UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
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Desenvolvimento de nanopartículas inovadoras a partir de constituintes da biodiversidade brasileira destinadas à aplicação tópica de antioxidantes

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Tese apresentada por **Letícia Marques Colomé** para obtenção do título de doutor em Ciências Farmacêuticas

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RESUMO

Desenvolvimento de nanopartículas inovadoras a partir de constituintes da biodiversidade brasileira destinadas à aplicação tópica de antioxidantes

Nanopartículas lipídicas têm sido desenvolvidas para aplicação tópica de fármacos e ativos cosméticos. Neste trabalho, foi proposta a primeira aplicação de um lipídeo natural não-refinado biodegradável e biocompatível - manteiga de cupuaçu (*Theobroma grandiflorum*) - para a preparação de nanopartículas lipídicas, as quais foram denominadas teosferas. As teosferas foram preparadas por emulsificação-evaporação do solvente (EES) e por homogeneização à alta pressão (HAP), apresentando tamanho nanométrico e distribuição granulométrica estreita quando preparadas por ambos os métodos. O trabalho teve continuidade com a preparação de teosferas pelo método de EES utilizando manteiga de cupuaçu ou sua mistura com óleo de castanha do Brasil (*Bertholletia excelsa*) - também derivado da biodiversidade Amazônica - visando a incorporação de antioxidantes. Idebenona (IDB) foi selecionada por sua conhecida ação antioxidante e pela sua utilização em formulações cosméticas antienvelhecimento. IDB foi incorporada nas teosferas com eficiência de encapsulação superior a 99%, sendo que os estudos de liberação *in vitro* mostraram que a liberação de IDB a partir das teosferas foi mais lenta em comparação à IDB livre. Estes experimentos foram capazes ainda de demonstrar as características elásticas das teosferas. Além disso, foi evidenciada *in vitro* a atividade antioxidante superior das teosferas contendo IDB em relação ao ativo livre. Visando possibilitar a aplicação tópica de teosferas contendo IDB, em um trabalho subseqüente, suspensões de teosferas preparadas por HAP foram incorporadas em géis hidrofílicos. As formulações apresentaram características pseudoplásticas e demonstraram efeito oclusivo *in vitro*, o qual foi dependente da composição dos colóides. Finalmente, os estudos de permeação *in vitro* utilizando pele humana demonstraram que teosferas e nanocápsulas de núcleo lipídico, utilizadas neste estudo de modo comparativo, modificaram a permeção da IDB, permitindo a acumulação do ativo nas camadas superficiais da pele.

Palavras-chave: nanopartículas lipídicas; teosferas; lipídeos naturais; manteiga de cupuaçu; óleo de castanha do Brasil; antioxidantes; idebenona; permeação *in vitro.*

ABSTRACT

Development of innovative nanoparticles using brazilian compounds intended for antoxidants topical application

Lipid nanoparticles have been developed for administration of active substances to the skin, both for pharmaceutical and cosmetic uses. In the present work, we proposed the first use of a none-refined natural biodegradable and biocompatible lipid – Cupuaçu seed butter (*Theobroma grandiflorum*) – for the preparation of lipid nanoparticles, which were called theospheres. Theospheres were prepared by emulsification-solvent evaporation (ESE) and by high pressure homogenization technique (HPH), presenting size in nanometrical range and narrow particle size distribution for both methods. Taking these results into account, the next step of this work was the preparation of theospheres by ESE method using Cupuaçu seed butter with or without Brazil nut (*Bertholletia excelsa*) seed oil - another ingredient derived from an Amazonian fruit - intending the encapsulation of an antioxidant. Idebenone (IDB) has been selected due to its known antioxidant action and because it has been used in antiaging cosmetic formulations. IDB was incorporated in the theospheres presenting encapsulation efficiency higher than 99%. The in vitro release evaluation demonstrated that the release of IDB from theospheres was lower than that of free drug. Besides, the in vitro release study highlighted the elastic characteristics of theospheres. Additionally, IDB-loaded theospheres showed higher antioxidant activity compared to free IDB. Viewing the cutaneous administration, theosphere suspensions prepared by HPH technique were incorporated into hydrogels. The rheograms of the semi-solid formulations exhibited a non-Newtonian behavior presenting pseudoplastic characteristics. In vitro occlusion study highlighted the dependence of the occlusive effect on the lipidic composition of the theospheres. Finally, in vitro human skin permeation studies showed that theospheres and lipid-core nanocapsules, used in this study in a comparative way, changed the permeation of IDB, increasing the accumulative amount of IDB in the upper skin layer.

Keywords: lipid nanoparticles; theospheres; natural lipids; cupuaçu seed butter; Brazil nut seed oil; antioxidants; idebenone, in vitro human skin permeation.

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INTRODUÇÃO

Nanopartículas lipídicas são sistemas coloidais derivados de emulsões O/A pela simples substituição do óleo por um lipídeo sólido. Estes sistemas representam uma alternativa a lipossomas e nanopartículas poliméricas, uma vez que podem contornar limitações existentes quanto à estabilidade e escalonamento do processo de produção. Esta importante e promissora alternativa repousa nas características das nanopartículas lipídicas que possibilitam conferir proteção a compostos lábeis contra degradação química e alcançar a liberação controlada da substância encapsulada (MÜLLER *et al*., 2000).

Muitas técnicas diferentes para a produção de nanopartículas lipídicas têm sido descritas na literatura (MEHNERT e MÄDER, 2001). Dentre estas, a técnica de homogeneização à alta pressão (HAP) é considerada a mais efetiva em produzir partículas com tamanho reduzido, o que se soma às vantagens de não utilizar solvente orgânico e ser passível de produção em grande escala, dada a ampla variedade de tamanho dos homogeneizadores comercialmente disponíveis (JOSHI e MÜLLER *et al*., 2009). Assim, a maioria dos trabalhos relatados na literatura trata da preparação de nanopartículas lipídicas pelo método de HAP, enquanto poucas exceções têm utilizado outras técnicas. O método de emulsificação-evaporação do solvente (EES), por exemplo, apresenta a vantagem de reduzir a exposição de compostos termolábeis à alta temperatura, sendo também uma técnica adequada à preparação de colóides lipídicos (CORTESI *et al.*, 2002).

Nanopartículas lipídicas foram planejadas primeiramente para a administração parenteral de fármacos. Contudo, atualmente são propostas para a administração de fármacos através de diferentes vias, como parental, oral e tópica. A partir da metade dos anos 90 o interesse foi ainda mais focado na aplicação de nanopartículas lipídicas pela via tópica, especialmente para uso cosmético. Para aplicação tópica, beenato de glicerila, palmitato-estearato de glicerila, trimiristato de glicerila e palmitato de cetila aparecem como os lipídeos sólidos mais utilizados. Como lipídeos líquidos, são utilizados principalmente triglicerídeos de cadeia média e, alternativamente, ácido oléico (SCHAFER-KORTING et al., 2007). Além disso, lipídeos sólidos naturais altamente purificados, tais como frações de estearina de frutos, têm sido utilizados (MANDAWGADE e PATRAVALE, 2008).

No cenário cosmético, novos ingredientes vêm sendo utilizados, a exemplo de materiais advindos da biodiversidade brasileira, destacando-se aqueles extraídos do

contexto amazônico. Representante desta classe, a manteiga de cupuaçu *(Theobroma grandiflorum)* é obtida de suas sementes por prensagem a frio. O cupuaçu apresenta vários compostos importantes como os ácidos graxos insaturados, aminoácidos e vitaminas (ROGEZ *et al*., 2004) e dois flavonóides glicosídeos específicos (teograndinas I e II), além de flavonóides antioxidantes como catequina, epicatequina, quercetina e kampferol (YANG *et al*., 2003), sendo interessante para o uso cosmético. Outro representante da biodiversidade amazônica com potencialidades cosméticas é o óleo de castanha do Brasil (*Bertholletia excelsa*) que contém cerca de 70% de gorduras insaturadas, incluindo os ácidos graxos palmítico, esteárico, linoléico e oléico, sendo ainda a maior fonte vegetal conhecida de selênio (GONÇALVES *et al*., 2002).

Além da inovação buscada na biodiversidade vegetal, pesquisas na área cosmética têm estudado possibilidades para contornar os processos envolvidos no envelhecimento, especialmente quando mediados por espécies reativas de oxigênio induzidas pela exposição à radiação UV. Neste contexto, muitas substâncias com capacidade antioxidante vêm sendo incorporadas em produtos com ação antienvelhecimento. Idebenona [2,3-dimetoxi-5-metil-6-(10 hidroxidecil)-1,4-benzoquinona, IDB] é um análogo sintético da coenzima Q10, com conhecidas propriedades antioxidantes. IDB tem sido estudada para o tratamento de doenças neurodegenerativas, como Parkinson, Alzheimer e ataxia de Friederich, uma vez que o cérebro pode ser particularmente vulnerável a danos oxidativos (PALUMBO *et al*., 2002). Contudo, assim como o cérebro, a pele está constantemente exposta a um ambiente pró-oxidante, especialmente pela radiação ultravioleta, que desempenha um papel fundamental no processo de envelhecimento extrínseco mediado por espécies reativas de oxigênio e fotocarcinogênese, o que faz da IDB uma alternativa interessante como componente de formulações tópicas *antiaging*.

Considerando as características promissoras para uso cosmético dos lipídeos naturais supra-citados, o objetivo deste trabalho foi primeiramente avaliar a viabilidade da aplicação inédita de um lipídeo natural não-refinado, biodegradável e biocompatível - manteiga de Cupuaçu - para preparação de nanopartículas lipídicas, denominadas teosferas, através de dois métodos diferentes (EES e HAP). Especificamente, o trabalho objetivou avaliar a concentração de lipídeo ideal para a formulação das teosferas e ainda conhecer a influência do método de preparação e os parâmetros de fabricação sobre as

características físico-químicas e a estabilidade dos colóides lipídicos. Em um segundo momento, este trabalho objetivou a incorporação de IDB em teosferas preparadas pelo método de EES utilizando manteiga de cupuaçu e sua mistura com óleo de castanha, a fim de avaliar a viabilidade de incorporação de uma substância antioxidante, bem como a influência de um lipídeo líquido nas características deste novo carreador. Avaliou-se ainda a atividade antioxidande das teosferas comparativamente ao ativo livre.

Dando continuidade aos estudos e visando a obtenção de formulações adequadas para aplicação tópica cutânea, teosferas preparadas pela técnica de HAP foram incorporadas em géis hidrofílicos que foram caracterizados quanto ao perfil reológico e o potencial oclusivo *in vitro*. Por fim, considerou-se pertinente a avaliação da capacidade das teosferas em modificar as características de permeação da IDB, para o que se recorreu à técnica de permeação *in vitro* em célula de difusão de Franz utilizando pele humana como membrana. Neste estudo, as teosferas contendo IDB foram avaliadas comparativamente às nanocápsulas de núcleo lipídico, outra formulação inovadora desenvolvida em nosso grupo de pesquisa.

Contemplando as referidas investigações, este trabalho é apresentado sob a forma de quatro publicações: a primeira intitulada “Theospheres Based on Theobroma Grandiflorum Seed Butter: Development of Innovative Nanoparticles for Skin Application”, publicada no periódico Soft Materials; a segunda intitulada “Idebenone loaded-theospheres for dermal application: preparation, physico-chemical characterization, *in vitro* release and *in vitro* antioxidant activity evaluation”, submetida à revista Macromolecular Bioscience; a terceira com o título de “Development and physicochemical characterization of idebenone-loaded theospheres in hydrogels intended for topical delivery” e quarta intitulada “Development and human skin permeation study of novel drug delivery systems for idebenone: lipid-core nanocapsules and theospheres”, ambas a serem submetidas para publicação em periódicos indexados.

Adicionalmente, este trabalho traz ainda em seu anexo, uma publicação realizada em paralelo às relativas ao doutorado, a qual se intitula “Polymeric Nanocapsules for Drug Delivery: an Overview” que constitui-se em um capítulo do livro "Colloids in Pharmaceutical and Biotechnological Applications".

Publicação 1: Theospheres Based on Theobroma Grandiflorum Seed Butter: Development of Innovative Nanoparticles for Skin Application

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THEOSPHERES BASED ON *THEOBROMA GRANDIFLORUM* SEED BUTTER: DEVELOPMENT OF INNOVATIVE NANOPARTICLES FOR SKIN APPLICATION

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□ *Lipid nanoparticles have been developed for application of active substances to the skin, both for pharmaceutical and cosmetic uses. In the present work, we proposed the first application of a non-refined natural biodegradable and biocompatible lipid—Cupuaçu seed butter (Theobroma grandiflorum)—for the preparation of lipid nanoparticles, which are called theospheres. Theospheres were prepared by two different methods, emulsification-solvent evaporation and high pressure homogenization technique, using Cupuaçu butter at different concentrations. Theospheres prepared by both methods showed size in the nanometrical range (around 200 nm) and narrow particle size distribution (0.2). Theosphere suspensions were stable for 30 days, excepting the systems containing 15 and 20% of butter prepared by emulsification-solvent evaporation method. Multiple light scattering analyses showed that physical instability was avoided by increasing the number of homogenization cycles during preparation in the systems prepared by high pressure homogenization. The DSC and X-ray analysis showed that the preparation method influenced the cristallinity of the lipid in the theospheres. The results showed that Cupuaçu butter is an alternative and promising compound for nanoparticle formulation, especially if it is used in concentrations up to 10%, irrespective to the preparation method.*

Keywords Colloidal carries, Lipid nanoparticles, Natural lipids, Theobroma grandiflorum seed butter, Theospheres

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INTRODUCTION

Since the beginning of the 1990s, colloidal carrier systems based on lipid nanoparticles have been intensively studied as drug delivery systems in the pharmaceutical field (1–3). Many different techniques for the production of lipid nanoparticles have been described in the literature, for example, high pressure homogenization (4), microemulsion technique (5), emulsification-solvent evaporation (6), emulsification-solvent diffusion method (7), ultrasonication (8), double emulsion technique (9), solvent displacement method (10), and phase inversion (11).

Several advantages are attributed to high pressure homogenization technique, such as easy scale up, avoidance of organic solvents, and short production time (1, 2). High pressure homogenizers are widely used in pharmaceutical industries, for example, to produce emulsions for feeding a patient intravenously bypassing the usual process of eating and digestion (parenteral nutrition). In addition, this technique can provide formulations for skin application concerning pharmaceutical or cosmetic purposes (12).

Most of the reported works have focused on the preparation of the lipid nanoparticles by high pressure homogenization method (13–17), while a few exceptions of those studies have used emulsification-solvent evaporation technique for the preparation of particles (6, 18). However, this method presents the advantage to reduce the exposure to high temperature of thermolabile compounds, such as proteins and nucleic acids (19). The solvent evaporation method is based on the evaporation of the organic solvent in which lipids are dissolved, allowing the formation of either solid microparticles or nanoparticles. To obtain nanoparticles, this process includes an additional step, such as high pressure homogenization (6) or sonication (18, 20). In this regarding, to the best of our knowledge no report has already been published concerning the production of lipid nanoparticles by emulsification-solvent evaporation using exclusively a rotor-stator homogenizer without needing an additional step for size reduction.

In therapeutics, lipid nanodispersions have been originally developed for intravenous administration and they have also gained interest for brain and liver targeting (2). Moreover, lipid nanoparticles have been proven valuable for drug application to the skin both for pharmaceutical or cosmetic uses (21). Concerning the dermal use, nanodispersions can be prepared using 5 to 40% of lipid (15). The higher concentrated preparations are of semisolid appearance (cream) being cosmetically acceptable as they are. The fluid dispersions, obtained with low lipid content, can be incorporated into a cream or a gel base to facilitate dermal application (22).

Lipid nanoparticles for skin application are made of lipids such as glycerol behenate, glycerol palmitostearate, or cetyl palmitate. For nanoparticles composed of lipid blends, oils such as medium chain triglycerides are

combined with solid lipids (3). Besides, highly purified natural solid lipids, such as stearine fractions of fruits have been used (5).

In the present work, we propose the first application of a non-refined natural biodegradable and biocompatible lipid—Cupuaçu seed butter—for the preparation of lipid nanoparticles. This butter is obtained from the seeds of Cupuaçu (*Theobroma grandiflorum*) by cold pressing. Cupuaçu is one of the most popular fruits on the Amazon market. The seed butter extraction is based in sustainable use of biodiversity by small communities in Amazon (23). Cupuaçu presents various important compounds as unsaturated fatty acids, amino acids and vitamins, being interesting for the cosmetic use (24). An important class of natural products is the flavonoids, which are polyphenolic compounds found in fruits, vegetables, and certain beverages that have diverse beneficial biochemical and antioxidant effects. Cupuaçu presents two specific sulfated flavonoid glycosides (theograndins I and II), beside known flavonoid antioxidants, for example, catechin, epicatechin, quercetin, and kaempferol (25).

