CHAPTER 3: PHEROIDS AND PRO-PHEROIDS – THE INS AND THE OUTS

3.1 The potential of Pheroid™ technology

Pheroid™ technology seen within the context of drug delivery and therapy is a complex polydisperse technology, based on colloidal emulsion systems and used for the delivery of pharmaceutical and other compounds (see Chapter 4). Studies on the Pheroid™ have shown that it has several unique advantages. The following advantages will become obvious through the course of this thesis, where the investigations into the applications of the technology are described (Chapters 4 to 8):

(i) Pheroid™ can be used to transfer molecules by a number of administration routes, such as orally, nasally or transdermally without the need for sophisticated procedures;

(ii) The particles show high cell penetration characteristics in all cell types investigated;

(iii) The Pheroid™ shows potentiating capabilities and can be used as a pro-delivery system, in analogy to a pro-drug;

(iv) It is a highly effective gene transfer vector and much more efficient than conventional products currently on the market;

(v) One of the most interesting and exciting properties is that it may be used to package ligands so that vesicles can be targeted to specific cell surface receptors for uptake by these cells;

(vi) It has been shown to be stable, both in terms of shelf life and in body fluids, solving one of the main production problems of peptide drugs and gene delivery.

3.2 Pheroid™ and patenting

3.2.1 From concept to product

A conscious decision was taken not to patent the Pheroid™ itself but rather to patent applications. Requirements for the creation of intellectual property (or unique knowledge) are generally uniqueness, inventive step and the invention or knowledge must be usable. The requirements for drug development are different - the main requirements are that the product must be effective and it must be safe. Thus if one wants to develop a patentable marketable drug the requirements are a summation of the requirements of the two approaches.

While the final aim of the research and development of any novel technology or formulation must be its practical application, the generation of a new concept that can be categorized as

intellectual property, is a valuable asset on the balance sheet of any institution and may be more valuable than individual products flowing from such a technology. Intellectual property is a generic term and refers to the innovative and/or creative efforts of individuals, as reflected in patents, industrial designs, trade marks, copy write, technical know-how and skills. Whereas concepts mostly fall under this category, products such as formulations may not belong to this category. Intellectual property (IP) is not tangible and its value lies in the fact that its owner is allowed a 20-year period within which the IP may be used unimpeded by competitors and with the advantages generated by that the specific know-how (Krattinger *et al.,* 2007; Adams and Adams, 2009; http://www.epo.org/ 2009, http://www.wipo.int, 2008).

Not all new products that are commercialized are patentable. The processes of both intellectual property development and pharmaceutical product development are many-faceted and quite complex, but the requirements of the development and/or registration of a product and of intellectual property are based on different approaches and are quite diverse. Since this thesis concerns itself with both processes, the section below will converse on some parallels and some differences between the two processes.

3.2.2 When is a product patentable?

According to the information provided by the World Intellectual Property Organization (WIPO; http://www.wipo.int, 2008), the European Patent Office (EPO; http://www.epo.org/ 2009), Anatole Krattinger (Krattinger *et al.,* 2007); and Adams & Adams (2009), a patent can be regarded as a monopoly granted by the state to a person, persons or legal entity for the exclusive exploitation of an invention. The summary below is compiled from the above named sources (Krattinger et al., 2007; Adams and Adams, 2009; http://www.epo.org/, http://www.wipo.int), as well as personal experience. Patents are subject to geographical and time limitations. In terms of time, patents are granted for a maximum period of 20 years from the date of filing of the patent application, provided payment of the necessary annual renewal fees is maintained. Patents are geographically limited to the specific territories (i.e. countries or regions) in which they are registered. Separate patent protection must be obtained in every country or region of the world and no such thing as a world wide patent exists (Krattinger *et al.,* 2007; Adams and Adams, 2009; http://www.epo.org/ 2009, http://www.wipo.int, 2008).

A granted patent gives the patentee the advantage of exclusivity for the duration of the patent, i.e. the right to prevent others from making, using, exercising, disposing of or importing the invention to the country or region within which the patent is granted. The patentee thus obtains a monopoly on the invention and should be able to enjoy the whole profit and advantage arising from the invention. Only the inventor or another entity, such as a university to whom the rights have been assigned by the inventor, may apply for a patent.

A granted patent is an asset that can be sold or in respect of which licenses may be granted. A patentee can institute legal proceedings against an infringer for an interdict to prevent further infringement of the patent. The patentee can also claim damages from such an infringer for any damages that the patentee had suffered as a result of such infringement. A patent application is not enforceable and does not confer any rights against infringers but once a patent is granted, the rights are enforceable in the country within which the patent has been granted.

According to the generally accepted patent acts of various countries throughout the world, any invention, which may include an article, an apparatus, a technology, a product, device, process, method or the like, an invention, a new product, devices design, or formulation is patentable in general if it satisfies three basic requirements, namely if it is

(i) absolutely novel,

(ii) involves an inventive step and

(iii) is useful in the trade, industry or agriculture.

Before going to the trouble of compiling a patent application, it behoves one to know what is patentable and what is not. In general, inventions contain technical content in the form of designs, processes for manufacturing, chemical substances and formulations, apparatus or technologies. The Patents Act defines the following as not being an invention for the purposes of the Act, mostly since they are not inventions in the real sense of the word: any animal or plant or any biological process for the production of animals or plants; a method of treatment of the human or animal body by surgery or by therapy or by diagnosis practiced on the human or animal body; a scientific theory or mathematical method or a computer program; any aesthetic creation, such as a literary, dramatic, musical or artistic work; a spiritually based scheme, rule or method for performing a mental or abstract act such as doing business (in most countries) or playing a game or the presentation of information or an invention that is expected to encourage offensive or immoral behaviour.

Although an item or method may in itself not be patentable, its application may in some countries be patentable. Microbiological processes and products found in fermentation procedures, and in the manufacture of antibiotics and substances or compositions for use in a method of treatment of the human or animal body, as well as new medical use of an old substance or composition are patentable provided the process, product, composition or substance is new, inventive and useful (see 3.2.4.2 below).

3.2.2.1 Absolute novelty

An invention is new if it does not form part of the so-called "state of the art" on the date on which a first patent application (e.g. provisional patent application) in respect of the invention is filed. The "state of the art" comprises of all matter that has been made available to the public anywhere in the world, through written or oral description, through use or in any other way. So, for example, earlier printed publications such as advertisements or earlier patents, commercial use, non-confidential discussions or tenders all qualify as "disclosure" and as such destroy subsequent patentability of the invention.

The state of the art also includes earlier patent applications ("pending patents") filed in South Africa, notwithstanding the fact that such earlier applications were not open to public inspection at the time when a subsequent patent application for the same or a similar invention is filed. Further included in the state of the art are patents that are filed in South Africa after a South African patent application has been filed by a third party, wherein the latter patent application claims priority from an earlier foreign patent application filed in a country affiliated to a regional patent office through a patent cooperation treaty. An invention used secretly, but on a commercial scale in the Republic of South Africa, also forms part of the state of the art and such an invention would not be patentable, even if it were not disclosed to the public.

3.2.2.2 Inventive step ("non-obviousness")

An invention involves an inventive step if it is not obvious to a person skilled in the art, having regard to all matter that was available to the public, anywhere in the world, immediately before the date of filing a patent application for the invention. The Patents Act does not define a quantitative test for inventiveness, but rather a qualitative test. In addition, the "person skilled in the art" is regarded as being a person with ordinary skills and expertise and not a specialist or highly qualified person in the art. The question: "Was it obvious to try?" should be answered by a person skilled in the art.

3.2.2.3 Usefulness

The Patents Act states that a patentable invention must be capable of being used or applied in trade or industry or agriculture. The Act also states that a patent may be revoked where the invention, as illustrated or exemplified in the patent specification (patent specifications are dealt with below), cannot be performed or does not lead to the results and advantages set out in the patent specification.

Closer to home; an invention that consists of a surgical or therapeutic method of treatment of the human or animal body, or the diagnoses of conditions in man or animal is not patentable

and non-patentable products are developed for this purpose. A substance or composition for use in a method of treatment or diagnoses is however patentable, if the method of treatment or diagnosis is new, even if the substance or composition itself is known. The development of an effective new formulation therefore does not necessarily mean that it will be patentable.

This thesis concerns itself with research regarding a technology that is needed by and useful to the industry.

3.2.3 Patentable research within the academic environment

According to Report RP25 of the Van Wyk de Vries Commission on Universities, the function of a university can be simplified to two basic functions, namely to teach and to generate new knowledge. According to this Commission, it is thus a function of the university to do research. If the knowledge generated is also needed by the community, it can be argued that it is the responsibility of the university to apply the needed the research. Thus the aim of a patent and the function of a university share some similarities:

> The generation of knowledge that is testable, that may be novel, that may be useful and that, above all else, is true.

3.2.4 The patenting process

The patenting process of a technology (as opposed to a device) generally contains the following phases:

3.2.4.1 Research and development (R&D)

The R&D phase includes the following:

a) The generation of a concept.

b) A study concerning current knowledge related to the concept. To verify the novelty of the concept, this study should include existing and proposed patents, scientific publications, public domain knowledge and existing products in the relevant field.

c) The conversion of the theoretical concept to reality. In this case it would include the formulation of the delivery system with specific classes of drugs.

d) An evaluation of the assays required to test the concept or article within its application framework, be it a prototype, a product or technology. In this study, the technology was evaluated for different classes of drugs and agricultural remedies (Chapters 4 to 8).

e) Formulation of various products in each of the categories of drugs and agricultural remedies.

f) Preclinical evaluations consisting of

- *in vitro* evaluations;
- *in situ* evaluations;
- Animal studies.

g) *In vivo* evaluations consisting of

- Absorption and kinetic trials (phase I trials);
- Efficacy trials.

Where positive results in support of the concept are obtained, the patentability is strengthened. Negative results may lead to further investigation in terms of formulations and retesting. Repeated negative results indicate the limits of the concept.

