side-effects reported when insulin is administered via eyedrops (Chiou *et al*., 1994). This route has a low incidence of immunological reactions when peptides are administered, the rate of systemic bioavailability is equal to that of subcutaneous injections and the first-pass hepatic metabolism is bypassed. Problems associated with this route of administration are irritation, blinking and tearing as well as rapid loss of the pharmaceutical ingredient through drainage (Kafagy *et al.,* 2007; Matteucci *et al.,* 2015). This route of administration can also be utilized for the Pheroid® delivery system as it is safe for ocular administration and might be a future prospect for this technology. A small amount of literature is available on effective strategies to utilize this route as more efforts seem to be focused towards inhalation, transdermal and oral formulations.

5.7.6 Rectal administration

This route is useful for drug delivery. Compared to the rest of the gastro-intestinal system, since the absorption is more stable and fewer enzymes are present. If suppositories are administered correctly some of the first pass hepatic metabolism may also be avoided. Various suppository formulations are in the preclinical development phase. Patient compliance and acceptance as well as possible local irritation due to formulations administered are to be considered (Kafagy *et al.,* 2007). This area of research was pursued extensively in the 1980's and 1990's (e.g. Aungust *et al.,* 1988; Ritschel & Ritchel, 1984; Yamamoto *et al.,* 1992; Watanbe *et al.,* 1992). Since then only a few publications were made available on the topic. Researchers at the North-West University investigated rectal insulin delivery through a system based on N-trimethyl chitosan chloride in rodents with positive results (Du Plessis *et al.,* 2010). No product involving rectal administration has researched the market yet.

5.7.7 Transdermal administration

Transdermal application is associated with good patient compliance, as well as the possibility of controlled release. It is however difficult to deliver APIs with a high molecular weight across this barrier. All the traditional methods for transdermal delivery has been applied to insulin with varied success and include the use of absorption enhancers, transdermal delivery systems (nanoparticles), sonophoresis, iontophoresis and the use of microneedles. Due to the large size of insulin molecules, the bioavailability achieved with these methods is still very poor compared to subcutaneous injections (Kafagy *et al,* 2007).

A product that is however currently gaining momentum is Macroflux® which is solid microneedles arranged in a unit that can create nanometer-scale holes in the stratum corneum through which

proteins including insulin can be delivered. This technology is however not yet at a stage to be used clinically (Martanto *et al.,* 2003).

5.8 The additional synergistic effect of the essential fatty acid components of Pheroid® on the treatment of DM

It has been established that DM 2 has a high concurrent incidence of other metabolic diseases including mostly hypertension, skewed cholesterol levels and other cardiovascular diseases. There is a direct link between dyslipidaemia and the development of DM 2 (see Figure 5-5). A diet that is low in essential fatty acids leads to a decrease in the availability thereof to be incorporated in skeletal-muscle membranes which in turn increase insulin resistance in skeletal muscle. The increase in insulin resistance leads to a great variety of metabolic defects.

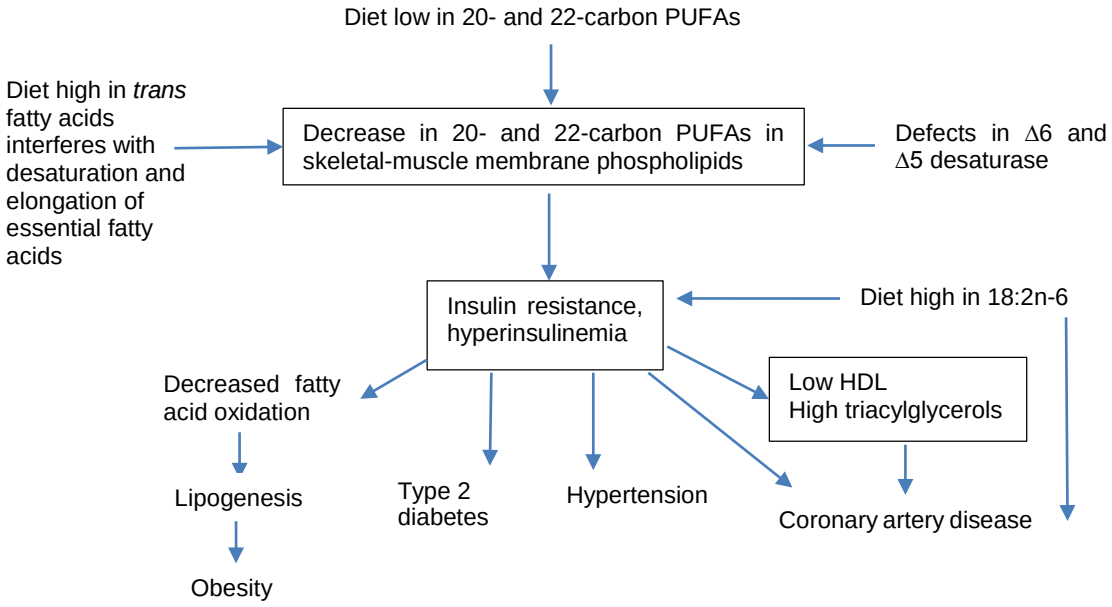

Figure 5-5: The interaction between dietary intake of essential fatty acids and DM 2 and other metabolic diseases (Reprinted with persmission from the Publisher. Original source: Simopoulos, 1999. The American Journal of Clinical Nutrition, 70: 560s-569s. ®1999 Oxford University Press).

The supplementation of essential fatty acids corrects the ratios of the different blood lipid levels in the patient. It is possible that some of the nerve damage present after prolonged DM disease can be reduced by supplementation with essential fatty acids (Stevens *et al.,* 1993; Das, 1995) Since Pheroid® is composed mainly of essential fatty acids (ethyl esters) and is indeed taken as such for supplementation, this can provide with a synergistic effect in the treatment of DM 2 patients with metabolic disease. An article is presented were data obtained in a primate

substantiate the viability of the Pheroid® system as a formulation that allows for the oral administration of insulin.

Oral delivery of insulin with Pheroid® technology: an evaluation in primates

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Abstract

Objective: The objectives of this study were to evaluate the effect (both efficacy and bioavailability) of an oral insulin Pheroid® and pro-Pheroid® formulation in primates. The evaluation was based on a comparison with the gold standard subcutaneous insulin (NovoRapid®) in terms of blood glucose levels and blood insulin levels.

Methods: NovoRapid® insulin was administered subcutaneously in the gold standard treatment group and the effect thereof was compared to recombinant insulin formulated in an oral Pheroid® emulsion or pro-Pheroid® capsules. Cynomolgus monkeys received a single dose and blood was collected over time (0, 30, 60, 90 and 300 minutes). A handheld glucometer was used to analyse blood glucose levels on the study day and collected blood samples were measured for insulin and glucose content by use of ELISA. The pre-administration and post-administration blood samples were also evaluated for changes in clinical biochemistry.

Results: The Pheroid® insulin emulsion demonstrated efficacy in lowering blood glucose in a less drastic but more prolonged manner when compared to the control insulin. The pro-Pheroid® capsule did not demonstrate blood glucose lowering in a useful manner but may demonstrate extended release characteristics. The effect of the Pheroid®-insulin emulsion on glucose levels of the primates extended beyond the 5-hour monitoring time of this study. Pheroid® -based insulin therefore has potential as a viable oral formulation for the administration of insulin and should be clinically investigated.

Keywords

Oral insulin, pharmacokinetics, drug delivery system, Pheroid®, cynomolgus primates, diabetes mellitus

1) Introduction

The incidence of diabetes mellitus (DM) is increasing at an alarming rate and it is expected to reach a total of 522 million cases worldwide in 2030. The incidence of this disease is also high amongst children and adolescents with a 21.1% increase in type 1 DM and a 30.5% increase in type 2 DM in the US alone (Chen *et al.,* 2012; Kenge *et al.,* 2013; Dabelea *et al.,* 2014). DM is

caused by either a reduction in the secretion of insulin or a deficiency in the action thereof, or both. Insulin is the hormone in the body responsible for the absorption of glucose into fat, the liver and skeletal muscle and it is produced by beta cells in the pancreas. The backbone of treatment for type 1 DM is insulin, but insulin is also important in type 2 DM where blood glucose cannot be controlled by other measures (Bastaki, 2005).

Many alternative routes for insulin administration is currently under investigation which includes the oral route, buccal application, nasal administration, pulmonary inhalation, topical ocular application and rectal administration. The oral route is particularly attractive due to less exhaustive regulatory requirements (regarding sterility and pyrogenicity) and is generally the more pleasing route of delivery from a patient's perspective (Khafagy *et al.,* 2007). An oral insulin formulation must be able to overcome all the barriers faced by proteins to reach the systemic circulation intact. The enzymes and acid content of the stomach, mucus and composition of the cellular lining of the gastrointestinal tract all contribute to block absorption of larger protein molecules through this route (Chen, 2011). Currently permeation enhancers (Krauland *et al.,* 2004; Mukhopadhyay *et al.,* 2012; Marais *et al.,* 2013) and enzyme inhibitors (Marschütz & Bernkop-Schnürch, 2000; Liu *et al.,* 2003) are under investigation to increase the oral absorption of insulin, but all these systems induce physiological adaptations that increase the risk of side-effects.