Taking into account the promising features of this natural lipid, the purpose of this study was to evaluate the feasibility of preparing novel lipid nanoparticles based on *Theobroma grandiflorum* seed butter—called theospheres—by two different methods: emulsification-solvent evaporation (using rotor-stator homogenizer) and high pressure homogenization. The influence of the preparation method and the fabrication parameters (e.g., the number of cycles in hot homogenization technique) on the physicochemical characteristics and on the stability of theospheres was investigated within 30 days by measurements of particle size, size distribution, zeta potential, pH, backscattering analysis, and viscosity. Additionally, DSC and X-ray analysis were carried out to evaluate the cristallinity of the Cupuaçu seed butter in the theospheres.

EXPERIMENTAL

Materials

Cupuaçu seed butter was gently gifted from Inovam (Brazil). Lipoid S45 (soybean phosphatidylcholine) was supplied by Lipoid (Germany). Poloxamer 188 was obtained from Sigma (USA). Diazolidinyl urea was supplied by Delaware (Brazil). Ethyl acetate was delivered from Tedia (USA). All the reagents were used as received.

Theospheres Preparation

Emulsification-Solvent Evaporation (ESE) Technique

Theospheres were prepared by a modified emulsification-solvent evaporation technique (19). Cupuaçu seed butter at different concentrations and

Lipoid S45 (2% w/w) were dissolved in ethyl acetate (25 mL) and this mixture was poured into an aqueous phase containing Poloxamer 188 (3% w/w) and diazolidinyl urea (0.5% w/w) using an Ultraturrax T-25 (Ika, USA) (24,000 rpm, 10 min). After emulsification, the organic solvent was evaporated at room temperature (2,000 rpm, 2 h). The theosphere suspensions were named 5E, 10E, 15E, and 20E, according to butter concentration (5, 10, 15, or 20% w/w, respectively) and the method used for preparation.

High Pressure Homogenization (HPH) Method

Theospheres were also prepared by high pressure homogenization method using a Panda 2K NS1001L homogenizer (Niro Soavi, Italy). The oil phase was composed of Cupuaçu seed butter (5, 10, 15, and 20% w/w) and Lipoid S45 (2% w/w) melted at 40°C. The aqueous phase was prepared by mixing Poloxamer 188 (3% w/w), diazolidinyl urea (0.5% w/w), and water (40°C). The aqueous phase was poured into the oil phase using an Ultraturrax T25 (Ika, Germany) (13,000 rpm, 2 min). The warm pre-emulsion was homogenized in the high pressure homogenizer at 500 bar. Samples were collected after 3, 6, and 9 cycles of homogenization. The theosphere suspensions were named 5H, 10H, 15H, and 20H, according to butter concentration (5, 10, 15, or 20% w/w, respectively) and the method used for preparation, in the same way of the emulsification-solvent evaporation technique.

Theospheres Characterization

Physico-Chemical Characterization of the Suspensions

Theospheres stored at room temperature were characterized by measurement of particle size, size distribution (polydispersity index—PDI), zeta potential, pH, and viscosity at 0, 15, and 30 days. The pH values of the suspensions were determined using a potentiometer B474 (Micronal, Brazil). Measurements of particle size and zeta potential were made at 25°C using a NanoSizer ZS (Malvern Instruments, UK). Particle size, size distribution, and polydispersity index were determined in diluted samples (500 times) with filtered water (0.45 μm). The zeta potential measurements were performed after diluting the samples (500 times) with 10 mM NaCl aqueous solution.

Multiple Light Scattering Analysis

In the Turbiscan Lab (Formulaction, France) the light source is an electro luminescent diode in the near infrared (λ = 880 nm) and two synchronous optical sensors receive, respectively, the light transmitted through the sample (180° from the incident light, transmission sensor), and the light backscattered by the sample (45° from the incident radiation, backscattering detector)

(26), acquiring transmission and backscattering data every 40 μm. The samples (20 mL) were placed in glass cells, without needing any sample dilution or filtration/centrifugation, and analyzed at 25°C using the scan mode.

Number of Particles per Volume in the Theosphere Formulations

Theosphere suspensions were analyzed using a Cary 50 UV-Vis spectrophotometer (Varian, USA) to determine the turbidity τ (cm^{-1}) [Eq. (1)] (27).

$$\tau = \frac{1}{b}\ln(10) \times A \quad (1)$$

where b (cm) is the optical cell path length and A is the instrumental absorbance signal.

In a sufficiently diluted sample of spherical particles, τ is a function of the particle diameter d (cm), the concentration of particles N (particles cm^{-3}) and the dimensionless quantity W, which is the extinction efficiency [Eq. (2)].

$$\tau = \frac{\pi}{4} d^2 \times N \times W \quad (2)$$

W is a function of the type $W(x,m)$, where x is the size parameter defined as $x = \pi d/\lambda$, in which λ(cm) is the incident wavelength in the dispersing medium, and the parameter m is the particle relative refractive index. At a fixed relative refractive index, W can be obtained by the relation below [Eq. (3)].

$$W = K \times 2\ln(10) \times \alpha \times d/3 \quad (3)$$

where d (cm) is the particle diameter, α(g cm^{-3}) is the particle density and K(cm^2 g^{-1}) is the sample extinction coefficient, which can be calculated using Eq. (4).

$$\tau = \ln(10) \times K \times c \quad (4)$$

where c(g cm^{-3}) is the sample concentration.

In this way, theosphere suspensions were diluted to a range of concentrations obeying the Lambert–Beer law. The diluted samples were analyzed at 395 nm, in which no absorption of the theospheres compounds occurs. The diameters d [Eq. (3)] were experimentally determined (Section *Physicochemical characterization of the suspensions*) (28).

Differential Scanning Calorimetry (DSC)

To evaluate the lipid crystallinity and the polymorphism, thermal behavior studies were performed using a DSC 822 (Mettler Toledo, Switzerland).

Before the analysis, theosphere suspensions were dried at room temperature. The samples weighing approximately 6 mg, based on the solid content, were placed in aluminum pans. An empty aluminum pan was used as reference. The heating runs were performed from –40 to 70°C and cooled to 25°C using the heating rate of 10°C/min by flushing with nitrogen at the rate of 30 mL/min. The melting point, enthalpies (ΔH), and onset temperatures were evaluated using the Star SW 9.10 software. The determination of crystallinity index was calculated using Eq. (5):

$$CI(\%) = \frac{\Delta H_{theospheres}}{\Delta H_{bulk} \times Conc_{lipid}} \quad (5)$$

X-Ray Analyses

X-ray analyses were carried out for evaluating the cristallinity of the theospheres. X-ray analyses were performed for Cupuaçu seed butter (raw material) and the formulations. The theosphere suspensions were dried at room temperature before the analysis. Diffraction solid material patterns were obtained with a D500 x-ray diffractometer (Siemens, Germany) using Cu Ka radiation at 35 kV.

Statistics

The data are presented as mean values ± standard deviation. Multiple comparisons were performed using LSD (Least Significance Difference) method or one-way ANOVA at the probability level of 0.05 using SigmaStat 3.1 Software.

RESULTS AND DISCUSSION

Theospheres composed of Cupuaçu seed butter (from 5 to 20%, w/w) were successfully prepared by both methods. We investigated the influence of both lipid concentration and preparation method (3, 6, or 9 cycles of high pressure homogenization-HPH or emulsification-solvent evaporation-ESE) on the size of theospheres. The high pressure homogenization technique rendered smaller nanoparticles than emulsification-solvent evaporation method for all lipid concentration studied (Fig. 1). Comparing the particle size of theospheres obtained by the different methods, it was found that the formulations prepared by HPH showed smaller particle sizes ($p < .05$) (144 ± 4, 155 ± 5, and 171 ± 17 nm for 10H, 15H, and 20H, respectively, which are medium values for different cycles) than those prepared by the ESE technique (218 ± 25, 217 ± 10, and 282 ± 53 nm for 10E, 15E, and 20E, respectively), except for the formulations composed of 5% of lipids (133 ± 6 for 5H and 167 ± 2 nm for 5E). In this case, there was no statistical difference

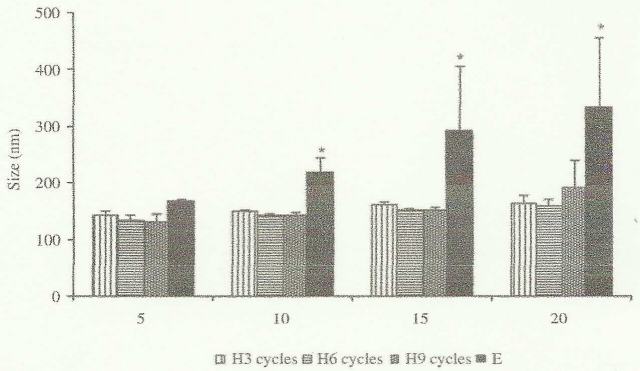

FIGURE 1 Mean particle size (nm) of theosphere formulations at day 0.

between the preparation methods. The increase in the lipid content did not increase the particle size ($p > .05$) using the HPH to prepare nanoparticles. Furthermore, the number of homogenization cycles had no influence on the size of the particles ($p > .05$). On the other hand, for ESE, significant increases on the particle size were verified with the increase of lipid content (from 167 ± 2 nm for 5E to 282 ± 53 nm for 20E).

The PCS results of the formulations stored at room temperature for 30 days (Fig. 2) showed no changes in the particle sizes for all formulations prepared by both the methods (ANOVA, $p > .05$). The values varied from 165 ± 4 to 168 ± 11, from 218 ± 25 to 233 ± 16, from 216 ± 12 to 250 ± 38, and from 282 ± 53 to 307 ± 83 for 5E, 10E, 15E, and 20E, respectively, when the ESE method was employed. Only the nanoparticles prepared by HPH technique using 6 homogenization cycles, which is the medium parameter, were evaluated in this study of 30 days. For these particles, the values varied from 126 ± 1 to 133 ± 9, from 141 ± 2 to 144 ± 2, from 149 ± 7 to 157 ± 9, and from 160 ± 11 to 236 ± 39. PDI values were close to 0.2 for all formulations indicating a relatively narrow size distribution just after preparation and within storage (Fig. 2).

The analysis of the zeta potential, which is the electric potential at the plane of shear, is a useful tool to predict the physical storage stability of colloidal systems. Zeta potential values higher than –30 mV show good physical stability of the colloids during the shelf-life, being optimal when they reach approximately –60 mV (29). In this study, the theospheres prepared by ESE presented high zeta potential values (Fig. 3A), which were around –60 mV, indicating that these formulations presented physical stability since the electrostatic repulsion between the particles avoids the particle aggregation. The

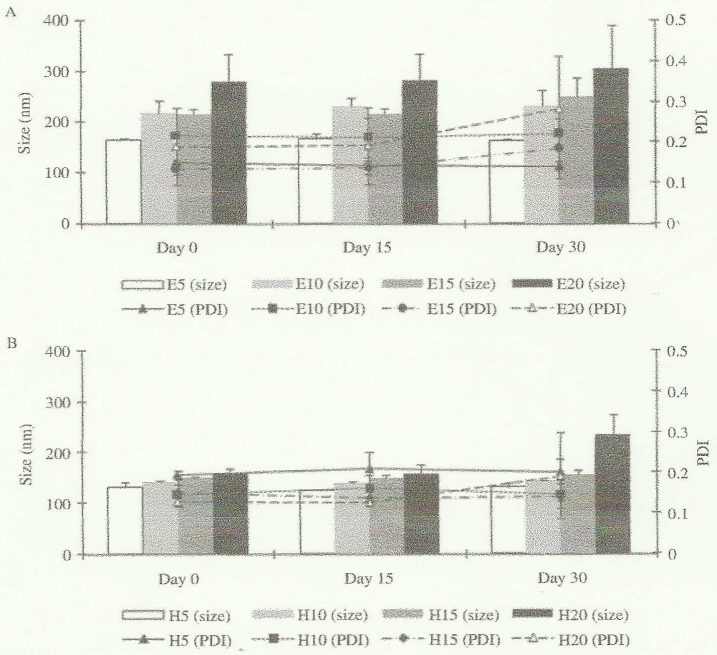

FIGURE 2 Mean particle size (nm) determined by PCS and PDI values of theosphere formulations obtained by ESE (A) and HPH (B) method within storage time.

lipid concentration did not influence the zeta potential values and no changes were observed within the storage time ($p > .05$) (Fig. 3).

Regarding the HPH method, the number of cycles did not influence the zeta potential ($p > .05$) (data not showed). However, the theospheres obtained by HPH method at lipid concentrations of 10, 15, and 20% (w/w) presented zeta potential values different compared to the one prepared by using 5% (w/w) of lipid ($p < .05$). For these formulations, two different values were observed by the eletrophoretic mobility analysis, one around –60 mV and another around –20 mV (Table 1), suggesting the simultaneous presence of two type of particles. The highest value is attributed to the presence of lecithin which is likely coating the particles, while the lowest value is due to the particles without the lecithin covering. Indeed, lipid nanoparticles in general present negative charge (22) and lecithin, like Lipoid S45, imparted a higher negative zeta potential to the nanoparticles

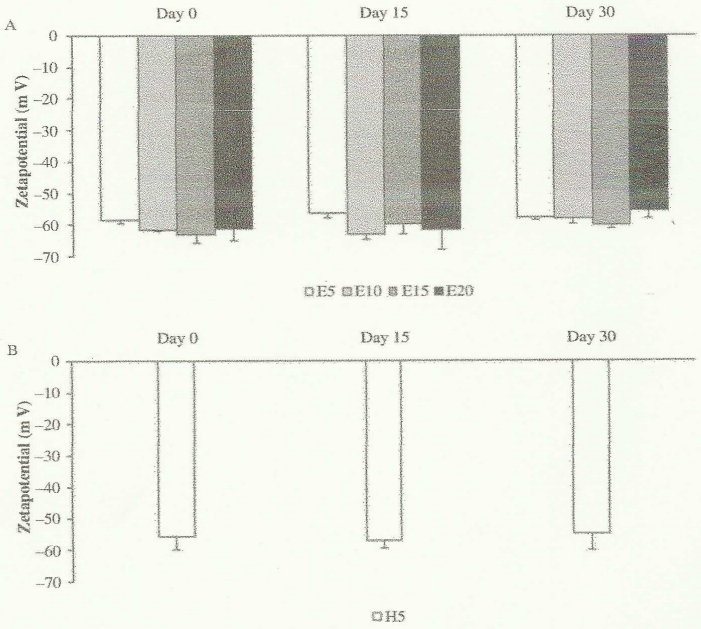

FIGURE 3 Zeta potential (mV) of theosphere formulations prepared by ESE (A) and HPH (B) method within storage time.

TABLE 1 Zeta Potential (mV) of Theosphere Formulations Prepared by HPH Method at Concentrations 10, 15, and 20% (w/w) of Lipid Within Storage Time

	Events	10H	15H	20H
Day 0	Peak 1	−18.4 ± 8.0	−15.1 ± 1.2	−15.4 ± 2.6
	Peak 2	−60.4 ± 2.9	−59.0 ± 9.5	−59.1 ± 7.1
Day 15	Peak 1	−20.2 ± 2.0	−20.9 ± 3.8	−13.7 ± 2.3
	Peak 2	−55.3 ± 1.7	−54.1 ± 1.6	−58.0 ± 3.7
Day 30	Peak 1	−27.3 ± 1.8	−25.1 ± 5.1	−12.2 ± 0.3
	Peak 2	−49.5 ± 3.5	−54.3 ± 4.2	−50.8 ± 1.1

(30). For the formulations 10H, 15H, and 20H, the particle concentration is higher than the values determined for the other formulations, as showed by turbidimetric analysis (Table 2), explaining the inefficient covering in these formulations.

