Mini Reviews in Medicinal Chemistry. 2016; 16(6):498-508

Microemulsions for colorectal cancer treatments: general considerations and formulation of methotrexate

Sergio E. Flores[1], M. Isabel Rial-Hermida[2], Jorge C. Ramirez[1], Alejandro Pazos[3], Angel Concheiro[2], Carmen Alvarez-Lorenzo[2] and René D. Peralta[1]

[1] Centro de Investigación en Química Aplicada, Departamento de Procesos de Polimerizacion, Saltillo, Coahuila, 25253 México; [2] Departamento de Farmacia y Tecnología Farmacéutica, Facultad de Farmacia, Universidad de Santiago de Compostela, 15782 Santiago de Compostela, Spain; [3] Department of lnformation and Communication Technologies, University of A Coruña, 15071 A Coruña, Spain

Abstract
Microemulsions combine the advantages of emulsions with those of nanocarriers, overcoming the stability problems of the former and providing facile scalable systems with compartments adequate for high drug loadings. Recently, microemulsions are gaining attention in the formulation of anticancer drugs not only for topical treatment, but also for systemic delivery as well as for the development of theranostic systems. The aim of this paper is two-fold. First, an updated review about general features, preparation, characterization and pharmaceutical applications, with a special focus on colorectal cancer, is provided. Second, a case study of formulation of methotrexate in microemulsions is presented. Various essential oils (menthol, trans-anethole, α-tocopherol) and surfactants (TPGS-l000, Maxemul 6112, Noigen RN-20) were investigated for the preparation of o/w microemulsions for the delivery of methotrexate, and the ability of methotrexate-loaded microemulsions to inhibit cancer cell growth was then evaluated. Disregarding the surfactants used, menthol and trans-anethole led to cytotoxic microemulsions, whereas α-tocopherol based-formulations induced cell proliferation. These findings highlight the role that the oily component may play in the efficacy and safety of the microemulsions.

Keywords
Antitumor therapy, colorectal cancer, essential oils, methotrexate formulation, microemulsion, phase diagrams

1. Microemulsions

1.1. General Issues

Microemulsions are ternary systems comprising an aqueous phase, an oily phase and a relatively large proportion of amphiphilic compounds and co-surfactants (usually short aliphatic alcohols), which enable the obtaining of a single optically isotropic and thermodynamically stable liquid dispersion [1-3]. Mean size of inner phase globules is in l0 100 nm scale and, therefore, visible light is not dispersed when passes through microemulsions [4]. Unlike conventional emulsions, typical characteristics of microemulsions are the ultra low interfacial tension between the immiscible phases, lower input of energy for preparation, and higher thermodynamic stability (Table 1) [5]. There are also important differences between microemulsions and nanoemulsions (also named sub-micron emulsions); the latter are prepared from emulsions that are subjected to high shear stress or mechanical extrusion processes and are thermodynamically unstable [6].

Table 1. Differences between microemulsions and emulsions [8]

Property	Microemulsion	Emulsion
Appearance	Transparent/translucent	Milky
Thermodynamic stability	Stable	Unstable (kinetically stabilized)
Formation	Spontaneous	Energy input
Interfacial tension	Towards 0 mNm^{-1}	~50 m N^{-1}
Microstructure	Dynamic (fluctuating surfaces)	Static (until coalescence)
Optical isotropy	Yes	No
Droplet size	10-100 nm	>500 nm

Combination of the above mentioned advantages while maintaining the capacity to solubilize hydrophobic drugs explains that microemulsions occupy a relevant position among other nanometric carriers [7, 8]. It should be noted that compared to conventional and reverse micelle systems, the inner compartment of microemulsion droplets is notably larger and so is its capability to host larger amounts of drugs and even therapeutic macromolecules.

Depending on the ratios between the components, three main types of microemulsions can be prepared. When the content in water is large, microemulsions consist of small oil droplets surrounded by an interfacial film of both surfactant and co-surfactant molecules and dispersed in the aqueous phase *(*o/w microemulsions). Oppositely, if the content in water is low, the situation is reversed and the system consists of water droplets dispersed in oil *(*w/o microemulsions). Those systems that exhibit a gradual transition from o/w to w/o microemulsion are known as bicontinuous microemulsions [9]. Since the 2000s, U-type microemulsions are receiving increasing attention [10]. These are highly water dilutable microemulsions that change progressively upon water addition from w/o to o/w without phase change [11].

Droplets in the microemulsions can adopt spherical (o/w, w/o) or tubular morphologies, depending on the magnitude of the surfactant packing parameter, $v/(a_o\ lc)$, where v is the volume occupied by the hydrocarbon tail of the surfactant, a_0 is the area of the polar head and lc is the length of the totally extended hydrocarbon chain of the surfactant [12-14]. In this mini-review, the focus will be on o/w microemulsions with spherical droplets. Following the concept of the packing parameter it is found that when $a_0 > v/lc$, then an o/w microemulsion forms; if $a_0 < v/lc$, then a w/o microemulsion is obtained, and if $a_0 \approx v/lc$, then a middle-phase (bicontinuous) microemulsion is the preferred structure [13].

Microemulsion formation is a spontaneous process, as indicated by a negative Gibbs free energy of formation, ΔG. For microemulsions, the equation can be written as:

$$\Delta G = \gamma \Delta A - T\Delta S$$

where γ is the interfacial tension, ΔA the change in interfacial area during the formation process, ΔS the change in entropy and T is the temperature. Microemulsion formation is accompanied by a significant increase in the interfacial area, A. In as much as the interfacial tension γ decreases notably (but remains positive all the time), a negative free energy is attained when the interfacial energy (γA) is compensated by a dramatic change in the dispersion entropy of the system [15, 16]. A very low interfacial tension required for microemulsion formation cannot always be accomplished by the selected surfactant and sometimes a co- surfactant is required. The co-surfactant penetrates the amphiphilic interfacial layer, and increases its curvature and fluidity [16, 17]. For this purpose and for reasons of biocompatibility, preferably non-ionic surfactants and short or medium chain alcohol s are used [8, 15, 16].

Phase equilibria in microemulsions can be studied through the construction of phase diagrams at a given temperature. In its simplest form, the diagram includes the proportions of oil, water and surfactant mixtures, and shows the combinations that lead to a single (apparent) phase [18]. A well-known classification of microemulsions is that of the Winsor type systems [19]. A Winsor I system consists of two phases, in which the o/w microemulsion phase placed at the bottom is in equilibrium with the supernatant oil in excess. Winsor II system comprises two phases also, but the upper microemulsion phase (w/o) is in equilibrium with excess water. Winsor III systems are characterized by the coexistence

of three phases: the microemulsion (o/w plus w/o, also known as bicontinuous) located in between oil (upper phase) and water (lower phase). Winsor IV systems exhibit a single phase domain, with oil, water and surfactant homogeneously mixed (microemulsion). These systems are schematically represented in (Fig. 1).

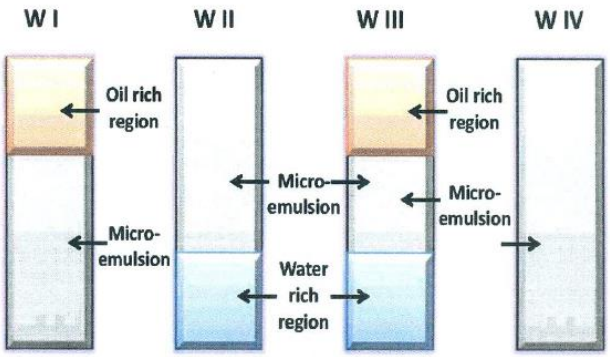

Fig. (l). Winsor classification of microemulsion systems [19].

1.2. Microemulsions Preparation

Preparation of a microemulsion to be used as drug nanocarrier system requires a careful selection of both qualitative and quantitative composition. The components should be chosen to fit the biocompatibility demands of the administration route and, therefore, adverse reactions and sensitizing or irritation potential of each compound have to be carefully considered. Moreover, the components should have adequate physicochemical properties, mainly regarding melting point, viscosity, chemical stability, ionization degree, and pH. On the other hand, the formation of thermodynamically stable microemulsions requires an adequate amount of the components to be mixed. As mentioned above, an important tool to correctly choose the compositions is the elaboration of ternary or pseudoternary phase diagrams.