The Pheroid® technology (including Pheroid® vesicles and pro-Pheroid® capsules) is a nano- or microemulsion system made by an environmentally conscious manufacturing method using inexpensive and non-toxic ingredients. The formulation consists of three phases: an oil phase (comprised of essential fatty acids), a water phase and a nitrous oxide gas phase. This technology has been proven to enhance the absorption of proteins through the oral route, without inducing physiological changes that are unsafe. Calcitonin, a peptide hormone regulating calcium metastasis, reached therapeutically effective concentrations upon intestinal administration in rodents. Previous investigations of an oral Pheroid® insulin formulation in Sprague-Dawley rats also demonstrated beneficial absorption (Oberholzer, 2009; Du Plessis *et al.,* 2010).

The aim of this study is to evaluate the efficacy of a Pheroid® insulin oral emulsion, as well as pro-Pheroid® insulin containing capsules when compared to the gold standard subcutaneously administered human insulin (NovoRapid®). Both the test formulations and the control formulations were administered to cynomolgus primates (*Macaca facicularis*) to evaluate the effect on blood glucose levels.

2) Materials and methods

2.1) Materials

The lipid phase of the Pheroid® vesicles and the pro-Pheroid® formulation were manufactured from vitamin F ethyl ester CLR (obtained from CLR Chemisches Laboratorium, IMCD), Incromega E3322 and E7010 (Croda chemicals, South Africa), dl-alpha tocopherol (Chempure, South Africa) and Kolliphor EL (BASF). The formulations were gassed with nitrous oxide gas obtained from Afrox situated in South Africa. The insulin used in test formulations was obtained from Sigma-Aldrich (recombinant human insulin, Zn containing 27.5 IU/mg). The gold standard control formulation was NovoRapid® (Novo Nordisk, 100 IU/ml). A gelatin based size 2 Licap™ capsule was used to dose the pro-Pheroid® and was provided by Capsugel (Belguim).

2.2) Pheroid® formulations

The pro-Pheroid® formulation was prepared by heating vitamin F Ethyl Ester (65%), Kolliphor EL (22%) together in a microwave. After cooling the mixture down, the remaining oil-phase ingredients were added (Incromega E3322 and E7101SR, dl-alpha tocopherol). The mixture was gassed with nitrous oxide (± 170 kPa) for 3 days. The human recombinant insulin was suspended in the pro-Pheroid® formulation and capsulated according to the individual weight of the primates. After manufacturing, the capsules were stored in a fridge at 5°C.

To manufacture the Pheroid®, sterile water was gassed with nitrous oxide (± 170 kPa) for 3 days. Human recombinant insulin was dissolved in the gassed water after which the insulin containing water was mixed thoroughly with previously manufactured and gassed pro-Pheroid® in a ratio of 1:9 of oil to water. The formulation was stored in a fridge at 5°C overnight, to allow for entrapment of insulin into vesicles to complete.

2.3) Analysis of formulations

The particle size of the vesicles composing the test formulations was measured by laser diffraction (Malvern Mastersizer Hydro 2000, Malvern instruments, Worcestershire) which is provided as a polydispersity index. During measurement the laser obscuration was retained between 10 and 20% and the pump speed at 1500 rpm. Samples were measured six times with the mean and standard deviation reported. To determine the stability of the formulation, the zeta potential of the samples was measured (Malvern Zetasizer Nano ZSP, Malvern instruments). To provide evidence of the correct morphological structure of vesicles, confocal laser scanning electron microscopy (CLSM, Nikon D-eclipse C1 confocal scanning microscope) was performed using the CLSM method outlined in a previous publication (Slabbert *et al,* 2011). To enable visualisation of

the vesicles formed by self-assembly of pro-Pheroid® after administration, the formulation was mixed with a 0.1 N hydrochloric acid diluent mimicking the pH of the gastric contents as described previously (Grobler *et al,* 2014). The confocal image is a merged from an image taken at wavelength 568 - 642 nm and an image taken at a wavelength of 500 - 530 nm.

2.4) Experimental animals

The study included 12 cynomolgus primates (*Macaca facicularis*), 6 females and 6 males. The Mauritius Cynomolgus monkey is Specific Pathogen Free (SPF) animals that are free from SRV, SIV, STLV, B-Virus, filoviruses, rabies, malaria, dengue and chickungunya. The animals were maintained at LCL-Cynologics (Les Champeches site) and this facility is AAALAC accredited. Ethical approval for this study was provided by the Minister of Agro Industry and Food Security, Mauritius (CYV/1117/002/17). The animals at LCL-Cynologics are group housed in open cages with temperatures, humidity and environment the same as that of their natural habitat. Temperatures range between 18 ˚C in the winter to 30 ˚C in the summer with a humidity of 80%. Pens are equipped with iron sheeting and sunshades to protect against the elements and provide shade. Cages are cleaned once a week with an environmentally safe disinfectant to reduce transmission of any illness. Regular feeding schedules are kept and *ad libitum* access to water is provided. Unnecessary noise near the monkey housing units are not allowed through strict internal regulations (e.g. limited vehicle access and noise equipment installed at fair distance from animals). Animals are housed according to weight and age and are routinely observed to monitor group behaviour and social hierarchy problems. In addition to standard perches, each cage has an addition of at least 3 enrichment structures consisting of the following: toys (e.g. plastic pipes, coloured balls, and suspended devices), hanging devices (e.g. barrels, hanging platforms or ladders) and a wheel placed on a fixed pivot. Other enrichments include ropes, swing feeders and foraging devices. All structures that are suspended are moved periodically to allow changes to the primary structure that the animals are kept in.

2.5) Study plan

All the animals enrolled were aged between 24 to 30 months and the weight variation was kept at no more than ± 300 g. The study animals were randomly assigned into the three study arms (Figure 1) ensuring that each group has an equal number of females and males. The groups were then randomly assigned to treatment. The international unit (IU) of insulin is a unit of biological equivalence (1 IU = 34.7 µg of pure crystalline insulin) and different insulin formulations can therefore be administered in the same amounts. Literature indicates a trend to use commercial preparations for the control animals in the same units as administered of the test formulation. This circumvents all the issues regarding sterility, stability and particle size that is associated with

parental preparations. Although the administration is subcutaneous, an adverse event with sub-par formulations (e.g. pain and allergic reactions) still comes into play. Grainger and co-workers (2004) gave a control dosage of 0.1 IU per kg of commercial insulin, administered subcutaneously, to healthy control cynomolgus monkeys. The starting dosage for the control animals during this study was therefore 0.1 IU per kg.

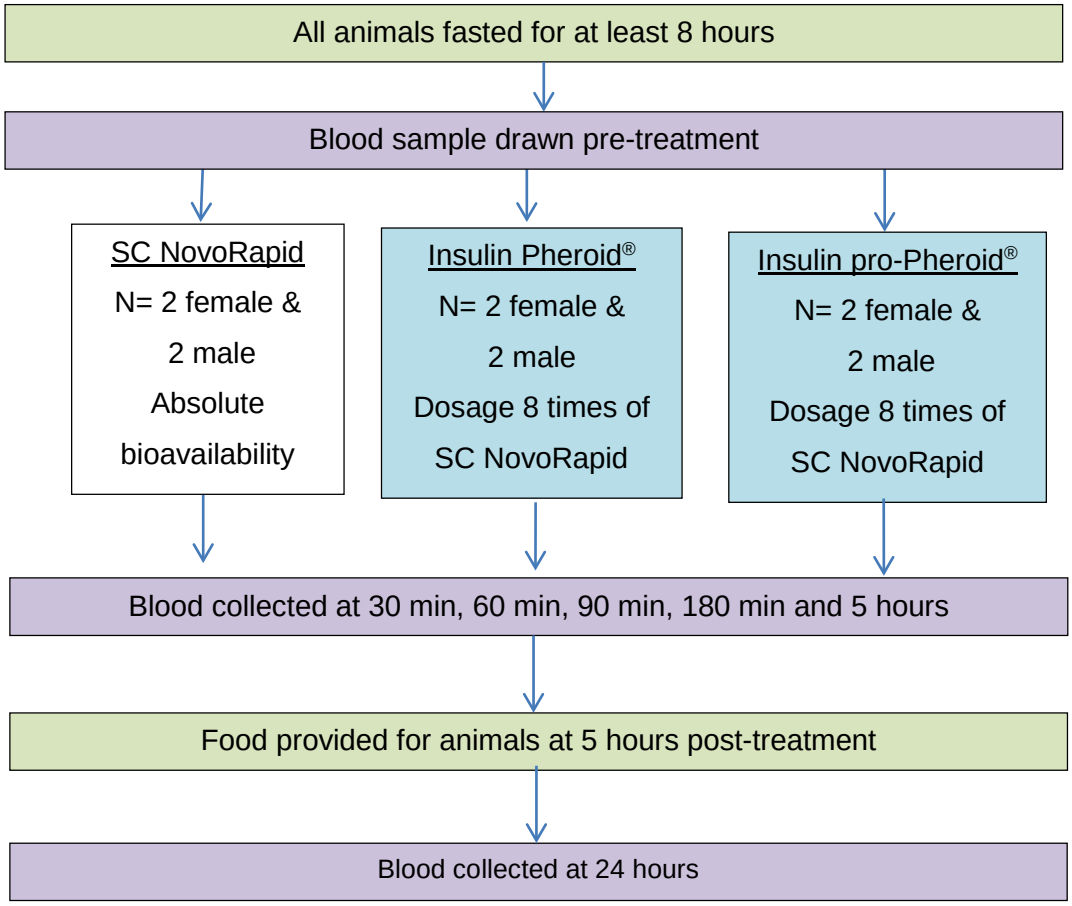

Figure 1: Study plan followed during this investigation

2.6) Analysis of blood samples

The glucose measurements were done by using a point-of care human glucometer (Accucheck-Active®). Additionally, the glucose levels were analysed in the laboratory together with all the other biochemical blood parameters (analysis performed by the MRC Harwell Institute, Oxforshire, UK). Insulin levels were determined with enzyme-linked immunosorbent assay (ELISA) for human insulin (RE53171, IBL, Germany).