TABLE 2 Number of Particles per Unit of Volume for Theosphere Formulations Determined by Turbidimetry at 395 nm

		Lipid concentration (%)			
N (particles. cm^{-3})		5	10	15	20
Preparation method	ESE	2.87×10^{13}	2.78×10^{13}	2.83×10^{13}	8.44×10^{12}
	HPH	6.91×10^{13}	1.15×10^{14}	1.04×10^{14}	1.03×10^{14}

TABLE 3 Viscosity (cP) of the Theosphere Formulations Prepared by ESE and HPH Method Within Storage Time *(n.d. = not determined)*

Theosome formulations	Number of cycles in the HPH method	Viscosity (cP)		
		Day 0	Day 15	Day 30
5E	—	2.98 ± 0.15	2.95 ± 0.09	2.79 ± 0.33
10E	—	2.95 ± 0.54	3.21 ± 0.20	3.30 ± 0.47
15E	—	5.05 ± 0.66	6.04 ± 0.42	n.d.
20E	—	5.41 ± 2.5	6.15 ± 1.8	n.d.
	3	2.37 ± 0.11		
5H	6	2.33 ± 0.03	2.25 ± 0.02	2.24 ± 0.01
	9	2.02 ± 0.38		
	3	2.30 ± 0.12		
10H	6	2.57 ± 0.38	2.91 ± 0.31	2.84 ± 0.51
	9	2.99 ± 0.23		
	3	3.49 ± 1.37		
15H	6	3.71 ± 1.07	3.88 ± 0.89	3.71 ± 0.83
	9	3.48 ± 0.05		
	3	5.29 ± 1.03		
20H	6	5.09 ± 0.97	5.22 ± 1.31	5.27 ± 1.6
	9	5.28 ± 1.25		

Concerning the rheological characterization, all theospheres presented Newtonian behavior (data not shown). The viscosity of the samples was evaluated (Table 3) and no difference ($p > .05$) was verified between the formulations prepared with 5 and 10% of lipids regardless of the preparation method or the number of the cycles in HPH technique. Besides, no changes were observed in the viscosity of those formulations during the evaluated period.

In the same way, no difference ($p > .05$) was observed between the theospheres prepared with 15 and 20% of lipids using both methods and different parameters in HPH. However, these lipid concentrations rendered formulations presenting higher viscosity ($p < .05$) than those prepared with 5 or 10% of lipid. Regarding the stability of the theospheres prepared using HPH method, no changes were observed in any concentration studied. Nevertheless, using the ESE technique, gelation phenomenon occurs after

30 days in 15E and 20E theospheres, preventing the measurements of viscosity by the same apparatus used for other formulations. This phenomenon is an unpredictably process and describes the transformation of a low-viscosity lipid nanoparticle suspension into a viscous gel (31). A similar process was reported by Schubert and co-workers (32) in lipid nanoparticles composed by 10% of lecithin based on 15% of the total lipid content.

Regarding acidity, the suspensions presented pH values varying from 4.83 to 5.80 and from 5.36 to 6.11 for theospheres prepared by the ESE technique and the HPH method, respectively. No difference ($p > .05$) was observed regarding the method of lipid nanoparticle preparation, the number of cycles in HPH method, the lipid concentration or the storage.

Concerning the multiple light scattering, all suspensions showed no transmission detected at 180° from the incident beam (data not shown). So, the backscattering variation was analyzed for each formulation (Fig. 4).

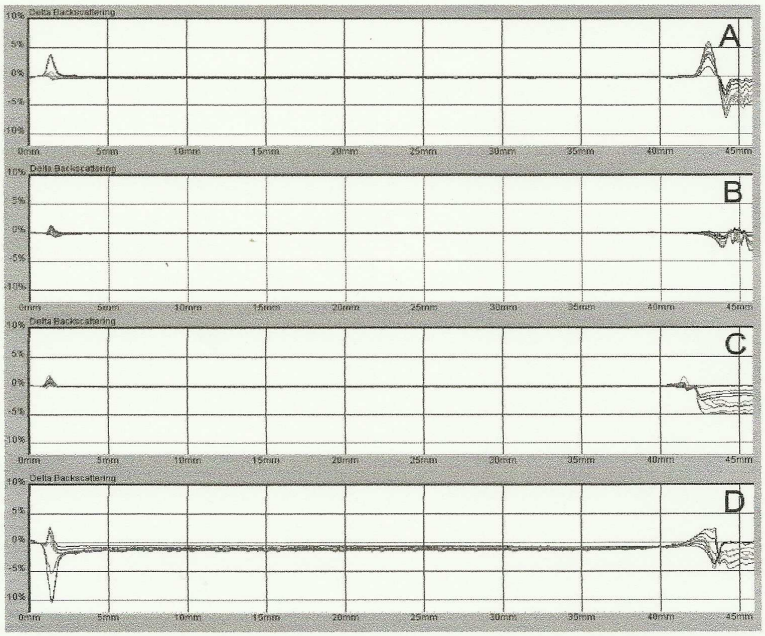

FIGURE 4 Backscattering curves of theospheres prepared by ESE method: 5E (A); 10E (B); 15E (C); and 20E (D).

Theospheres 20E showed the most intense physical instability considering all the formulations tested. This formulation presented a decrease in backscattering of about 10% at the bottom of the glass cell due to a decrease of the concentration of the particles (clarification). For the formulations 5E, 10E, and 15E, it was observed a slight increase in backscattering at the bottom due to sedimentation phenomena, which did not reach 5%. For the formulations prepared by HPH (Fig. 5) the backscattering variations were lower than 5% for 5H and 10H theospheres, considering all the cycles studied. The decrease in backscattering observed for the formulations 15H (3 cycles) and 20H (3 cycles), which reached –6% and –8%, respectively, were avoided by increasing the number of homogenization cycles during preparation.

In order to characterize the melting behavior of Cupuaçu seed butter, DSC analyses were carried out exemplarily for E10 and H10 theospheres (Fig. 6). DSC curves showed that for both formulations the melting peaks were detected at 35.7°C and 37.2°C for E10 and H10, respectively, close to the melting range of the bulk material (36.7°C). Moreover, the formulation obtained by HPH showed a reduction in the onset temperature as compared to bulk lipid (28.4°C and 33.3°C, respectively). This shift could be taken as an indication of the interference of theospheres formation in the

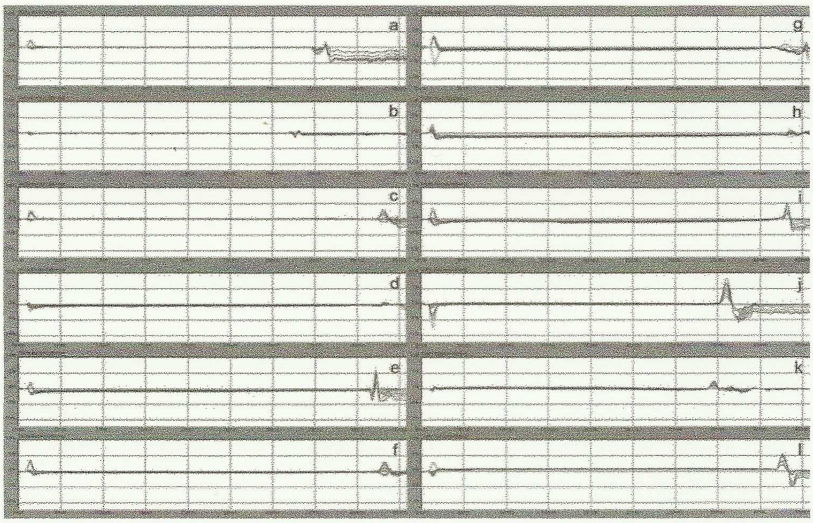

FIGURE 5 Backscattering curves of theospheres prepared by HPH method: 5H 3 cycles (A); 5H 6 cycles (B); 5H 9 cycles (C); 10H 3 cycles (D); 10H 6 cycles (E); 10H 9 cycles (F); 15H 3 cycles (G); 15H 6 cycles (H); 15H 9 cycles (I); 20H 3 cycles (J); 20H 6 cycles (K); and 20H 9 cycles (L).

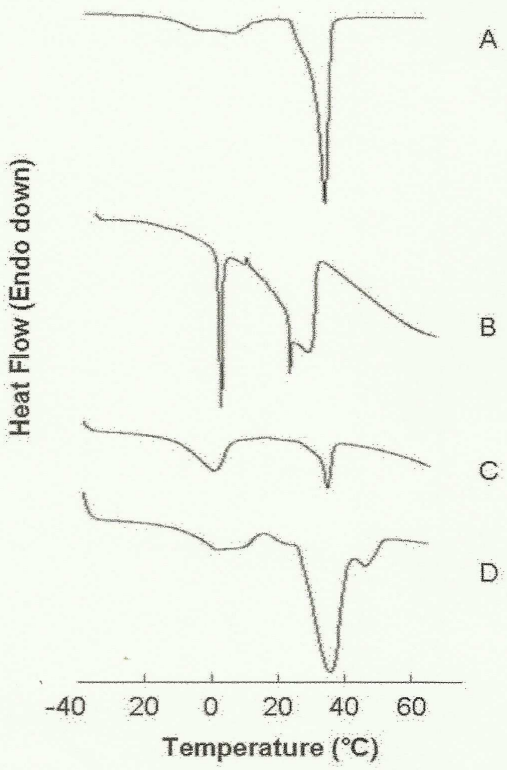

FIGURE 6 DSC heating curves of the Cupuaçu seed butter (A), lecithin (B), and air-dried theosphere suspensions E10 (C) and H10 (D).

crystallization of Cupuaçu seed butter, due to the small particle size, high specific surface area, and the presence of surfactants (33). However, this phenomenon was not observed for E10 theospheres. In fact, theospheres prepared by HPH present lower size than the ones prepared by ESE ($p < .05$, Fig. 2), and, consequently, higher specific surface area. Besides, a decrease in melting enthalpy (Table 4) was observed for both formulations (73.8 $J.g^{-1}$ for the bulk lipid to 25.7 or 37.7 $J.g^{-1}$ for E10 and H10, respectively). This is in agreement with its lipid proportion in theospheres, indicating a lower degree of crystallinity compared to the bulk lipid (33).

TABLE 4 Melting and Crystallization Behavior of the Cupuaçu Butter and the Theospheres Prepared by ESE and HPH Method.

	Onset temperature (°C)	Melting temperature (°C)	Melting enthalpy ($J.g^{-1}$)	Crystallinity index (%)
Bulk cupuaçu butter	33.3	36.7	73.8	—
E10 theospheres	33.3	35.7	25.7	54.5
H10 theospheres	28.4	37.2	37.6	79.6

Beside the melting properties, the crystallization features of colloidal dispersed lipid differ from those of their bulk materials (32). In this study, the preparation method influenced the crystallinity of the theospheres (Table 4). The lower crystallinity obtained in the formulation prepared using ESE method (54.5%) in comparison with the suspension prepared using the HPH method (79.6%) might be presumably attributed to the use of organic solvent and high shear forces.

The decrease in crystallinity was corroborated by the X-ray analysis (Fig. 7). Schubert and co-workers (32) observed a decrease in crystallinity with the increase in the lecithin content in the lipid matrices indicating a loss of

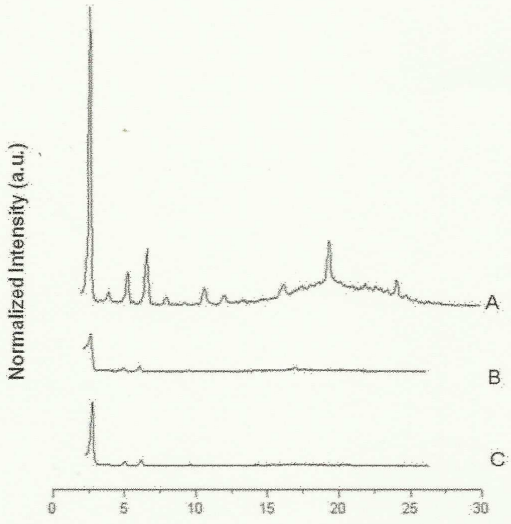

FIGURE 7 X-ray analysis of the Cupuaçu seed butter (A) and air dried-theosphere suspensions E10 (B) and H10 (C).

crystallinity probably caused by the reduced triglyceride content. In this study, we observed the same phenomenon—the peak intensities decreased or disappeared in the theosphere formulations when compared to the Cupuaçu seed butter, probably due to a reduction of Cupuaçu butter bulk in the formulations.

CONCLUSIONS

We described the first application of a natural biodegradable and biocompatible lipid—Cupuaçu seed butter (*Theobroma grandiflorum*)—for the preparation of lipid nanoparticles, which were called theospheres. Cupuaçu seed butter showed to be an alternative and advantageous compound for lipid nanoparticles preparation since the stable theospheres were successfully prepared by two different methods, both rendered formulations presenting particle size in the nanometrical range. The HPH method rendered the smallest nanoparticles and the homogenization with at least 6 cycles avoided physical instabilities as observed in the multiple light scattering analysis. The ESE method also showed narrow values in terms of size and size distribution, especially using 5 and 10% of lipid in the formulations. Besides, in these concentrations no changes were observed in viscosity, such as gelation phenomenon, or in physical stability of the theospheres. In general, concentrations up to 10% of lipid, irrespective of the preparation method, showed better results in terms of the studied parameters within the evaluated period.

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Publicação 2: Idebenone loaded-theospheres for dermal application: preparation, physico-chemical characterization, *in vitro* release and *in vitro* antioxidant activity evaluation

Publicação 2- Idebenone loaded-theospheres for dermal application: preparation, physico-chemical characterization, in vitro release and in vitro antioxidant activity evaluation

O artigo científico intitulado *"Idebenone loaded-theospheres for dermal application: preparation, physico-chemical characterization, in vitro release and in vitro antioxidant activity evaluation"* de autoria de Letícia Marques Colomé, Irene Clemes Külkamp, Eduardo André Bender, Denise Soledade Jornada, Guilherme Soares dos Santos, Taís Lusa Durli, Adriana Raffin Pohlmann e Silvia Stanisçuaski Guterres foi submetido para publicação no periódico *Macromolecular Bioscience*, sendo assim suprimido temporariamente da presente tese, conforme resuloção 002/2009 do Programa de Pós-Graduação em Ciências Farmacêuticas.

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O artigo científico intitulado "*Development and physicochemical characterization of idebenone-loaded theospheres in hydrogels intended for topical delivery*" de autoria de Letícia Marques Colomé, Renata Platcheck Raffin, Kelly Bueno da Silva, Guilherme Soares dos Santos, Adriana Raffin Pohlmann e Sílvia Stanisçuaski Guterres será submetido para publicação em periódico indexado, sendo assim suprimido temporariamente da presente tese, conforme resuloção 002/2009 do Programa de Pós-Graduação em Ciências Farmacêuticas.

Publicação 4: Development and human skin permeation study of novel drug delivery systems for idebenone: lipid-core nanocapsules and theospheres

O artigo científico intitulado "*Development and human skin permeation study of novel drug delivery systems for idebenone: lipid-core nanocapsules and theospheres*" de autoria de Letícia Marques Colomé, Eduardo André Bender, Guilherme Soares dos Santos, Taís Lusa Durli, Adriana Raffin Pohlmann e Sílvia Stanisçuaski Guterres será submetido para publicação em periódico indexado, sendo assim suprimido temporariamente da presente tese, conforme resuloção 002/2009 do Programa de Pós-Graduação em Ciências Farmacêuticas.

DISCUSSÃO

A preparação de nanopartículas lipídicas utilizando compostos alternativos aos já amplamente conhecidos e utilizados mostrou-se viável com o emprego da manteiga de cupuaçu (*Teobroma grandiflorum)*, que possibilitou a preparação de nanopartículas denominadas *teosferas*. A utilização da manteiga de cupuaçu permitiu a obtenção inédita de nanopartículas utilizando um lipídeo natural não-refinado. Além disso, concentrações ideais para a utilização desta manteiga foram determinadas em função das características físico-químicas das partículas formadas, da estabilidade dos colóides em suspensão e de acordo com o método de preparação utilizado.

Em se tratando do método produtivo, duas técnicas foram avaliadas: a homogeneização à alta pressão, já estabelecida e amplamente utilizada para a preparação de colóides lipídicos, e uma técnica alternativa, a emulsificação-evaporação do solvente, pouco explorada para a formulação de sistemas nanométricos baseados em lipídeos. É importante ressaltar ainda que para este segundo método, descreveu-se a preparação inédita de nanopartículas lipídicas utilizando somente um rotor-estator, sem etapa adicional de redução de tamanho que se faz normalmente necessária para a obtenção de faixas nanométricas.

A caracterização de nanopartículas lipídicas pode abranger diversos parâmetros, sendo que, alguns deles têm maior impacto na estabilidade e na cinética de liberação do fármaco a partir das partículas. Assim, considera-se fundamental avaliar o tamanho de partícula, a polidispersão, o potencial zeta e o grau de cristalinidade dos lipídeos (MÜLLER *et al*., 2000). Os métodos de preparação utilizados, juntamente com as concentrações de lipídeo empregadas, demonstraram exercer influência no tamanho das partículas, no número de partículas por unidade de volume, no potencial de recobrimento dos colóides pelo sistema tensoativo, na estabilidade física das suspensões e na cristalinidade da manteiga de cupuaçu constituinte das teosferas.