1.2.1. Components

Oil phase. Most microemulsions performing as drug nanocarriers are o/w systems and, consequently, the main criterion that the oil phase must accomplish is that the drug should have high solubility in it. The oil also affects the curvature of the microemulsion droplet, as this depends on the ability of the surfactant tails to penetrate and swell in the interface. Since the drug delivery system should be biocompatible, excipients to be used in the formulation are restricted to some natural oils (com, cottonseed, orange, clove, peppermint, eucalyptol, coconut, soybean and jojoba oils) [20-24], triglycerides and esters of fatty acids (e.g., isopropyl myristate (IPM), ethyl oleate) [25-27].

Aqueous phase. It usually contains regulators of pH (buffers) and ionic strength (salts) as well as certain cosolvents (mainly alcohols).

1.2.1.1. Surfactants

The role of the surfactant in the formulation is to lower the interfacial tension between oil and water as much as possible in order to facilitate the spontaneous formation of the microemulsion and to provide mechanical flexibility to the droplets. The surfactant should have the appropriate lipophilic character to give the correct curvature at the interfacial region. The emulsifying capability of an agent may be classified according to the hydrophilic-lipophilic balance (HLB) in its molecule. This is a function of the weight percentage of hydrophilic groups in the molecule [28]. Generally low HLB (< 12) surfactants lead to w/o microemulsions, while high HLB (> 12) ones are mostly suitable for o/w microemulsions. Commonly used surfactants are Aerosol OT and non-ionic molecules, such as polysorbates, alkyl

polyethers, and sorbitan monoesters [29]. Surfactants are commonly included at proportions as high as 25% and, therefore, they should be carefully chosen to avoid toxicity problems when administered through a given route. In general, surfactants cannot be assumed to be inert excipients since they can modify the transfer of drugs across biological membranes. Further, surfactants might produce significant changes in the biological activity of drugs by exerting an influence on metabolizing enzymes or on the binding to receptor proteins [30].

1.2.1.2. Co-surfactants

They are short to medium chain length alcohols or amines (C3-C8) that reduce the interfacial tension and increase the fluidity of the interface [29, 31]. Both surfactants and co-surfactants migrate to the liquid-liquid interface, which helps to decrease more the interface tension.

1.2.1.3. Drugs

Commonly the drug is incorporated to the inner oil phase, but if the drug is oily, it may constitute itself the inner phase. Hydrophilic drugs could be also dissolved in the aqueous phase, and the microemulsion could allow formulating together immiscible/incompatible drugs or may enable a rapid release of the drug from the aqueous phase and a sustained release of the drug incorporated into the oil phase.

1.2.2. Preparation Methods

There are two main methods to prepare microemulsions, as described below.

1.2.2.1. Phase Titration Method

First a ternary or pseudoternary diagram is constructed. Each comer represents 100% of a particular component, as shown in (Fig. 2). The regions can be separated into w/o or o/w microemulsions by simply considering whether it is oil rich or water rich. If only water and surfactant predominate, conventional micelles are formed. Oppositely, the surfactant molecules arrange in the form of reverse micelles if oil and surfactant are the major components.

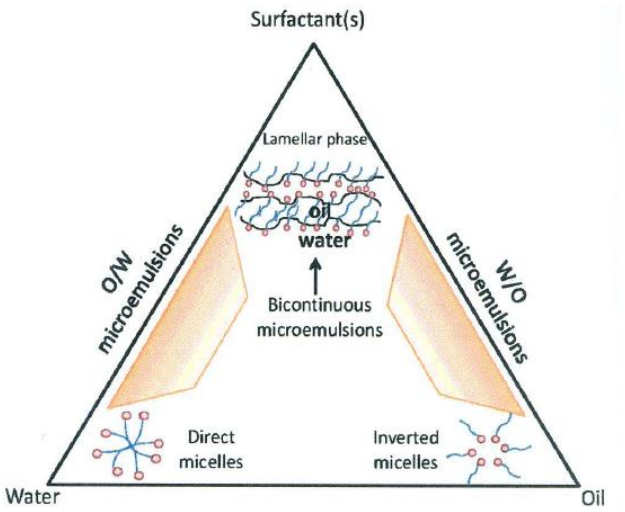

Fig. (2). Schematic phase diagram showing the regions where microemulsions are formed.

1.2.2.2. Phase Inversion Method

lt consists in increasing the temperature to cause loss of water of the head groups of the surfactants [32]. Shrinking the head group area of the non-ionic surfactant causes a phase inversion from o/w to w/o microemulsions in two-phase mixtures with either excess oil or excess water (phase inversion temperature, PIT) [33]. The changes in the curvature of non-ionic surfactant packing force a transition from an o/w microemulsion at low temperature to a w/o microemulsion at higher temperature (transitional phase inversion) [34]. Alternatively, phase inversion can occur adding the dispersed phase in excess. Phase inversion is accompanied by marked changes in physical properties including droplet size.

1.3. Microemulsions Characterization

The characterization of microemulsions is a difficult task due to their complexity and the variety of structures and components involved in these systems. The starting point is the construction of a ternary diagram (as depicted in Fig. 2). More complex and sophisticated techniques are then used to investigate in detail physicochemical properties and their dependence on the composition and variables such as pH or temperature. Some of the techniques most frequently used are electron microscopy, nuclear magnetic resonance, spectroscopy, rheology, electrical conductivity, and radiation scattering.

Scattering techniques involve X-rays, neutrons and light and provide information about droplet size and internal structure. These techniques rely on the application of an incident beam of radiation to the sample, and subsequent registration of the intensity and the angle of the scattered beam. The scattering originates when the radiation interacts with regions of different refractive index (light scattering), electron density (X-ray scattering) or nuclear composition (neutron scattering) [18, 35]. Dynamic light scattering (DLS), quasielastic light scattering and photon correlation spectroscopy have been applied to analyze microemulsion droplet size via determination of the hydrodynamic radius, which can be estimated from measurements of the diffusion coefficients of the dispersed phase (droplets) undergoing Brownian motion. The equipment employed in this technique is rather simple and experimental times are very short [36, 37]. In the small-angle x-ray scattering (SAXS) technique, the scattering profile at low angles is fitted to adequate models to obtain reports about the shape, size and nanostructure of scattering elements; in the case of microemulsions, angles must be lower than 10, because of the scale of study is > 10 nm [18].

Comprehensive information about characterization techniques can be found in recent reviews [18, 35] and the interested reader is referred to them for details.

2. Microemulsions as drug delivery systems

2.1. Application Field

In the past 20 years, oil-in-water and water-in-oil microemulsions have been intensive1y evaluated as carriers for drug delivery [37]. Microemulsion systems allow sustained or controlled release of drugs for transdermal, topical, oral, nasal, ocular, parenteral and other administration routes [5]. The shelf-life of the microemulsions largely depends on their thermodynamic stability and the chemical stability of the drug in the dispersed phase. Stability in a wide range of temperatures, viscosity sufficiently low to allow filtration sterilization, acceptance of a variety of additives, removal from the application site or c1earance from the body are key factors for the use of these systems in the pharmaceutical field [38, 39]. Most research has focused on applications related to skin therapy and transdermal delivery [40, 41] due to concerns raised by the use of so large amounts of surfactants. Nevertheless, if the composition is adequately tuned, microemulsions can offer important advantages for systemic delivery of drugs.

Since microemulsions possess both polar and non-polar microdomains, it is possible to solubilize both hydrophilic and lipophilic drugs in these microscopically heterogeneous but macroscopically homogeneous systems [27, 42-44]. Their large content in surfactants al so contributes to the solubilization capability of the microemulsions. In fact, microemulsions can increase up to several orders of magnitude the apparent solubility of drugs considered of Class II of the Biopharmaceutical Classification System (BCS), notably enhancing their oral bioavailability [20, 45, 46]. Encapsulation in the droplets also protects the drug from adverse environments, improving the stability against hydrolytic and oxidative processes. Additionally, since the small droplets can rapidly extend on epithelia (including the

gastrointestinal one) and the surfactants can increase cell permeability, microemulsions have been shown to favor the permeability/absorption of a variety of drugs, inc1uding peptides and proteins [47]. Accumulation in intestinal lymph and absorption via the lymphatic route can also play a role in the oral absorption of drugs encapsulated in lipid droplets [48].