2.7) Statistical analysis

Descriptive statistics were reported for each formulation tested as the mean of the values obtained from blood samples for the relevant treatment group per time (mean ± SD). Statistical comparison was done through analysis of variance (ANOVA) and a level of $p<0.05$ was considered as significant. For toxicity the Mann-Whitney U test was performed and the Wilcoxon Matched Pairs Test as further analysis of any finding that was statistically significant.

3) Results and discussion

3.1) Characterization of test formulations

Both formulations were evaluated for particle-size distribution, zeta potential and morphology. The particle size of the Pheroid® insulin emulsion had a span of 7.199 µm and a median particle size of 3.184 µm. The distribution (Figure 2) was in a way that 80% of the particles were distributed between 0.202 µm and 8.364 µm. The zeta-potential of this formulation was -18.9 mV. The pro-Pheroid® capsules demonstrated a span of 6.011 µm upon dilution (Figure 2) and the median particle size was 3.637 µm. The distribution of 80% of the particles was between 0.292 µm and 9.786 µm. The zeta-potential of the pro-Pheroid® formulation was -9.99 mV. The CLSM images (Figure 2) demonstrated the successful formation of Pheroid® vesicles that contained internal structure where the insulin could be entrapped.

Although there are some differences in the size distribution of the two formulations, both demonstrates a typical distribution for Pheroid® formulations including a bimodal distribution. The zeta-potential of the formulation is relatively low, although this might be attributed to the entrapment of the insulin.

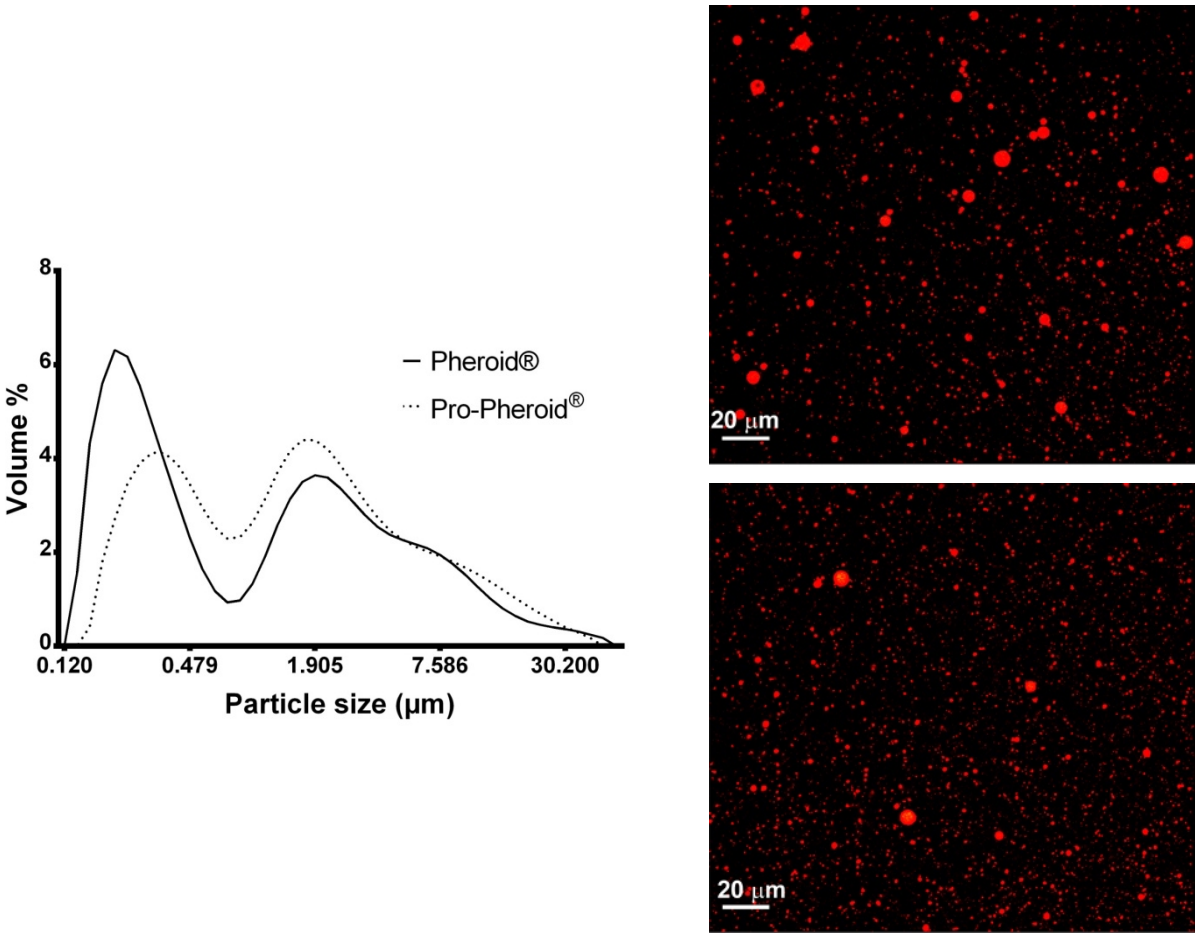

Figure 2: The particle size distribution (mean provided for 6 measurements per sample) with the pro-Pheroid® formulation and the Pheroid® formulation, both with insulin included. CLSM pictures on the right are of the Pheroid® formulation (top) and the pro-Pheroid® formulation (bottom).

3.2) Changes in blood glucose, insulin levels, creatinine kinase and ketone body levels

The first pilot study animal was injected 0.1 IU per kg of animal, subcutaneous control (NovoRapid®) and demonstrated sufficient lowering of blood glucose levels with an average drop of 2 mmol/L at 90 minutes post injection, measured with the hand-held glucose monitor. The Pheroid® and pro-Pheroid® capsules were loaded with 0.8 IU per kg of animal to allow for a substantial loss in bioavailability. The changes in blood sugar level from the pre-dose blood sample (handheld data provided in Figure 3 and laboratory values in Figure 4) demonstrated a lowering in glucose of short duration as expected from the subcutaneous control injection. The pro-Pheroid® capsules did not provide significant lowering in blood glucose and further development needs to be done. All the original blood glucose values was in line with literature (3.9-5.6) – values varied between 4-6 mmol/L for fasted animals with the lowest value recorded after treatment as 2.7 (Margliano *et al.,* 2011).

The Pheroid® emulsion demonstrated a significant decrease in blood glucose, possibly extended further beyond the 5 hour mark (blood samples were only taken again at 24 hours to ensure animal health and not for pharmacokinetic purposes). The Pheroid® causes retention of insulin in blood circulation (refer to Figure 5 and Table 1) as can be deducted from the fact that the insulin levels in the blood is significantly higher than that of the control (with low levels measured in pro-Pheroid® indicating non-absorption). In contrast, the blood glucose lowering effect is not as pronounced, indicating a steady release of insulin out of the drug carrier system and a prolonged physiological effect. Since the control insulin is short acting, this might indicate that the Pheroid® insulin formulation is demonstrating the behaviour of a longer acting insulin formulation that can be used to effectively control basal glucose levels.

The use of intra-nasal or pulmonary administration to deliver insulin through a non-parenteral route demonstrates a quick onset of action and short duration (Haruta *et al*., 2003; Patton *et al.,* 2004; Grainer *et al,* 2004). During this study the T_{max}, C_{max} and AUC of the Pheroid® emulsion formulation were not determined since the predetermined evaluation period of 5 hours did not allow for the complete excretion of the Pheroid® formulated insulin. In consultation with the statistical analyst it was decided that a further investigation with additional samples per time frame as well as a longer evaluation time is necessary before accurate pharmacokinetic assumptions can be made. It is however clear and statistically significant that this formulation will allow for the continuous suppression of blood glucose levels over an extended period, rather than demonstrating the response of a short acting insulin.