3.2.4.2 Patenting

Die process of patenting consists of the following phases:

a) Defining of the field of the invention and the specifications of the patent in the form of a provisional patent.

b) Filing of the provisional patent at a patent office. A provisional patent does not have to contain specific formalized claims and is generally followed by an expansion of the research regarding the subject of the patent with the purpose of strengthening the formal claims that are to be made. The date on which the provisional patent application is filed is called the "priority date". The provisional patent is usually filed in the country of origin and has the advantage of setting the priority date, while allowing a time period within which the invention can be refined and additional supporting evidence obtained. To protect the novelty, no disclosure of the invention may be made before the priority date. After filing, the invention can be disclosed to the public. This allows academics to file a patent and then to publish the research inherent to the invention.

c) Conducting infringement searches in each country where the possible patent may be exploited to determine whether the exploitation infringes existing public or patent rights. Infringement may be punishable since remedy may include damages, an interdict and destruction of all infringing products by court order.

d) The provisional examination of the patent to verify that the patent complies with a variety of stipulated requirements. The requirements may differ between various patent offices and are founded on legal articles, the best known of which is probably the "European Patent Convention".

e) A complete patent application is then filed with the inclusion of formal claims when the invention is in its final form and when no further technical developments seem to be required. The complete patent application must generally be filed within 12 months of the priority date, by which time the provisional application lapses. This time period is not valid for all countries and is for instance not allowed in Taiwan. The filing of either the provisional or complete patent application does not imply in the least that a patent will be granted.

f) A South African patent is only valid in South Africa and application must be made in each country within which the invention is to be protected. It is preferable that the patent is submitted to an office that is recognized by the countries within which the application of the concept requires protection. Although regional patent applications can be filed in Europe, Africa and for certain eastern countries, patents cannot be granted regionally. Such applications do however, leverage additional time before the national phase – that is the phase during which applications have to be filed in the countries where patent protection is sought. Regional examinations may also give an indication as to the patentability of the invention.

g) Instead of regional application, a patent may also be filed under the Patent Co-operation Treaty (PCT), to which nearly 150 countries throughout the world belong. A PCT patent application can be filed directly or within 12 months from the priority date. Search reports are provided by the PCT office. Originally, patents were not granted by the PCT, but this situation may change within the foreseeable future. At the moment, national applications still have to be filed in all countries where patent protection is sought, although a time period of 30 months is allowed from the first PCT filing of a patent. The national offices generally rely on the searches conducted for the PCT application. The filing costs of national applications can thus be staggered or postponed. For filing an application, the European Patent Office sets the following requirements: a request indicating that a European Patent is sought using the specified format (the form and content of the request for grant); sufficient particulars to identify the applicant and the designation of the inventor;

at least one claim with its description in English, German or French; designation of at least one European contracting state and payment of fees due. The European Patent Office examines compliance to these requirements, any claim to priority and the appointment of a professional representative. This procedure is called the formalities examination.

h) A regional application, such as a PCT application, should ideally contain the claims, an abstract, background to the invention with inclusion of the "state of the art", a description of the invention, supporting evidence that the invention complies with the novelty, inventiveness and usefulness as follows:

- The first examination of the filed application includes a search of all records of published patents and databases that include scientific and commercial publications, technology abstracts, the internet, magazines, newspapers and any other publications according to keywords determined by the examiners. Disclosures about items or processes to the public through a medium other than publication, such as commercial exploitation, may not always be revealed during patentability searches.
- The examiner(s) then screen the patents and publications to determine the novelty of the invention. If the examiner(s) find that there is an overlap between the patent application and existing patents, publications or products, the examiner rejects the application and supplies a search report and written opinion within which the existing patent(s), publication(s) or product(s) are sited. Novelty is therefore a prerequisite to the patentability of an invention – it has to be 'new' according to the Act. The generation of such novel concepts/products is per definition therefore the generation of new knowledge.

- State of the art and 'prior art': The examiner investigates the patent in terms of the "state of the art". The invention should not be obvious to a person skilled in the relevant art; i.e. a qualified scientist should not have been able to compile or predict the proposed formulation from the knowledge available at that stage. If the examiner and/or expert feel that the invention suffers from obviousness, then the application is rejected. An invention is therefore only regarded as 'novel' if it does **not** form part of the 'state of the art' at the time directly preceding the priority date. 'State of the art' consists of all material (be it about a product, a process, information about either or any other relevant information) made known to the public in South Africa or anywhere in the world through either a verbal or written description or by any other means. The state of the art upon which the novelty is based also include material in pending patent applications with earlier priority

dates. An invention that is used in secret on a commercial scale also forms part of the state of the art, should awareness regarding the product / process exist.

- The invention has to contain an inventive step and in the case of multiple claims, the application has to show unity of the invention. This requirement for unity of the invention is often problematic when a technology is pluri-potent and the patent application contains multiple claims.

- The invention has to be usable within the context of its time. The prerequisite of usefulness was instituted because of the patenting of inventions of which the cost or the value for humankind was suspect.

- All claims of the invention must be supported by evidence.

(h) When the EPO receives the patent, it initiates two different international searches; the first to investigate the patentability of the invention and the second to verify that the application is not in contravention of an existing patent, an applied for patent not yet granted or an invention accepted as being within the public domain (infringement search). In addition, the application is subject to several prescriptions that assure the quality of the application. Applications that do not comply with these requirements are returned to the applicant by the Receiving Office. Quite a number of patent applications are sifted out and is not continued beyond this stage. However, the applicant may respond and motivate why either of the search reports may not be valid or may not be specifically applicable and ask for a modification of the search report.

(i) After about 6 months the results of the searches are forwarded to the applicant together with a copy of the documents cited in the search. The searches, together with the patent application and specifications are published in the "Patent Journal". A granted European patent needs to be validated by the patent office of each designated state in order for it to take effect and bestow enforceable rights on the patentee in that state. Validation may include filing a translation of the specification in an official language of the contracting state. Other requirements may also need to be fulfilled depending on the national law of the state.

(j) The application, together with the search report, and any correspondence of the applicant regarding the results of the two search reports, are handed over to the examining division of the patent office if the applicant requests the examining office to continue with the application. For the so-called **formal** examination, three examiners are appointed, one of which carries the overall responsibility. The examiners, who are experts in the field of the patent application, i.e. the pharmaceutical industry or biological

material or agriculture, once again verify that the application complies with their requirements of a patent. This time the examination are in-depth and subject specialists, as well as the applicant, may at this stage be asked for verbal or written opinions or responses. The evidence in support of the patent claims is examined. Each claim has to be supported by studies, reasonable arguments, etc. In the case of apparatuses, diagrams may be sufficient, while evidence of efficacy may be required in the case of therapeutic compounds. Most often, the duration of the process of examination by a patent office, such as the EPO, is an average of two years, but it can be much longer. For instance, the patent based on the research described in Chapter 6 was submitted to the EPO in 2004 and has been granted in China in May 2009, while it still has not been granted in the United States of America.

(k) Should the examining division find that the patent cannot be granted, the applicant is so informed and the patent and claims are not published. The applicant has the opportunity of appellation by written documentation. The appellation documentation may consist of supporting arguments or studies, or arguments against the interpretation of the claims by the examining division or their finding that the invention is not novel or obvious. Should questions still exist, a verbal defence may be possible at a hearing of the relevant patent office. An example of a successful appeal is applicable: an anti-inflammatory patent application based on Pheroid™ technology was filed in 1999. Although the patent offices of the USA and Canada approved the respective national applications, the European patent office found that the evidence provided in the application was insufficient to prove the claims. More pre-clinical and clinical evidence were provided several times before the appeal division was finally convinced of the veracity of the claims in 2005.

(l) If the examining division decides to grant the patent, the application with claims and specifications and the examining report are published in the official journal of the relevant patent office, e.g. "Official Patent Journal of the EPO". Third parties now have a time period within which to oppose the granting of the patent. The time period depends on the patent office, but is mostly 9 months. For instance, a pharmaceutical company may argue that a similar drug delivery system is already patented by them. Or academics may show that they have published or presented similar inventions. A hearing will now take place, under the auspices of three examiners making up the Opposition Division. The applicant and opposing parties have a chance to state their case and/or to defend their case. At such a hearing, the relevant examination division as well as subject specialists are present. The decisions taken at this hearing can be appealed and will then be heard by appointed independent boards of appeal.

(m) Any patent can be revoked on the grounds, *inter alia*, that

- the invention was not new at the priority date and/or
- the invention does not involve an inventive step and/or
- the invention cannot be applied in trade or industry or agriculture.

The novelty of an invention can be destroyed by the actions of the applicant: a patentee or inventor can inadvertently make the invention or knowledge about the invention available to the public before the priority date of the patent. Novelty can also be destroyed by disclosure of the invention to the public by an independent third party anywhere in the world before the priority date of the patent. A patent can also be revoked on certain formal grounds relating to the manner in which the complete specification and claims were prepared and to the information disclosed in the specification. The preparation of a patent specification is a skilled task and it is essential that there is close co-operation between the inventor and his/her patent attorney and that the inventor provides the patent attorney with full and clear information on all aspects of the invention.

In the case of the patents based on Pheroid™ technology, the inventor or writer of the patents thus had to show absolute novelty, an inventive step and usefulness (Chapters 6, 7 and 8).

3.2.4.3 Phases of patenting

The patents and patent applications presented in this thesis are in different phases of the patenting process. For instance, the vaccine patent has been granted in South Africa, but is still pending in the USA, where it is now being defended against a second Office Action or examination report. The patent on the application of Pheroid™ as delivery system for plants have been granted in South Africa. The status of the various national phases of the vaccine and plant applications is reported on in Chapters 6 and 8. The duration of the procedure, with inclusion of lodging the application to grant, is usually between 3 and 5 years. A search report may be received after 6 months, but may take much longer. The examiner may request the applicant to advise on selection criteria, as is the case with the vaccine patent. Although accelerated search, examination or publication can be requested, the first communication from the examination division may be expected after about 27 months and a patent is typically granted after about 44 months. A summary of the patenting process and its timelines are reflected in figure 3.1.

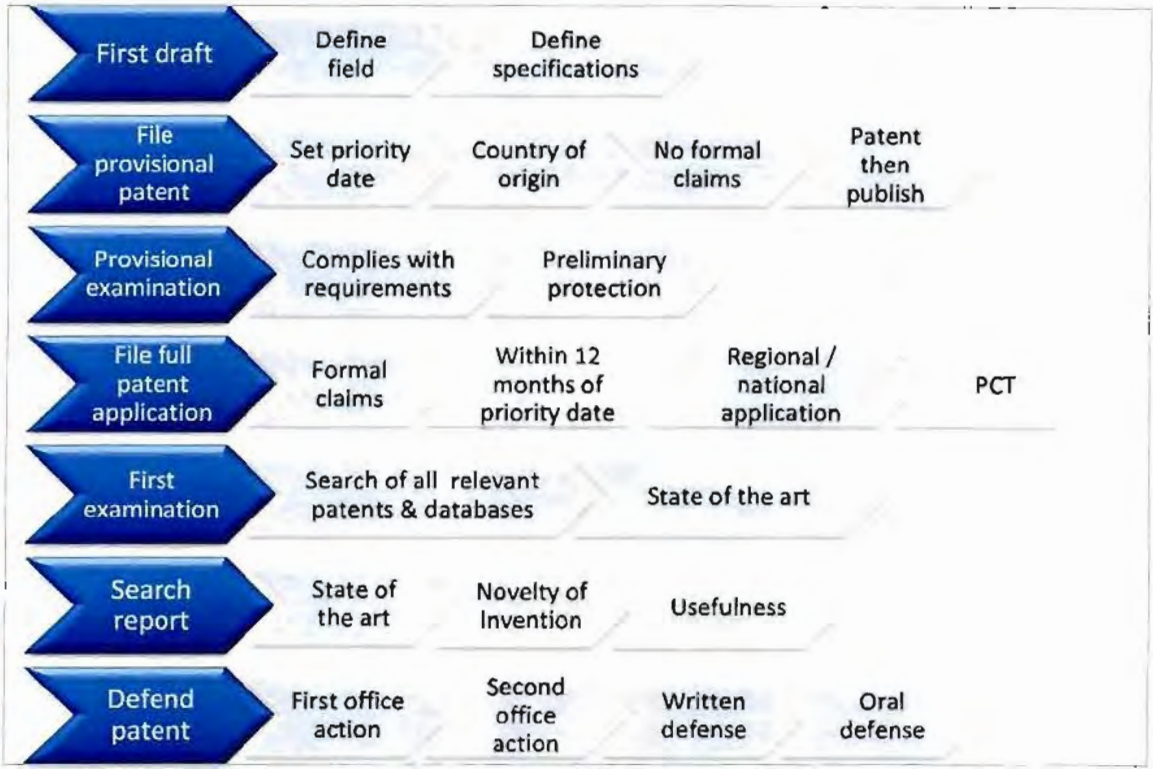

Figure 3.1 is a general scheme that reflects the various stages (blue) of the patenting process used by the EPO and most of the countries not affiliated to WIPO.