Regarding parenteral delivery, o/w microemulsions have been largely tested as depots of hydrophobic drugs when administered subcutaneously or intramuscularly. A similar role can be played by w/o microemulsions encapsulating hydrophilic drugs. Intravenous administration of hydrophobic antitumoral, antimicrobial and antiinflamatory drugs has been shown to be also notably facilitated when formulated as o/w microemulsions [49]. Microemulsions enable the preparation of liquid formulations (with the drug totally solubilized in the droplets) avoiding the use of ethoxylated castor oil (Cremophor® EL) which is commonly employed to prepare injectable solutions of hydrophobic therapeutic agents. Additionally, if PEGylated surfactants are used, microemulsions show high physical stability in plasma and great resistance to leaching of drugs through the oil phase, which in turn facilitates a sustained re1ease of the drug in the blood stream [50-52]. The small size of the microemulsion droplets ensures that the probability of emboli formation in the blood is insignificant and, if the surface is neutral or anionic, a prolonged blood circulation time can be achieved [49]. All together these features make microemulsions attractive for passive targeting of drugs towards tissues showing enhanced capillary permeability, such as those affected by inflammatory, infectious or tumoral processes. Sizes between 10 and 100 nm (which are the typical ones of microemulsion droplets) have been pointed out as the optimum for exploiting the enhanced permeability and retention (EPR) effect [53]. Selective extravasation of drug- loaded droplets can allow for greater accumulation of the drug in the sites of the body where it is required. Therefore, therapeutic efficacy is improved while total systemic dose can be notably diminished [5]. For example, phospholipid- based microemulsions loaded with all-trans-retinoic acid have been proved to be suitable for treatment of patients with acute promyelocytic leukemia [54]. Although much less investigated than other nanocarriers, microemulsion droplets could be al so susceptible of decoration with ligands able to recognize specific cells, enabling active targeting as recently demonstrated for nanoemulsions [55]. Moreover, microemulsions droplets are suitable starting cores for functionalization with diagnostic agents (magnetic resonance imaging (MRI), X-ray computed tomography (CT) imaging) and may lead to theranostic systems [56].

Drug release rate from microemulsions largely depends on the composition of each specific formulation and on the environmental conditions (e.g., pH, stirring, ionic strength, enzymes). In o/w microemulsions, the oily droplets behave as cores and the lipophilic drugs have to cross the surfactant layer (which may behave as an additional, intermediate phase) and then diffuse towards the aqueous barrier and partition into it [57]. The strength of the interactions between microemulsion components has been shown to play a role in the release rate of ketoprofen and octylmethoxycinnamte [58, 59]. For drugs showing poor affinity for the aqueous environment, drug solubility in the aqueous phase may become the rate limiting step of the release process. If surfactant species are present in the release medium (as occurs when microemulsions are orally administered), interaction of the drug with the surfactants may also play a relevant role in the release process, and profiles typical of diffusion through a thick membrane can be obtained [60, 61]. Temperature has been shown to notably affect the release rate of Vitamins C and E from isopropyl myristate, Tween 80 and Imwitor 308 o/w microemulsions [62] and from isopropyl myristate, Span 20, Tween 20 microemulsions which also incorporated Pluronic FI27 in the aqueous phase [63].

2.2. Microemulsions for Colorectal Cancer Treatments

In the last two decades intensive research has been focused on the formulation of different anticancer drugs as microemulsions. Paclitaxel (PTX), methotrexate (MTX), curcumin (CUC), and doxorubicin (DOX) have been shown to be soluble in a variety of oils (essential and edible) and also to be incorporated with high encapsulation yield in microemulsions [64-76]. These systems have been tested in different cell lines such as breast, cervical, colon and colorectal cancer cells. In some reports it has been noted that blank microemulsions themselves have certain activity against the cancer cells. Thus, drug-loaded microemulsions inhibit cell growth with lower dose of the chemotherapeutic agent. Relevant microemulsions loaded with anticancer drugs that have been successfully employed either in *in vitro* or *in vivo* studies are reported in Table 2.

Table 2. Relevant microemulsion systems loaded with different drugs and tested *in vitro* or *in vivo* for the treatment of several types of cancer.

Surfactant/Ce-surfactant	Oil phase	Drug	*In vitro* studies	*In vivo* studies	Ref.
PEG-20 glycerol monooleate[1]	Pelemol©	CUC	-	Human	[64]
Lecithin/butanol[1] Capmul[1]	My vacet oil	PTX	MDA-M231 breast cancer cells		[65]
Tween 80/isopropanol[2] Tween 80/propylenglycol[2]	Isopropylmyristate Ethyl oleate	Cynanine IR-768	Human red blood cells. MCF-7/WT and MCF-7/DX breast cancer cells		[66]
Tween 80/transcutol[2]	Capmul MCM	Melphalan	Human cervical (HeLa)cancer	Mice	[67]
Lecithin/bencil alcohol[1]	Decanol	MTX	Hairless mouse skin	-	[68]
Labrasol/Plurol isostearique[2,4]	Ethyl oleate	MTX	-	Pig	[69]
Tween80/lecithin[2,3]	Soybean oil	CUC	Oral squamous cell (carcinoma cell lines)	-	[70]
Cremophor EL/Transcutol[2]	Capryol 90	Docetaxel	Caco 2 cells	Rats	[71]
Cremophor EL/Span 80/isopropyl alcohol[1]	Corn oil	MTX	MCF-7 cellline, DU-145 cell line and OVCAR cell line	-	[72]
Polysorbate 80/capmul[2]	My vacet oil	PTX	lntestine rats	Rats	[73]
Egg phosphatidylcholine Cremophor EL[2]	Cremophor EL/pluronic F68/alcohol	PTX	-	Hypersensivity (in pigs) and pharmacokinetic profile (in rats)	[74]
TPGS/lsopropyl alcohol[2]	IPM/Oleic acid	Temozolomide acid hexyl ester	Rat skin	-	[75]
Soya phosphatidylcholine/ Eumulgin HRE 40/ sodium oleate[2]	Cholesterol	DOX	-	-	[76]

As can be seen in Table 2 most of the surfactants are quite biocompatible (e.g., polysorbates, soya derivatives and lecithin) and may partially or even totally replace Cremophor EL.

Compared to other cancer cells, the studies about the use of microemulsions (loaded or non-loaded with anticancer drugs) for inhibiting the growth of colorectal cancer cells is much limited. Reports in the literature are still scarce. Microemulsions based on 37% Cremophor EL/transcutol mixture 2:1, 29% Capryol 90, and 34% water were loaded with docetaxel and tested in colon cancer cells (HCT-116), [77] Droplets without drug (blank microemulsion) had sizes in the 89±25 nm range, while after loading the size increased up to 205± 13 nm, All microemulsions showed high cytotoxic effects on colon cancer cells compared with the reference Taxotere, but caused less detrimental effects on non-tumoral human epithelial cells. Therefore, docetaxel-loaded microemulsions showed improved therapeutic efficacy and safety [78]. In other work, microemulsions were prepared combining Tween 80, Span 20, ethanol, isopropyl myristate and water and loaded with gemcitabine [78], a chemotherapeutic agent effective against many solid tumors and also employed in colorectal cancer treatment [79]. Drug-loaded microemulsions had high hemolysis activity (17-21 %) although below that exhibited by a drug solution of similar concentration (37%). Blank microemulsions showed a relevant cytotoxic activity (30-50% growth inhibition) against HCT-116 colon cancer cells. Moreover, cells treated with drug-loaded microemulsions showed more intense apoptosis than those exposed to the drug solution [78].