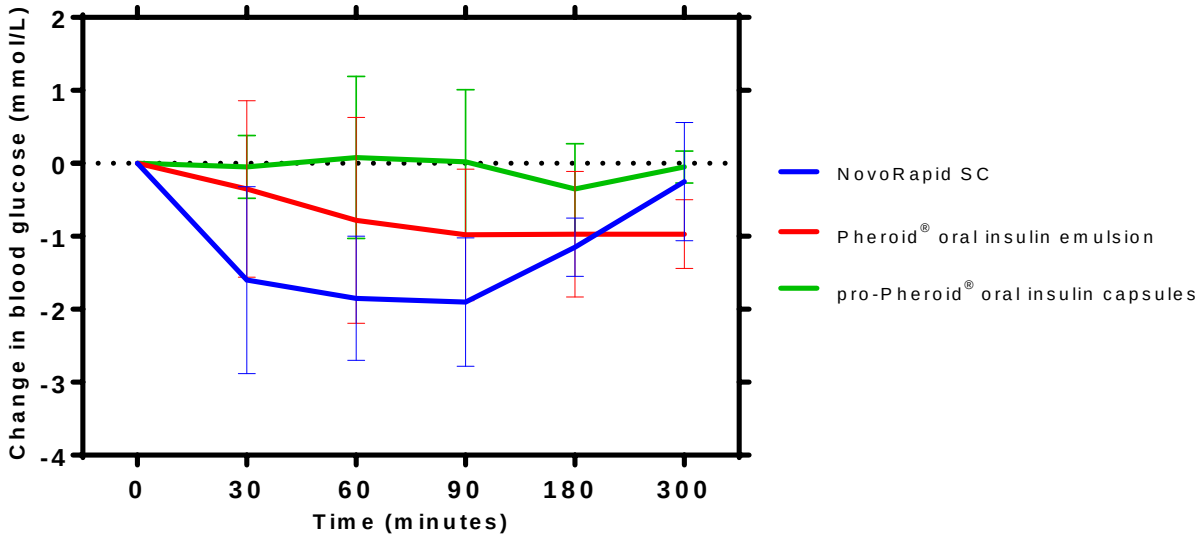

Figure 3: Change in blood glucose levels (mmol/L) per time when compared to pre-administration levels as measured by the Accu-Check Active™ handheld meter. Data is presented as mean ± SD for each interval.

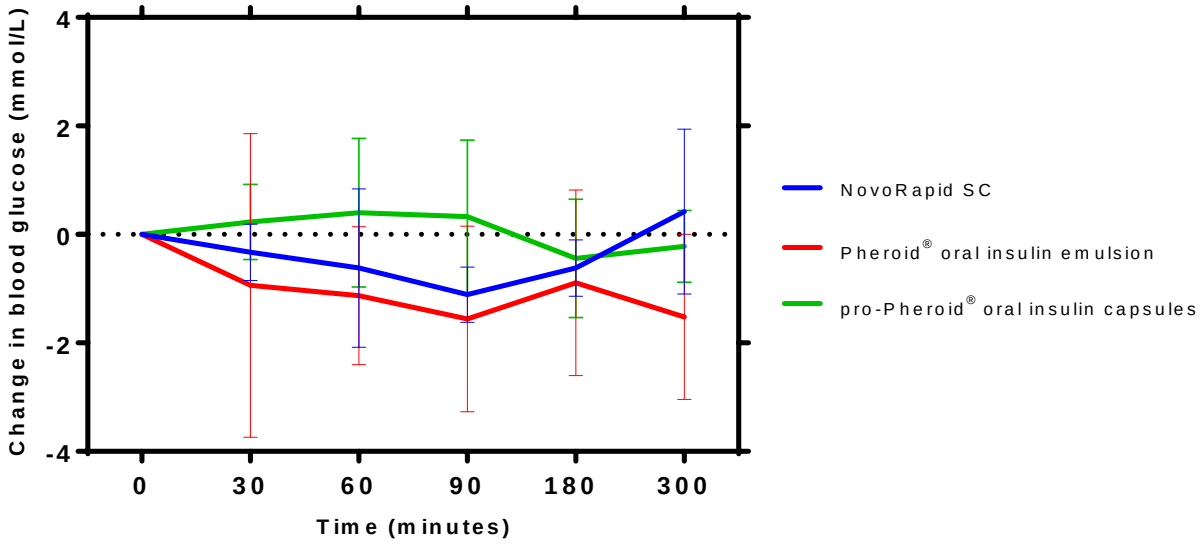

Figure 4: Change in glucose levels from pre-administration per time as measured post-study. Data is expressed as the mean ± SD for each interval.

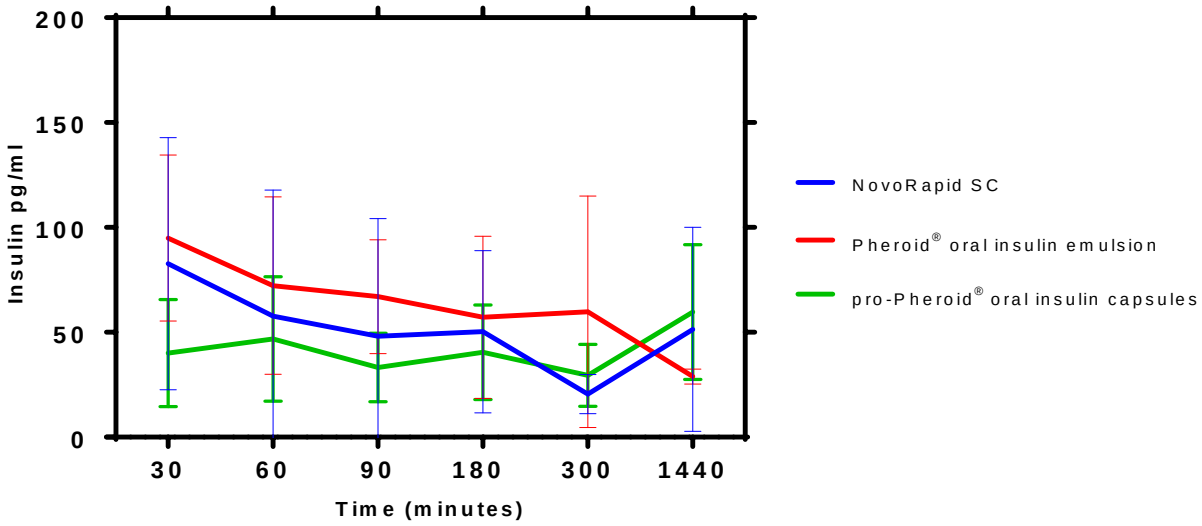

Figure 5: Blood insulin concentration per time frame as measured by ELISA. Data is expressed as the mean ± SD for each interval

Table 1: The plasma insulin levels at different blood sampling times.

Time (min)	SC NovoRapid® (pg/ml ± SE)	Pheroid® oral insulin emulsion (pg/ml ± SE)	Pro-Pheroid® oral insulin caps (pg/ml ± SE)
30	82.7 ± 34.7	94.9 ± 17.7	40.1 ± 10.4
60	57.8 ± 34.7	72.3 ± 18.9	46.8 ± 12.1
90	48.1 ± 28.0	67.0 ± 12.1	33.2 ± 6.7
180	50.3 ± 19.3	57.1 ± 17.3	40.5 ± 9.2
300	20.6 ± 4.6	59.8 ± 24.7	29.5 ± 6.0

Table 2: Change in glucose levels from pre-administration per time as measured post-study. Data is expressed as the mean change ± SD for each interval.

Time (min)	SC NovoRapid®	Pheroid® oral insulin emulsion	Pro-Pheroid® oral insulin caps
30	-1.60 ± 1.28	-0.35 ± 1.21	-0.05 ± 0.36
60	-1.85 ± 0.85	-0.78 ± 1.41	0.08 ± 1.108
90	-1.9 ± 0.88	-0.98 ± 0.92	0.02 ± 0.99
180	-1.15 ± 0.21	-0.96 ± 0.34	-0.35 ± 0.26
300	-0.25 ± 1.62	-0.97 ± 1.14	-0.05 ± 0.51

3.3) Blood chemistry data

The differences between the pre-administration and post-administration values of the clinical biochemistry of the animal blood samples are presented in Table 2. The pre-administration levels of the subcutaneous control were compared to the pre-administration levels of the test formulations. The levels of parameters at 5 hours for the subcutaneous control were also compared to the levels at 5 hours for the test formulations. No statistical significant changes were present (consult the supplementary data for values calculated). Figure 6 and 7 is included to demonstrate the level of ketone bodies and the creatinine kinase levels that were measured over time in the study animals. The levels of ketone bodies increased over time after insulin administration, indicating a state of hypoglycaemia in the animals. This could be attributed either to the fasted state of the animals or to the effect of the administered insulin. A reduction in the production of ketone bodies upon insulin administration is well known and this is conclusive evidence that all the formulations (NovoRapid, Pheroid®-insulin and pro-Pheroid®-capsules) lead to a reduction in blood glucose levels (Bieberdorf *et al.,* 1970). It is suspected that the process of formation of vesicles as well as release out of capsules contribute to an even longer lag-time in physiological effect of pro-Pheroid® and this needs to be investigated.

Serum creatinine kinase for non-human primates is reported to be 498 ± 91 units/L (with a maximum level of 1869) and 334 ± 225 units/L (with a maximum of 1054) for females (Park *et al.,* 2016). A unit is the amount of enzyme that will catalyse 1 µmol of substrate under standard specified conditions. Physiological impact is suspected during the administration of insulin with increased insulin levels suppressing the concentration of creatinine kinase. When the blood sugar levels return to normal these values stabilized. This phenomenon was present in both the gold standard treatment and the Pheroid® group and it is not suspected to be a formulation related adverse effect.