Apesar das diferenças observadas na caracterização e na estabilidade das teosferas preparadas por diferentes métodos, ambos mostraram-se adequados para a obtenção de sistemas nanométricos lipídicos utilizando manteiga de cupuaçu, quando esta foi utilizada em concentrações de até 10%. Assim, a técnica de emulsificação-evaporação

do solvente foi empregada para a avaliação da influência de um lipídeo líquido nas características físico-químicas das teosferas, bem como na investigação da potencialidade destes sistemas para veiculação de antioxidantes.

IDB foi selecionada pela sua potente ação antioxidante e pelo fato de atualmente fazer parte de estudos de utilização tópica em formas de administração convencionais. IDB foi incorporada às teosferas com alta eficiência de encapsulação, o que é atribuído às características lipofílicas da molécula, denotando sua afinidade pelos lipídeos utilizados. Um dos maiores desafios no desenvolvimento tecnológico de nanopartículas lipídicas é a reduzida capacidade de incorporação de ativos, que freqüentemente limita-se a 10 % em relação ao conteúdo lipídico, resultando em concentrações de aproximadamente 1 % na dispersão final (SCHÄFER-KORTING et al., 2007). Este fato pode ser atribuído à natureza específica da matriz lipídica sólida, pois devido ao limitado espaço nas camadas cristalinas pode ocorrer expulsão do ativo da matriz lipídica durante a cristalização, o que é mais comumente observado com lipídeos homogêneos que formam camadas praticamente perfeitas (MÜLLER *et al*., 2002).

Ao contrário, misturas de diferentes glicerídeos formam camadas de empacotamento menos densas, podendo favorecer a incorporação de substâncias. Isto também é válido para sistemas lipídicos constituídos por lipídeos líquidos, juntamente aos lipídeos sólidos, onde o ativo pode dissolver-se no nanocompartimento oleoso, localizado na matriz sólida ou aderido à superfície do lipídio solidificado. A alta capacidade de carga apresentada pelas teosferas em relação a outros colóides foi atribuída à natureza da manteiga de cupuaçu, formada por diferentes tipos lipídicos, o que denota características mais amorfas à matriz lipídica do carreador. Como discutido acima, características amorfas devem também ser alcançadas com a presença do óleo de castanha do Brasil (*Bertholletia excelsa*), lipídeo líquido escolhido para a formulação de um segundo tipo de teosferas. Essa capacidade de carga elevada permitiu que a dose de idebenona veiculada pelas suspensões estivesse dentro da faixa utilizada em formulações convencionais, sem alteração do diâmetro de partícula ou estabilidade física das suspensões. Assim, IDB foi incorporada nas teosferas obtendo-se uma concentração final de 2,5 mg/mL, dose que foi

subsequentemente aumentada para 5 mg/mL, sem prejuízo na eficiência de incorporação do ativo que apresentou valores superiores a 99% em todos as formulações preparadas.

Diferentes sistemas tensoativos foram utilizados com sucesso para a preparação das teosferas incluindo poloxamer, lecitina, álcol láurico etoxilado, polissorbato 80 e monoestearato de sorbitano, além de sistemas auxiliares para redução da tensão interfacial como o etanol. O emprego de diferentes tensoativos ou combinações destes resultou na obtenção de teosferas de tamanho variado, embora todas as formulações apresentassem diâmetro nanométrico e adequada distribuição de tamanho.

A presença do óleo de castanha nas teosferas demonstrou não influenciar características como tamanho, distribuição granulométrica, potencial zeta, viscosidade, número de partículas por unidade de volume e eficiência de encapsulação do ativo cosmético incorporado às teosferas. Contudo, a presença deste óleo influenciou a liberação *in vitro* do ativo encapsulado.

Ainda que o uso pretendido seja a aplicação tópica, a avaliação dos perfis de liberação permite determinar a capacidade de retenção do ativo pelas matrizes lipídicas e identificar fases de liberação sustentada (TURSILLI *et al.*, 2006). Assim, o estudo da liberação *in vitro* demonstrou que as teosferas foram capazes de controlar a liberação de idebenona, sendo que as teosferas constituídas por manteiga de cupuaçu sem a presença do óleo demonstraram perfil de liberação ainda mais lento. Além disso, características elásticas foram identificadas nas teosferas, especialmente naquelas constituídas por óleo de castanha, conforme foi elucidado pela passagem dos colóides através da membrana de diálise durante os ensaios de liberação *in vitro*. Adicionalmente, foi verificado que a presença da idebenona influenciou o grau de cristalinidade dos lipídeos nas teosferas, influência esta que foi dependente da composição lipídica do carreador.

Pesquisas na área cosmética têm dirigido atenção aos processos envolvidos nos danos anatômico-funcionais da pele que caracterizam o processo de envelhecimento. Desse modo, diversas possibilidades para contornar os efeitos arrolados neste processo têm sido discutidas, dentre as quais está o estudo de substâncias antioxidantes capazes de prevenir injúrias cutâneas mediadas pela ação de radicais livres, especialmente quando gerados pela radiação UV (SAIJA *et al*., 2002). Além disso, alguns estudos têm avaliado a possibilidade de melhorar a atividade de substâncias antioxidantes por sua incorporação

em sistemas nanoparticulados (PALUMBO *et al*., 2002; SCHAFFAZICK *et al.*, 2005). Neste estudo, as teosferas demonstraram potencial para aumentar a atividade antioxidante da idebenona, em concentrações a partir de 0.4 mM, através de um mecanismo ainda não elucidado, mas que considera as hipóteses de efeito do nanoencapsulamento e/ou sinergismo existente entre a idebenona e flavonóides antioxidantes constituintes da manteiga de cupuaçu.

Suspensões de nanopartículas lipídicas geralmente necessitam ser incorporadas em formulações semissólidas a fim de se obter consistência adequada para administração tópica cutânea. Vários estudos têm utilizado formulações com base em goma xantana, hidroxietilcelulose, quitosana, Carbopol® 934 (SOUTO *et al*., 2004), Carbopol® 940 (ALVES *et al*., 2005) Carbopol® Ultrez 10 (MANDAWGADE e PATRAVALE, 2008) e Carbopol® ETD 2020 (LIU *et al*., 2008) para incorporação de suspensões de nanopartículas. Neste sentido, este estudo concentrou-se ainda na obtenção de hidrogéis contendo teosferas, os quais demonstraram comportamento reológico pseudoplástico, demonstrando-se adequados para aplicação cutânea. As formulações semissólidas demonstraram ainda efeito oclusivo, o qual foi dependente da composição quali-quantitativa dos colóides, intimamente relacionado com seu grau de cristalinidade. Este efeito oclusivo, que ocorre devido à formação de um filme na superfície cutânea, é capaz de promover um aumento dos espaços inter-corneócitos, facilitando a permeação de ativos topicamente aplicados (JUNYAPRASERT *et al*., 2009).

Com o intuito de aprofundar as investigações neste sentido, as teosferas foram avaliadas quanto à sua capacidade de modificar a permeação cutânea da IDB, comparando-se essa característica com aquela apresentada por nanocápsulas de núcleo lipídico carreando o mesmo ativo. Ambas as partículas demonstraram habilidade para modificar a permeação do ativo promovendo seu acúmulo nas camadas mais superficiais da pele. Esse resultado, somado aos anteriormente descritos, vem confirmar a adequabilidade das teosferas como carreadores de ativos destinados à aplicação tópica cutânea.

CONCLUSÕES

Este trabalho descreveu a primeira aplicação de um lipídeo natural não-refinado - manteiga de cupuaçu – para a preparação de nanopartículas lipídicas, as quais foram denominadas teosferas. A manteiga de cupuaçu demonstrou ser uma alternativa promissora para a preparação de colóides lipídicos, especialmente por suas propriedades benéficas para aplicação tópica. Suspensões de nanopartículas lipídicas com adequada estabilidade puderam ser obtidas através de dois diferentes métodos, ambos resultando em formulações de tamanho nanométrico e distribuição granulométrica estreita. De maneira geral, concentrações de até 10% de manteiga de cupuaçu, independentemente do método de preparação utilizado, demonstraram os melhores resultados com relação aos parâmetros físico-químicos avaliados nas suspensões de teosferas.

Adicionalmente, a incorporação de óleo de castanha à manteiga de cupuaçu, demonstrou ser igualmente viável para a preparação de colóides lipídicos, os quais apresentaram tamanho, distribuição granulométrica e estabilidade similares aos primeiros sistemas preparados. A incorporação de IDB nas teosferas mostrou-se viável, não ocorrendo alterações na distribuição de tamanho ou na estabilidade do sistema quando da sua incorporação. As teosferas foram capazes de controlar a liberação da IDB, a qual foi influenciada pela presença de óleo como constituinte lipídico do carreador. As características elásticas das teosferas foram elucidadas durante os ensaios de liberação *in vitro*, demonstrando sua potencialidade para atuar como sistemas carreadores para aplicação tópica. Além disso, teosferas contendo IDB apresentaram melhores resultados em ensaio de peroxidação lipídica *in vitro*, em comparação com o ativo livre, demonstrando que as teosferas podem ser vantajosas para a administração tópica de moléculas antioxidantes.

Por fim, a incorporação das teosferas em formulações semissólidas demonstrou ser uma estratégia viável, resultando em formulações adequadas à aplicação cutânea, sem prejuízo para a estabilidade física dos colóides, apresentando ainda efeito oclusivo. Ensaios de permeação *in vitro* demonstraram a capacidade das teosferas em modificar a permeação da IDB encapsulada, acumulando-se nas camadas superficiais da pele.

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ANEXO: PUBLICAÇÃO COMPLEMENTAR

3 Polymeric Nanocapsules for Drug Delivery
An Overview

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3.1 INTRODUCTION

The pharmacological response to a drug is directly related to its concentration at the action site in the organism. However, the biodistribution of a substance is determined by its physicochemical properties, • • • implying a high drug concentration at the sites that are not the target one. This is strictly related to the adverse effects of drugs. To circumvent this problem, different strategies have been proposed in the last few decades to control the distribution of a drug within the organism. Examples of these strategies include the chemical modification of molecules to prodrugs and drug entrapment in delivery systems.

Submicron devices are more advantageous as drug carriers compared with conventional formulations, such as drug targeting and the possibility of intravenous administration without any risk of embolization. Oily-core polymeric nanocapsules are a particular class of submicron devices, which are composed of an oil core surrounded by a polymeric wall. Their mean sizes are generally around 200–300 nm with a monomodal and narrow size distribution. There are reports of nanocapsules being proposed for therapeutic applications dating from the 1970s. Over the last 40 years, several academic investigations furnished insights into their properties and supramolecular architecture. This nanoscience permits the tuning of nanocapsule characteristics in order to improve their efficacy as a drug delivery system. This chapter focuses on the raw materials, architecture, preparation methods, and physicochemical characterization of oily-core nanocapsules. The main biomedical applications of these systems are also described according to the therapeutic classes of nanoencapsulated drugs.

3.2 NANOCAPSULE ARCHITECTURE AND RAW MATERIALS

The theoretical model for a polymeric nanocapsule is a vesicle, in which an oily or an aqueous core is surrounded by a thin polymeric wall (Couvreur et al., 2002). These devices are stabilized by surfactants, such as phospholipids, polysorbates, and poloxamers, and by cationic surfactants (Schaffazick et al., 2003a). Nanocapsules composed of different raw materials have been described, including polyesters and polyacrylates as polymers and triglycerides, large-sized alcohols, and mineral oil as oily cores. Numerous bioactive molecules have been loaded into these systems, such as antitumorals, antibiotics, antifungals, antiparasitics, antiinflammatories, hormones, steroids, proteins, and peptides. As a general rule, the type of raw material used to compound nanocapsules can influence their morphological and functional characteristics, which may influence *in vitro* release, *in vivo* response, or both.

3.2.1 Oily Core

An advantage of oily-core nanocapsules over matrix systems is the higher drug loading capacity, especially when the lipophilic core is a good solvent for the drug. Other advantages are the reduction of burst release, the protection of drugs from degradation, and the reduction of drug side effects (Couvreur et al., 2002). A wide range of oils is suitable for the preparation of nanocapsules, including vegetable or mineral oils and pure compounds such as ethyl oleate (Mosqueira et al., 2000). In some cases, the oily core is the active component, such as the chemical sunscreen octyl methoxycinnamate (Alvarez-Román et al., 2001; Weiss-Angeli et al., 2008). Oil selection criteria include the absence of toxicity, risk of degradation and/or dissolution of the polymer, and high capacity to dissolve the drug (Couvreur et al., 2002; Guterres et al., 2000). The nanocapsule oily core should be compatible with the administration route (Table 3.1).

3.2.2 Polymeric Wall

3.2.2.1 Organic Polymers

Synthetic and natural biodegradable polymers are reported to make up the polymeric wall of nanocapsules (Table 3.1). The most common are hydrophobic polyesters, such as poly(lactide) (PLA), poly(lactide-*co*-glycolide) (PLGA), and poly(•-caprolactone) (PCL). These polymers have been widely used in drug delivery systems because of their biocompatibility and degradability in forming

TABLE 3.1
Examples of Raw Materials, Methods, and Administration Routes for Oily-Core Nanocapsules

Component	Examples	Preparation Method	Drug/Bioactive Molecule	Administration Route	Reference	
Oil	Triglycerides	Interfacial deposition of preformed polymers	Nimesulide	Topical	Alves et al., 2007	
	Miglyol 812N	*In situ* interfacial polymerization	Insulin	Oral	Pinto-Alphandary et al., 2003	
	Mineral oil	Interfacial deposition of preformed polymers	Indomethacin	—	Pohlmann et al., 2002	
	CCT	Interfacial deposition of preformed polymers	Melatonin	Intraperitoneal	Schaffazick et al., 2008	
	Octylsalicylate	Emulsification–solvent diffusion	Hinokitiol	Topical	Hwang and Kim, 2008	
	Miglyol 810 or ethyl oleate	Interfacial deposition of preformed polymers	—	—	Mosqueira et al., 2000	
Monomer[a]/polymer[b]	Isobutylcyanoacrylate (monomer)	*In situ* interfacial polymerization	Insuline	Oral	Courtarie et al., 2002	
	PLA	Interfacial deposition of preformed polymers	Diclofenac	Intravenous and oral	Guterres et al., 1995a,b	• •••
	PCL	Interfacial deposition of preformed polymers	Chlorhexidine	Topical	Lboutounne et al., 2002	
	PLGA	Interfacial deposition of preformed polymers	Insulin	Oral	Saï et al., 1996	
	Eudragit S100	Interfacial deposition of preformed polymers	Melatonin	—	Schaffazick et al., 2005	
	Cellulose acetate phthalate	Emulsification–solvent diffusion	Octyl methoxycinnamate (chemical sunscreen)	Topical	Olvera-Martinez et al., 2005	• •••
Surfactants	Epikuron 170[a] and Poloxamer 188	Interfacial deposition of preformed polymers	Halofantrine	Intravenous	Leite et al., 2007	
	Tween 80[a]	*In situ* interfacial polymerization	Idebenone	—	Palumbo et al., 2002	
	CTAC	Emulsification–solvent diffusion	Hinokitiol	Topical	Hwang et al., 2008	• •••
	Poly(vinyl alcohol)	Emulsification–solvent diffusion	—	—	Quintanar-Guerrero et al., 1998	
Organic solvent	Ethanol, acetone, or acetonitrile	*In situ* interfacial polymerization	Ethosuximide, 5,5-diphenyl hydantoin and carbamazepine	—	Fresta et al., 1996	
	Acetone	Interfacial deposition of preformed polymers	Ketoprofen	Intraperitoneal	Matoga et al., 2002	
	Ethyl acetate	Emulsification–solvent diffusion	—	—	Quintanar-Guerrero et al., 1996	

Note: CTAC—cetyltrimethylammonium chloride.

[a] For chemical methods.

[b] For physicochemical methods.

nontoxic residues. They have been approved by the U.S. Food and Drug Administration in several medicines, including those administered systemically. In addition, the release kinetics of entrapped drugs can be controlled by varying the molecular weight (MW) of the polymers (Cha and Pitt, 1988; Sinha et al., 2004; Wise et al., 1987). The *in vitro* and *in vivo* degradation kinetics and biological behavior of the polymers have been extensively characterized (Middleton and Tipton, 2000; Sinha et al., 2004). They present slow degradation, which can be catalyzed by lipases, causing minimal immune response. The MW influences the degradation kinetics. The hydrolytic degradation of low MW PLA polymers starts in a few days, whereas it takes much longer for high MW PLA polymers (Andreopoulos et al., 1999). Considering this, PLA, PLGA, and PCL are the first choices for producing nanocapsules for parenteral routes. Other polymers, such as polyacrylates, polyacrylamides, and polyureas, have also been described as alternatives for producing nanocapsules because of their adequate biocompatibility (Montasser et al., 2007; Vauthier et al., 2007).