Microemulsions have been also shown to be particularly suitable to encapsulate plant extracts whose antitumoral activity is limited by a poor aqueous solubility [80, 81]. For example, *Flammulina velutipes* sterol (active against gastric and colon cancer cells) was encapsulated in a microemulsion prepared with 0.3% medium chain triglycerides, 5.0% ethanol, 21.0% Cremophor EL and 71.0% water, leading to an apparent solubility of the sterol of three orders of magnitude higher with respect to the solubility in water [80]. lnterestingly, the microemulsion exhibited a good physical stability and no loss of drug was observed in the first 24 hours after 100-fold dilution with distilled water. When orally administered to healthy rats, microemulsions increased more than 2-fold the bioavailability of the sterol. Similarly, quercetin was formulated in a microemulsion prepared with 7% ethyl oleate, 48% Tween 80 and 45% dehydrated ethanol (co-surfactant) [82]. Compared to quercertin-loaded micelles (without ethyl oleate), the microemulsion notably enhanced intestinal absorption of the drug. lnterestingly, site-specific absorption of the drug- loaded emulsion was observed *in vivo;* the uptake percentage being greater in colon followed by ileum. These findings can be related to the thinner mucous layer of the colon and to the predominance of M cells and lymphatic vessels of ileum.

3. Formulation of methotrexate microemulsions

In the last part of this paper, a case study of formulation of MTX in microemulsions is presented. The aim was to investigate the suitability of essential oils for the preparation of o/w microemulsions for the delivery of methotrexate and to elucidate the ability to inhibit cancer cell growth.

3.1. Materials

Balb 3T3 done A31 mouse embryonic fibroblast cells (CCL-163) and HeLa cervical cancer cells (CCL-2) from American Type Culture Collection (USA). DMEM F12-HAM, RPMI 1640 with and without folic acid, D-α- tocopherol polyethylene glycol 1000 succinate (TPGS- 1000), 2-methyl-1-propanol (isobutanol), D-L-α-tocopherol (vitamin E) and trans-anethole from Sigma-Aldrich (Germany); mentha piperita (peppermint) essential oil (menthol main component) from Eladiet (Spain); penicillin/ streptomycin from Hyclone Thermo Scientific (UK), fetal bovine serum (FBS) from Biowest (France), Maxemul 6112 from Croda (UK), Noigen RN-20 from Dai-Ichi Kogyo Seiyaku (Japan), methotrexate (MTX) from AK Scientific (USA), and MTT proliferation Kit from Roche (Germany). Ultrapure MilliQ water was obtained using a Millipore equipment (Spain). Other reagents were analytical grade.

3.2. Methods

3.2.1. Preparation of'o/w Microemutsions

Solutions of Maxemul 6112 (10% w/w; anionic surfactant) and TPGS-1000 (10% w/w; non-ionic surfactant) were prepared separately in phosphate buffer pH 7.4 (PBS). Then, isobutanol was added dropwise and magnetic stirring was maintained until the solutions became transparent again. In the case of MTX-loaded microemulsions, fue drug was first dissolved in isobutanol. Following, oil phase was added (compositions in Table 3) and the systems were kept under magnetic stirring until the turbidity disappeared. MTX final concentration was 0.3 mg/mL in microemulsions prepared with D-L-α-tocopherol (vitamin E) and 0.6 mg/mL in microemulsions with trans-anethole or menthol. Additionally, mixtures (50/50 w/w) of Maxemul 6112 and Noigen RN-20 (non-ionic surfactant) with TPGS-1000 were also tested to prepare the microemulsions.

Table 3. Composition of microemulsions (in percentage in the final formulation). MTX-loaded microemulsions were identified with the same codes as placebo but ending in b (e.g., l3b, 14b, 15b, 16b, 17b, 1 9b, 20b, 21 b, 22b, 23b, 24b

Code	PBS	TPGS-1000	Maxemul 6112	Noigen RN-20	lsobutanol	Menthol	Trans-anethole	α- Tocopherol
13	81.60	9.07	---	---	8.61	0.73	--	---
14	79.89	8.98	---	---	8.43	2.80	--	---
15	80.68	8.96	---	---	8.52	---	--	1.79
16	81.73	8.17	---	0.91	8.63	0.56	--	---
17	79.47	7.95	---	0.88	8.39	---	3.31	---
19	79.89	---	8.88	---	8.43	2.80	---	---
20	81.10	---	9.01	---	8.56	---	1.33	---
21	80.34	---	8.93	---	8.48	---	--	2.26
22	81.71	4.54	4.54	---	8.62	0.54	--	---
23	81.01	4.50	4.50	---	8.55	---	1.44	---
24	80.35	4.46	4.46	---	8.48	---	--	2.21

3.2.2. Proliferation Cell Assay

Balb 3T3 were cultured in DMEM F12-HAM medium containing 10% FBS and 1% penicillin (10,000 units/mL)/ streptomycin (10,000 ug/ml.) solution, and HeLa cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 1 % antibiotics solution. Both cell lines were seeded in 96-well plates at a density of 200,000 cells/mL (100 µL) in RPMI 1640 without folic acid and supplemented with 10% FBS and 1 % antibiotics solution, and incubated for 24 hours at 37°C, 5% CO_2 and 90% relative humidity. Aliquots of microemulsions (100 µL) were added to each well. Plates were incubated during 24 and 48 hours. All experiments were carried out in triplicate.

Components of the microemulsions were separately dispersed phosphate buffered saline (PBS, Sigma-Aldrich) and tested at the same concentration as in the formulations. To do that, 2 µl of component dispersion was added to each well and incubated as indicated above. After 24 and 48 h of exposition, cell proliferation was measured using a MTT Kit (Roche, Gennany), following the manufacturer instructions. Absorbance was measured at a wavelength of 550 nm using an ELISA plate reader (BJORAD Model 680 Microplate Reader, USA).

3.3. Results and Discussion

3.3.1. Preparation of microemulsions

Several essential oils were investigated for the preparation of MTX-loaded o/w microemulsions. Peppermint oil (having menthol as main component) has been tested in the preparation of microemulsions [83, 84] loaded with celecoxib [85] or other anti-inflammatory drugs such as aceclofenac and diclofenac [86]. On the other hand, trans-anethole (1-methoxy-4-(l E)-1-propenylbenzene) is the major component (>99 %) of the naturally occurring essential oils of fennel and star anise, and is also present in dill, basil, and tarragon plants. It is widely used as an aniseed flavoring agent in a variety of confectioneries and beverages. Also, it has been used as an important ingredient of herbal medicines for centuries. Further, it has been shown that 1 mM trans-anethole causes death of MCF-7 human breast cancer cells after three days exposition [87]. Just recently, liposomal formulations of trans-anethole have been reported to be eight to nine times more cytotoxic than free trans- anethole in T47D and MCF-7 breast cancer cell lines. Therefore, the use of trans-anethole as oil carrier of antineoplastic agents may strengthen the cytotoxic

effects. Finally, a -tocopherol and its derivatives have been pointed out to also have antitumor and anti-inflammatory activity [88].

TPGS-1000 is a biocompatible surfactant widely used as solubilizer and emulsifier. It may provide microemulsion droplets with prolonged circulation half-life and improved tumor accumulation, as previously observed for related surfactants such as distearoylphosphatidylethanolamine (DSPE)-PEG 2000 or 5000 [89]. Furthermore, it has been reported that TPGS inhibits P-glycoprotein efflux pump increasing oral absorption and cell accumulation of a variety of drugs, including antitumoral drugs [90-92]. Other surfactant tested was Maxemul 6112, an anionic phosphate ester (oleyl alcohol ethoxylate phosphate, concentration between 50 and 100 % w/w) with a polymerizable double bond in its main hydrocarbon chain, which may allow to form a stimuli responsive polymeric shell coating the drug-loaded oleic core. Similarly Noigen RN-20 (polyoxyethylene 4-nonyl-2-propylene-phenol) is a nonionic polymerizable surfactant (HLB 15.4), which has been previously shown useful for the stabilization of non-aqueous emulsions [93].

As a first step, the phase diagrams of ternary systems combining each essential oil, water and a mixture of TPGS- 1000 (surfactant) and isobutanol (co-surfactant) were obtained (Fig. 3). For the three essential oils tested, proportions of surfactant and co-surfactant above 10% were required. Microemulsions with peppermint oil (menthol) only formed when the oil was at very low proportion, which may represent a limitation for the incorporation of hydrophobic drugs. Microemulsions prepared with the compositions disclosed in Table 3 were proven to be transparent and homogeneous. Drug-loaded formulations were yellow due to drug color.