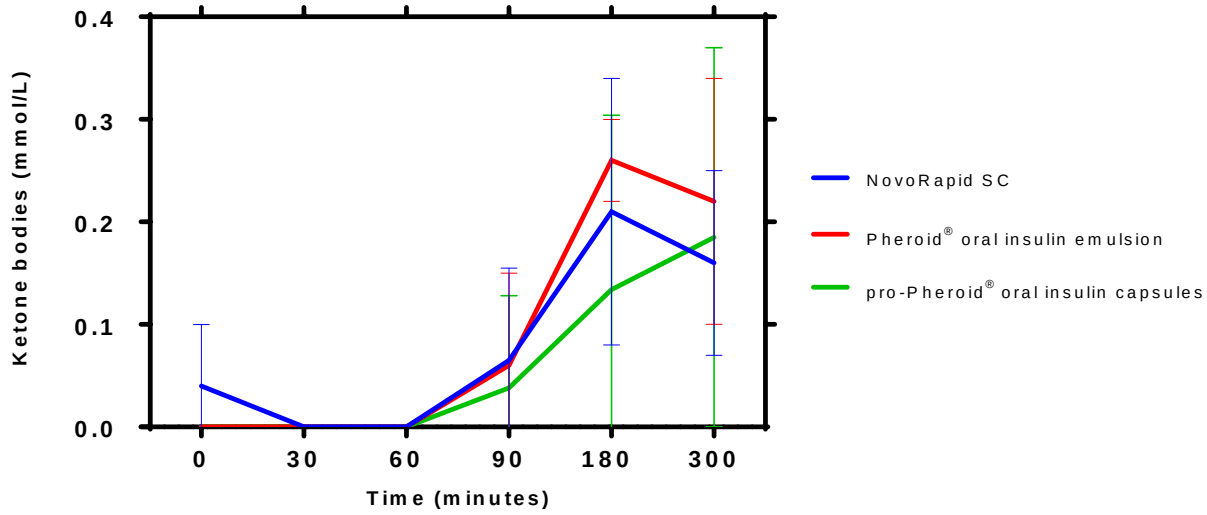

Figure 6: Levels of ketone bodies over time. Data expressed as the mean ± SD for each interval.

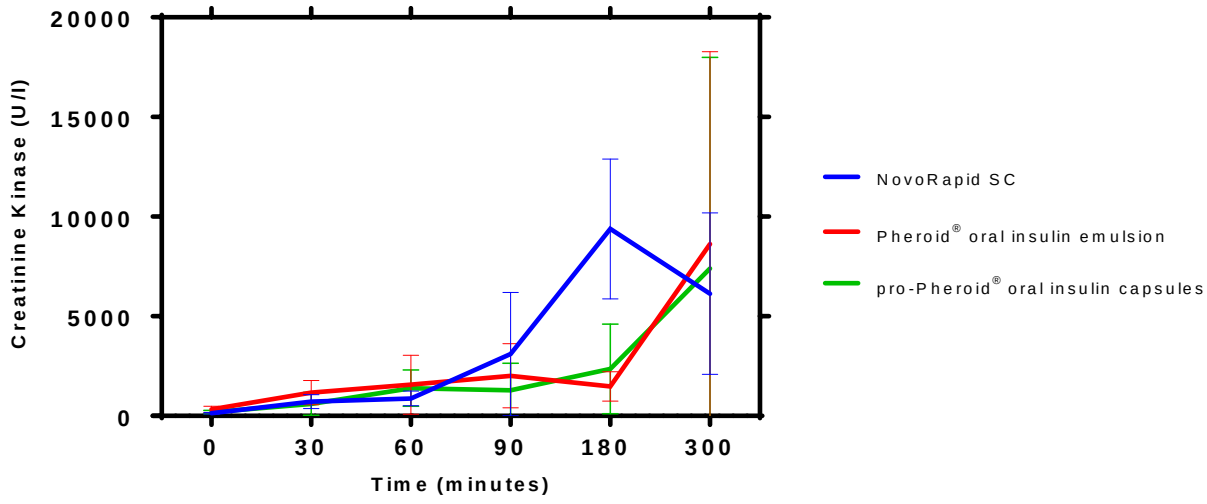

Figure 7: Levels of creatinine kinase over time. Data expressed as the mean ± SD for each interval.

Table 2: Blood chemistry of pre-administration samples and 5 hour post-administration samples harvested from primates

	Intravenous control group		Pheroid® oral insulin emulsion		Pro-Pheroid® oral insulin capsules	
Time →	Pre-admin	5 hours	Pre-admin	5 hours	Pre-admin	5 hours
Serum proteins						
Total proteins (g/L)	68.33 ± 13.86	71.0 ± 4.97	78.97 ± 4.33	75.45 ± 3.33	81.00 ± 4.55	74.67 ± 7.004
Albumin (g/L)	40.30 ± 7.56	43.63 ± 1.20	44.65 ± 1.91	45.48 ± 1.76	44.55 ± 1.64	42.93 ± 4.08
Total bilirubin (µmol/L)	2.13 ± 0.90	4.58 ± 1.47	2.10 ± 0.89	4.78 ± 1.54	1.80 ± 0.36	3.18 ± 0.36
Hepatic function						
ALT (U/L)	51.0 ± 30.81	56.25 ± 19.78	44.0 ± 40.63	37.75 ± 30.51	60.50 ± 38.65	60.83 ± 37.19
ALP (U/L)	22.3 ± 17.92	21.75 ± 10.81	18.0 ± 5.67	16.75 ± 2.06	23.5 ± 6.61	22.33 ± 6.80
AST (U/L)	47.0 ± 10.44	169.25 ± 41.87	66.0 ± 21.38	134.5 ± 70.05	90.75 ± 77.95	136.0 ± 102.37
Kidney function						
Inorganic phosphates (mmol/L)	0.95 ± 0.19	1.10 ± 0.29	1.32 ± 0.47	1.16 ± 0.38	1.15 ± 0.08	1.32 ± 0.12
Urea (mmol/L)	7.03 ± 2.27	10.02 ± 1.03	8.18 ± 0.56	8.5 ± 1.70	6.70 ± 3.08	8.52 ± 0.83
Creatinine (µmoll/L)	52.74 ± 13.26	52.50 ± 10.19	54.65 ± 16.33	57.33 ± 8.55	57.50 ± 13.26	53.80 ±12.64
Pancreatic function						
Amylase (U/L)	195.67 ±86.90	221.0 ± 95.90	215.67 ± 18.58	280.0 ± 88.37	266.75 ± 95.75	341.17 ± 58.08
Serum lipids						
High density lipids (mmol/L	0.71 ± 0.12	0.67 ± 0.13	0.76 ± 0.10	0.78 ± 0.07	0.84 ± 0.15	0.82 ± 0.16
Total cholesterol (mmol/L)	1.89 ± 0.44	2.07 ± 0.28	2.58 ± 0.12	2.22 ± 0.24	2.39 ± 0.38	2.39 ± 0.33
Glycerol	90.33 ± 62.12	125.25 ± 50.51	168.67 ± 186.04	154.75 ± 70.01	66.50 ± 20.7	93.83 ± 49.15
Free fatty acids	0.62 ± 0.23	1.34 ± 0.28	0.51 ± 0.16	1.42 ± 0.40	0.38 ± 0.14	1.05 ± 0.45
Fructose	146.67 ± 28.18	173.50 ± 9.47	192.0 ± 33.42	201.75 ± 46.51	166.0 ± 11.14	165.0 ± 30.39

4) Conclusion

The Pheroid® drug delivery system proved a viable oral formulation for the delivery of insulin by the gastro-intestinal route. This system demonstrated a longer acting pharmacokinetic profile when compared to the ultra-short acting control insulin injected subcutaneously. The pro-Pheroid® capsules did not demonstrate effective blood glucose lowering and this might be ascribed to a longer lag-time. Further investigation is needed to evaluate if this system is not a long-acting insulin preparation. The primates demonstrated no adverse effects and changes in blood chemistry. This system can now be evaluated in a first in human clinical trial.

5) Acknowledgements

Financial assistance was received from the National Research Foundation (NRF) of South Africa (grant number SFH 14070573914), the Innovation Fund. Opinions expressed, and conclusions arrived at, are those of the authors and are not necessarily to be attributed to the NRF. Further sponsorship for this study was provided by BGM Pharmaceuticals. Various patents regarding applications of the Pheroid® technology are registered by the authors. The authors declare that they have no additional conflicts of interest. We also thank Dr. Matthew Glynn and Mr. Lesley Masetle for assisting with the analysis of the various formulations. We would also like to thank LCL-Cynologics and MRC Harwell for services rendered. Lastly, we would like to thank Prof. Faans Steyn for assisting with the statistical analysis of data.