3.2.2.2 Functionalized Surfaces

In recent years, engineering approaches have been devised to create "smart" nanocapsules. In this context, functionalized-surface nanocapsules present interesting properties such as stealth ability and active targeting via ligand binding to specific cell receptors. This is especially important for some molecules that are considered as "undeliverable" compounds, such as nucleic acids, which need to reach an intracellular target to achieve their therapeutic effect (Behr et al., 1989; Felgner et al., 1987), or highly toxic drugs (Couvreur et al., 2002).

Functionalization strategies are divided into two groups: the ligand is incorporated into the nanocapsule during or after its preparation. In the former case, the polymer is chemically bound to the ligand and this complex material is used as the raw material to produce the nanocapsules. In the latter case, nonfunctionalized nanocapsules are produced earlier and the ligand is attached to the nanocapsule polymeric wall by physicochemical or chemical processes. An example of the first strategy is nanocapsules that are covalently attached to poly(ethylene glycol) (PEGylated), which are prepared using a diblock copolymer as the polymeric wall. The second strategy includes post-insertion of whole antibodies and antibody fragments into the surface of nanocapsules (Figure 3.1).

Bioadhesive properties can be obtained by coating the nanocapsule surface with polymers presenting positive charge, such as the polysaccharide chitosan and its derivatives. The adsorption process of chitosan onto nanocapsules occurs via ionic interactions between chitosan and

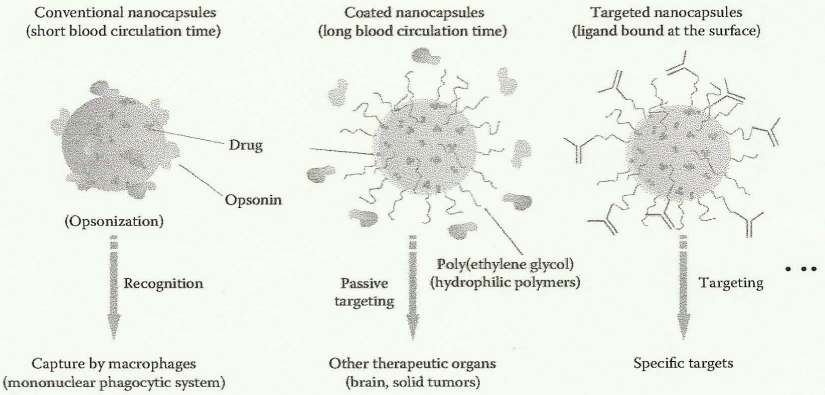

FIGURE 3.1 Conventional versus surface-functionalized nanocapsules.

specimens containing negative charges on the particle surface (such as lecithin), which is reflected by an inversion in the zeta potential signal (Calvo et al., 1997; Prego et al., 2006a). Bioadhesion can increase the residence time of the drug at the site of absorption, which allows improvement in drug penetration (Kriwet et al., 1998). Polyacrylates also have bioadhesive properties, which occur through hydrogen bonding between the carboxylic acid groups of the polymer and the hydroxyl groups of the mucous membrane (Dodou et al., 2005).

Active targeting of specific organs and tissues is based on molecular recognition processes. Examples of relevant targets are the folate receptor or the integrin surface receptor for tumor cells (Couvreur et al., 2002) and mannose and fucose recognizing receptors for macrophages, which are the host cells of the *Leishmania* sp. parasite (Date et al., 2007). A particularly interesting example of surface-functionalization devices is the stealth nanocapsule. The rapid removal of conventional nonfunctionalized-surface nanocapsules from the bloodstream by the mononuclear phagocytic system decreases their effectiveness as site-specific drug delivery devices (Gref et al., 1994; Owens and Peppas, 2006). Macrophages remove the nanocapsules because they can recognize specific opsonin proteins that bind at their surfaces (Frank and Fries, 1991). In this way, the introduction of long hydrophilic polymer chains and nonionic surfactants at the nanocapsule surface can provide slow opsonization because of a steric effect that delays electrostatic and hydrophobic interactions to bind opsonins onto nanoparticle surfaces (Owens and Peppas, 2006). As a consequence, the nanoparticle blood circulation half-life increases (Gref et al., 1994; Kaul and Amiji, 2004). Steric shielding can be obtained using polymers such as polysaccharides, polyacrylamides, poly(vinyl alcohol), poly(*N*-vinyl-2-pyrrolidone), PEG, PEG block copolymers, and PEG-containing surfactants such as poloxamines, poloxamers, and polysorbates. PEGylation of the particles is the most effective and commonly used strategy to obtain stealth nanoparticles (Owen et al., 2006; Veronese and Pasut, 2005). PEG can be introduced at the surface in two ways: by the adsorption of surfactants (Illum et al., 1987; Moghimi et al., 1993) or by the use of block or branched copolymers, usually with PLA or poly(alkyl cyanoacrylate) (PAC; Peracchia et al., 1999).

Functionalized surfaces provide obvious advantages for nanocapsule performance in comparison with those that are nonfunctionalized. However, formulating nanocapsules with new materials that have not been well characterized may make their preclinical and eventual clinical evaluation difficult. The toxicity profiles of these materials must be established before the nanocapsule clinical trials (Jabr-Milane et al., 2008). Furthermore, it is noteworthy that not only the surface characteristics of nanocapsules but also their size and morphology play a key role in their biological fate (Couvreur and Vauthier, 2006; Gaumet et al., 2008).

3.2.3 Supramolecular Structure Models

Nanocapsule supramolecular organization may differ for each particular combination of raw materials because of the complexity of structures (Mosqueira et al., 2000; Müller et al., 2001). Different organizations can be obtained by selecting specific raw materials. Therefore, supramolecular structure models for nanocapsules can only be proposed in each case after extensive physicochemical investigation.

The theoretical model for oily-core nanocapsules is a vesicle, in which the oil is surrounded by a polymeric wall (Couvreur et al., 2002). However, a careful combination of polymers and oil is required to obtain truly vesicular nanocapsules. For instance, swelling experiments of polyesters (PLA or PCL) immersed in benzyl benzoate demonstrated the dissolution of polymers, indicating that nanocarriers prepared using these materials are nanoemulsions instead of nanocapsules (Guterres et al., 2000). Conversely, PCL was not dissolved by octyl methoxycinnamate, suggesting the presence of a polymeric wall in the nanocapsules composed of this polymer–oil combination (Weiss-Angeli et al., 2008).

Materials structured at the submicron level may present properties different from those at the macroscopic level. In this way, drug release experiments, light scattering analyses, and nuclear magnetic resonance spectroscopy are valuable tools for determining the supramolecular structures of nanocapsules.

Dynamic light scattering (DLS) studies have demonstrated that nanocapsules prepared with PCL, mineral oil, sorbitan monostearate, and polysorbate 80 present low interactions with water because of the presence of sorbitan monostearate dispersed in the polymer (Pohlmann et al., 2002). In contrast, sorbitan monostearate was dispersed in the oil phase of nanocapsules prepared with the same composition but using caprylic/capric triglyceride (CCT) as the oil core instead of mineral oil (Cruz et al., 2006a; Müller et al., 2001). Hence, the organization of the wall and core domains depends on the nature of the polymer, oil, and surfactants. Insights concerning the supramolecular structure of nanocapsules using nuclear magnetic resonance spectroscopy have also been reported (Hoffmann and Mayer, 2000; Mayer et al., 2002). The nanocapsule polymer wall structure composed of poly(*n*-butyl cyanoacrylate) did not significantly differ from the structure of the solid compact polymer (bulk) used as the reference. In contrast, the triglyceride (core) and Pluronic® F68 (surfactant) presented high mobility and were temporarily adsorbed on the nanocapsule wall. Another study investigated nanocapsule particle–water interface interactions using fluorescent dyes chemically bound to poly(methyl methacrylate), which constituted the polymeric wall (Jäger et al., 2007). The chemical environment-sensitive fluorescent dyes showed that the particle–water interface was composed of oil, surfactant, polymer, and water.

The nature of the surfactant used to stabilize the nanocapsules can also influence their morphology. PLGA capsules containing the ultrasound contrast agent perfluorooctyl bromide (PFOB) as the oily core were prepared with different surfactants (Pisani et al., 2008). Sodium taurocholate generated capsules with an acorn morphology in which a hemisphere of liquid PFOB coexisted with a hemisphere of solid polymer, whereas sodium cholate provided liquid PFOB perfectly encapsulated within a PLGA wall. The use of poly(vinyl alcohol) resulted in the coexistence of both morphologies in the same suspension.

In vitro drug release profiles can be useful in determining drug localization in the nanocapsule structure. A comparative study conducted with nanocapsules, nanospheres, and nanoemulsions showed similar indomethacin release behavior; an ethyl ester derivative showed different release profiles (Cruz et al., 2006b). Kinetic data indicated that indomethacin was adsorbed on the nanocarriers, whereas the indomethacin ethyl ester was entrapped within the nanocarriers.

3.3 PREPARATION METHODS

3.3.1 Physicochemical Methods

3.3.1.1 Solvent Displacement-Based Methods

The interfacial deposition of preformed polymers was the first method described (Fessi et al., 1989) for the preparation of nanocapsules, and it is a combination of the spontaneous emulsification of oily droplets and the simultaneous precipitation of polymer onto the water–oil interface. Spontaneous emulsification occurs because of the initial nonequilibrium states of two miscible liquids when they are brought into close contact with each other. Depending on the specific conditions used to carry out spontaneous emulsification, nanodroplets can also be formed (Bouchemal et al., 2004). This process increases the entropy of the system and, as a consequence, decreases its Gibbs free energy. The motive force is the interface tension gradient induced by the diffusion of solutes between the two liquid phases (Marangoni effect). Katz and coworkers (Ganachaud and Katz, 2005; Vitale and Katz, 2003) provided new insights into the physical phenomenon behind spontaneous emulsification. They named this phenomenon the ouzo effect after a beverage with the same name, which is common in Greece: this phenomenon is where an ethanol extract of anise seeds becomes instantaneously cloudy when diluted with water. In the ouzo effect, the addition of water to a solution of oil in a totally water-soluble solvent causes supersaturation of the oil in the mixture, which leads to its nucleation in small droplets. The mean droplet diameter is exclusively a function of the oil/solvent ratio at a given temperature.

Considering the above, a fundamental requirement for the interfacial deposition of preformed polymers is the high miscibility between the organic and aqueous phases. The most commonly used

solvents for preparing nanocapsules by this method are acetone and ethanol. In addition, the organic solvent must be a good solvent for the polymer, but water must not (Fessi et al., 1989). The interfacial tension between oil and the aqueous phase seems to have a greater effect on nanocapsule size than oil viscosity (Mosqueira et al., 2000). A decrease in interfacial tension values causes a reduction in nanocapsule sizes. The advantages of the interfacial deposition of preformed polymers are its simplicity and robustness at small scale and high reproducibility among different batches (Fessi et al., 1989; Teixeira et al., 2005).

3.3.1.2 High-Shear Techniques

High-shear techniques involve the emulsification of an organic phase with an aqueous phase by rotor/stator devices or ultrasound generators. The most common high-shear technique of preparing nanocapsules is emulsification–solvent diffusion, which involves the formation of a conventional oil-in-water emulsion within a partially water-soluble solvent (Quintanar-Guerrero et al., 1996). Water and solvent are previously saturated with each other. The subsequent addition of a second aqueous phase to the system makes the solvent diffuse into the external phase, resulting in the formation of nanocapsules.

Several preparative variables can affect nanocapsule size, such as the type and concentration of the stabilizers, stirring rate of the primary emulsion, internal/external phase ratio of the primary emulsion, polymer concentration in the organic phase, pH, and viscosity of the external phase (Quintanar-Guerrero et al., 1996). Examples of solvents that are used to prepare nanocapsules are ethyl acetate, propylene carbonate, and benzyl alcohol (Quintanar-Guerrero et al., 1998). The sizes of nanocapsules prepared by emulsification–solvent diffusion are a function of thermodynamic parameters such as the mutual diffusion coefficients of water and solvent and the solvent–polymer interaction parameters (Choi et al., 2002). In addition, the sizes of nanocapsules prepared by this technique are related to the chemical composition of the organic phase and the size of the primary emulsion droplets by a simple geometrical relationship (Moinard-Checot et al., 2008). Therefore, most of the properties of the nanocapsules are decided at the emulsification step. A modification of this technique involving the use of ethanol as cosolvent in the organic phase was proposed to produce controlled-size poly(hydroxybutyrate-*co*-hydroxyvalerate) nanocapsules containing CCT or mineral oil as the oily core (Poletto et al., 2008a). In this study, the control of nanocapsule diameters was achieved by adjusting the surface tension of the organic phase.

The advantages of emulsification–solvent diffusion involve high yields of encapsulation, high reproducibility, control of particle size, and easy scale-up (Moinard-Checot et al., 2008). However, a large amount of water has to be removed to concentrate the suspensions.

3.3.2 Chemical Methods

3.3.2.1 *In Situ* Interfacial Polymerization

In situ polymerization to obtain nanocapsules consists of the polymerization of a monomer at the interface between the organic and the aqueous phase of an emulsion. The monomers can be added into the aqueous phase or organic phase. In the former case, the monomers must have good solubility in the external phase, but the polymer must be immiscible in both phases (Zhang et al., 2008). The drug is dissolved in the polymerization medium either before the addition of the monomer or at the end of the reaction. Hence, drugs are encapsulated within or adsorbed onto the particles (Couvreur et al., 1990). The polymerization reaction occurs because of the presence of initiators and specific process conditions, such as pH (Soppimath et al., 2001). The morphology of the polymeric wall is a function of the monomer's initial concentration and reaction time (Anton et al., 2008; Turos et al., 2007). With the addition of monomers in the organic phase, the solvent serves as a vehicle for the monomer. This organic phase is emulsified with an aqueous phase containing water and a hydrophilic surfactant. Nanodroplets are formed to give a milky suspension, and the organic solvent is then

removed under reduced pressure. The polymer is synthesized by changing some process parameters (temperature or pH), and it segregates toward the water–oil interface, thereby generating the nanocapsules. The polymerization must be very fast in order to allow efficient formation of the polymer envelope around the oil droplet and thus achieve an effective encapsulation of drugs (Couvreur et al., 2002; Gallardo et al., 1993). Alkyl cyanoacrylates are commonly employed as monomers because of their very fast polymerization rates when they come into contact with water (Gallardo et al., 1993).

Covalent bonds may be created between the active molecule and the polymer during polymerization. Thus, their potential mutual reactivity must be taken into account to obtain the designed nanocapsules (Anton et al., 2008). In addition, nanocapsule formation and structure may be highly affected by the nature of the reaction initiator in chemical methods (Han et al., 2003).

3.4 PHYSICOCHEMICAL CHARACTERIZATION

3.4.1 Morphology

Different microscopy techniques can be used to observe the nanocapsule morphology and structure. Scanning electron microscopy (SEM) allows observation of the sample after drying and coating with a thin layer of gold or platinum. This method gives information about the size, shape, and surface aspects of nanocapsules. However, particles smaller than 100 nm may be difficult to observe (Gaumet et al., 2008). Freeze–fracture SEM has also been helpful in visualizing different organizations of lipophilic surfactant in nanocapsule suspensions, which can simultaneously form vesicles, micelles, bilayers, or monolayers, depending on the concentration (Mosqueira et al., 2000). This technique can also be applied to identify the presence and morphology of nanocapsules on animal tissues (Lboutounne et al., 2002) or in a gel matrix after storage (Milão et al., 2003).

Transmission electron microscopy (TEM) is a technique in which a beam of electrons is transmitted through the sample. The sample is dried and stained with contrast agents, such as uranyl acetate or phosphotungstic acid, and then analyzed. TEM provides information about the size, shape, and integrity of nanocapsules and permits observation of their vesicular structure (Mosqueira et al., 2000). In addition, TEM performed after freeze–fracture has given helpful information about the polymeric wall and the core, allowing the wall thickness to be estimated (Fresta et al., 1996). There have been other studies to determine the thickness of the nanocapsule polymeric wall. The wall thickness of nanocapsules prepared with PCL and Miglyol® 812 was estimated to be about 2.0 nm (Guinebretière et al., 2002). Moreover, the polymeric wall can also be characterized using neutron scattering techniques. A wall thickness of about 10 nm for nanocapsules composed of PLA and triglycerides was determined by small-angle neutron scattering (Rübe et al., 2005).