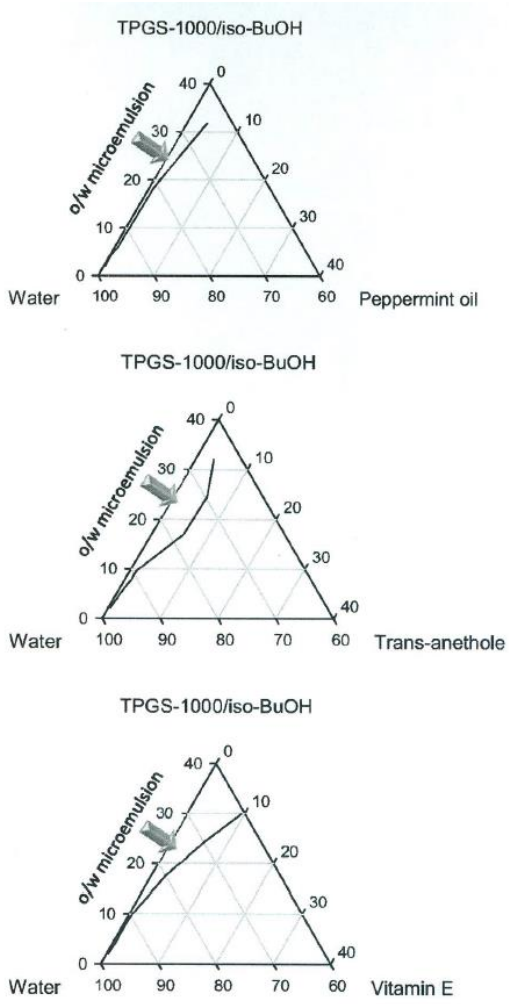

Fig. (3). Partial phase diagrams of ternary systems combining an essential oil, water and a mixture of TPGS-1000 (surfactant) and isobutanol (iso-BuOH, co-surfactant).

3.3.2. Proliferation Cell Assay

Microemulsions were tested regarding cytocompatibility using murine fibroblasts (Balb 3T3 cells), and regarding bioactivity using adenocarnicoma cells (HeLa cells) (Fig. 4). In the case of fibroblast cells, drug loaded and non-loaded microemulsions prepared with a -tocopherol significantly increased cell proliferation, both in the tests carried out with 100 µl of microemulsion. Formulations with menthol and trans-anethole proved to be less cytocompatible, as expected from the previously reported data about cytotoxicity of these essential oils [87]. No relevant differences were observed between microemulsions with and without MTX. As observed with fibroblast cells, microemulsions were bioactive against tumor cells except those prepared with α- tocopherol. Interestingly, menthol and trans-anethole microemulsions led to similar cytotoxicity leve\s.

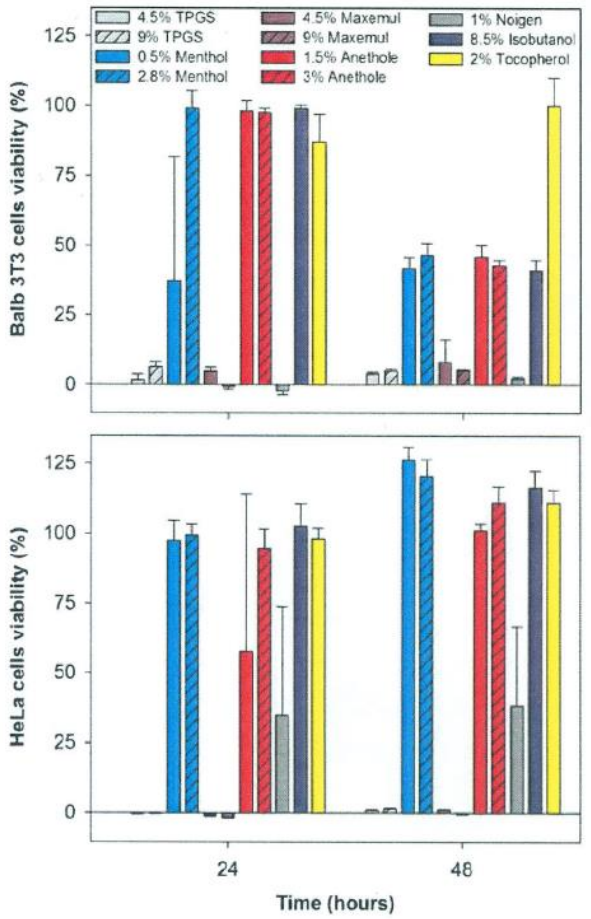

Fig. (4). Proliferation (in percentage) of murine fibroblasts (Balb 3T3 cells) and adenocamicoma cells (HeLa cells) after 24 and 48 hours of incubation with 100 µl, of each microemulsion. Codes with "b" refer to MTX loaded formulations (compositions in Table 3). Blue, yellow and red bars identify microemulsions baving mentbol, α-tocopherol or trans-anethole as oily pbase, respectively.

To gain further insight into the origin of the cytotoxic effects of the microemulsions, each component was tested in separate at the same concentration as it is present in the formulations. Isolated surfactants, particularly TPGS and Maxemul, were found to be extreme\y toxic for both fibroblasts and tumor cells (Fig. 5). It is known that surfactants exhibit concentration-dependent effects on cell viability and, in the absence of oils, TPGS and Maxemul unimers and micelles can readily interact with cell membranes and intracellular enzymes [94]. According to the suppliers, the oral LD_{50} (for rats) values of TPGS-I000, Maxemul 6112 and Noigen RN-20 are >7000, 1530 and 2500 mg/kg, respectively. Menthol and trans-

anethole led to detrimental effects on fibroblast viability after 48 h of incubation, but these essential oils did not significantly alter the viability of tumor cells. As expected, n-tocopherol showed an excellent cytocompatibility with fibroblasts and tumor cells. The proliferative effect of n-tocopherol explains its capability to counteract the toxicity of the surfactants.

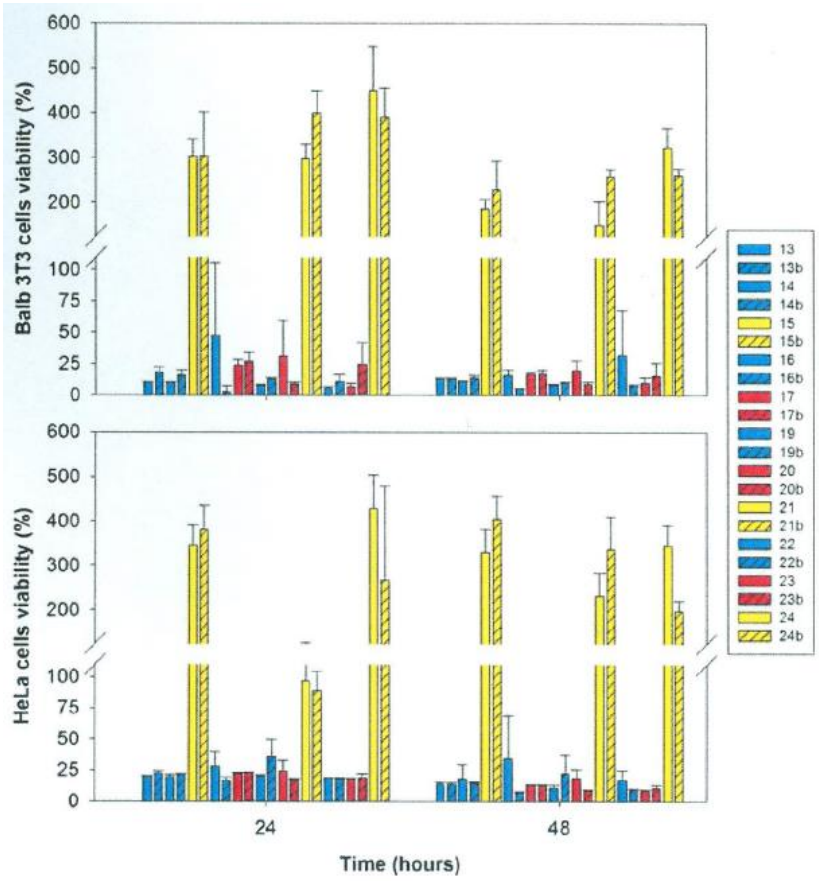

Fig. (5). Proliferation (in percentage) of Balb 3T3 cells and HeLa cells after 24 and 48 hours of incubation with each microemulsion component (2 µL dispersion in PBS) in separate.