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Supplementary data

Variable	Mann-Whitney U Test (w/ continuity correction) (Toxicity) By variable Group Marked tests are significant at p <.05000				
	Rank Sum Subcut Control group	Rank Sum Oral Pheroid®	U	Z	p-value
Urea_pre	10.00000	18.00000	4.000000	-0.53033	0.595883
Urea_300	25.00000	20.00000	5.000000	1.10227	0.270345
Creatinine_pre	4.00000	6.00000	1.000000	-0.38730	0.698536
Creatinine_300	9.00000	12.00000	3.000000	-0.43644	0.662521
Inorg_phos_pre	8.00000	13.00000	2.000000	-0.87287	0.382734
Inorg_phos_300	17.00000	19.00000	7.000000	-0.14434	0.885234
ALP_pre	9.50000	11.50000	3.500000	-0.21822	0.827259
ALP_300	20.00000	16.00000	6.000000	0.43301	0.665006
ALT_pre	11.00000	10.00000	4.000000	0.00000	1.000000
ALT_300	21.00000	15.00000	5.000000	0.72169	0.470487
AST_pre	8.00000	13.00000	2.000000	-0.87287	0.382734
AST_300	22.00000	14.00000	4.000000	1.01036	0.312322
Tot_protein_pre	7.00000	14.00000	1.000000	-1.30931	0.190431
Tot_protein_300	14.00000	22.00000	4.000000	-1.01036	0.312322
Albumin_pre	8.00000	7.00000	2.000000	-0.28868	0.772830
Albumin_300	12.00000	24.00000	2.000000	-1.58771	0.112352
Tot_Chol_pre	6.00000	15.00000	0.000000	-1.74574	0.080857
Tot_Chol_300	14.00000	22.00000	4.000000	-1.01036	0.312322
High_DL_pre	8.50000	12.50000	2.500000	-0.65465	0.512691
High_DL_300	13.00000	23.00000	3.000000	-1.29904	0.193932
Glycerol_pre	10.00000	11.00000	4.000000	0.00000	1.000000
Glycerol_300	16.00000	20.00000	6.000000	-0.43301	0.665006
Free_fatty_acids_pre	13.00000	8.00000	2.000000	0.87287	0.382734
Free_fatty_acids_300	21.00000	15.00000	5.000000	0.72169	0.470487
Tot_Bilirubin_pre	10.00000	11.00000	4.000000	0.00000	1.000000
Tot_Bilirubin_300	17.00000	19.00000	7.000000	-0.14434	0.885234
Amylase_pre	9.00000	12.00000	3.000000	-0.43644	0.662521
Amylase_300	14.00000	22.00000	4.000000	-1.01036	0.312322
CK_pre	7.00000	14.00000	1.000000	-1.30931	0.190431
CK_300	9.00000	6.00000	3.000000	0.28868	0.772830
Ketone_pre	14.00000	14.00000	4.000000	0.53033	0.595883
Ketone_300	13.00000	8.00000	3.000000	-0.23146	0.816961
Fructose_pre	7.00000	14.00000	1.000000	-1.30931	0.190431
Fructose_300	16.00000	20.00000	6.000000	-0.43301	0.665006

Variable	Mann-Whitney U Test (w/ continuity correction) (Toxicity) By variable Group Marked tests are significant at p <.05000				
	Z adjusted	p-value	Valid N IV Control group	Valid N Oral Pheroid®	2*1sided exact p
Urea_pre	-0.55035	0.582080	3	4	0.628571
Urea_300	1.10689	0.268341	4	5	0.285714
Creatinine_pre	-0.38730	0.698536	2	2	0.666667
Creatinine_300	-0.43644	0.662521	3	3	0.700000
Inorg_phos_pre	-0.87287	0.382734	3	3	0.400000
Inorg_phos_300	-0.14434	0.885234	4	4	0.885714
ALP_pre	-0.22140	0.824778	3	3	0.700000
ALP_300	0.44371	0.657255	4	4	0.685714

Variable	Mann-Whitney U Test (w/ continuity correction) (Toxicity) By variable Group Marked tests are significant at p <.05000				
	Z adjusted	p-value	Valid N IV Control group	Valid N Oral Pheroid®	2*1sided exact p
ALT_pre	0.00000	1.000000	3	3	1.000000
ALT_300	0.72602	0.467826	4	4	0.485714
AST_pre	-0.87287	0.382734	3	3	0.400000
AST_300	1.01036	0.312322	4	4	0.342857
Tot_protein_pre	-1.30931	0.190431	3	3	0.200000
Tot_protein_300	-1.01036	0.312322	4	4	0.342857
Albumin_pre	-0.28868	0.772830	3	2	0.800000
Albumin_300	-1.58771	0.112352	4	4	0.114286
Tot_Chol_pre	-1.74574	0.080857	3	3	0.100000
Tot_Chol_300	-1.01036	0.312322	4	4	0.342857
High_DL_pre	-0.66421	0.506556	3	3	0.400000
High_DL_300	-1.31478	0.188583	4	4	0.200000
Glycerol_pre	0.00000	1.000000	3	3	1.000000
Glycerol_300	-0.43301	0.665006	4	4	0.685714
Free_fatty_acids_pre	0.87287	0.382734	3	3	0.400000
Free_fatty_acids_300	0.72169	0.470487	4	4	0.485714
Tot_Bilirubin_pre	0.00000	1.000000	3	3	1.000000
Tot_Bilirubin_300	-0.14434	0.885234	4	4	0.885714
Amylase_pre	-0.43644	0.662521	3	3	0.700000
Amylase_300	-1.01036	0.312322	4	4	0.342857
CK_pre	-1.30931	0.190431	3	3	0.200000
CK_300	0.28868	0.772830	3	2	1.000000
Ketone_pre	0.86603	0.386477	3	4	0.628571
Ketone_300	-0.23483	0.814338	4	2	0.800000
Fructose_pre	-1.30931	0.190431	3	3	0.200000
Fructose_300	-0.43301	0.665006	4	4	0.685714

Variable	Mann-Whitney U Test (w/ continuity correction) (Toxicity) By variable Group Marked tests are significant at p <.05000				
	Rank Sum IV Control group	Rank Sum pro-Pheroid®	U	Z	p-value
Urea_pre	14.00000	22.00000	7.00000	0.00000	1.000000
Urea_300	30.50000	24.50000	3.50000	1.70561	0.088082
Creatinine_pre	4.00000	11.00000	1.00000	-0.86603	0.386477
Creatinine_300	13.00000	23.00000	7.00000	0.00000	1.000000
Inorg_phos_pre	8.00000	20.00000	2.00000	-1.23744	0.215926
Inorg_phos_300	14.00000	41.00000	4.00000	-1.59901	0.109820
ALP_pre	10.00000	18.00000	4.00000	-0.53033	0.595883
ALP_300	20.50000	34.50000	10.50000	-0.21320	0.831171
ALT_pre	11.00000	17.00000	5.00000	-0.17678	0.859684
ALT_300	22.00000	33.00000	12.00000	0.10660	0.915106
AST_pre	10.00000	18.00000	4.00000	-0.53033	0.595883
AST_300	29.00000	26.00000	5.00000	1.38580	0.165808
Tot_protein_pre	7.50000	20.50000	1.50000	-1.41421	0.157300
Tot_protein_300	16.00000	39.00000	6.00000	-1.17260	0.240956
Albumin_pre	10.50000	17.50000	4.50000	-0.35355	0.723674
Albumin_300	22.00000	33.00000	12.00000	0.10660	0.915106
Tot_Chol_pre	9.00000	19.00000	3.00000	-0.88388	0.376760
Tot_Chol_300	15.00000	40.00000	5.00000	-1.38580	0.165808

Variable	Mann-Whitney U Test (w/ continuity correction) (Toxicity) By variable Group Marked tests are significant at p <.05000				
	Rank Sum IV Control group	Rank Sum pro-Pheroid®	U	Z	p-value
High_DL_pre	8.00000	20.00000	2.00000	-1.23744	0.215926
High_DL_300	15.00000	40.00000	5.00000	-1.38580	0.165808
Glycerol_pre	12.00000	16.00000	6.00000	0.17678	0.859684
Glycerol_300	30.00000	25.00000	4.00000	1.59901	0.109820
Free_fatty_acids_pre	16.00000	12.00000	2.00000	1.23744	0.215926
Free_fatty_acids_300	30.00000	25.00000	4.00000	1.59901	0.109820
Tot_Bilirubin_pre	13.00000	15.00000	5.00000	0.17678	0.859684
Tot_Bilirubin_300	29.50000	25.50000	4.50000	1.49241	0.135594
Amylase_pre	9.00000	19.00000	3.00000	-0.88388	0.376760
Amylase_300	13.00000	42.00000	3.00000	-1.81221	0.069955
CK_pre	10.00000	26.00000	4.00000	-0.89443	0.371094
CK_300	9.00000	12.00000	3.00000	-0.43644	0.662521
Ketone_pre	16.00000	20.00000	5.00000	0.59628	0.550985
Ketone_300	21.00000	24.00000	9.00000	0.12247	0.902523
Fructose_pre	8.50000	12.50000	2.50000	-0.65465	0.512691
Fructose_300	24.50000	30.50000	9.50000	0.42640	0.669816