3.2.5 Summary

The examination of a patent application is more complex and harsh than that usually associated with academia because:

(i) the granting of a patent recognizes the commercial value of an invention within the context of its time and

(ii) the granting of a patent can be opposed by outside parties on an academic basis or by parties with a commercial interest, and is thus not only dependent on the evaluation by subject specialist 'reviewers'.

A patent is property that can be sold. It can also be licensed to a licensee by the patent holder, the licensor. The nature of such licenses differ: they can be sole, where the license provides for both the licensee and the licensor to exploit the patented invention in an exclusive or non-exclusive fashion. An exclusive license guarantees the exclusive right to the licensee to exploit the invention, with the exclusivity normally subject to minimum performance clauses.

Figure 3.2 shows some inherent developmental processes as discussed in 3.3. Whereas the generic patenting process has been described above, the process of drug development as exemplified on the hand of Pheroid™-based drug development is described below. The

different components of patent creation, product development and commercialization of idea/product are illustrated in figure 3.2, in which an effort was made to reflect the process of development, from the conceptualization of an idea to the registration of the resulting product. Three very definite stages are present. Phases A and B are required for the patenting process as described above.

The requirements of medicinal drug development, as also outlined in Chapter 1, revolve mainly around two aspects: efficacy and safety. Novelty is not a requirement. Medicinal product development therefore requires phase B and C only. For product development based on new applications of Pheroid™ technology, all three phases needed to be addressed.

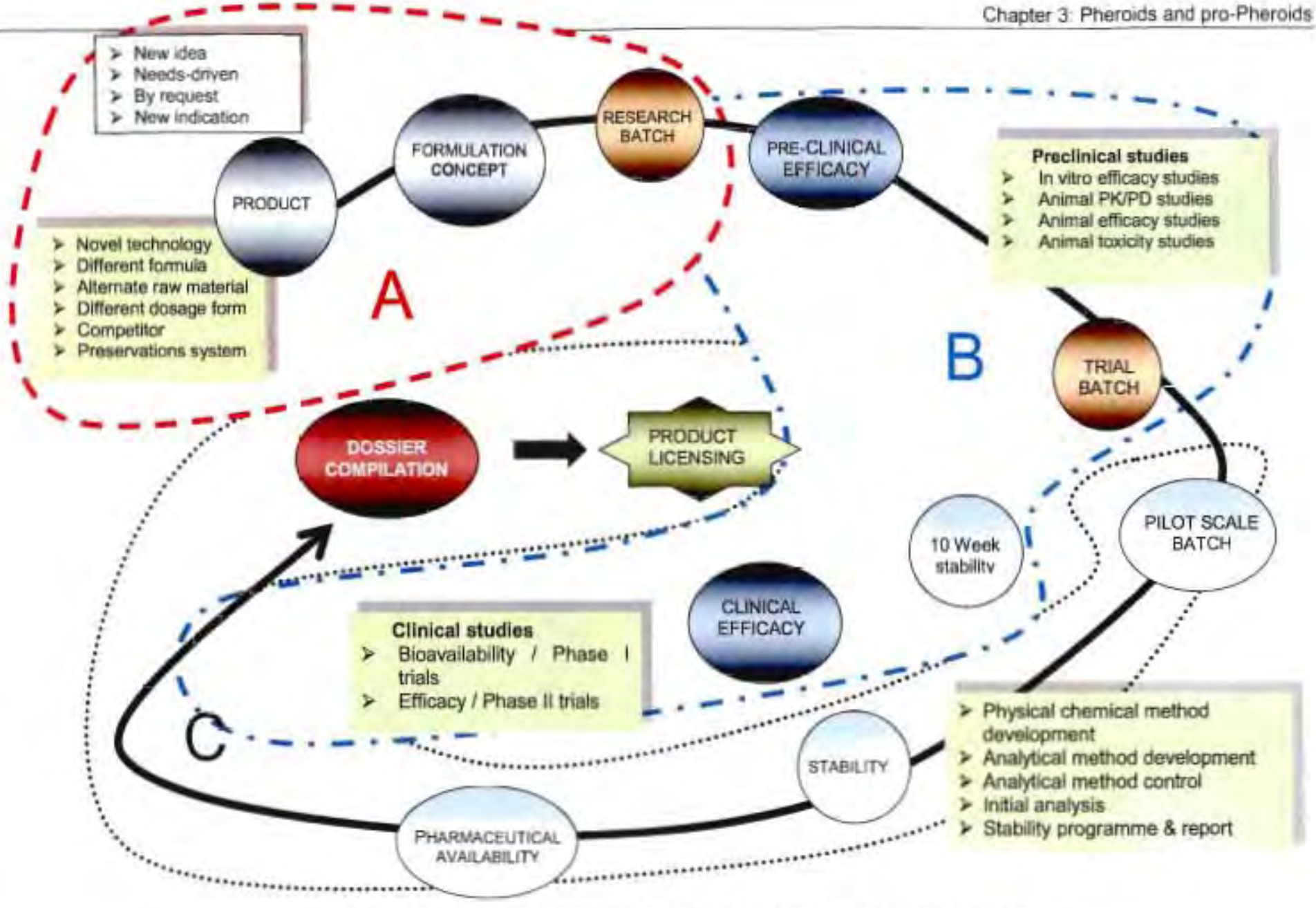

Figure 3.2: Processes inherent in patenting and product development

3.3 Pheroid™ and product development

In developing a product not based on a new patent, the marketer generally looks for a characteristic that differentiates the product from other "me too's". Besides safety and efficacy, that characteristic may be based on price, cost-effectiveness, user-friendliness and even smell and feel. In the pharmaceutical industry, companies are challenged with developing products that will reach clinical studies quickly. A further advantage could be a cost-effective manufacturing process.

As discussed in Chapter 2, the challenge for the pharmaceutical scientists lies in the development of an effective therapeutic. The efficacy of the therapeutic is dependent on a number of factors, such as class of pharmaceutical compound, mode of administration, the severity of the condition (related to safety and risk/benefit ratio, e.g. streptomycin can cause deafness but can also cure multidrug-resistant tuberculosis), and the type of disease i.e. chronic, acute, and latent.

The components forming the basis of therapeutic efficacy are the (a) effective delivery of (b) the therapeutic form of a drug (c) at the site of action. The process of drug delivery can in its turn be broken down into absorption, distribution, release, metabolism, clearance. Each of these components and the process supporting each may differ between the various drug categories as summarized in Table 3.1 below.

Table 3.1: Summary of factors impacting in the Pheroid™ drug delivery

Delivery	Absorption
	Distribution
	Release from carrier
	Metabolism
	Clearance
Molecular form	Physical: stereochemical e.g. isomers
	Spatial orientation e.g. quarternary orientation of proteins
	Biological intactness: status of degradation, e.g. - protein degradation by proteases - nucleic acid degradation by nucleases
Site of action	Concentration
	Duration: therapeutic window

3.3.1 Historical perspective of Pheroid™-based drug development

Pheroid™ technology has its origin in Emzaloid™ technology. Emzaloid™ technology was first unknowingly used in a quick and dirty product formulated by Piet Meyer and Steven Zall in an effort to treat or cure the psoriasis of Meyer. The psoriasis product proved to be more effective with fewer side effects than any comparable product then on the market. The company MeyerZall Laboratories was established with the aim of commercializing the psoriasis product. With rare insight, they initiated a research programme, initially in collaboration with the South African Medical Research Council (SAMRC), to try to pinpoint the reason for the success of their product. Research at the SAMRC showed that the very effective psoriasis topical product contained, besides a lot of waxes, crystals and other particulates also micro- and nano-sized vesicles that may entrap an active ingredient. The active ingredient used in the psoriasis formulation, coal tar, was autofluorescent and could therefore be visualised. It seemed possible that the entrapment of the coal tar in the vesicles may result in enhancement of both the amount and rate of transport of the coal tar into the skin. The work described in this thesis thus has its origin in 1999 and is based fundamentally on the original research done by myself at the MRC at that time.

A hypothesis was formulated that the vesicles in fact constituted a delivery system with wider application than the single topical product and that the system may be optimized to package and deliver a number of active ingredients. MeyerZall Laboratories established their own research team in 2000 with myself as head of the team to explore the potential of the delivery system and the term Emzaloid was coined to describe the system (Saunders *et al.,* 1999). The word Emzaloid was derived from the 'm' of Meyer, the 'za' of Zall and the word "colloid" and has been trade-marked. In comparative randomized multi-centre and controlled double-blind clinical trials the Emzaloid™-based topical coal tar psoriases product proved to be superior or at least equivalent to the reference products in substantial patient populations [n=327, n=60]. These trials were done in the UK, Austria, the Netherlands and Boston USA (Goodfield *et al.;* 2003; Tzaneva *et al.,* 2003).

Various topical products based on the so-called Emzaloid™ technology have since been developed, registered, manufactured and marketed. A number of medicines based on this technology have been registered with the South African Medicines Control Council. Two of the products have also been registered with the medicine regulatory authorities in the UK, EU, Australia, USA and Canada. In these topical products, the Emzaloid™ is used as a delivery vehicle to enhance the absorption of active ingredients such as coal tar, diclofenac, miconazole nitrate, and salicylic acid. A number of transdermal *in vitro* studies (Saunders *et al.,* 1999) have shown that the absorption of the active compound through the skin is faster and deeper when

formulated in Emzaloid™. As often happens with successful new ideas, both the two founding members, Meyer and Zall, have subsequently left MeyerZall Laboratories and the research department has been closed down. Nevertheless, the research has shown functionalities and possible applications for pharmaceutical products other than those already developed. For that reason the North-West University (NWU) has obtained all intellectual property with regards to the Emzaloid™ in 2003.