4. Conclusions

Although the literature on microemulsions as carriers of antitumor agents is still incipient, recent papers have pointed out their suitability not only for topical or mucosal application but also for systemic delivery. Versatile preparation, high drug loading and droplet size adequate for drug targeting are the most relevant advantages of microemulsíons compared to other nanocarriers. However, concerns about the toxicity of the surfactants still prevent from the systemic use. The MTX formulation study points out that in addition to surfactants, the essential oils used as internal phase of o/w microemulsions may notably determine the cytocompatibility. Blank microemulsions prepared with menthol and trans-anethole inhibit cell proliferation as much as those loaded with methotrexate. By contrast, n-tocopherol leads to microemulsions that strongly promote proliferation of both fibroblasts and adenocarcinoma cells, being totally inadequate for cancer treatment. Therefore, much research is still required for identifying suitable oily components that enable a good balance between compatibility with healthy cells and growth inhibition of cancer cells.

Conflict of interest
The authors have no conflicts of interest.

Acknowledgements
Work supported by CONACYT (grant No. CB-2011/ 168472) Mexico, MINECO (SAF2014-52632-R) Spain, FEDER, and the "Galician Network for Colorectal Cancer Research (REGICC)" (Ref. R2014/039) funded by the Xunta de Galicia. S.E. Flores acknowledges a fellowship from CONACYT, Mexico, to pursue his Doctoral studies at CIQA.

References

[1] Hoar, J.H.; Schulman, J.H. Transparent water-in-oil dispersions: the oleopathic hydro-micelle. *Nature,* 1943,152,102-103.
[2] Lindman, B.; Danielsson, L The definition of microemulsion. *Colloids Surf A,* 1981, 3, 391-392.
[3] Burguera, J.L; Burguera, M. Analytical applications of emulsions and microemulsions. *Talanta,* 2012,96,11-20.
[4] Sharma, M.K.; Shah, D.O. In: *Macro- and microemulsions: theory and applications;* Shah, D.O., Ed.; American Chemical Society: Washington O.C., 1985, pp. 1-18.
[5] Fanun, M. Microemulsions as delivery systems. *Curro Opino Colloid Interf Sci.,* 2012, 17,306-313.
[6] Ganta, S.; Talekar, M.; Singh. A.; Coleman, T.P.; Amiji. M.M.
Nanoemulsions in translational research- Opportunities and challenges in targeted cancer therapy. *AAPS Pharm. Sci. Tech.,* 2014,15,694-708.
[7] Bonacucina, G.; Cespi, M.; Misici-Falzi, M.; Pamieri, G.F. Colloidal soft matter as drug delivery system, *J. Pharm. Sci., 2009,* 98,1-42.
[8] Heuschkel, S.; Goebel, A.; Neubert, RH. Microemulsion: modem colloidal carrier for dermal and transdermal drug delivery. *J. Pharm. Sci.,* 2008, 97, 603-631.
[9] Kogan, A.; Shalev, O.E.; Raviv, U.; Aserin, A.; Garti, N. Formation and characterization of ordered bicontinuous rnicroemulsions. *J. Phys. Chem. B,* 2009,113,10669-10678.
[10] Garti, N.; Amar- Yuli, L; Spemath, A.; Hoffman, RE. Transitions and loci of solubilization of nutraceuticals in U-type nonionic microemulsions studied by self-diffusion NMR *Phys. Chem. Chem. Phys.,* 2004, 6,2968-2976.
[11] Kalaitzaki, A; Xenakis, A.; Papadimitriou, V. Highly water dilutable microemulsions- a structural study. *Colloid Polym. Sci* . 2015,293,1111-1119.
[12] Najjar, R. In: *Microemulsions - An introduction to properties and applications;* Najjar, R., Ed.; Intech. 2012, pp. 1-30. Available from: http://www.intechopen.com/books/microemulsions-an- introduction-to-properties-and-applications/microemulsions-a- brief-introduction.
[13] Eastoe, J.; Dong, J.; Hetherington, KJ.; Steytler, D.C.; Heenan, R.KJ. Structure in microemulsions of di-chained surfactants. *J. Chem. Soco Faraday Trans.,* 1996, 92, 65-72.
[14] Israelachvili, J.N.; Mitchell, DJ.; Ninham, B.W. Theory of self- assembly of hydrocarbon amphiphiles into micelles and bilayers. *J. Chem. Soco Faraday Trans.,* 1976,2, 1525-1568.
[15] Lawrence, M.J.; Rees, G.D. Microemulsion-based media as novel drug delivery systems. *Adv. Drug Deliv. Rev* .. 2000, 45, 89-121.
[16] Lopez, F.; Cinellia, G.; Ambrosone, L.; Colafemmina, G.; Ceglie, A.; Palazzo, G. Role of the cosurfactant in water-in-oil microemulsion: interfacial properties tune the enzymatic activity of lipase. *Colloid Surf. A.,* 2004, 237, 49-59.
[17] De Gennes, P.G.; Taupin, C. Microemulsions and the flexibility of oil/water interfaces. *J Phys. Chem.,* 1982,6,2294-2304.
[18] Syed, H.K.; Peh, K.K. Identification of phases of various oil, surfactantlco-surfactants and water system by ternary phase diagram. *Ae/a Polo Pharm. Drug Res.,* 2014, 71,301-309.
[19] Acharya, D.P.; Hartley, P.G. Progress in microemulsion characterization. *Cun·. Opino Coll.Lnterf. Sci.,* 2012, /7,274-280.
[20] Constantinides, P.P. Lipid microemulsions for improving drug dissolution and oral absorption: physicaJ and biopharmaceuticals aspects. *Pharm. Res.,* 1995, 12,1561-1572
[21] Mukhopadhyay, L.; Mitra, N.; Bhattacharya, P.K.; Moulik, S.P. Thermodynamics of formation of biological microemulsion with cinnamic alcohol, AOT, Tween-20 and water and kinetics of alkaline fading of crystal violet in them. *J Colloid Interf. Sci.,* 1997, 186, 1-8.
[22] Acharya, A.; Moulik, S.P.; Sanyal, S.K.; Mishra, B.K.; Puri, P.M. Physicochemical investigations of microemulsification of coconout oil and water using polyoxyethylene 2-etyl ether (Brij-52) and isopropanol or ethanol. *J. Colloid Interf Sci.* 2002,245, 163-170.
[23] Majhi, P.; Moulik, S.P. PhysicochenúcaJ studies on biologicaJ macro and microemulsions. Mixing behavior of eucalyptus oil, water and polyoxyethylene sorbitan monolaurate (Tween 20) assisted by n-butanol and cinnamic alcohol. *J. Disp. Sei. Technol.,* 1999,20,1407-1427.
[24] Gupta, S.; Sanyal, S.K.; Datta, S.; Moulik, S.P. Preparation of prospective plant oil derived microemulsions vehic1es for drug delivery. *Ind. J. Biochem. Biophys.,* 2006,43,254-257.