Variable	Mann-Whitney U Test (w/ continuity correction) (Toxicity) By variable Group Marked tests are significant at p <.05000				
	Z adjusted	p-value	Valid N IV Control group	Valid N Oral pro-Pheroid®	2*1sided exact p
Urea_pre	0.00000	1.000000	3	5	1.000000
Urea_300	1.72133	0.085193	4	6	0.066667
Creatinine_pre	-0.86603	0.386477	2	3	0.400000
Creatinine_300	0.00000	1.000000	3	5	1.000000
Inorg_phos_pre	-1.24864	0.211799	3	4	0.228571
Inorg_phos_300	-1.59901	0.109820	4	6	0.114286
ALP_pre	-0.53513	0.592561	3	4	0.628571
ALP_300	-0.21385	0.830664	4	6	0.761905
ALT_pre	-0.17678	0.859684	3	4	0.857143
ALT_300	0.10692	0.914849	4	6	1.000000
AST_pre	-0.53033	0.595883	3	4	0.628571
AST_300	1.38580	0.165808	4	6	0.171429
Tot_protein_pre	-1.42701	0.153577	3	4	0.114286
Tot_protein_300	-1.17617	0.239526	4	6	0.257143
Albumin_pre	-0.35675	0.721277	3	4	0.628571
Albumin_300	0.10660	0.915106	4	6	1.000000
Tot_Chol_pre	-0.88388	0.376760	3	4	0.400000
Tot_Chol_300	-1.38580	0.165808	4	6	0.171429
High_DL_pre	-1.23744	0.215926	3	4	0.228571
High_DL_300	-1.39002	0.164523	4	6	0.171429
Glycerol_pre	0.17678	0.859684	3	4	1.000000
Glycerol_300	1.59901	0.109820	4	6	0.114286
Free_fatty_acids_pre	1.23744	0.215926	3	4	0.228571
Free_fatty_acids_300	1.60387	0.108743	4	6	0.114286
Tot_Bilirubin_pre	0.17678	0.859684	3	4	0.857143
Tot_Bilirubin_300	1.50153	0.133219	4	6	0.114286
Amylase_pre	-0.88388	0.376760	3	4	0.400000
Amylase_300	-1.81221	0.069955	4	6	0.066667
CK_pre	-0.89443	0.371094	3	5	0.392857

Variable	Mann-Whitney U Test (w/ continuity correction) (Toxicity) By variable Group Marked tests are significant at p <.05000				
	Z adjusted	p-value	Valid N IV Control group	Valid N Oral pro-Pheroid®	2*1sided exact p
CK_300	-0.43644	0.662521	3	3	0.700000
Ketone_pre	1.03280	0.301700	3	5	0.571429
Ketone_300	0.13553	0.892196	4	5	0.904762
Fructose_pre	-0.66421	0.506556	3	3	0.400000
Fructose_300	0.43301	0.665006	4	6	0.609524

5.9 Summary of this chapter

There is currently a serious need for the provision of an alternative method of insulin delivery than that of the subcutaneous route. A preclinical evaluation was undertaken in primates and this study served to demonstrate the efficacy of Pheroid® as an oral delivery system for proteins normally degraded by the gastrointestinal system. The Pheroid® delivery system is manufactured by an uncomplicated method and all the ingredients in this system is proven non-toxic. The primates also demonstrated no side-effects during this study. This application can therefore be evaluated in a first in human clinical trial.

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CHAPTER 6: CONCLUSIN BASED ON NEW DATA PRESENTED IN THIS THESIS

It is a well-known fact that patients prefer the oral administration route above parenteral routes. Furthermore, many formulations fail to excel on the market due to poor patient compliance (e.g. Exhubera® which is an inhalable insulin). The Pheroid® drug delivery system may potentially allow for the oral administration of traditional parenteral formulations and has the ability to improve the targeting of the API. The aim of this study was to investigate Pheroid® technology as a tool to change the administration route of selected pharmaceuticals from the parenteral route to the oral route. The emphasis of this project was strongly focused on the proposed clinical outcomes (the end goal is to better patient quality of life) as well as to progress this technology along the drug development pipeline (Figure 6-1).

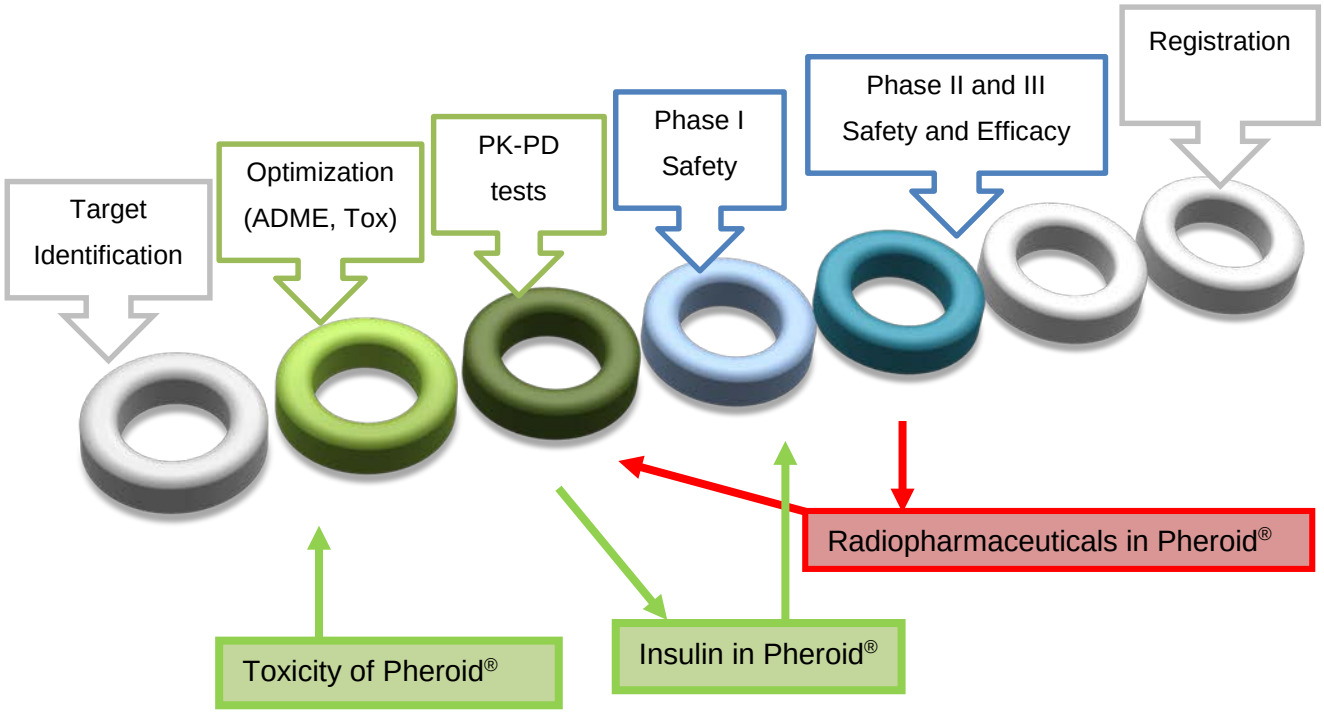

Figure 6-1: This figure is an adapted version of Figure 2-2 to demonstrate the impact of the research presented in this thesis on the drug development pipeline with reference to Pheroid®. The investigations indicated in green indicate progression and the investigation in red demonstrate the need to revisit previous preclinical work.

The optimization phase of Pheroid® technology was addressed by an evaluation of the toxicity profile of this system, both in terms of genotoxicity and acute and subchronic toxicity. This is a prerequisite for any preclinical and clinical studies and this will contribute greatly to the literature and understanding of this drug carrier system. The toxicity of this system was evaluated *in vitro* (AMES Assay) as well as *in vivo* (subchronic and acute in BALB/c mice and Sprague-Dawley rats). The guidelines for evaluation stated by the OECD were followed. The animals showed no adverse reactions at the maximum intravenous dosage of 2000 mg/kg and clear data (physiological, chemical and hematological) is provided to substantiate that Pheroid® (not considering the API) will not contribute to toxicity of future formulations. This study also provides clear parameters to which the delivery system (especially when administered intravenously) should adhere in order to assure its safety.

The clinical evaluation of an oral radiotracer formulation was evaluated in a first-in-human hybrid phase I/II clinical trial. This formulation proved a lack of effectivity and should be reverted to an earlier stage of the drug delivery pipeline; a re-think about the design of this formulation is required. The importance of pH in the dissociation of ^{99m}Tc-MDP with resulting artefactual labeling of the thyroid was illustrated. In addition, knowledge was gained about the interaction between the tracer and the Pheroid® delivery system and this will contribute to further development.

The preclinical evaluation of insulin formulated in Pheroid® was furthered by the evaluation of efficacy in primates. The study in non-human primates was a follow-on from previous rodent evaluations. The effect of insulin administration when formulated in a Pheroid® emulsion and pro-Pheroid® capsules was evaluated and compared to the subcutaneously injected NovoRapid® commercial insulin. The blood glucose lowering effect of the Pheroid® insulin formulation was substantial enough for possible clinical utilization and an additional prolonged action when compared to the subcutaneous injection was present. Pro-Pheroid® was demonstrated as ineffective, although it is suspected that this is because the absorption of the capsules adds additional hours before blood sugar is lowered. The next step will be to evaluate the effect of insulin administered in Pheroid® as a chronic study in primates having existing DM2. The pro-Pheroid® capsules could be further developed preclinically.

The following future studies are suggested based on the outcomes of this dissertation:

- The evaluation of the toxicity of each new API-Pheroid® formulation. Although the system is proven non-toxic, it influences the biodistribution of the entrapped API and can therefore change the accumulation of the API.
- The application of Pheroid® in nuclear medicine should be re-evaluated with a different radiotracer (possibly ^{153}Samarium with a half-life of 1.9 days) and such an *in vivo* investigation in Sprague Dawley rats by means of micro-SPECT is currently being planned for application with the Animcare ethics committee of the North-West University.

- Since the IV toxicity study proved that Pheroid® is safe for IV administration, the formulation of radiotracers in Pheroid® for other applications (e.g. multi-modal imaging and radiochemotherapeutics) should be investigated.
- The pharmacokinetics of insulin entrapped in Pheroid® should be evaluated, preferably in a non-human primate model of DM 2 on a more long-term basis.