3.3.2 A Pheroid™ is not an Emzaloid™

There is a general misconception that the Emzaloid™ has been renamed to Pheroid™ by the NWU. Pheroid™ technology is based on Emzaloid™ technology but the two technologies are not quite equal. The term Emzaloid™ was erroneously and indiscriminately used by MeyerZall Laboratories for formulations manufactured by two different procedures. The basic delivery system and products manufactured for research purposes and stability were manufactured in the company's pilot plant in George, South Africa, according to a different manufacturing protocol than that used for the manufacturing of their commercial products. The commercial products are manufactured by (a) third party manufacturer(s) in Johannesburg, South Africa. In other words, the technology used for research purposes by MeyerZall Laboratories was not identical to the technology used by them for producing their commercial products. With the transfer of the intellectual property regarding this technology to the NWU at the end of 2003, it was decided to use and trademark the term Pheroid™ to describe those formulations manufactured according to the manufacturing protocol used in the research laboratories and pilot plant. The term Emzaloid™ is still used by MeyerZall Laboratories in their topical commercial products.

The word Pheroid is a conjugation of the word colloid and the Greek words "apo" and "phero", which quite literally mean "to move", "to ferry" or "to deliver". Several differences exist in the manufacturing protocols of Emzaloid™ and Pheroid™ systems, the main difference being that Emzaloid™-containing products are manufactured using low pressure gas exposure (80kPa) for 4 hours only, resulting in under-saturation of the formulation with the gas, whereas Pheroid™ formulations are saturated with nitrous oxide at higher than 150kPa for 3 to 4 days. Furthermore, all Pheroid™-based formulations contain D/L-α-tocopherol whereas the same is not true for all Emzaloid™-based products. The question is whether the differences in manufacturing are meaningful and result in different end products. To answer the question, it is necessary to look at the basic principles and components in the manufacturing of these two systems (see Figure 3.2 below), the roles played by both nitrous oxide and D/L-α-tocopherol and their relative amounts (see Chapters 4 and 5). Furthermore, the ratios of the various components constituting the Emzaloid™ and Pheroid™ systems differ from each other and

have an effect on the end product. In this document, when talking about the formulations under-saturated with nitrous oxide, the term Emzaloid™ is consistently used, while the term Pheroid™ is used for the research, pilot and clinical trial batches that were and still are consistently oversaturated with nitrous oxide. All results reported on in this document were obtained with research or trial products manufactured according to the research manufacturing protocol, either in the pilot plant in George or in the manufacturing laboratory of the NWU. Similarly, the applications described in this document for which patents have been submitted and/or granted, concern only the application of Pheroid™ technology and not that of the Emzaloid™.

3.3.3 The concept of Pheroid™ and pro-Pheroid™

The Pheroid™ basically consists of an oil phase, a water phase and a gas phase. The pro-Pheroid™ formulations, as devised by myself, contain no water phase and has no particles; macroscopically it looks like an oil phase. Pheroid™ micro- and nano-particles form spontaneously upon addition of a water phase to the pro-Pheroid™. While this spontaneous reaction occurs, the APIs present are packaged into the particles. When the water phase is added externally, it can contain electrolytes and may be buffered.

The Pheroid™ is a fatty acid-based delivery system and a number of the terms used in colloidal drug delivery is applicable and will be used. The concept of a pro-delivery system has originally been described by Payne *et al* (1986) when pro-liposomes were described as precursor of liposomes. The pro-liposome were described as a free-flowing product "which, on addition of water, disperses/dissolves to form an isotonic liposomal suspension". Whereas references to liposomes abound, references to pro-liposomes in databases such as Science Direct are surprisingly rare, independent of whether it is spelt with of without a hyphen. A number of papers describe enhanced delivery of peptides by pro-liposomes (Paine *et al.,* 1986; Ganter and Volker, 1997, see also Chapter 7). In US Patent 5635206 (Ganter and Volker, 1997), entitled "Process for liposomes or proliposomes", Ganter and Volker wrote: "Accordingly, there is a need for a process for the preparation of liposomes and pro-liposomes which can be operated under mild conditions and without great dilution. The process should permit the preparation, under mild conditions, of liposomes or pro-liposomes having a regular structure in which one or more active substances, which may be added, are uniformly dispersed. In addition, the process should be readily operable on an industrial scale" (Ganter and Volker, 1997). It does therefore seem as if the repeatable preparation of homogenously sized pro-liposomes is fairly problematic.

The pro-Pheroid™ system unlocks the potential of this technology for administration routes other than the topical route. Like pro-liposomes, it is based upon the intrinsic property of hydrated membrane fatty acids to form vesicles and/or other lipid aggregates on dilution with

water. Pro-Pheroid™ is especially important in the case of drugs or APIs that are unstable in the presence of moisture, such as rifampicin. As will be shown (3.3), the manufacturing procedure is simple and side-steps many of the difficulties generally encountered with the manufacturing of lipid-based delivery vesicles by the generation of the actual delivery vehicle at the site with required biological milieu. Figure 3.3 depicts the main components of both the basic Pheroid™ and pro-Pheroid™ and also illustrates the difference between the two.

Originally a triangular concept was hypothesized with the fatty acids as one corner, nitrous oxide the second corner and the active compound to be packaged as the third corner of the triangle. Clearly, this was an oversimplification, as the presence of a tocopherol is required and the amount of tocopherol may change the morphology of the vesicle. The unsaturated fatty acid component of the Pheroid™ received a lot of attention since it gave the Pheroid™ the added dimension of inherent therapeutic qualities. In addition, changes in the fatty acid component by the addition of fatty acids not present in Vitamin F ethyl ester, led to changes in the morphology of the particles. However, the use of anti-oxidants and preservatives may have an impact on the size of the particle formed, and contributes to the stability of the vesicles.

In the case of Pheroid™s, the ingredients of the water phase contribute to the final packaging. The components or raw materials used in the production of Pheroid™s and pro-Pheroid™s will be discussed in some depth below. The various factors influencing the Pheroid™ type and sizes are discussed in the book chapter incorporated in Chapter 4.

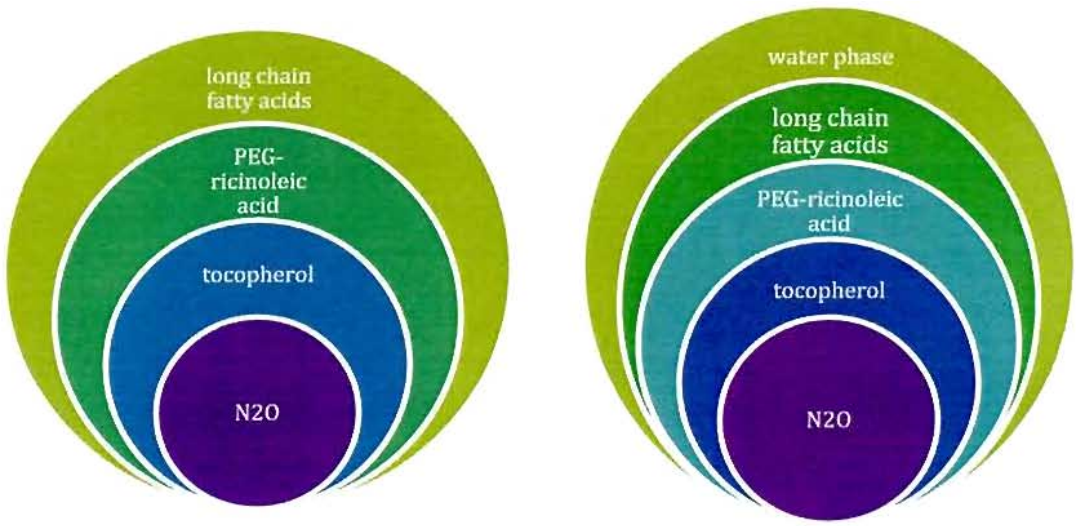

Figure 3.3: The obvious difference between the components of the Pheroid™ on the right and pro-Pheroid on the left is the presence or absence of a water phase - no water phase is

present in the pro-Pheroid™. The water phase can be added externally by the formulator or manufacturer or can consist of body fluid, such as fluid present in stomach /intestinal content.

An understanding of the colloid and interfacial perspective of a delivery event enables one to manipulate such delivery systems as colloidal systems. The product developer will take into account that certain *in vivo* processes may alter the surface of particles to the advantage or disadvantage of the biodistribution of the particular product. An example is the opsonization of particles. Pegylation on the other hand is one of the artificial processes that may be used to prevent some of the problems caused during the *in vivo* circulation of particles.

The formation of Pheroid™ from pro-Pheroid™ can be visualized on plain glass or dextran-coated glass. Simply place 5µl pro-Pheroid™ that has been fluorescently-labelled with 10nM Nile red on the slide and 10µl of a water phase such as 0.1N HCl a small distance away. Rapid spreading of the pro-Pheroid™ formulation occurs with fingering at the edges, probably because of pinning defects. When the leading edge of either the water phase or the pro-Pheroid™ oil phase encounters defects on the surface, the front flows around rather than over the defect. Because the amount of pro-Pheroid™ on the glass surface is limited, the spreading slows and then halts, presumably because further spreading would result in the loss of intermolecular interactions within the oil phase. When a reservoir of pro-Pheroid™ is available, spreading continues while spreading is self-limiting in the absence of a reservoir. The Pheroid™ vesicles form spontaneously where the two moving fronts (the pro-Pheroid™ and the water phase) meet and Pheroid™ formation can be captured by confocal laser scanning microscopy as shown in figure 3.4. It is possible to analyze the formation kinetics using a theoretical model that accounts for the competition of favourable interactions between the oil phase, the water phase and the solid support with hydrodynamic shear flow and inter-monolayer friction, but such analysis is not included here.

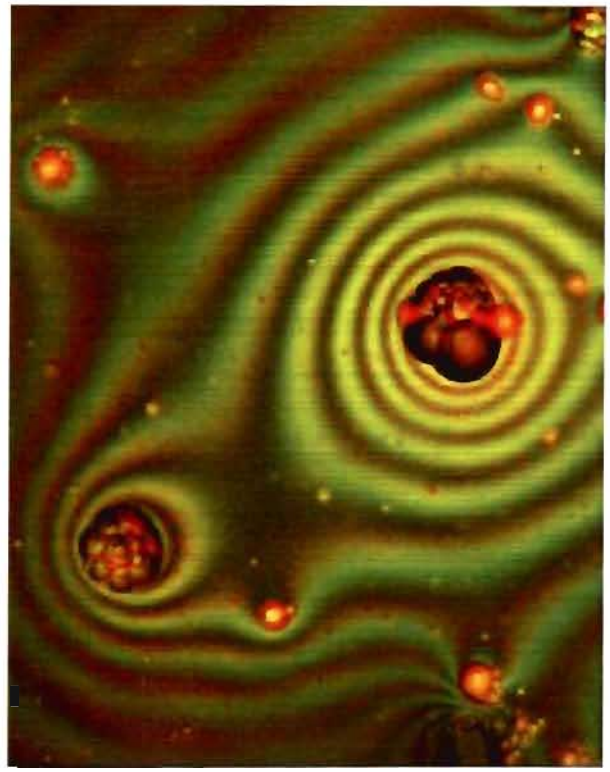

Figure 3.4: A micrograph of the formation of Pheroid™ vesicles from captured by confocal laser scanning microscopy (CLSM) according to the procedures described in 3.4.