Atomic force microscopy is a useful tool for determining the simultaneous characterization of particle shape, surface structure, and interparticle organization by tridimensional images. In some cases, atomic force microscopy may differentiate nanocapsules according to their composition (e.g., presence vs. absence of PEG as coating; de Assis et al., 2008). The drawback of this technique is the complexity of the sample preparation process before the microscopy analysis.

3.4.2 Mean Average Size and Particle Size Distribution

Many tools based on different physical principles are currently available to measure the size of submicron particles. DLS, also called photon correlation spectroscopy, is based on the interaction of the particle with an incident light beam. The intensity of scattered light detected at a fixed angle provides the mean size, size distribution, and polydispersity index (PI) of the sample. Because the calculation model is based on the equivalent sphere principle, the presence of aggregates greatly increases the mean size. In addition, parameters such as the viscosity or pH of the suspension medium, temperature, concentration, and particle sedimentation may influence the data. Microscopic methods (SEM and TEM) are also described to evaluate the nanocapsule mean size. Particle sizing

with microscopic techniques requires image treatment of a large number of particles, thus making them subjective and time consuming. Furthermore, nanocapsules may shrink during the drying step, leading to an underestimation of their actual diameter (Gaumet et al., 2008).

Size distribution is a parameter as important as mean particle size in nanotechnology. Size distribution can be monomodal (one population) or multimodal (several populations) and monodisperse (narrow distribution) or polydisperse (broad distribution). Generally, the size distribution of nanocapsules is evaluated on the basis of the PI, which takes into account the mean particle size, the refractive index of the solvent, the measurement angle, and the variance of the distribution (Koppel, 1972). However, a linear correlation between the PI value and the true polydispersity of a sample cannot be drawn (Gaumet et al., 2008). None of the methods available to determine nanoparticle size and distribution are fully satisfactory and a combination of at least two methods, one of which should be a microscopic method, is highly recommended.

3.4.3 Zeta Potential

The zeta potential is the electric potential at the interfacial double layer between the dispersion medium and the stationary layer of fluid attached to the dispersed particle. This parameter can be determined by electrophoresis, in which an electric field is applied across the dispersion. Particles migrate toward the electrode of opposite charge with a velocity proportional to the magnitude of the zeta potential (Delgado et al., 2005). The most common experimental electrophoretic technique used to determine the zeta potential of nanocapsules is electrophoretic light scattering.

The zeta potential is influenced by the charge of the different components of nanocapsules, especially surfactants located at the interface with the dispersion medium, as well as the composition of this dispersion medium (Couvreur et al., 2002). Lecithin, poloxamers, and polymers are the major components that affect this parameter. For instance, the polysaccharide chitosan gives a positive zeta potential to nanocapsules (Prego et al., 2006a). By contrast, many polymers, especially • -hydroxy acids (such as PLA) and lecithin, contribute a negative charge to the surface, which is reflected in the zeta potential value. Nonionic surfactants, such as poloxamer and poly(vinyl alcohol), also tend to reduce the absolute zeta potential value (Mosqueira et al., 2000). A zeta potential of 30 mV (positive or negative) is indicative of the adequate stability behavior of nanocapsules that is attributable to the charge repulsion between particles, which may be sufficient to prevent their aggregation (Couvreur et al., 2002). However, surface-coated nanocapsules can be stable despite a zeta potential close to zero because of the steric effect of surfactants or PEG copolymers.

Zeta potential measurements may also be used to investigate whether a biologically active compound is encapsulated within the nanocapsule oily core or adsorbed onto the particle surface (Aboubakar et al., 1999) and to confirm nanocapsule coating by a specific material (Preetz et al., 2008). Zeta potential values may be generally associated with the pH values of the bulk (Sussman et al., 2007).

3.4.4 Drug Content

The total content of a drug in the nanocapsule suspension is determined using high-performance liquid chromatography or another analytical technique after dissolving or extracting the drug from the system using an adequate solvent. Free drug (nonencapsulated) is usually determined in the ultrafiltrate after separation of the nanocapsules by the ultrafiltration–centrifugation technique. The amount of encapsulated drug (associated within or adsorbed onto the nanocapsules) is calculated from the difference between the total and the free drug concentrations determined in the nanocapsule suspension (after dissolution) and in the ultrafiltrate, respectively. The amount of drug encapsulated in the nanocapsule expressed as the percentage of the total amount of drug in the suspension is commonly called the "encapsulation efficiency." Aggregates of the pure drug (stabilized by surfactants) are retained with the nanocapsules in the upper compartment.

Although the encapsulation efficiency of lipophilic drugs is generally related to drug solubility in the oily core (Fresta et al., 1996), hydrophilic compounds such as peptides have also been successfully encapsulated in oily-core nanocapsules prepared by chemical methods. One explanation for this may be the extremely rapid polymerization of the polymer occurring at the surface of the oil droplet, which limits the diffusion of the peptide toward the aqueous phase and therefore leads to higher entrapment in the nanocapsules (Aboubakar et al., 1999).

3.4.5 *In Vitro* Drug Release

The composition of nanocapsules influences the mechanism of drug encapsulation (adsorption on the polymer or entrapped within the core; Couvreur et al., 2002). The localization of drug in the nanocapsule structure affects its release kinetics (Cruz et al., 2006a). Therefore, *in vitro* drug release experiments can give valuable information about the interactions between the drug and the nanocapsule. This concept was illustrated by a comparative study carried out with nanoencapsulated 3-methoxyxanthone and xanthone (Teixeira et al., 2005). Similar kinetics were observed for 3-methoxyxanthone released from nanocapsules and nanoemulsions (without polymer), indicating that drug release was mainly governed by the partition between the oily core and the external aqueous medium. In contrast, the release of xanthone from nanocapsules was significantly slower than that from nanoemulsions, suggesting an interaction of the drug with the polymer.

The release of drugs from nanocapsules depends on drug desorption, drug diffusion, or polymer erosion (Lopes et al., 2000; Soppimath et al., 2001). Similar *in vitro* rapid release kinetics were observed for benzathine penicillin G from PLGA nanocapsules and from nanoemulsions without polymer (Santos-Magalhães et al., 2000). Although experimental data indicate that the polymeric wall is an important factor for drug release kinetics (Pisani et al., 2006; Poletto et al., 2008b), hydrophilic drugs and peptides are generally adsorbed on the polymer surface rather than encapsulated within the oily core. In this case, the drug does not need to cross the polymeric wall to be released, and the release profile from nanocapsules and nanoemulsions may be quite similar. Conversely, the controlled release of 4-nitroanisole from different types of PLA nanoparticles (oily-core nanocapsules and matrix nanospheres) was explained by a Fickian diffusion mechanism (Romero-Cano and Vincent, 2002), in which the exact particle morphology (presence of an oily core, concentration of polymer, and localization of drug in the particle) influenced the release rate kinetics.

To determine the drug release profiles from nanocapsules, the drug may be separated from the nanostructures using ultracentrifugation, ultrafiltration–centrifugation, or dialysis techniques. However, these methods are limited to the determination of the drug partition coefficient between nanoparticles and the continuous phase, and an experimental sink condition was not achieved (Washington, 1990).

3.4.6 Physicochemical Stability of Nanocapsule Suspensions

The stability of nanocapsules can be evaluated in terms of macroscopic aspects, free and total drug contents, zeta potential, pH, and mean particle size as a function of time (Guterres et al., 1995a; Schaffazick et al., 2007). Previous reports demonstrated that the reduction in the pH values of nanocapsule suspensions is related to a partial hydrolysis of the polymer during storage (Calvo et al., 1996a; Guterres et al., 1995a). Nanocapsules may aggregate because of attractive forces among particles. Steric stabilization and electrostatic stabilization are needed to overcome these attractive interactions. Steric stabilization is based on the osmotic stress created by encroaching steric layers of bulk polymers and surfactants at the surface of nanocapsules, whereas electrostatic stabilization provides charge repulsion caused by charged species onto the particle surface. Generally, steric stabilization alone is sufficient to prevent irreversible aggregation. However, attractive forces among particles may still remain to cause reversible flocculation in some cases. Steric stabilization combined with electrostatic stabilization may circumvent flocculation due to the additional repulsive

contribution (Kesisoglou et al., 2007; Vauthier et al., 2008). Aggregation of nanocapsules during storage can be monitored by DLS. Nevertheless, this technique requires a dilution of suspensions before the measurement, and reversible flocculation may not be detected. Multiple light scattering analyses have been carried out recently to evaluate the physicochemical instability phenomena of nanodevices, such as precipitation, creaming, and aggregation (Daoud-Mahammed et al., 2007; Lemarchand et al., 2003). This technique permits analysis of the sample without further dilution. Variations in zeta potential values during storage can also be indicative of aggregation.

The reduction in the drug content of nanocapsules can occur because of its chemical degradation after storage, which is caused by external agents such as oxygen, temperature, and ultraviolet irradiation. The degradation products can be identified and quantified by chromatography or other analytical techniques (Müller et al., 2004). Drug nanocrystals stabilized by surfactants can also be concomitantly formed with nanocapsules (Calvo et al., 1996a; Guterres et al., 1995a). Drug oversaturation of nanocapsules causes the formation of nanocrystals, which agglomerate and precipitate during storage (Pohlmann et al., 2008). The consequence is the reduction of drug content within the nanocapsules. Although ultrafiltration–centrifugation cannot separate nanocapsules and drug nanocrystals, their simultaneous presence in suspension can be detected by static light scattering analysis.

3.5 APPLICATIONS IN THERAPEUTICS

3.5.1 Antiinflammatories

Nonsteroidal antiinflammatory drugs (NSAIDs), such as diclofenac and indomethacin, are known to cause gastrointestinal side effects such as irritation and mucosal damage, which are due to local contact between the mucosa and solid drug particles and by an indirect effect (inhibition of prostaglandins and prostacyclin; Reynolds, 1993). Thus, these drugs are excellent models to evaluate the potentiality of nanoencapsulation to protect biological mucosae against the ulcerative effect of NSAIDs (Friedrich et al., 2008). One of the first works to consider this issue focused on nanocapsules containing diclofenac (free acid) and indomethacin prepared by the deposition of PLA (Guterres et al., 1995b). After oral administration, a significant reduction of gastrointestinal toxicity in rats was observed for the nanoencapsulated drugs. The protective effect of nanocapsules was attributed to a reduction in the local irritation caused by the direct contact of these drugs.

Indomethacin-loaded nanocapsules prepared by the interfacial polymerization of isobutyl cyanoacrylate exhibited a 10-fold increase in antiinflammatory activity compared to free indomethacin (Gursoy et al., 1989). Nanoencapsulation of indomethacin using PLA and poly(isobutyl cyanoacrylate) (PIBC) as polymers induced a protective effect for the jejunal tissue compared to the ulcerative effect of a commercial indomethacin solution (Ammoury et al., 1991). No difference was observed in the pharmacokinetic parameters.

Indomethacin was also encapsulated in PLA by nanoprecipitation using benzyl benzoate as the oil core (Ammoury et al., 1993). The drug release from nanocapsules occurred within minutes, indicating that the nanocapsules were not able to retain the drug in the oil core. Regarding gastrointestinal tolerance, these nanocapsules significantly reduced the ulceration caused by indomethacin in solution. A subsequent work (Guterres et al., 2000) verified that benzyl benzoate completely dissolves both PLA and PCL, showing that nanocapsules prepared with this lipophilic phase and these polyesters do not have a polymer wall. In the same work, the authors used Miglyol 810 as the oil core, which is a nonsolvent for PLA and PCL. The results indicated that the polymer wall acted as a barrier and the oil remained in the spray-dried powder, in contrast to the results observed for the benzyl benzoate formulation (Guterres et al., 2000). In addition, nanocapsules prepared using Miglyol 810 reduced the indomethacin gastrointestinal side effects after oral administration in rats (Raffin et al., 2003).

Despite the advantage of protecting the gastrointestinal mucosae, some authors (Guterres et al., 1995a; Saez et al., 2000; Schaffazick et al., 2003b) stated that the nanocapsules in aqueous

suspensions are unstable formulations because of the risk of ingredient degradation, microbiological growth, or both. To improve the stability of nanocapsule formulations, the spray-drying technique was applied to dry the suspensions and produce solid dosage forms (Müller et al., 2001). The production of a nanocapsule spray-dried powder involves droplet formation from the atomized suspension, followed by their solidification driven by water evaporation. This one-step process is easy to scale-up and equipment is readily available, so the processing costs can be reduced (Masters, 1991). This strategy was used for the first time to dry diclofenac-loaded nanocapsules using colloidal silica as the drying adjuvant (Müller et al., 2000, 2001). Biological evaluation of the powder demonstrated the maintenance of gastrointestinal tolerance observed for diclofenac-loaded nanocapsule suspensions (Guterres et al., 2001). A pharmacokinetic study in rats demonstrated that the drug was completely absorbed after oral administration of diclofenac-loaded nanocapsule powder (reconstituted in water and administered by gavage), presenting a higher half-life in plasma than the sodium diclofenac aqueous solution.

An organic–inorganic system based on polymeric nanocapsules prepared by the spray-drying technique has also been described. The system consists of microagglomerates containing the drug dispersed in the core (Aerosil® 200) and polymeric nanocapsules as the coating material (Beck et al., 2004, 2006). Nanoparticle-coated microparticles were prepared using Eudragit® S100 nanocapsules as the coating material of a core composed of diclofenac and silicon dioxide (Beck et al., 2005). This powder showed a protective effect against mucosal diclofenac damage in rats, indicating that this coating strategy presents a potential use for the oral administration of drugs.

The efficient control of drug release from dexamethasone-loaded nanoparticle-coated microparticles was shown by an *in vitro* drug transport study across Caco-2 cell monolayers (Beck et al., 2007). In accordance with the *in vitro* drug release studies at pH 7.4, nanocapsule-coated microparticles presented lower permeability coefficient values across this human intestinal cell line compared to the free dexamethasone solution. Furthermore, cytotoxicity studies showed that the nanoparticle-coated microparticles were nontoxic to membranes of Caco-2 cells. This recent study reinforced that nanocapsule-coated microparticles represent a promising platform for the development of controlled oral NSAID delivery systems.

In a subsequent study, silica xerogel was used as the core material instead of Aerosil 200 to prepare nanocapsule-coated microagglomerates encapsulating sodium diclofenac as the hydrophilic drug model (da Fonseca et al., 2008). The new system showed gastroresistance, and it was efficient in reducing burst release and in sustaining the drug dissolution profile.

An indomethacin derivative, indomethacin ethyl ester, was used as a drug model in order to determine the nanocapsule architecture and drug release mechanism (Pohlmann et al., 2004). The strategy was based on the interfacial alkaline hydrolysis of indomethacin ester simulating a sink condition of release. Indomethacin ethyl ester loaded nanocapsules were prepared, varying the PCL concentration to evaluate the influence of the polymeric wall in drug release (Cruz et al., 2006b). The increase in polymer concentration enhanced the drug release sustained half-lives. The antiedematogenic activity of indomethacin ethyl ester loaded nanocapsules in rats showed a significant pharmacological effect in comparison with an indomethacin ethyl ester loaded nanoemulsion (Cruz et al., 2006a). The pharmacokinetics of the nanocapsule formulation were evaluated in rats (Cattani et al., 2008), demonstrating a fast *in vivo* release of ester from the nanocapsules and its conversion to indomethacin independently of the administration route (intravenous or oral). Hence, indomethacin was the entity responsible for the antiedematogenic activity after indomethacin ethyl ester loaded nanocapsule dosing.

In addition to the oral route, polymeric nanocapsules were evaluated by alternative routes of administration. Indomethacin-loaded nanocapsules were administered rectally (Fawaz et al., 1996) and showed 100% bioavailability. Further, the nanocapsule formulation was effective in protecting the rectal mucosa against the local toxic effects of indomethacin.

Indomethacin *in vitro* corneal penetration was evaluated using nanocapsules as drug carriers (Calvo et al., 1996b). The transcorneal flux of the drug through isolated rabbit cornea showed a

considerable increase of 4–5 times the penetration rate of the nanoencapsulated drug compared to commercial eyedrops. In addition, PCL nanocapsules containing indomethacin were coated with chitosan, poly(L-lysine), or both in order to combine the features of nanocapsules with the advantages of a cationic mucoadhesive coating (Calvo et al., 1997). Chitosan-coated nanocapsules provided an optimal corneal penetration of indomethacin and displayed good ocular tolerance.