The evidence provided in this thesis presents the conclusion that Pheroid® technology is indeed an attractive drug carrier system to allow the oral administration of parenteral agents.

ANNEXURES

- Author Information Pack: Toxicology Reports
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- Author Information Pack: Journal of Controlled Release
- Proof of article submission and reviewers comments
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Animcare approval letter for animal study

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Institutional Research Ethics Regulatory Committee
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Email : Ethics@nwu.ac.za

ETHICS APPROVAL CERTIFICATE OF STUDY

Based on approval by **AnimCare Animal Research Ethics Committee (AREC-130913-015)** on **29/11/2016** after being reviewed at the meeting held on **23/11/2016**, the North-West University Institutional Research Ethics Regulatory Committee (NWU-IRERC) hereby **approves** your study as indicated below. This implies that the NWU-IRERC grants its permission that provided the special conditions specified below are met and pending any other authorisation that may be necessary, the study may be initiated, using the ethics number below.

Study title: **In vivo evaluation of the acute intravenous toxicity of Pheroid®.**

Study Leader/Supervisor: **Prof Anne Grobler**
Student: **Ms Janke Kleynhans**

Ethics number:

N	W	U	-	0	0	4	9	3	-	1	6	-	A	5
Institution				Study Number						Year			Status	

Status: S = Submission; R = Re-Submission; P = Provisional Authorisation; A = Authorisation

Application Type: New Application - Standard Project
Commencement date: 2016-11-29 **Category:** 3

Continuation of the study is dependent on receipt of the annual (or as otherwise stipulated) monitoring report and the concomitant issuing of a letter of continuation up to a maximum period of three years.

Special conditions of the approval (if applicable):

- Any research at governmental or private institutions, permission must still be obtained from relevant authorities and provided to the AnimCare. Ethics approval is required BEFORE approval can be obtained from these authorities.

General conditions:

While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, please note the following:

- *The study leader (principle investigator) must report in the prescribed format to the NWU-IRERC via AnimCare:*
  - *annually (or as otherwise requested) on the monitoring of the study, and upon completion of the study*
  - *without any delay in case of any adverse event or incident (or any matter that interrupts sound ethical principles) during the course of the study.*
- *Annually a number of studies may be randomly selected for an external audit.*
- *The approval applies strictly to the proposal as stipulated in the application form. Would any changes to the proposal be deemed necessary during the course of the study, the study leader must apply for approval of these amendments at the AnimCare, prior to implementation. Would there be deviated from the study proposal without the necessary approval of such amendments, the ethics approval is immediately and automatically forfeited.*
- *The date of approval indicates the first date that the study may be started.*
- *In the interest of ethical responsibility the NWU-IRERC and AnimCare retains the right to:*
  - *request access to any information or data at any time during the course or after completion of the study;*
  - *to ask further questions, seek additional information, require further modification or monitor the conduct of your research or the informed consent process.*
  - *withdraw or postpone approval if:*
    - *any unethical principles or practices of the study are revealed or suspected,*
    - *it becomes apparent that any relevant information was withheld from the AnimCare or that information has been false or misrepresented,*
    - *the required amendments, annual (or otherwise stipulated) report and reporting of adverse events or incidents was not done in a timely manner and accurately,*
    - *new institutional rules, national legislation or international conventions deem it necessary.*
- *AnimCare can be contacted for further information or any report templates via Ethics-AnimCare@nwu.ac.za or 018 299 2197.*

The IRERC would like to remain at your service as scientist and researcher, and wishes you well with your study. Please do not hesitate to contact the IRERC or AnimCare for any further enquiries or requests for assistance.

Yours sincerely

Linda du Plessis

Digitally signed by Linda du Plessis
DN: cn=Linda du Plessis, o=NWU, ou=Vaal Triangle Campus, email=linda.duplessis@nwu.ac.za, c=ZA
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Prof Linda du Plessis
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Pharmachen Research Committee approval letter of PhD proposal

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To whom it may concern,

RE: APPROVAL OF RESEARCH PROPOSAL BY SCIENTIFIC RESEARCH COMMITTEE

The PhD research proposal of Ms J Kleynhans was reviewed and discussed at the meeting of the Scientific Research Committee of Pharmacen on 15 May 2015 where it was conditionally approved. The revised proposal was finally approved on 18 May 2015 by a selected reviewer sub-committee of the Scientific Research Committee (consisting of Prof JH Hamman, Prof LH Du Plessis and Dr J Viljoen).

Details of the research proposal that was approved:

Title of study: Investigation into the impact of the entrapment of selected ^{99m}Technetium radiotracers in the Pheroid® delivery system on diagnosis.

Candidate: J Kleynhans

Supervisor/promoter: Prof A Grobler

Co-supervisors/promoters: Prof JR Zeevaart, Prof M Sathekge

Degree: PhD (Pharmaceutics)

Yours sincerely,

PROF J H HAMMAN (Chair of the Scientific Research Committee)

UP Faculty of Health Sciences Ethics Committee approval letter for clinical trial

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 22 May 2002 and Expires 20 Oct 2016.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 22/04/2017.

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Faculty of Health Sciences Research Ethics Committee

28/05/2015

Approval Certificate
New Application

Ethics Reference No.: 159/2015

Title: Investigation into the clinical impact of the entrapment of selected 99mTechnetium radiotracers in the Pheroid® delivery system

Dear Janke Kleynhans

The **New Application** as supported by documents specified in your cover letter dated 18/05/2015 for your research received on the 21/04/2015, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 27/05/2015.

Please note the following about your ethics approval:
- Ethics Approval is valid for 3 years
- Please remember to use your protocol number (**159/2015**) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

Ethics approval is subject to the following:
- The ethics approval is conditional on the receipt of 6 monthly written Progress Reports, and
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

*** Kindly collect your original signed approval certificate from our offices, Faculty of Health Sciences, Research Ethics Committee, H W Snyman South Building, Room 2.33 / 2.34.*

Dr R Sommers; MBChB; MMed (Int); MPharMed.
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

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HREC approval for Clinical Trial letter

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2016/04/15

ETHICS APPROVAL CERTIFICATE OF PROJECT

Based on approval by **Health Research Ethics Committee (HREC)** at the meeting held on **15/07/2015**, the North-West University Institutional Research Ethics Regulatory Committee (NWU-IRERC) hereby **approves** your project as indicated below. This implies that the NWU-IRERC grants its permission that, provided the special conditions specified below are met and pending any other authorisation that may be necessary, the project may be initiated, using the ethics number below.

Project title: Investigation into the impact of the entrapment of selected 99mTechnetium radiotracers in the Pheroid® delivery system on diagnosis.

Project Leader/Supervisor: **Prof AF Grobler**
Student: **J Kleynhans**

Ethics number:

N	W	U	-	0	0	1	8	3	-	1	5	-	A	1
Institution				Project Number						Year			Status	

Status: S = Submission; R = Re-Submission; P = Provisional Authorisation; A = Authorisation

Application Type: Full Single

Commencement date: 2016-04-14 **Expiry date**: 2017-04-13 **Risk:** High

Special conditions of the approval (if applicable):

- Translation of the informed consent document to the languages applicable to the study participants should be submitted to the HREC (if applicable).
- Any research at governmental or private institutions, permission must still be obtained from relevant authorities and provided to the HREC. Ethics approval is required BEFORE approval can be obtained from these authorities.

General conditions:

While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, please note the following:

- *The project leader (principle investigator) must report in the prescribed format to the NWU-IRERC via HREC:*
  - *annually (or as otherwise requested) on the progress of the project, and upon completion of the project*
  - *without any delay in case of any adverse event (or any matter that interrupts sound ethical principles) during the course of the project.*
  - *Annually a number of projects may be randomly selected for an external audit.*
- *The approval applies strictly to the protocol as stipulated in the application form. Would any changes to the protocol be deemed necessary during the course of the project, the project leader must apply for approval of these changes at the HREC. Would there be deviated from the project protocol without the necessary approval of such changes, the ethics approval is immediately and automatically forfeited.*
- *The date of approval indicates the first date that the project may be started. Would the project have to continue after the expiry date, a new application must be made to the NWU-IRERC via HREC and new approval received before or on the expiry date.*
- *In the interest of ethical responsibility the NWU-IRERC and HREC retains the right to:*
  - *request access to any information or data at any time during the course or after completion of the project;*
  - *to ask further questions, seek additional information, require further modification or monitor the conduct of your research or the informed consent process.*
  - *withdraw or postpone approval if:*
    - *any unethical principles or practices of the project are revealed or suspected,*
    - *it becomes apparent that any relevant information was withheld from the HREC or that information has been false or misrepresented,*
    - *the required annual report and reporting of adverse events was not done timely and accurately,*
    - *new institutional rules, national legislation or international conventions deem it necessary.*
- *HREC can be contacted for further information or any report templates via Carolien.VanZyl@nwu.ac.za or 018 299 1206.*

The IRERC would like to remain at your service as scientist and researcher, and wishes you well with your project. Please do not hesitate to contact the IRERC or HREC for any further enquiries or requests for assistance.

Yours sincerely

Prof LA Du Plessis

Digitally signed by Prof LA Du Plessis
DN: cn=Prof LA Du Plessis, o=North-West University, ou=Campus Rector, email=Linda.DuPlessis@nwu.ac.za, c=ZA
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May 16, 2017

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