[25] Von Corswant, C,; Engstrom, S.; Soderman, O. Microemulsions based on soybean phosphatidy1choline and triglycerides phase behavior and microstructure. *Langmuir,* 1997, 13,5061-5071.
[26] Von Corswant, e.; Thoren, P.; Engstrom, S. Triglyceride based microemulsion for intravenous administration of sparingly soluble substances. *J. Pharm. Sei.,* 1998, 87, 200-208.
[27] Kahlweit, M.; Busse, G.; Faulhaber, B. Preparation nontoxic microemulsions with alkyl monoglucosides and the role of alkane diols as cosolvents. *Langmuir,* 1996, 12,861-862.
[28] Kralova, l.; Sjüblom, J. Surfactants used in food industry: a review. *J Disper. Sci. Tech* .. 2009,30, 1363-1383.
[29] Rosen, M.J. *Surfactants and interfacial phenomena,* 3,d ed., John Wiley and Sons: New Jersey, 2004.
[30] Attwood, D.; Florence, A.T. In: *Surfactant Systems: Their chemistry, pharmacy and biology;* Attwood, D.; Florence, A.T., Ed.; Springer: New York, 1983; pp. 388-467.
[31] Myers, D. *Surfactant science and technology surfactant science,* 3rd ed .. John Wiley and Sons: New Jersey, 2005.
[32] Saito, H.K.S. The stability of o/w type emulsions as functions of temperature and the HLB of emulsifiers: the emulsification by PIT- method. *J Colloid Interf. Sci., 1969,30,258-263.*
[33] Robb, LD. *Specialist surfactants,* 1st ed., Blackie Academic and Professional: London, 1997.
[34] Singh, V.; Bushettii, S.S.; Raju, A.S.; Ahmad, R.; Singh, M.; Bisht, A. Microemulsions as promising delivery systems: a review. *Ind. J. Phm. Ed. Res.,* 2011, 45,392-401.
[35] Gradzielski, M. Recent developments in the characterization of microemulsions. *Curr. Opino Coll. Interf. Sci.,* 2008, 13,263-269.
[36] Silva, EJ.; Zaniquelli, M.E.D.; Loh, W. Light-scattering investigation on microemulsion formation in mixtures of diesel oil (or hydrocarhons) plus ethanol plus additives. *Energy Fuel. 2007,* 21, 222-226.
[37] Lemyre, J.L.; Lamarre, S.; Beaupre, A.; Ritcey, A.M. A new approach for the characterization of reverse micellar systems by dynamic light scanering. *Langmuir,* 2010, 26, 10524-10531.
[38] Gupta, S.; Moulik, S.P. Biocompatible microemulsions and their prospective uses in drug delivery. *J. Pharm. Sei.,* 2008, 97,22-45.
[39] Bagwe, R.P.; Kanicky, J.R.; Palla, BJ.; Patanjali, P.K.; Shah, D.O. lmproved drug delivery using microemulsions: rationaJe, recent progress and new horizons. *Crit. Rev. Ther. Drug Carr. Syst., 2001,* 18,77-140.
[40] Paolino, D.; Ventura, C.A.; Nistico, S.; Puglisi, G.; Fresta, M. Lecithin microemulsions for the topical administration of ketoprofen: Percutaneous adsorption through human skin and *in vivo* human skin tolerability. *Int. J. Pharm.,* 2002,244,21-31.
[41] Rhee, Y.S.; Choi, 1. G.; Park, E.S.; ehi, s.e.c. Transdermal delivery of ketoprofen using microemulsions. *Int. J. Pharm., 2001,* 228, 161-170.
[42] Watnasirichikul, S.S.; Davies, N.M.; Rades, T.; Tucker, LG. Preparation of biodegradable insulin nanocapsules from biocompatible microemulsions. *Pharm. Res.,* 2002, 17, 684-689.
[43] Lawrence, M.J. Surfactant systems: their use in drug delivery. *Chem. Soc. Rev.,* 1994,23,417-424.
[44] Cortesi, R.; Nastruzzi, C. Liposomes, micelles and microemulsions as a new delivery system for cytoioxic alkaloids. *Pharm. Sei. Technol. Today.* 1999,2,288-298.
[45] Kaur, G.; Mehta, S.K.; Kumar, S.; Bhanjana, G.; Dilbaghi, N Coencapsulation of hydrophobic and hydrophilic antituberculosis drugs in synergistic Brij 96 microemulsions: a biophysical characterization. *J. Pharm. Sci.,* 2015, *104, 2203-2212.*
[46] Araya, H.; Tomita, M.; Hayashi, M. The novel formulation design of O/W microemulsion for improving the gastrointestinal absorption of poorly water soluble compounds. *Int. J. Pharm.,* 2005,305,61-74.
[47] Gao, Z.G.; Choi H.G.; Shin, H.J.; Park, K.M.; Lim SJ.; Hwang KJ.; Kim C.K. Physicochemical characterisation and evaluation of a microemulsion system for oral delivery of cyc1osporin A. *Int. J. Pharm.,* 1998, 161,75-86.
[48] Shantha Kumar, T .R.; Chawla, S.; Nachaegari, S.K.; Singh, S.K.; Srinivas, N.R. Validated HPLC analytical method with programmed wavelength UV detection for simultaneous determination of DRF-4367 and phenol red in rat in situ intestinal perfusion study.1. *Pharm. Biomed. Anal.,* 2005,38,173-179.
[49] Date, *AA.;* Nagarsenker, M.S. Parenteral microemulsions: an overview. *Inl. J. Pharm.,* 2008, 355,19-30.
[50] Jha, S.K.; Dey, S.; Karki, R. Microemulsions: potential canier for improved drug delivery. *Asian J. Biomed. Phann. Sci.,* 2011, 1, 5-9.
[51] Park, K.M.; Kim, C.K. Preparation and evaJuation of flurbiprofen- loaded microemulsion for parenteraJ delivery. *Int. J. Pharm. 1999,* 181,173-179.
[52] Brime, B.; Moreno, M.A.; Frutos, G.; Ballesteros, M.P.; Frutos, P. Amphotericin B in oil-water lecithin-based microemulsions: Formulation and toxicity evaluation. *J. Pharm. Sci..* 2002, 91, 1178-1185.
[53] Darthier, F.; Feron, O.; Préat, V. To exploit the tumor microenvironment: passive and active tumor targeting of nanocarriers for anti-cancer drug delivery. *J. Control. Release,* 2010,148,135-146.
[54] Hwang, K.J.; Lim, S.J.; Park, J.S.; Kim, C.K. Phospholipid-based microemulsion formulation of all-trans-retinoic acid for parenteral adnúnistration. *Int. J. Pharm.,* 2004,276, 175-183.
[55] Ganta, S.; TaJekar M.; Singh, A.; Coleman, T.P.; Amiji, M.M. Nanoemulsions in translational research. Opportunities and challenges in targeted cancer therapy. *AAPS Pharm.* Sci. *Tech.,* 2014, 15, 694-708.
[56] Liu, F.; He, X.; Lei, Z.; Liu, L.; Zbang, J.; You, H.; Zhang, H.; Wang, Z. Facile preparation of doxorubicin-loaded upconversion@polydopamine nanoplatforms for simultaneous *in vivo* multimodality imaging and chemophotothermal synergistic therapy. *Adv. Healthc. Mater.,* 2015, 4, 559-568.
[57] Friedman, D.; Benita, S. A mathematical model for drug release from o/w emulsion: Application to controlled release morphine emulsion. *Drug Dev. Ind. Pharm.,* 1987, 13, 2067-2085.
[58] Podlogar, F.; Bester Rogac, M.; Gasperlin, M. The effect of internal structure of selected water- Tween 40-lmwilor 308-IPM microemulsions on ketoprofene release. *Int. J. Pharm.,* 2005, *302,* 68-71.