It is hypothesized that upon contact with the gastric fluid, the same process as described above occurs, with the formation of a Pheroid™ forming zone. As vesicles are formed, active compounds in suspension in the pro-Pheroid™ become entrapped in the vesicles, which move away from the formation zone, allowing new vesicles to form. When selecting an appropriate oil phase for vesicle formation, the specific application and bulk properties such as permeability, diffusional characteristics and degradation rate must be considered. A bio-resorbable material is preferable for drug delivery. Classifying the properties of delivery systems for their selection as biomaterials is challenging, because a wide variety of particular applications exist.

No single, simple set of methods can be used to characterize all available forms of Pheroid™. A rationalization of macromolecular design for a function-specific application is therefore required. In this chapter no detailed discussion of preparation and characterization methods is given, nor are examples of other applications given. In such applications as that discussed in Chapters 4, 5, 6, 7 and 8, some investigation is needed with regards to the specific delivery mode, the dosage required, the type of cells targeted, the surface of the vesicles and other properties.

3.3.4 Pheroid™ types and components

The essential fatty acids are necessary for various cell functions but cannot be manufactured by human cells and have to be ingested. The Western diet has been shown to be limited in its supply of these basic lipid molecules. The fatty acids component typically used in the manufacturing of Pheroid™s are procured in the form of Vitamin F ethyl ester. The fatty acid profile of Vitamin F Ethyl ester is given in the patents/patent submissions incorporated in Chapters 6, 7 and 8. Some of the functions of this component of the Pheroid™ system are the maintenance of membrane integrity of cells, energy homeostasis, modulation of the immune system through amongst others the prostaglandins/leukotriens and some regulatory aspects of programmed cell death as discussed in Chapter 4. Specific physiological and formulatory functions of the different components of Pheroid™ are discussed in the chapters following on this chapter.

Various analytical, pre-clinical and clinical procedures have been used by myself in the various studies described and these will be addressed as and when relevant in the next chapters. The original observations of Pheroid™ particles relied mainly on confocal laser scanning microscopy (CLSM) and the results obtained have now been confirmed by other analytical techniques. CLSM is still used to visualize Pheroid™s, and to determine some of the structural characteristics and morphology of the particles. In addition, it has been used in the studies herein described to monitor quality, to determine particle size distribution and crystallization, to optimize formulations, to determine drug loading and efficiency of entrapment and for just about any analytical determination possible during the course of this study. Since it is not a generally used analytical procedure in the pharmaceutical industry, some aspects of CLSM are summarized here. A flow associated cell sorter (FACS Calibur), also based on laser imaging, and was used to differentiate between various Pheroid™ populations (results not included).

3.4 Research methodology

3.4.1 Confocal laser scanning microscopy (CLSM)

The first paper describing the use of CLSM in drug delivery was that by Mohsen *et al.* to study liposomal uptake mechanisms and intracellular distribution (Mohsen *et al.*, 2009). Since CLSM is the qualitative and quantitative investigative and analytical tool most used in these studies a short discussion of the principles and application of this form of microscopy was thought fitting. Confocal microscopy is well-known and often used in the three-dimensional analysis of industrial materials and biological structures. As far as could be ascertained, there are very few references to the use of CLSM in the investigation, analyses and quality control of

drug delivery systems. A large amount of data and scientific information can be acquired within a relatively short time and from a single properly prepared or archived sample with CLSM. High resolution investigations of live biological processes at the micro- and nano-scale, including investigations into the therapeutic actions of new pharmaceutical products can be investigated, since CLSM allows the semi-quantitative analysis of images of dynamic interactions within the spatial and dynamic context of the biochemical setting of cells, tissues and organisms. The technique itself will not be described here; several sources are available in the literature and on the internet. Some of the characteristics that make CLSM a tool *par excellence* for the 3D investigation of micro-particles:

- Because of the use of lasers, it is possible to penetrate samples up to a specific depth. Using available software, the data from the optical sections can by integrated to enable the 3D-reconstruction of intact samples in their natural environment. Samples can therefore be investigated in the x, y and z-axes.
- Samples can be viewed in planes running parallel and perpendicular to the investigative angle. High resolution images can be obtained in this manner.
- It is possible to regulate the intensity of illumination of each of the lasers used to allow for the density of the sample investigated and the depth of penetration required.
- For each specific field of depth, images are acquired point-by-point and reconstructed. Since a single sample will contain hundreds to thousands of micro- or nano-particles at each field of depth or optical section, sharp focused images of single particles are nearly impossible to obtain with conventional microscopy and imagery.
- CLSM has the capacity for direct, non-invasive imaging – it is possible to optically section a sample without causing artefactual disturbances in the sample.
- The presence of a pinhole allows very specific focusing at a very specific depth while extraneous light from the surrounding sample is excluded. Since most images are based on fluorescence, in which light scattering takes place, the use of a pinhole and point-to point capturing increases the contrast and transverse definition of the images, and the images are very clean and sharp.
- The use of fluorescence adds the dimension of specificity to this form of microscopy, but with the added ability to discriminate features against out-of-focus background fluorescence.
- Different fluorophores can be excited at various wavelengths with resultant emission of photons at different wavelengths. Specific features can in this way be identified and spatially be related to the rest of the sample.
- The fluorescence intensity can be translated into semi-quantitative measurements.

- A minimum of sample preparation is required. This is particularly advantageous when the technique is used for quality assurance. In addition, no harsh solvents or resins are used and sample aberration is not caused by the application of vacuum.
- CLSM can be used in various modes, such as reflectance, emission, differential interference contrast (DIC) and fluorescence and overlays of the two modes can be computed.
- The use of low energy lasers in combination with high resolution detection allows imaging of live cells and/or organisms over an extended time period without causing cellular damage or phototoxiciy.
- By mathematically processing the captured photon emissions obtained from more than one fluorophore, clearer and repeatable images with little cross-talk can be obtained. This is particularly useful in observations of multi-stained specimens.
- Most biological material contains autofluorescence. For instance, the chloroplasts in plants fluoresce strongly when exposed to both blue (Kr/Ar) and green (He/Ne) lasers and it is impossible to perform conventional fluorescent microscopy of multiple stained samples with success. Separation of probe signals from autofluorescent signals is now possible, particularly with spectral confocal microscopy, as mathematical unmixing allows separation of fluorescence spectra.
- Acquisition speed of image capturing can be set, and time series analysed. This is especially useful in time-lapse or real time observation of living cells or tissue.
- Quantitative spectral imaging allows the accurate quantification of both exogenous/external and endogenous/internal fluorescence signals in 3-D by the spectral separation of photons emitted by molecules which normally emits photons at overlapping wavelengths. Precise quantitative analysis of nearly all information contained within a sample during imaging is possible in a number of modes, including transmission, DIC, fluorescent and confocal mode. This could provide a direct approach to investigate the pharmacokinetics of a drug at the cellular level. A few Pheroid^{TM} samples were analysed with this technique.

3.5 Pheroid^{TM} and pro-Pheroid^{TM} manufacturing

A number of more and less sophisticated manufacturing processes are used in the preparation of pharmaceutical lipid-based colloidal systems. Storm and Crommelin (1998) summarizes the procedures in an amazingly insightful article (see Figure 3.5).

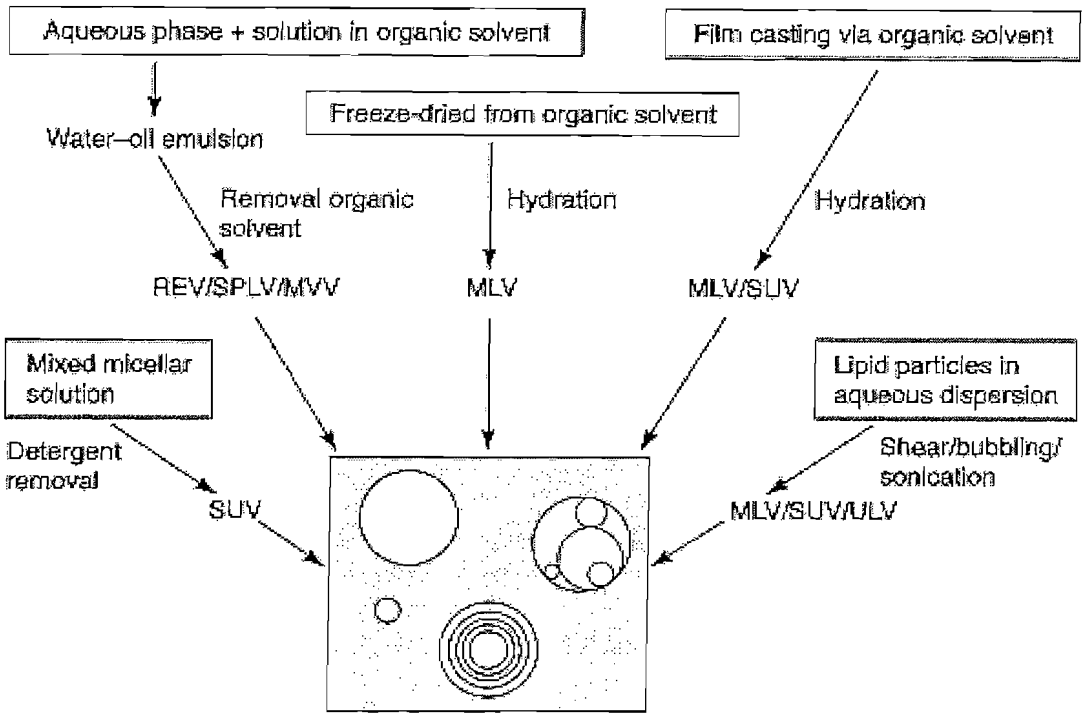

Figure 3.5: Schematic diagram of regularly used methods for liposome preparation. The commonly obtained types of vesicles are indicated. MLV, (classical) multilamellar vesicles; MVV, multivesicular vesicles; REV, reverse-phase evaporation vesicles; SPLV, stable plurilamellar vesicles; SUV, small unilamellar vesicles; ULV, unilamellar vesicles, with S, M and L prefixes added for small, medium and large vesicles respectively; OLV, oligolamellar vesicles. Reprinted with permission (Storm and Crommelin, 1998).