NSAIDs, apart from their classical peripheral site of action, display a central analgesic effect (Matoga et al., 2002). In this sense, indomethacin-loaded nanocapsules were tested in glioma cell lines, because this drug presents an antiproliferative effect because of the arrest of cell cycle progression (Bernardi et al., 2008). Indomethacin-loaded nanocapsules were at least 2 times more cytotoxic than indomethacin in solution for glioma cell lines, indicating that nanoencapsulation improved the indomethacin effect without the undesirable side effects of conventional chemotherapy.

Other NSAIDs have been the focus of nanoencapsulation studies. The pharmacokinetics of ketoprofen in solution and in nanocapsules were evaluated in plasma and cerebrospinal fluid (Matoga et al., 2002). Nanocapsules were prepared with PLA and injected intraperitoneally in rats. The extent of absorption was similar for both solution and nanocapsule suspension, but with different plasma profiles. Ketoprofen administered in solution was rapidly absorbed, and for the nanoencapsulated formulation two peaks of concentrations were noted after administration. The second and lower peak was attributed to a progressive release of the drug from nanocapsules during the elimination phase.

Nimesulide is an NSAID that selectively inhibits cyclooxygenase-2. A semisolid topical formulation containing nimesulide-loaded nanocapsules was evaluated using Franz diffusion cells and a tape-stripping technique in order to investigate whether nanoencapsulation is able to modify the drug distribution in the different strata of full-thickness human skin (Alves et al., 2007). Gels containing nimesulide-loaded nanocapsules were able to promote drug penetration in the stratum corneum compared to a conventional formulation, and they allow higher penetration in the skin compared to the nimesulide-loaded nanospheres and nimesulide-loaded nanoemulsion.

3.5.2 Anticancer Drugs

Conventional chemotherapeutics are often limited by the inadequate delivery of therapeutic concentrations to tumor target tissue. Therefore, it is important to develop new nanotechnologies for targeted delivery to tumors at both the cellular and tissue levels, thereby improving the therapeutic index of the carried anticancer molecules. Strategies for developing new efficient targeted nanocarriers of anticancer drugs may result from the combined knowledge of cancer physiopathology features, drug characteristics, and *in vivo* fate and behavior of nanocarriers (Couvreur and Vauthier, 2006).

Nanotechnological devices can be crucial approaches for the stability of the carried drug. Gemcitabine, for example, is an anticancer drug that suffers from rapid plasmatic metabolization. To overcome this problem, both physical and chemical protection of gemcitabine were developed (Stella et al., 2007). Lipophilic derivatives of gemcitabine [4-(*N*)-valeroylgemcitabine, 4-(*N*)-lauroylgemcitabine, and 4-(*N*)-stearoylgemcitabine] were synthesized and incorporated into poly[aminopoly(ethylene glycol)cyanoacrylate-*co*-hexadecyl cyanoacrylate] nanocapsules by nanoprecipitation of the copolymer. The cytotoxicity assay showed that 4-(*N*)-stearoylgemcitabine was more toxic than gemcitabine on two cell lines (human cervix carcinoma cell line KB3-1 cells, and human breast adenocarcinoma MCF-7 cells). Moreover, the incorporation of 4-(*N*)-stearoylgemcitabine in nanocapsules did not change its half-maximal inhibitory concentration values, showing that the cytotoxic activity of 4-(*N*)-stearoylgemcitabine was not modified by the nanoencapsulation.

Besides protecting the carried chemotherapeutic, nanoparticles can also be used to avoid cellular mechanisms such as multidrug resistance, because they allow entry into the cancer cells and act as an intracellular anticancer drug reservoir. Taking this into account, an approach was proposed using lipid nanocapsules (LNCs) consisting of Labrafac®, lecithin, and PEG-660 hydroxystearate (Lamprecht and Benoit, 2006). Etoposide-loaded LNCs reverted multidrug resistance and reduced

the cell growth in glioma cell lines, showing higher efficiency than the drug solution. The mechanism of action proposed for etoposide LNC was cell uptake followed by sustained drug release in combination with intracellular P-glycoprotein inhibition, ensuring a higher anticancer drug concentration inside the cancer cells.

The importance of developing new formulations for carrying anticancer drugs emerges from the failure of established therapies. Tamoxifen is an antiestrogenic molecule, which is the endocrine therapy of choice for the treatment of estrogen receptor (ER) positive breast cancer. Unfortunately, tamoxifen resistance often occurs, and in that case a blockade of human prostaglandin E_2 synthesis by aromatase inhibitors is sometimes of benefit. However, no response to aromatase inhibitors can occur. Thus, despite encouraging improvements in breast cancer treatment, the prognosis of metastatic disease is dramatic, stressing the need for new drugs and new administration strategies (Maillard et al., 2005).

With this in mind, biodegradable PLA and PEG–PLA nanocapsules containing either 4-hydroxytamoxifen (4-HT) or RU 58668 (RU), both antiestrogens, were prepared using the interfacial deposition of preformed polymer following solvent displacement (Renoir et al., 2006). The small sizes of the particles (233–246 nm) were compatible with their extravasations through the discontinuous endothelium of tumor vasculature. This allowed their accumulation in MCF-7 cell xenografts and led to prolonged exposure of the tumor to antiestrogen in athymic nude mice bearing established xenografts. In these tumors and in MCF-7/Ras xenografts, RU- and 4-HT-loaded nanocapsules inhibited tumor growth. In addition, RU-loaded nanocapsules promoted ER• subtype loss in the tumor cells, according to the immunohistochemistry assay. The same *in vivo* tumor models had already been used by the authors to evaluate the antitumoral activity of RU encapsulated within PEG–PLA nanoparticles (Ameller et al., 2003). RU-loaded PEG–PLA nanocapsules were more active than free RU or RU entrapped with PEGylated nanospheres at an equivalent dose.

Approaches of coating nanocapsules with PEG have been widely described in anticancer therapy. At the tissue level, upon intravenous injection, colloids without this coating are opsonized and rapidly cleared from the bloodstream by the normal reticuloendothelial defense mechanism, irrespective of particle composition (Nguyen et al., 2008). Hence, in order to increase the circulation time in the bloodstream and to enhance the probability of the molecule to extravasate in tumor tissues, a great deal of work has been devoted to developing so-called stealth particles, which are "invisible" to macrophages (Figure 3.1). For this, PEG (MW • 1000–5000 Da) placed at the nanoparticle surfaces reduces the opsonization process, thus enhancing the blood circulation time (Couvreur and Vauthier, 2006). This approach is the so-called passive targeting approach and subsequent drug accumulation in the tumor interstitium is due to the known enhanced permeability and retention effect (as a result of the gaps of the discontinuous endothelium of cancer cells, which are richly vascularized; Lozano et al., 2008; Maillard et al., 2005).

Another system, based on the incorporation of RU in PLA nanocapsules and in PEG–PLA nanocapsules, potentiated the RU effect of increasing the population of MCF-7 cells in sub-G1 by blocking cell cycle progression (Maillard et al., 2005). In addition, the nanocapsules enhanced the activity of the free drug, inducing MCF-7 apoptosis and supporting the notion that the incorporation of RU within the nanocapsules increased their antitumor activity. However, RU-loaded nanocapsules were not able to inhibit the E_2-induced tumor growth rate through intravenous administration in nude mice bearing MCF-7 cell tumors.

Cases of intrinsic and acquired resistance, when the insensitive ER tumor cells point out the limitation of hydroxytamoxifen, highlight the need for new active molecules with broader therapeutic scopes. In this context, a potentially cytotoxic moiety (the organometallic group ferrocene) was added to the competitive bioligand hydroxytamoxifen scaffold (Nguyen et al., 2008). This attachment enhanced hydroxytamoxifen cytotoxicity, increasing the lipophilicity of the compound to facilitate its passage through the cellular membrane. Two organometallic triphenylethylene compounds, 1,1-di(4•-hydroxyphenyl)-2-ferrocenylbut-1-ene (Fc-diOH) and 1,2-di(4•-hydroxyphenyl)-1-[4•-(2•-ferrocenyl-2•-oxoethoxy)phenyl]but-1-ene (DFO), which present strong antiproliferative activity in

breast cancer cells but are insoluble in biological fluids, were synthesized and incorporated in PEG–PLA nanocapsules. The influence of the encapsulated drugs on the cell cycle and apoptosis was studied by flow cytometry analyses. Whether free or encapsulated in the nanocapsules, Fc-diOH arrested the cell cycle in the S-phase. However, free DFO had no significant effect on MCF-7 cells, whereas nanoencapsulated DFO slightly increased the number of cells in the S-phase.

One group recently incorporated Fc-diOH in LNCs (Allard et al., 2008) with high drug loading capacity because of a larger oily core in their structure. The cytostatic activity of Fc-diOH was conserved after its encapsulation in LNCs, which were taken up by glioma cells. Fc-diOH-loaded LNCs were very effective on 9L-glioma cells, showing low toxicity levels when in contact with healthy cells. In addition, Fc-diOH LNC treatment significantly reduced both tumor mass and volume evolution after 9L-cell implantation into rats, indicating the *in vivo* efficacy of this kind of organometallic compound.

A nanosystem based on oil-encapsulating poly(ethylene oxide)-*b*-poly(propylene oxide)-*b*-poly(ethylene oxide) (PEO–PPO–PEO)/PEG nanocapsules conjugated with folic acid was synthesized using PEO–PPO–PEO and amine-functionalized six-arm branched PEG (MW • 20,000 Da; Bae et al., 2007). The shell encapsulating an oil phase was developed as a target-specific carrier for a water-insoluble drug, paclitaxel. Folate-mediated targeting significantly enhanced the cellular uptake and apoptotic effect on KB cells overexpressing folate receptors. The anticancer effect of paclitaxel-loaded nanocapsules was comparable to that of a clinically available formulation of paclitaxel (Taxol®). However, the cytotoxicity of Taxol was mainly caused by the toxicity of the Cremophor® EL vehicle rather than the drug. In contrast, the folate-conjugated nanocapsules exhibited far greater cytotoxicity against KB cells at a lower dosage than Taxol (Bae et al., 2007).

Besides paclitaxel, docetaxel belongs to the taxane class that is characterized by its hydrophobic character, resulting in the necessity of using solubilizers for its intravenous administration. These solubilizers, however, are responsible for severe side effects, which limit the amount of drug that can be safely administered. To overcome these problems, an alternative formulation based on chitosan colloidal carriers (nanocapsules) was prepared by the solvent displacement technique (Lozano et al., 2008). Chitosan nanocapsules were rapidly internalized by human tumor cells. Docetaxel-loaded chitosan carriers had an effect on cell proliferation, which was significantly greater than that of free docetaxel. Another work demonstrated that the encapsulation of docetaxel within LNCs dramatically increased the drug biological half-life, providing substantial accumulation at the tumoral site in mice bearing subcutaneously implanted C26 colon adenocarcinoma (Khalid et al., 2006).

Paclitaxel-loaded LNCs were used to elucidate whether LNCs were able to improve anticancer hydrophobic drug bioavailability and overcome multidrug resistance (Garcion et al., 2006). The results revealed an interaction between LNCs and efflux pumps, which results in an inhibition of multidrug resistance in rat glioma cells both in culture and in cell implants in animals. Paclitaxel-loaded LNCs were also more efficient than Taxol.

Nanocapsules with an external layer made up of PLA, PLA grafted with PEG (PLA–PEG), and PLA coated with poloxamer 188 (PLA–polox) have been proposed to incorporate photosensitizers for tumor tissue in photodynamic therapy (Bourdon et al., 2002). The cellular uptake, localization, and phototoxicity of *m*-tetra(hydroxyphenyl)chlorine (mTHPC) encapsulated in submicron colloidal carriers were studied in macrophage-like J774 cells and HT29 human adenocarcinoma cells. Cellular uptake by J774 was reduced with mTHPC encapsulated within surface-modified nanocapsules (PLA–PEG and PLA–polox) compared to naked PLA, indicating a possible limitation of the clearance of such carriers by the reticuloendothelial system. A specific punctate fluorescence pattern was revealed with PLA–PEG and PLA–polox nanocapsules, in contrast to a more diffuse distribution with solution, indicating that photodamage targeting could be different.

The same formulations were studied to evaluate the biodistribution of mTHPC in nude mice bearing HT29 human tumors (Bourdon et al., 2002). Compared to PLA nanocapsules, incorporation of mTHPC in surface-modified nanocapsules resulted in strong modifications of drug biodistribution and tumoral retention with an increase in drug levels. Reduced liver uptake was observed,

indicating that surface-modified nanocapsules are effective in limiting reticuloendothelial system uptake and are potential carriers for enhancing the therapeutic ratio of lipophilic photosensitizers.

3.5.3 Hormones, Proteins, and Peptides

Hormones are substances that present low therapeutic doses and require chronic administration, so they are suitable molecules to nanoencapsulate. Melatonin was associated with Eudragit S100 nanocapsules in order to improve its protective properties against lipid peroxidation induced by ascorbyl free radicals using liposomes and microsomes as substrates (Schaffazick et al., 2005). The antioxidant capacity of melatonin was significantly increased when it was nanoencapsulated. The *in vivo* acute antioxidant capacity (lipid peroxidation, total antioxidant reactivity, and free radical levels in the brain and liver of mice) showed that lipid peroxidation significantly decreased in the cortex and in the hippocampus when melatonin-loaded nanocapsules were administered. In contrast, a melatonin aqueous solution did not exert any significant activity against lipid peroxidation (Schaffazick et al., 2008).

Peptide drugs are poorly absorbed after oral administration because of their susceptibility to enzymatic degradation and their low permeability across the intestinal epithelium. Keeping these important biopharmaceutical limitations in mind, many pharmaceutical scientists have taken the challenge of designing new delivery strategies intended to enhance the oral absorption of these macromolecules (Prego et al., 2006b).

A challenge in ocular drug delivery is to enhance the permeation of macromolecules across the cornea. PCL nanocapsules containing cyclosporin A, an immunosuppressive drug, were developed for ocular delivery in order to reduce its systemic side effects (Calvo et al., 1996b). The nanocapsules promoted the penetration of cyclosporin A to a very high degree.

Novel drug delivery systems for insulin administration, avoiding injectable formulations, have been the focus of much research for over 20 years. As a hypothesis, oral formulations containing insulin provide the peptide directly to the liver by hepatic portal circulation. This is a major advantage because this pathway mimics the physiological traffic of insulin when it is secreted by the pancreas of healthy individuals (Saffran et al., 1997). However, mucosal routes are extremely challenging for the administration of peptides and proteins because these generally hydrophilic macromolecules are unable to overcome mucosal barriers by themselves and are degraded before reaching the bloodstream (Couvreur and Vautier, 2006).

Insulin-loaded nanocapsules have been studied since 1988 (Damgé et al., 1988), when it was proved that PAC nanocapsules preserve the therapeutic effect of insulin in rats when administered orally, prolonging its effect. Insulin-loaded nanocapsules controlled glycemia for at least 13 days in streptozotocin-induced diabetic rats (Michel et al., 1991). *In vitro* nanocapsules protect insulin against proteolysis from pepsin, chymotrypsin, and trypsin (Michel et al., 1991). In addition, nanocapsules administered orally induce several beneficial persistent effects in both normal and diabetic dogs (Damgé et al., 1995). This formulation was tested as a prophylactic strategy to prevent diabetes in nonobese diabetic mice via oral administration. In humans, this form of prophylactic insulin administration was claimed to be less constraining than insulin injections (Saï et al., 1996). The early administration of insulin nanocapsules reduced diabetes and insulitis in the nonobese diabetic mouse model that mimics human Type 1 diabetes.

More recently, PIBC nanocapsules containing Texas Red® labeled and gold labeled insulin were studied (Pinto-Alphandary et al., 2003). Insulin was located inside nanocapsules that were observed on both sides of the jejunum. In the lumen, the environment was suitable to protect insulin from degradation. Nanocapsules were absorbed by portions of the M-cell-free epithelium and were highly degraded in M-cell-containing epithelium.

After oral administration, insulin from PIBC nanocapsules was very quickly but heterogeneously absorbed. Furthermore, nanocapsules allowed the delivery of noticeable levels of insulin into the blood of diabetic rats (Cournarie et al., 2002). Nevertheless, high levels of plasma insulin were

necessary to produce efficient hypoglycemic activity, and they were not reached after oral administration of nanocapsules.

The activity of insulin-loaded PAC nanocapsules was evaluated after subcutaneous administration, showing a reduction in blood glucose in diabetic rats, which was delayed by the nanoencapsulation (Watnasirichaikul et al., 2002). The formulation of insulin-loaded nanocapsules dispersed in a water-in-oil microemulsion showed a significant increase in the oral bioavailability of insulin in diabetic rats (Watnasirichaikul et al., 2002).