[59] Montenegro, L.; Carbone, C.; Puglisi, G. Vehicle effects on *in vitro* release and skin permeation of octylmethoxycinnamate from microemulsions. *Int. J. Pharm.,* 2011, *405, 162-168.*
[60] Grassi, M.; Coceani, N.; Magarotto, L. Mathematical modelling of drug release from microemulsions: Theory in comparison with experiment. *J. Colloid Interf. Sci.,* 2000,228,141-150.
[61] Sirotti C, Coceani N, Colombo 1, Lapasin R, Grassi M. Modeling of drug release from microernulsions: A peculiar case. *J. Membr. Sci.,* 2002, *204, 401-412.*
[62] Rozman, B.; Zvonar, A.; Falson, F.; Gasperlin, M. Temperature- sensitive microemulsion gel: an effective topical delivery system for simultaneous delivery of vitamins C and E. *AAPS Pharm. Sci. Tech..* 2009, *10,54-61.*
[63] Zhao, X.Y.; Xu, J.; Zheng, L.Q.; Li, X.W. Preparation of temperature-sensitive microemulsion-based gels formed from a triblock copolymer. *Colloid Surf. A, 2007,307,100-107.*
[64] Teichmann, A.; Heuschkel, S.; Jacobi, U.; Presse, G.; Neubert, R.; Sterry, W.; Lademann, J. Comparison of stratum comeum penetration and localization of a lipophilic model drug applied in an o/w microemulsion and an amphiphilic cream. *Eur. J. Pharm. Biopharm.,* 2007, 67, 699-706.
[65] Nornoo, A.O.; Chow, D.S. Cremophor-free intravenous m icroemulsions for paclitaxel II. Stability, *in vitro* release and pharmacokinetics. *Int. J. Pharm.,* 2008,349, 117-123.
[66] Pietkiewicz, J.; Zieliáska, K.; Saczko, 1.; Kulbacka, J.; Majkowski, M.; Wilk, K..A. New approach to hydrophobic cyanine-type photosensitizer delivery using polymeric oil-cored nanocarriers: hemolytic activity, *in vitro* cytotoxicity and localization in cancer cells. *Eur. J. Pharm. Sei.,* 2010, 39, 322-335.
[67] Rajpoot, P.; Bali, V.; Pathak, K. Anticancer efficacy, tissue distnbution and blood pharmacokinetics of surface modified nanocarrier containing melphalan. *Int. J. Pharm.,* 2012, 426, 219- 230.
[68] Trotta, M.; Pattarino, F.; Gasco, M.R. Influence of counter ions on the skin permeation of methotrexate from water-oil microemulsions. *Pharm. Acta Helvetiae,* 1996, 71, 135-140.
[69] Alvarez-Figueroa, M.J.; B1anco-Mendez, J. Transdermal delivery of methotrexate: iontophoretic delivery from hydrogels and passive delivery from núcroemulsions. *Int. J. Pharm.,* 2001, 215, 57-65.
[70] Lin, H.; Thomas, J.L.; Chen, H.; Yang, W. 111 *vitro* suppression of oral squamous cell carcinoma growth by ultrasound-mediated de1ivery of curcumin microemulsions, 1111. *J. Nanomed.,* 2012, 7, 941-951.
[71] Yin, Y M.; Cui, F.D.; Mu, C.F.; Choi, M.K.; Kim, J.S.; Chung, S.J.; Shim, C.K.; Kim, D.D. Docetaxel microemulsion for enhanced oral bioavailability: preparation and *in vitro* and *in vivo* evaluation, 1. *Control. Release,* 2009,140,86-94.
[72] Karasulu, H.Y.; Karabulut, B.; Goker, E.; Güneri, *T.;* Gabor, F. ControUed release of methotrexate from *w/o* microemulsion and its *in vitro* antitumor activity. *Drug Deliv.,* 2007, 14, 225-233.
[73] Nornoo, A.O.; Zheng, H.; Lopes, LB.; Johnson-Restrepo, B.; Kannan, K.; Reed, R. Oral microernulsions of paclnaxel: in situ and pharmacokinetic studies. *Eur. J. Pharm. Biopharm.,* 2009, 71, 310-317.
[74] He, L.; Wang, G.; Zhang, Q. An alternative paclitaxel microemulsion formulation: hypersensitivity evaluation and pharmacokinetic profile. *Int. J. Pharm.,* 2003, *250, 45-50.*
[75] Suppasansatorn, P.; Nirnmannit, U.; Conway, B.R.; Du, L.; Wang, Y. Microemulsions as topical delivery vehicles for the anti- melanoma prodrug, temozolomide hexyl ester (TMZA-HE). *J. Phann. Pharmacol.,* 2007, 59,787-794.
[76] Formariz, T.P.; Chiavacci, L.A.; Sarmento, V.H.; Santilli, C.V.; Tabosa do Egito, E.S.; Oliveira, A.G. Relationship between structural features and *in vitro* release of doxorubicin from biocompatible anionic microemulsion. *Colloid Surf. B.,* 2007, *60,* 28-35.
[77] Alkhatib, M.H.; Al-Qaidi, W.A. Cytotoxicity effect of docetaxel- loaded microemulsion in A549 non-small cell lung cancer and HCTlI6 colon cancer cells. *Int. J. Pharm. BioSci.,* 2014, 5, 300- 315.
[78] Alkhatib, M.H.; AJkhayyal, N.S. Cytotoxicity of gemcitabine- loaded-microemulsions in breast and colon cancer cells. *Tropical J. Pharm. Res.,* 2014, 13, 217-224.
[79] Rial-Hermida, M.l.; Alvarez-Lorenzo, C.; Concheiro, A. In: Abordaje muitidisciplinar de cáncer colorectal, Pazos, A.; Ulla, J.L.; Valladares, M, eds. Andavira: Santiago de Compostela, 2015; pp. 299-313.
[80] Yi, C.; Zhong, H.; Tong, S.; Cao, X.; Firempong, C.K.; Liu, H.; Fu, M.; Yang, *Y.;* Feng, *Y.;* Zhang, H.; Xu, X.; Yu, J. Enhanced oral bioavailability of a sterol-loaded microemuJsion formulation of *Flammulina velutipes,* a potential antitumor drug. *Int. J. Nanomed.,* 2012, 7,5067-5078.
[81] Qu, D.; He, J.; Liu, C.; Zhou, J.; Chen, Y. Triterpene-loaded microemulsion using Coix lacryma-jobi seed extraer as oil phase for enhanced antitumor efficacy: preparation and *in vivo* evaluation. *Int. J. Nanomed.,* 2014,9,109-119.
[82] Gao, *Y.;* Wang, *Y.;* Ma, *Y.;* Yu, A.; Cai, F.; Shao, W.; Zhai G. Formulation optimization and in situ absorption in rat intestinal tract of quercetin-loaded microemulsion. *Colloid Surf. B,* 2009,71, 306-314.
[83] Fanun, M. Microemulsions with nonionic surfactants and mint oil. *Open Colloid Sci.* J., 2010, 3, 9-14.
[84] Fanun, M. Formulation and characterization of microemulsions based on mixed nonionic surfactants and peppermint oil. *J. Colloid Interf. Sci.,* 2010, 343, 496-503.
[85] Fanun, M. Solubilization of celecoxib in microemulsions based on mixed nonionic surfactants and peppermint oil. *J. Dispers. Sci. Technol.,* 2010, 31,1140-1149.
[86] Pol, A.S.; Patel, P.A.; Hedge, D. Peppermint oil based drug delivery system of aceclofenac with improved anti-inflammatory activity and reduced ulcerogenecity. *InI. J. Phann. Biosci. Technol.,* 2013, *1, 89-101.*
[87] Nakagawa, *Y.;* Suzuki, T. Cytotoxic and xenoestrogenic effects via biotransformation of trans-anethole on isolated rat hepatocytes and cultured MCF-7 human breast cancer cells. *Biochem. Pharmacol.,* 2003,66,63-73.
[88] Mazzini, F.; Betti, M; Canonico, B.; Netscher, *T.;* Luchetti, F.; Papa, S.; Galli, F. Anticancer activity of vitamin e-derived compounds in mourine C6 glioma cells. *Chem. Med. Chem., 2010,* 5,540-543.

[89] Khalid, M.; Simard, P.; Hoarau, D.; Dragomir, A.; Leroux, J.C. Long circulating poly(ethylene glycol)-decorated lipid nanocapsules deliver docetaxel to solid tumors. *Pharm. Res., 2006,* 23,752-758.
[90] Ho, P.Y.; Yeh, T.K.; Yao, H.T.; Lin, H.L.; Wu, H.Y.; Lo, Y.K.; Chang, Y.W.; Chiang, T.H.; Wu, S.H.W.; Chao, Y.S.; Chen, Ch. T. Enhanced oral bioavailability of paclitaxel by d-α tocopheryl polyethylene glycol 400 succinate in mice. *Int. J. Pharm., 2008,* 359,174-181.
[91] Sokol, R.J.; Johnson, K..E.; Karrer, F.M; Narkewicz, M.R..; Smith, D.; Kam, I. lmprovement of cycIosporin absorption in children after liver transplantation by means of water-soluble vitamin E. *Lancet,* 1991, 338. 212-214.
[92] Boudreaux, J.P.; Hayes, D.H.; Mizrahi, S.; Maggiore, P.; Blazek, J.; Dick, D. Use of water-soluble liquid vitamin E to enhance cycIosporine absorption in children after liver transplant. *Transplant. Proc.,* 1993, 25,1875.
[93] Dyab, A.K.F.; Atta, A.M. Microgel-stabilised non-aqueous emulsions. *RSC Adv.,* 2013,3,25662-25665.
[94] Anderberg, E.K.; Nystrom, C.; Artursson, P. Epithelial transport of drugs in cell culture. Vil: Effects of pharmaceutical surfactant excipients and bile acids on transepithelial permeability in monolayers of human intestinal epithelial (Caco-2) cells. *J. Pharm. Sci., 1992,81,879-887.*