Methods for preparing emulsions are usually simple and fairly repeatable and can consist of single or multiple stages. Generally no hi-tech equipment is needed. The preparation process of liposomes on the other hand, is generally more complex and typically consists of at least three stages: a hydration stage, a sizing stage and a purification stage (Shah *et al.,* 2006; Storm and Crommelin, 1998). The different procedures that can be used to accomplish each stage are reflected in the schematic diagram in Figure 3.6. Whichever way you look at it, a lot can go wrong with both process and equipment in the upscaled manufacturing of such a delivery system.

3.5.1 The process

Several problems are generally applicable to the production of any lipid-based system. These may include the cost of production, the poor quality of the raw material (phospholipids) and the poor characterization of the physicochemical properties of the produced lipid particles. Specific problems that have been encountered were low entrapment efficiencies, problems with

upscaling and lack of stability with resultant short shelf life of the product (Shah *et al.,* 2006; Mohsen *et al.,* 2009; Storm and Crommelin, 1998).

Most of the stability, entrapment and production problems associated with liposomal formulations are not applicable to the patented Pheroid™ technology with its simple method of preparation in a vessel designed for this purpose. The procedure or protocol for the preparation or manufacturing of Pheroid™s has been progressively simplified since the original batch was produced in 2000. The currently used protocol is shown in Figure 3.7. The basic protocol does not reflect changes in fatty acid ratios, the addition of other long chain fatty acids or the inclusion of anti-oxidants and preservatives. These factors are discussed where and when relevant. A protocol for repeatable basic pro-Pheroid™ preparation has in the meantime been established and is also portrayed in Figure 3.7.

Pheroid™s prepared according to the protocol illustrated in the schematic in figure 3.7 are vesicular in form, submicron in size and bi-layered in terms of morphology. In the pro-Pheroid™ formulation, no particles are present before the addition of a water phase, but vesicles formed immediately upon the addition of water. Generally pro-Pheroid™s are prepared with polyethylene glycol (PEG) 400 but PEG of other sizes or polymeric units may also be used. A comparison between the processes portrayed in figures 3.6 and 3.7 will immediately make it clear that the Pheroid™ is not prepared according to liposomal preparatory principles. Its preparation is quite similar to that of an emulsion – it is simple and easy to prepare if the protocol is followed precisely.

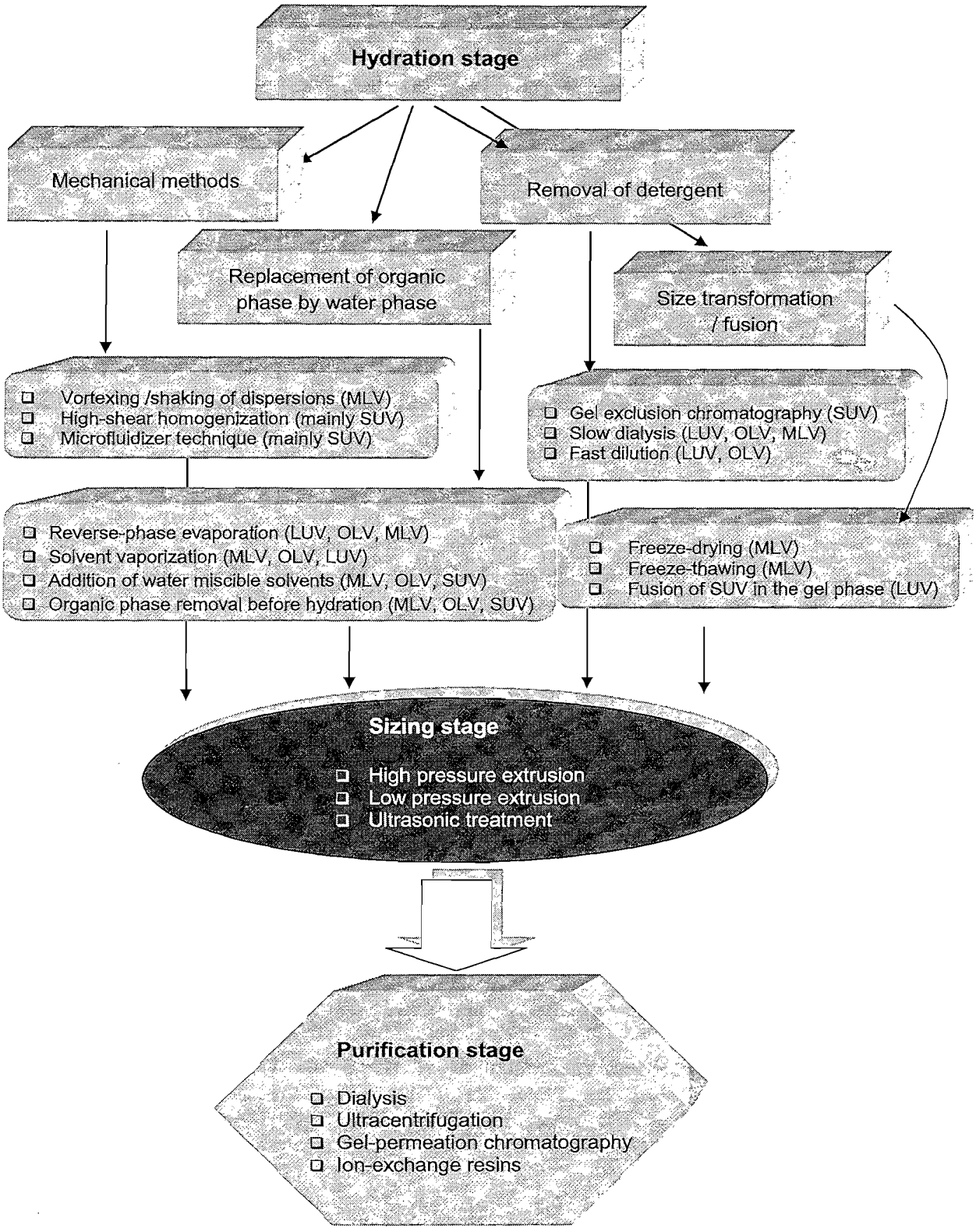

Figure 3.6: Schematic representation of the manufacturing processes used for the preparation of liposomes.

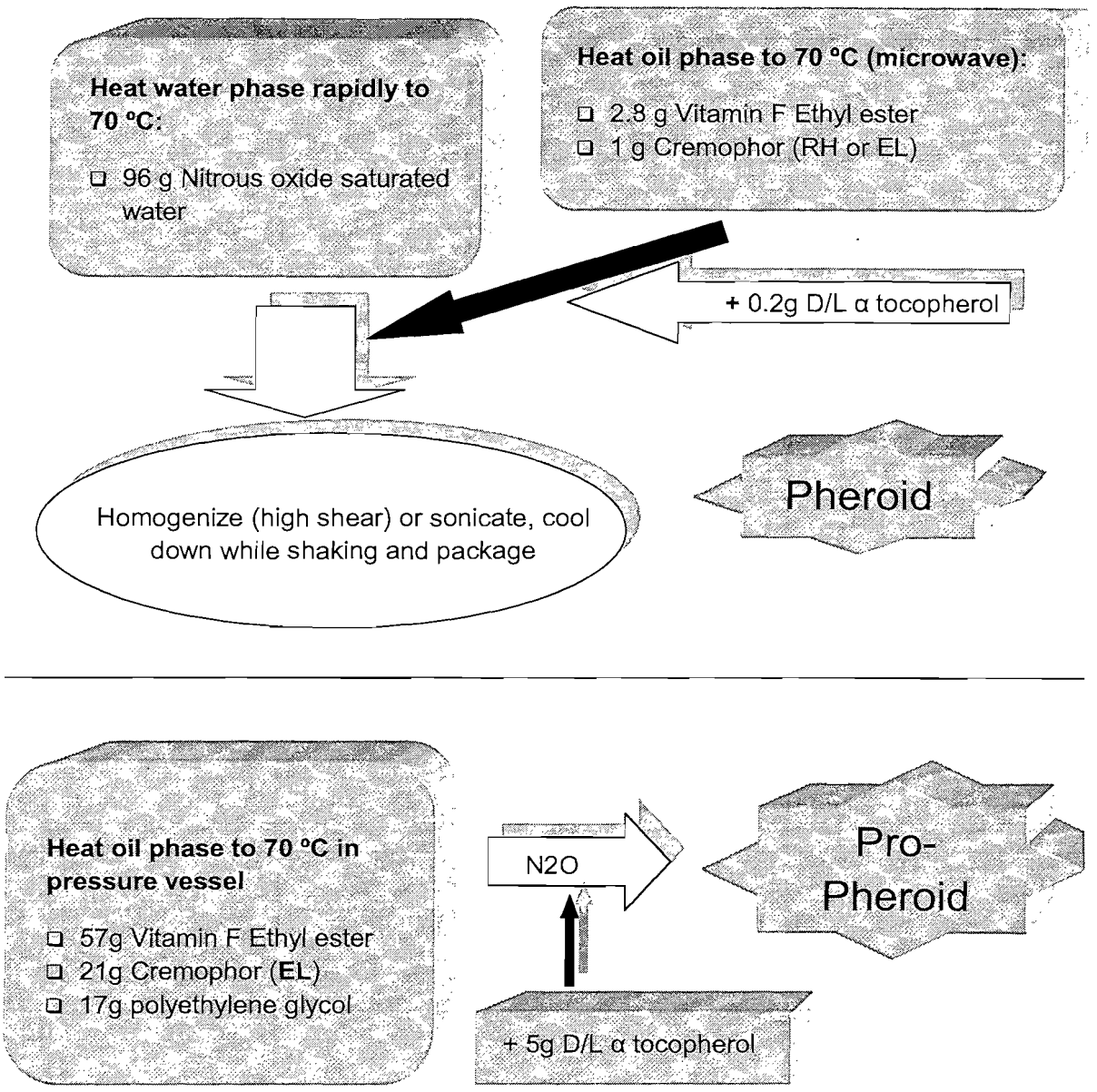

Figure 3.7: Schematic representation of the manufacturing process of the Pheroid™ and pro-Pheroid™.

A typical manufacturing batch document for basic Pheroid™s is shown below. The precise amount of raw material used has been optimized. The quality of the Pheroid™s produced have been analysed by CLSM in terms of particle counting and morphology for a number of concentration series of each of the raw materials.