Salmon calcitonin (model peptide) was encapsulated in chitosan–PEG nanocapsules prepared by the solvent displacement technique (Prego et al., 2006a). The PEGylation of the chitosan coating facilitated the retention of peptide in the nanocapsules. The hypocalcemic effect after the oral administration of nanocapsules was significantly higher than the nanoemulsion and free peptide aqueous solution. The study with Caco-2 cells indicated that nanocapsules were able to interact within the cells, showing a random distribution by being able to enter the cells by a transcellular pathway. The mucoadhesive character of chitosan nanocapsules was the determinant for interaction with intestinal mucosae, facilitating the intestinal absorption of salmon calcitonin (Prego et al., 2006a).

3.5.4 Antifungals, Antibiotics, and Antiparasitics

The encapsulation of antifungal agents in nanoparticulate carriers was proposed with the objective of modifying the pharmacokinetics of drugs, resulting in more efficient treatments with fewer side effects (de Assis et al., 2008). In this way, fluconazole was radiolabeled and encapsulated in PLA–PEG nanocapsules, demonstrating a fast release of radioactivity. The PEG layer around the nanocapsules probably reduced the amount of drug released by impairing protein binding at the nanocapsule surface. Another antifungal, griseofulvin, is rarely used because of its high lipophilicity, which makes both formulation and delivery difficult (Zili et al., 2005). Griseofulvin was very rapidly released from PCL nanocapsules, which was probably due to the dissolution of the polymer by the oily phase (benzyl benzoate). Griseofulvin nanocapsules showed a higher dissolution rate, which indicated that lower doses of this molecule can be used for oral applications, thus reducing its side effects (Zili et al., 2005).

Several antiseptics can be incorporated in hand-washing agents. However, their frequent use induces contact dermatitis and allergies. To improve and sustain antimicrobial activity, chlorhexidine-loaded nanocapsules were tested *in vitro* against some microorganisms (Lboutounne et al., 2002). The activity of the nanocapsules was also tested *ex vivo* in porcine ear skin. The encapsulation maintained the chlorhexidine effect, sustaining the *ex vivo* topical antimicrobial activity against *Staphylococcus epidermidis*. Chlorhexidine was incorporated in a hydrophilic gel and tested as a hand-rub gel against resident skin flora. This product had immediate antibacterial effect, explained by the rapid desorption of chlorhexidine from the nanocapsule wall, subsequent diffusion within bacteria, and sustained antibacterial effect. This immediate effect was a consequence of the slow release of chlorhexidine from the nanocapsule core against further bacterial colonization (Nhung et al., 2007).

Injectable formulations of benzathine penicillin G were developed in the 1950s and consisted of intramuscular depot formulations because of the low solubility of the drug. New formulations (nanoemulsion and PLGA nanocapsules containing benzathine penicillin G) were developed that were stable over 120 days when stored at 4°C, exhibiting *in vitro* antimicrobial activity against *S. pyogenes* (Santos-Magalhães et al., 2000).

The emergence of chloroquine resistance in *Plasmodium falciparum* has increased the search for new antimalarial drugs, such as halofantrine hydrochloride, one of the most active antimalarial drugs against *P. falciparum in vitro* but presenting serious cardiotoxicity (Mosqueira et al., 2004). Considering that the cardiac epithelium is continuous, stealth nanocapsules containing halofantrine have been developed to reduce drug side effects and to increase drug circulation (Mosqueira et al., 2004). No signs of toxicity or abnormal behavior were observed after intravenous administration of halofantrine-loaded nanocapsules in mice, and the maximum tolerated dose was higher than that of

the free drug in solution. Poloxamer-coated nanocapsules provided a fast effect, whereas PEG-coated nanocapsules provided a more sustained effect because of their long blood circulation. PEG-coated nanocapsules provided a reduced halofantrine cardiotoxic profile when compared to the free drug, showing that drug distribution seemed to be modified by these nanocarriers (Leite et al., 2007).

The potential of colloidal drug carriers in the targeted and controlled delivery of antileishmanial compounds has also received much attention. Leishmania are obligate intracellular parasites in mammals that live exclusively in the cells of the mononuclear phagocyte system (Cauchetier et al., 2003a). Taking into consideration that conventional nanocapsules undergo phagocytosis by macrophages after opsonization (Stolnik et al., 1995), atovaquone-loaded nanocapsules were prepared by the interfacial deposition of preformed polymer using different polyesters (Cauchetier et al., 2003b). Atovaquone was released from PLA nanocapsules by diffusional transport of the drug through the polymer, associated with the first stage of polymer degradation. *In vivo* nanocapsules were significantly more effective than the free drug in the treatment of mice with visceral leishmaniasis. The dose–response data indicated that livers were cleared of parasites if the nanocapsule preparation was administered in three doses, whereas the maximum suppression possible with the free drug is about 61%, whatever the dose (Cauchetier et al., 2003a).

3.5.5 Other Drugs

Nanocapsules can be used to increase the accessibility of drugs to the receptors localized in specific areas. They can serve as vehicles for use in the treatment of ophthalmic pathologies, because increased corneal penetration and prolonged therapeutic response have been achieved for some drugs (Losa et al., 1993). Several nanocapsule formulations containing metipranolol were tested for drug release and the ability to prevent conjunctival absorption. The nanoencapsulation was capable of reducing bradycardia, showing lower systemic toxicity. Another drug used as eyedrops, pilocarpine, was encapsulated in PIBC nanocapsules incorporated in a Pluronic F127 gel (Desai and Blanchard, 2000). The formulation increased the contact time of the drug with the absorbing tissue in the eye and improved ocular bioavailability.

Many authors have focused on different drugs used to control drug release and improve drug bioavailability and stability (Calvo et al., 1996c; Fresta et al., 1996; Ourique et al., 2008). Antiepileptic drugs (5,5-diphenyl hydrotoin, carbamazepine, and ethosuximide) were encapsulated in poly(ethyl-2-cyanoacrylate) nanocapsules and zero order release was achieved, providing controlled drug release (Fresta et al., 1996).

Idebenone is a lipophilic benzoquinone electron carrier, which behaves as an antioxidant free radical scavenging molecule, that is active in central nervous system disorders (Palumbo et al., 2002). Idebenone-loaded poly(ethyl cyanoacrylate) nanocapsules showed a greater effectiveness for the antioxidant effect *in vitro*, under different stress conditions, toward human fibroblasts than the free drug.

Tretinoin is the active form of a metabolic product of vitamin A, which is indicated in the topical treatment of different skin diseases such as acne vulgaris, ichtyose, and psoriasis. However, this drug presents some drawbacks such as poor solubility, high chemical instability and photoinstability (which gives inactive metabolites), and irritation of the treated area. Nanocapsules containing tretinoin were developed, which were aimed at reducing drug side effects and drug photoinstability (Ourique et al., 2008). Tretinoin-loaded nanocapsules improved tretinoin photostability, independently of the type of oil core used.

Spironolactone is a specific steroid antagonist that is used as a potassium-sparing diuretic in premature infants to reduce lung congestion, but no liquid formulations are available because of its low solubility (Blouza et al., 2006). Different parameters were tested in order to obtain an optimized formulation of nanocapsules. The release of spironolactone from nanocapsules was rapid and complete in a simulated gastric fluid. Therefore, recourse to spironolactone nanoencapsulation should enhance its oral bioavailability and probably its efficiency. Concerning drug therapies based on nanocapsules, Table 3.2 gives an overview of the systems carrying different molecules and their main results.

TABLE 3.2
Oily-Core Polymeric Nanocapsules Proposed for Therapeutic Goals

Therapeutic Class	Drug	Nanocapsule Composition	Main Result	Reference	
Antiinflammatory	Indomethacin and diclofenac	PLA and Miglyol 810	Reduction of gastrointestinal toxicity	Guterres et al., 1995a,b	• • •
	Indomethacin	PIBC and benzyl benzoate	10-fold increase in antiinflammatory activity	Gursoy et al., 1989	
		PLA, PIBC, and benzyl benzoate	Protective effect on jejunal tissue	Ammoury et al., 1991	
		PLA and benzyl benzoate	Reduction of ulceration in intestine and rectum	Ammoury et al., 1993; Fawaz et al., 1996	
		PCL and Miglyol 840	Increases corneal penetration rate 4–5 times	Calvo et al., 1996c	
		PCL coated with chitosan or poly(L-lysine) and Miglyol 840	Optimal corneal penetration and good ocular tolerance	Calvo et al., 1997	
		PCL and CCT	Twice as cytotoxic for glioma cell lines	Bernardi et al., 2008	
	Indomethacin ethyl ester	PCL and Miglyol 810	Increase in antiedemadogenic activity	Cruz et al., 2006b	
	Ketoprofen	PLA and benzyl benzoate	Progressive release of the drug	Matoga et al., 2002	
	Nimesulide	PCL and CCT	Higher penetration in the skin	Alves et al., 2007	
Diuretic	Spironolactone	PCL and Labrafac hydro	Fast and complete release	Blouza et al., 2006	
Hormone	Melatonin	PCL and Miglyol 810	Protective properties against lipid peroxidation induced by ascorbyl free radical in cortex and in hippocampus	Schaffazick et al., 2005, 2008	
	Insulin	PAC and Miglyol	Preserves therapeutic effect orally in rats and dogs	Damgé et al., 1988, 1995	
		PIBC and Miglyol	Glycemia for at least 13 days in rats	Michel et al., 1991	
		PIBC and Miglyol	Reduced diabetes and insulitis in mice	Saï et al., 1996	• • •
		PIBC and Miglyol 812N	Nanocapsules at both sides of the jejunum	Courniarie et al., 2002; Pinto-Alphandary et al., 2003	
		PAC and Capmul® MCM		Watnasirichaikul et al., 2002	
Peptide	Cyclosporine A	PCL and Miglyol 840	Promotes penetration of cyclosporine A across cornea	Calvo et al., 1996b	
	Salmon calcitonin	Chitosan–PEG and Miglyol 812	Higher hypocalcemic effect and penetration by transcellular pathway	Prego et al., 2006a,b	
Antifungal	Fluconazole	PLA–PEG and CCT	Controlled release	Assis et al., 2008	• • •
	Griseofulvin	PCL and benzyl benzoate	Higher dissolution rate	Zili et al., 2005	
Antibiotics	Chlorhexidine	PCL and Labrafac hydrophile WL 1219	Sustained effect against *Staphylococcus epidermidis*	Lboutounne et al., 2002; Nhung et al., 2007	

continued

TABLE 3.2 (continued)
Oily-Core Polymeric Nanocapsules Proposed for Therapeutic Goals

Therapeutic Class	Drug	Nanocapsule Composition	Main Result	Reference
	Benzathine penicillin G	PLGA and sunflower oil and benzyl benzoate	Stability over 120 days and activity against *Streptococcus pyogenes*	Santos-Magalhães et al., 2000
Antimalarial	Halofantrine	PLA, PLA–PEG, and Miglyol 810	No signs of toxicity after IV administration	Mosqueira et al., 2004
		PCL and Miglyol 810	Reduced halofantrine cardiotoxic	Leite et al., 2007
Antileishmanial	Atovaquone	PLA, PCL, PLGA, and benzyl benzoate	More effective in visceral leishmaniasis in rats	Cauchetier et al., 2003a
Antiepileptic	5,5-Diphenyl hydrotoin, carbamazepine, and ethosuximide	PEC and Miglyol 812	Controlled drug release	Fresta et al., 1996
Antioxidant	Idebenone	PEC and Miglyol 812	Greater *in vitro* antioxidant activity	Palumbo et al., 2002
Vitamin	Tretinoin	PCL and Miglyol 810	Improved tretinoin photostability	Ourique et al., 2008
Anticancer drug	Gemcitabine lipofilic derivate	Poly(H_2NPEGCA-*co*-HDCA) and Miglyol 812N	Higher cytotoxicity than gemcitabine, not modified by nanocapsule incorporation	Stella et al., 2007
	Etoposide	PEG–HS and Labrafac	Reverts multidrug resistance, reduce tumor growth	Lamprecht and Benoit, 2006 • ••
	4-HT and RU	PLA, PLA–PEG, and Miglyol 810	Block cell cycle progression in tumor cells, increase antitumor activity, promote loss ER• in tumor cells	Ameller et al., 2003; Maillard et al., 2005; Renoir et al., 2006
	Organometallic tamoxifen derivatives	PLA–PEG and Miglyol 810	Increase number of cells in S-phase	Nguyen et al., 2007 • •••
	Organometallic tamoxifen derivative	PEG–HS and Labrafac	Reduce tumor mass *in vivo*	Allard et al., 2008
	Paclitaxel	PEO–PPO–PEO/PEG folic acid conjugated and Lipiodol®	Enhance cellular uptake and apoptotic effect on cells overexpressing folate receptors	Bae et al., 2007
	Docetaxel	Chitosan and Miglyol 812	Rapidly internalized by human tumor cells, promotes reduction of cell proliferation	Lozano et al., 2008
	Docetaxel	PEG–HS and tricaprylin	Increase drug biological half-life and accumulate at tumoral site *in vivo*	Khalid et al., 2005 • •••
	Paclitaxel	PEG–HS and Labrafac	Inhibition of multidrug resistance *in vivo* and *in vitro*	Garcion et al., 2006
Photosensitizer	Tetra(hydroxyphenyl)chlorin	PLA, PLA–PEG, PLA–polox, and Miglyol 812N	Reduce cellular uptake and clearance with surface-modified nanocapsules	Bourdon et al., 2000, 2002

Note: HS—hydroxystearate; PEC—poly(ethyl cyanoacrylate); poly(H_2NPEGCA-*co*-HDCA)—poly[aminopoly(ethylene glycol)cyanoacrylate-*co*-hexadecyl cyanoacrylate].

CONCLUSION

Investigations concerning polymeric nanocapsules are generally a multidisciplinary study involving engineering, chemistry, pharmacy, and pharmacology. Challenges have been presented during the past 40 years about the physicochemical evaluation of these formulations. The complexity of these systems is a consequence of both submicron-size and soft-matter properties. However, successful applications using complementary techniques to elucidate the nanocapsule supramolecular structure have been reported since the 1970s. The improvement of knowledge concerning the supramolecular organization of nanocapsules has been accomplished, tuning their characteristics for specificities of use, such as the administration route, drug release time, and drug target site in the organism. Major reports in the literature about the therapeutic applications of nanocapsules describe the delivery of antiinflammatory drugs, chemotherapeutic agents, hormones, peptides, and other agents, with increases of drug efficacy and reduction of drug toxicity. Considering this, nanocapsule science and technology open new and interesting perspectives for human health.

ABBREVIATIONS

CCT	Caprylic/capric triglyceride
DFO	1,2-Di(4•-hydroxyphenyl)-1-[4•-(2•-ferrocenyl-2•-oxoethoxy)phenyl]but-1-ene
DLS	Dynamic light scattering
E_2	Type of human prostaglandin
ER	Estrogen receptor
Fc-diOH	1,1-di(4•-hydroxyphenyl)-2-ferrocenylbut-1-ene
4-HT	4-Hydroxytamoxifen
KB3-1	Human cervix carcinoma cell line
LNC	Lipid nanocapsules
MCF-7	Human breast adenocarcinoma cell line
mTHPC	Meta-*tetra*(hydroxyphenyl)chlorine
MW	Molecular weight
NSAID	Nonsteroidal antiinflammatory drug
PAC	Poly(alkyl cyanoacrylate)
PCL	Poly(• -caprolactone)
PEG	Poly(ethylene glycol)
PEO	Poly(ethylene oxide)
PFOB	Perfluorooctyl bromide
PIBC	Poly(isobutyl cyanoacrylate)
PI	Polydispersity index
PLA	Polylactide
PLGA	Poly(lactide-*co*-glycolide)
PPO	Poly(propylene oxide)
RU	RU 58668 steroidal antiestrogen
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy

TRADEMARKS

Aerosil® (Evonik Degussa GmbH, Frankfurt am Main, Germany)
Capmul® (Abitec Corporation, Columbus, Ohio, USA)
Cremophor® (BASF Corporation, Florham Park, New Jersey, USA)
Eudragit® (Evonik Degussa GmbH, Frankfurt am Main, Germany)
Epikuron® (Degussa GmbH, Hamburg, Germany)

Labrafac® (Gattefossé, Saint-Priest, France)
Lipiodol® (Guerbet S.A., Roissy Charles-de-Gaulle Cedex, France)
Miglyol® (SASOL Germany GmbH, Witten, Germany)
Pluronic® (BASF Corporation, Florham Park, New Jersey, USA)
Taxol® (Bristol-Myers Squibb, New York, USA)
Texas Red® (Molecular Probes, Leiden, The Netherlands)
Tween® (ICI Americas Inc., London, England)

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