PRODUCT: **PHEROID™ (unpreserved)**	PRODUCT TYPE: Research batch
PRODUCT CODE: PH 1/2004	BATCH SIZE:
BATCH NO.	DATE:

MANUFACTURING FORMULA:

APPROVED NAME OF RAW MATERIALS	BATCH NUMBER	QUANT /100g (%)	BATCH QUANT (g)	ACTUAL QUANT WEIGHED.	ACTION SIGN	CHECK SIGN
Vitamin F ethyl ester		2.8				
Cremophor RH-40 **or**						
Cremophor Etocas		1.0				
dL-α tocopherol		*0.2*				
N_2O Saturated water		*86.0*				

METHOD OF MANUFACTURE:

PROCEDURE	SIGNATURE	
	Manufacturer	Scientist
1. Place slightly more than the desired volume of water in a beaker and saturate with nitrous oxide under pressure of 1.6kPa for 4 days in pressure vessel. _____kPa		
2. In a glass beaker, measure nitrous oxide water, close top and heat on warm plate to 70°C.		
3. In a closable container, weigh off Vitamin F Ethyl ester, Cremophor RH-40 or Cremophor Etocas = Oil phase. Mix and heat Oil phase to 70^0C in a microwave. Temp _____°C. Add dL-α tocopherol and swirl.		
4. While maintaining the N_2O saturated water at 70^0C, add the heated Oil phase from Step 2 to the water. Work quickly.		
5. Mix with Braun, speed 2 until homogenous. Work quickly. Transfer to glass Schott bottle(s) and shake until room temperature is reached. Temp _____°C		

CALCULATIONS:

Please show all calculations used.

METHOD OF PACKAGING AND CONSOLIDATION:

1. Pour Pheroid™ into supplied container.		
2. Fill Jars / Bottles / Tubes		
3. Record fill volume:_____.		
4. Record quantity of jars/ bottles filled: _____		
5. Label with appropriate labels indicating batch number and expiry date.		

Person responsible for making batch _____

Name of responsible scientist: _____ Date: _____

Signature: _____

3.5.2 The equipment

Initially, water was saturated with nitrous oxide by placing a beaker containing the desired water phase in a pressure cooker. The pressure cooker was modified to house a gas inlet. Using a regulator, nitrous oxide was supplied through the inlet until a pressure of between 160 to 200kPa was reached. The water phase was left under pressure for 4 days to achieve saturation. However, the air that was present in the vessel was also forced into the water phase, diminishing the amount of nitrous oxide. For that reason, a manifold with an escape or sparging valve was introduced to the vessel to allow the replacement of most if not all air by nitrous oxide, which is heavier than air. With this system, batches of up to 5kg can be prepared and the system is still used to prepare small research scale batches.

A simple way of testing saturation was to fill balloons with nitrous oxide and fix these tightly over the neck of a bottle containing the water phase. If the balloon deflates overnight, the water phase is not saturated, since the gas still moves from the balloon to the water phase.

The manufacturing batch document in 3.5.1 above is written for this system. One of the disadvantages of this system is that the nitrous oxide has to be forced into the water from the top and the surface of the water phase is limited; complete saturation of the water phase with the nitrous oxide is difficult to achieve.

To prepare larger batches, a stainless steel circular tank 30cm in diameter and 0.6m in length was mounted on a stand with the long axes horizontal to the floor. A gas inlet and manifold with escape valve was fitted to one side of the tank with a tap with a pressure seal on the other side. A length of tubing with 1mm holes all over the tube was fitted inside to the gas inlet on the inside of the tank to enable sparging of the gas through the water phase; the sparger dramatically increased the water phase surface exposed to the nitrous oxide and saturation could be achieved in a shorter time period. Both of the above systems suffer from the drawback that the water phase has to be moved to another vessel to be heated and in the process it loses some of its gas component.

Three different mechanical processes are incorporated into the production process: gas saturation, heating and high shear mixing. It seemed a good idea to design a vessel that could accommodate all three these processes, and where nitrous oxide is not lost during the process. Figure 3.8 contains photographs of a rough pilot scale home-built prototype vessel. This vessel can accommodate a volume of 150 litres and is still in use for the preparation of trial material for testing on plants (see Chapter 8). This home-built vessel evolved into a sophisticated pilot scale vessel that complies with the European Union, EMEA and South African Medicines Control Council (SAMCC) regulations. It was designed in conjunction with the Head of Instrument Makers at the NWU and built by an engineering company named Falcon Engineering. Figure 3.9 is an illustration of this vessel. With both these vessels, it is now possible to heat and mix under pressure. With the home built vessel, it is necessary to release some pressure manually during the heating step, as the pressure increase beyond the safety limits for the vessel. Subsequently, a vessel with a capacity of 600 litres was commissioned and is now being used for commercial purposes. This vessel also contains an in-line water regulator for accurate measurement of the water volume. Table 3.2 shows the difference in procedure between the research batch manufacturing and the upscaled pilot scale manufacturing.

In the newly designed pilot scale and commercial vessels, the complete process happens within the enclosed space, with very little chances of contamination of the product from the environment or of the environment by the product.

Table 3.2: Difference in manufacturing for research and pilot scale batches	
PILOT SCALE MANUFACTURING	RESEARCH BATCH MANUFACTURING
1. Measure the desired volume of water into the pressure vessel and apply nitrous oxide at a pressure of between 1.8 – 2.0kPa for 24 hours. Pressure _____ kPa. Set thermostat of vessel to 70°C and heat. Temp_____°C.	1. Place slightly more than the desired volume of water in a beaker and saturate with nitrous oxide under pressure of 1.6kPa for 4 days in pressure vessel. _____kPa. In a glass beaker, measure nitrous oxide water, close top and heat on warm plate to 70°C.
2. Weigh off Vitamin F Ethyl ester, Cremophor RH-40 or Cremophor Etocas = Oil phase. Heat each separately to 70°C in microwave and then mix by stirring. Temp Vitamin F Ethyl ester _____°C; Cremophor temp _____°C. Add cold dL-α tocopherol and stir. Note: work quickly.	2. In a closable container, weigh off Vitamin F Ethyl ester, Cremophor RH-40 or Cremophor Etocas = Oil phase. Mix and heat Oil phase to 70^0C in a microwave. Temp _____°C. Add dL-α tocopherol and whirl.
3. Relieve pressure of vessel (step 1) to enable the addition of the oil phase from step 2 to the heated water phase and homogenize in the following manner: Homogenize for 30 seconds, leave for 2 minutes, homogenize for 1 minute, leave for 2 minutes, homogenize for 2 minutes.	3. While maintaining the N_2O saturated water at 70^0C, add the heated Oil phase from Step 2 to the water. Work quickly.
4. Apply nitrous oxide again at a pressure of between 1.8 – 2.0kPa Pressure _____ kPa. Cool the preparation down quickly by circulating cold water through water jacket. Saturate with nitrous oxide at the above pressure for another 24 hours.	4. Mix with Braun, speed 2 until homogenous. Work quickly. Transfer to glass Schott bottle(s) and shake until room temperature is reached. Temp _____°C

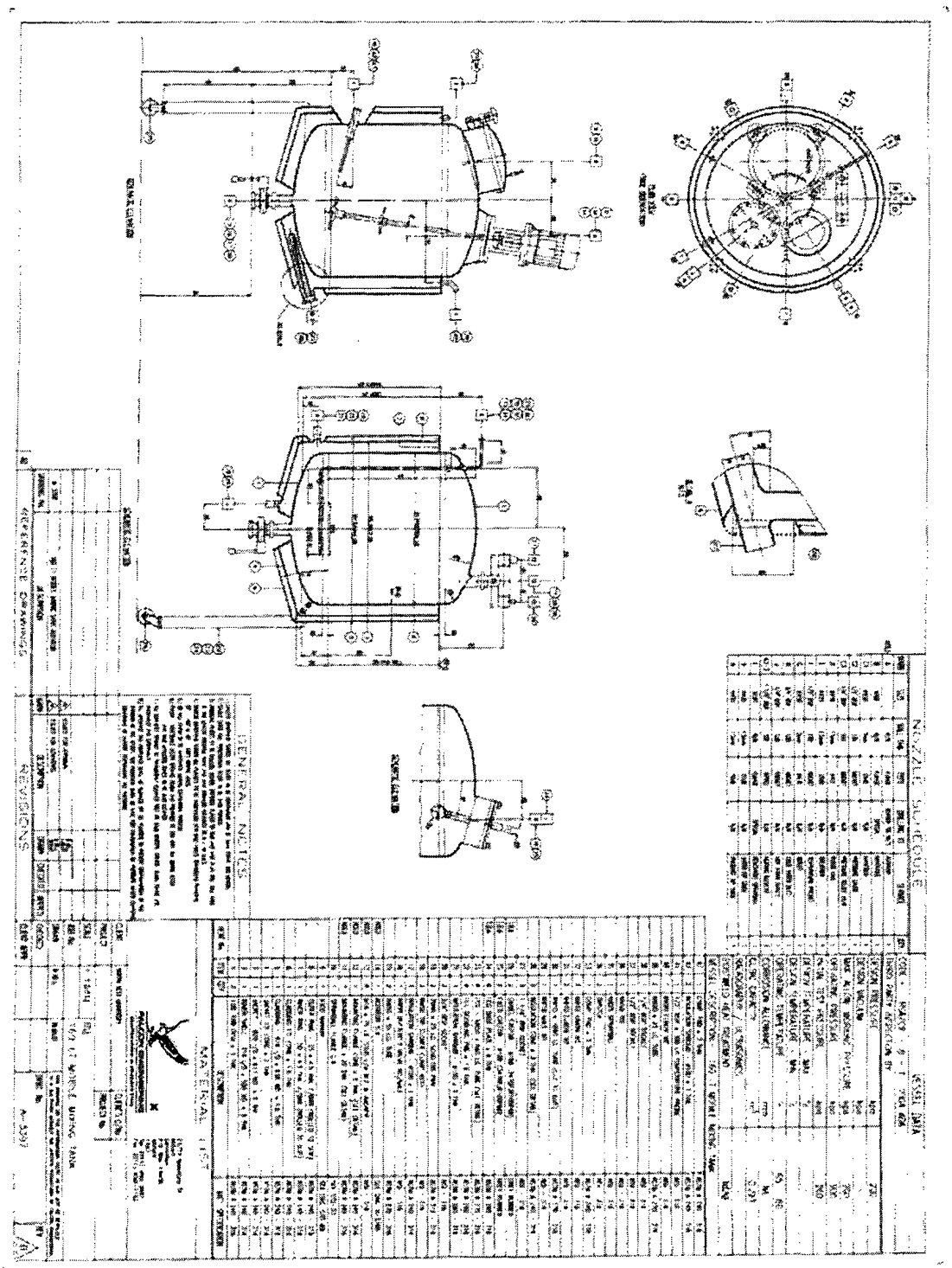

Figure 3.9: Blueprint of manufacturing vessel with a capacity of 150 litres as discussed above (copyright NWU and Falcon Engineering).

3.6 Investigative procedures

3.6.1 Microscopical investigative process

Since a substantial part of the research and quality assurance presented here included microscopical analysis, the procedure of sample preparation and investigation was incorporated into a standard operating procedure (SOP) that is included below.

3.6.1.1 SOP of microscopical sample investigation and analysis

1. **OBJECTIVE:**

1.1 To investigate the attributes of Pheroid™-based formulations

1.2 To determine the effect of specific Pheroid™ formulations on biological samples

1.3 To investigate/ or develop different types of Pheroid™ applicable to the indication or aim of a project.

1.4 All investigations into the effect of Pheroid™ formulations on biological samples need to include control and/or comparative samples.

1.5 All products, formulations and samples to be investigated must be accompanied with the stipulated information necessary to determine the procedural details (see Product specifications, SOPs and Annexure 1).

1.6 All procedures and methods using light microscopy, fluorescence microscopy, or confocal laser microscopy may be performed only after training and adequate experience in the operation of the necessary equipment and specific procedures.

2. PRINCIPLES:

2.1 No single procedure is applicable to all formulations. The procedure used is dependent on *inter alia* the aim of the investigation, the type of formulation, the therapeutic or indicated active compound used, the physical characteristics of the sample, product or formulation, the type and quantity of the proposed Pheroid and fatty acids, the administration route and the dedicated treatment environment. Product-specific procedures are based on the specifications of each individual product.

2.2 Experimental formulation and biological sample investigative procedures are developed according to the requirements and aims of each investigation or project.

2.3 A high degree of sterility during preparation of samples must be maintained.

3. RESPONSIBILITY:

3.1 Head of Department

3.2 Research Scientists

Trainee, under supervision of Research Scientists

4. ACTION:

Products, formulations and samples are processed according to the Confocal-booking schedule unless otherwise decided by the management or project team.

4.1 Sample identity and concomitant documentation

- Products, samples and formulations must be clearly labeled with the necessary information as per relevant SOP.
- Sign for receival of samples on departmental sheets.
- Note sample information and documentation as well as general aim of investigation/analysis in sample log record sheet. (Annexure 2).

4.2 Sample Processing

4.2.1 Determine the exact nature of the investigation, and applicable procedure and equipment to be used:

- Polarized light-, phase- or reflected light microscopy
- Autofluorescense microscopy
- Fluorescence microscopy
- Confocal laser scanning microscopy
- Particle or structure size determinations
- Quantification
- Morphology analysis

4.2.2 Fluorescent Labeling Procedures:

Follow the labeling procedure as specified for each product, sample or formulation as set out in applicable SOPs, specifications, and manufacturer instructions. Where no procedure exists, develop and document as a new procedure.

4.2.3 Sample preparation for microscopy:

NO PERSON NOT QUALIFIED MAY TURN ON OR OFF THE LASERS, OR OPERATE THE EQUIPMENT. THE ENERGY SOURCES, INCLUDING THE LASERS CAN BE FUSED BY INCORRECT OPERATIONAL PROCEDURES. THE 4x, 10x and 40x OBJECTIVES ARE DRY OBJECTIVES AND OIL SHOULD NEVER BE USED WITH THESE OBJECTIVES.

The type of sample and the nature of the investigation to be carried out determine the sample preparation procedure. The procedure below describes the simplest general Pheroid™ analysis procedure for low viscosity fluid samples, using glass slides for microscopy. Samples, controls, and comparators will hereafter be referred to as samples.

a) Label applicably sized microfuge tubes with the sample information.

b) Measure or weigh correct amount of each of the samples, controls and/or comparators into the microfuge tubes.

c) Dilute the samples as necessary and according to specifications.

d) Incubate samples with the required calculated amount of applicable fluorophore stock solution(s) using the procedures determined for the specific product, sample or formulation.

e) Mix samples by means of vortexing, pipetting, plastic spatula stirring and / or sonication according to sample specifications.

f) On toweling paper, lay out a clearly labeled glass slide for each sample.

g) Measure / weigh out incubated labeled samples onto middle glass slide according to the sample specifications.

h) Cover glass slide with cover slip.

i) Turn glass slide face down so that the cover slip is facing the toweling paper.

j) Cover with second paper towel and press for between 1 and 2 seconds when stipulated by procedure.

k) Seal edges of prepared slide with non-fluorescent quick-drying nail polish.

4.3 Microscopy and image capturing

4.3.1 Objective selection: Depending on the magnification required, select the applicable objective. If an oil objective is selected, put a drop of non-fluorescent microscope oil on the cover slip side of the slide.

4.3.2 Slide placement: Place glass slide in the slide holder with the cover slip facing the objective.

4.3.3 Record the time from start of sample preparation to actual image capturing.

4.3.4 Select the appropriate energy source for the investigation.

4.3.5 When using the fluorescent mercury lamp or lasers as energy source, select the applicable excitation and emission windows for the fluorophore used.

4.3.6 Capture the image, using the correct setting for the investigation; e.g. still images, average images, time series, and 3D investigations.

4.3.7 Store images in project-specific dated files as image cytometric data. Store images to be printed or processed also as tiff or bmp files.

4.3.8 When necessary, quantify particles, Pheroid™s or structures with the use of Scion Image.

4.4 Results

4.4.1 Record all the applicable information on CLMS Analysis Sheets (Annexure 3).

4.4.2 Print selected images as required.

4.4.3 Process and interpret results and write conclusion of investigation/ analysis.

4.4.4 Get result record and images checked and signed off by second Research Scientist or Head of Research.

4.4.5 Send result record(s) and images to appropriate department, and log receival of documents by that department.

5. LIST OF ANNEXURES:

5.1 Formulation record

5.3 Microscopy Sample Investigation Record

5.4 Microscopy Cell Investigation Record

The Formulation record and Microscopy Cell Investigation Record are shown below. The Microscopy Sample Investigation Record is not included since it is a simple list.

5.1 ANNEXURE1: FORMULATION RECORD

An example of a product formulation sheet is attached below.

PROJECT	Product X
TRIAL NO	2008/02
OBJECTIVE	To formulate a _____ for _____, based on Pheroid™ technology, allowing for subsequent incorporation of active compound _____.
DATE	19/05/2009

Ingredients	%	200 g
A. Vitamin F Ethyl Ester	6,000	12,00 g
Cremophor RH-40	2,000	4,00 g
Cetearyl Octanoate	3,000	6,00 g
Light Mineral Oil	1,000	2,00 g
Cetyl alcohol	2,000	4,00 g
Vitamin E	0,300	0,60 g
Propyl Paraben	0,050	0,10 g
Butylated hydroxytoluene	0,005	0,01 g
Carbopol Ultrez 21	0,300	0,60 g
B. Propylene Glycol	3,000	6,00 g
Methyl Paraben	0,300	0,60 g
Butylated hydroxyanisole	0,015	0,03 g
Triethanolamine	0,170	0,34 g
Purified N_2O Water	82,660	168,70 g (incl. 2% overage)
C. Perfume Unisex 45.051	0,200	0,40 g

Procedure:

Heat (A) to 65°C and (B) to 70°C.

Add (B) to (A) while stirring by hand (spatula).

Cool down to room temperature, then add (C) while stirring by hand.

Measure and record pH: _____

Measure and record viscosity when possible: _____

Pack into n x y ml aluminium tubes and m x z ml jar, and label Technician _____

ANNEXURE 5.2 MICROSCOPY SAMPLE INVESTIGATION RECORD

SAMPLE _____ SCIENTIST: _____ SIGN: _____ DATE : QA:

AIM/ OBJECTIVES OF INVESTIGATION: _____

Project/ Formulation				Conditions		Sample details
Project details				Time		Date receive
Formulation name		Manufac. date		Temp		Date completed
Batch nr				% RH		

Labeling procedure:				Pheroid Counts								Lazer power	
Fluorophores	Mixing	Time	Temp			Vesicles		Sponges/ Depots					
SOP:	Sonicate ☐			File	Total	Submicron	> 1µm	1 –2 µm	2-4µm	5-9 µm	>10 µm	Red	Green
	Vortex ☐												
	Pipetting ☐												
				Average:									

Physical appearance:

Light ☐	Fluorescence ☐		Confocal ☐		Pinhole	Objectives	Filters	Colour	
Polarized ☐	Blue	☐	Lasers		Small ☐			Odour	
	Green	☐	Green	☐	Medium ☐			Homogeneity	
	Red	☐	Red	☐	Large ☐			Other	

Particles/ Crystals		Observations	Conclusions
Presence			
Number in 10 fields			
Size range			
Details			
Save: Light:			
CLSM:			

5.3 MICROSCOPY CELL INVESTIGATION RECORD

SAMPLE _____ SCIENTIST: _____ SIGN: _____ DATE : QA:

AIM/ OBJECTIVES OF INVESTIGATION:

Project/ Formulation			Cells/Organisms		Sample details
Project details			Time		Date receive
Formulation name		Manufac. date	Temp		Date completed
Batch nr			% RH		

Labeling procedure:				Details of Formulation	Details of treatment	Other
Fluorophores	Mixing	Time	Temp			
SOP:	Sonicate ☐					
	Vortex ☐					
	Pipetting ☐					
	Other					

Light ☐	Fluorescence ☐		Confocal ☐		Pinhole	Objectives	Excitation filters	Emission filters	Zoom factor
Polarized ☐	Blue	☐	Lasers		Small ☐				
Phase ☐	Green	☐	Green	☐	Medium ☐				
	Red	☐	Red	☐	Large ☐				

Cells/Organism/Pheroid/TMs/Particles/Structures		Observations	Conclusions
Presence			
Number in 10 fields			
Size range			
Details			
Save: Light:			
CLSM:			
Comments			

3.7 Conclusion

Despite the decades of research on liposomes, the molecular mechanisms of liposome-cell interactions have not been elucidated completely. The interactions of drug carriers with cells are complex as it involves different processes such as surface binding, internalization and possible release of its contents. These processes are difficult to mimic *in vitro*. Furthermore, the composition, charge type, size and fluidity also have an impact on cellular uptake of carriers.

It is necessary to understand carrier uptake mechanisms and intracellular distribution in order to design more efficient drug carrier systems. The interaction of carriers with cells is a developing area in pharmaceutics since it holds the possibility of introducing new material into the cytoplasm and plasma membranes of the cell.

For each formulation, a specified range for the number and size of Pheroid™, as well as a maximum crystal size for the active compound was determined. These specifications were summated in a Certificate of analysis and were also used during the quality control process. As can be deduced from the microscopical sample investigation record, the average number of Pheroid™ per 23µl, obtained from 5 fields of each Nile red labelled sample, as well as the average sizes of Pheroid™ in each field was recorded and measured against the ranges set for that formulation before the batch was released.

CLSM was used to generate information on the degree of association of various vesicles to a number of cell types, as well as to estimate of the amount of vesicle fatty acid associated with the cell and the intracellular disposition of Pheroid™. Some of these results will be discussed in the subsequent chapters.

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